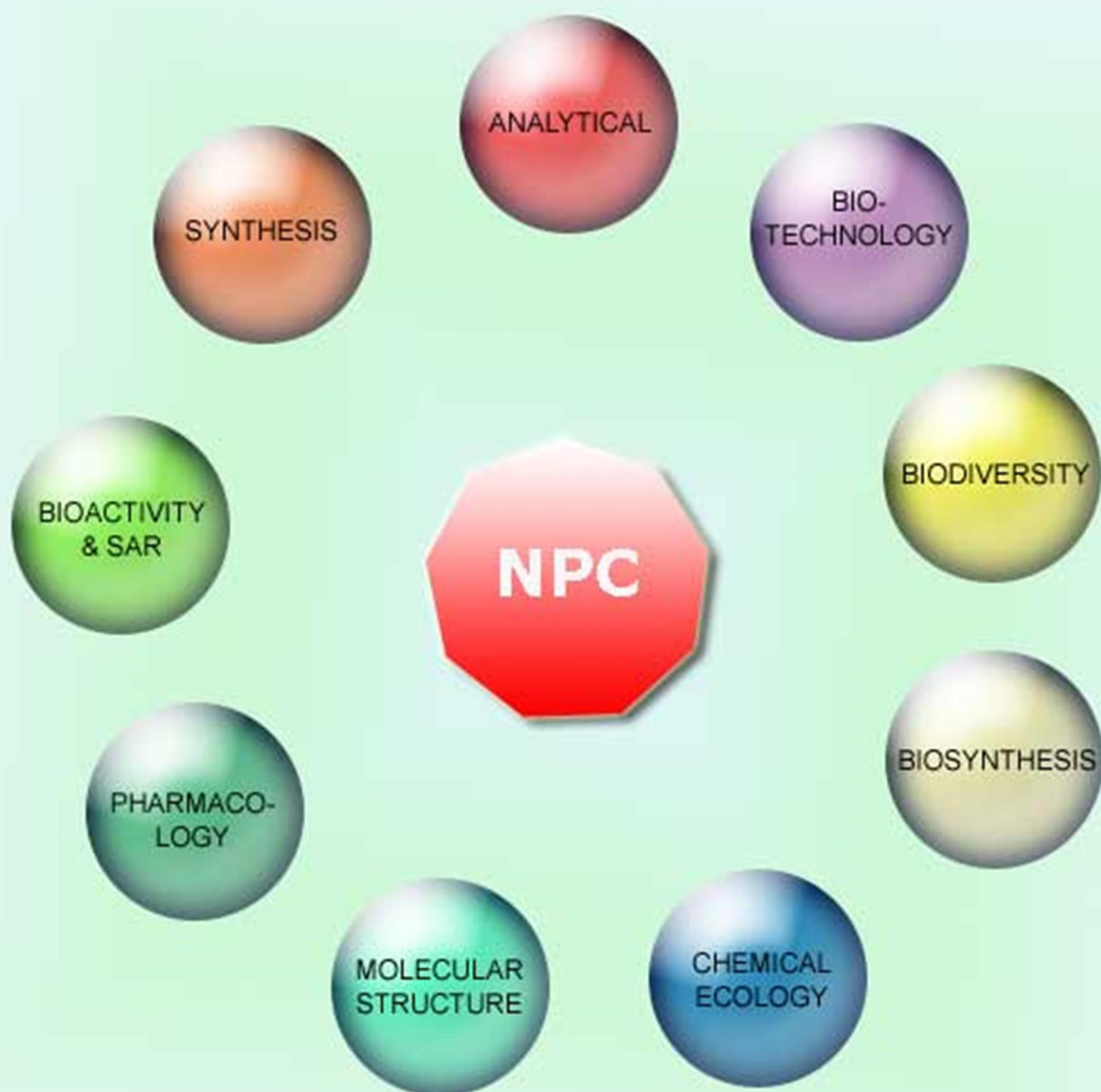


# NATURAL PRODUCT COMMUNICATIONS

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Aspects of Natural Products Research



**This Issue is Dedicated to  
Professor Feng-Peng Wang  
On the Occasion of his 70th Birthday**

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## Professor Feng-Peng Wang

### Editorial

It is my privilege and pleasure to introduce this issue, which is dedicated to Professor Feng-Peng Wang, West China School of Pharmacy, Sichuan University, China, on the occasion of his 70<sup>th</sup> birthday. The editors join me in paying tribute to Professor Wang for his outstanding contributions to natural products chemistry and diterpenoid alkaloids in particular.

He has published over 255 research papers, book chapters, reviews, and three books. He is on the Editorial Advisory Boards of several journals including *Journal of Asian Natural Products Research*, *Acta Pharmaceutica Sinica B*, the *Chinese Journal of Natural Medicines*, and *Natural Product Research and Development*.

I am extremely grateful for his contributions to *Natural Product Communications* from the very beginning. He has already published 16 articles in the journal, his first appearing in 2006 [Two new C<sub>18</sub>-diterpenoid alkaloids from *Aconitum piepunense*. *Natural Product Communications*, **1**, 191-194.] and the most recent in 2015 [Recent advances in the synthesis of *Stemona* alkaloids. *Natural Product Communications*, **10**, 1093-1102]. I am also looking forward to his further publications in *NPC*, as well as his continued support.

It is a great pleasure to honor the outstanding achievements of Professor Wang on the occasion of his 70<sup>th</sup> birthday and to send him warm wishes from all his colleagues and friends. Special thanks to Professor Geoffrey A. Cordell (Natural Products Inc., Evanston, IL, USA) and Professor Qiao-Hong Chen (Department of Chemistry, California State University-Fresno, Fresno, CA, USA) for the preface. My thanks go to the authors and reviewers who have made this issue of *Natural Product Communications* possible.

We all express our appreciation for Prof. Wang's excellent contributions to natural products research and wish him all the best for his future.

Pawan K. Agrawal  
Editor-in-Chief



## Preface

It is a very great pleasure and honor to be invited to write a Preface to this issue of *Natural Product Communications* in recognition of the 70<sup>th</sup> birthday and the scientific achievements of my dear friend and illustrious colleague Professor Feng-Peng Wang of the West China College of Pharmacy at Sichuan University in Chengdu. He was born on May 11, 1945 in Changwu County, ShanXi Province. He received a B.S. degree in Pharmacy in 1969 from Sichuan Medical College, and was awarded a M.S. degree in 1981, and a Ph.D. degree in Medicinal Chemistry in 1984, from the Institute of Materia Medica, Peking Union Medical College & Chinese Academy of Medical Sciences. His M.S. supervisor was Dr. Qi-Cheng Fang and his Ph.D. supervisor was Academician Xiao-Tian Liang, himself a world-class natural product chemist and a pioneer in the field of natural products in China. In 1985, Professor Wang travelled to the United States and worked for two years as a postdoctoral research fellow in the laboratory of Professor S. William Pelletier at the Natural Products Institute in the University of Georgia. Professor Pelletier was a well-known alkaloid chemist with a substantial interest in the structure elucidation and chemistry of the diterpene alkaloids.

When he returned to China, Professor Wang resumed his lecturer position at the School of Pharmacy of West China University of the Medical Sciences where his talents, drive, and hard work led him through the academic and administrative ranks to become Professor and Dean of the School of Pharmacy, WCUMS, which in 2000 became assimilated with Sichuan University. He is presently a Professor of Medicinal and Natural Products Chemistry, and a highly regarded academic leader in Sichuan Province.

Professor Wang has been working in the field of natural products for over 50 years, and his research interests have consistently focused on the various groups of diterpenoid alkaloids, in particular, those which are derived from traditional Chinese medicines. His research involves the isolation, structure determination, chemistry, structural modifications, and synthesis of alkaloids, and in this process he has characterized 207 new natural products from a variety of plants. As a chemical transformation approach to the taxane diterpene system, he developed the process for the synthetic conversion of abundantly available diterpenoid alkaloids to taxane derivatives. These studies led to the development of numerous intriguing reactions, the formation of over 1000 diterpene alkaloid derivatives, and to his substantial reputation in the field of alkaloid chemistry.

He has also applied his talents in natural product chemistry to drug discovery and development, where he has made significant contributions, including four patents. Particularly, he was involved in the assessment of the effects of several traditional Chinese medicines, including *Fritillaria cirrhosa*, *Codonopsis tangshen*, and *Rhodiola rosea*. At the same time, he was responsible for the development of several standardized preparations of traditional Chinese medicines, including Huo Xiang Zheng Qi San, Wendan Decoction, Yupingfengsan, and Ziziphi Spinosae Decoction. The first three preparations have already entered the Japanese market. In collaboration with a pharmaceutical company, he has also successfully developed the first, third-generation, multidrug resistance reversal agent (XiuTaiJun) in China. This drug was approved by the Chinese FDA to enter clinical trials, and a phase I study is almost completed.

Professor Wang has assumed responsibility for the development of 18 national and provincial research projects, and has supervised 64 Masters and Doctoral students and several postdoctoral research associates. Over the years, these results have been published in 223 peer-reviewed research articles and in 23 reviews. He is the author or co-author of 11 book chapters, and is an editor of three books. He has been a consistent and outstanding contributor to *The Alkaloids, Chemistry and Biology* series, with chapters on various aspects of the diterpene alkaloids in Volumes 42, 59, 67, and 69. These contributions established him as the world expert on diterpenoid alkaloids. Indeed, his last chapter on the C<sub>19</sub>-diterpenoid alkaloids was a true “tour de force”, and constituted the whole volume of 578 pages! Two books which he has edited in Chinese, *The Chemistry of Alkaloids* and *Modern Chemistry of Natural Products*, are widely recognized by the natural product community in China as being essential texts.

As a result of his contributions to the chemistry of natural products, Professor Wang and his students have received a number of important awards. The doctoral dissertation of Qiao-Hong Chen, one of his graduate students, received an award as one of the Top 100 National Excellent Doctoral Dissertations in 2003. In 1991, Professor Wang received an Award for a Chinese Ph.D. with Outstanding Contributions from the Ministry of Education in China, the National Evaluation Institute of Degrees and Grade Education, and Peking Union Medical College & Chinese Academy of Medical Sciences. Since 1993, Professor Wang has received special government allocations from the State Council in China, and in 1995 he received the first prize of Wu JiePing Medical Research Award - Paul Janssen Phytochemistry Research Award. In 2002, and again in 2015, he achieved a First Place Natural Science Award from the Ministry of Education in China, and a first place Technology Invention Award from the Ministry of Education in 2004.

Professor Wang has also held a number of professional positions locally and nationally. He was the Chair of the Chengdu Pharmaceutical Association from 1995 to 2005, and the Associate Director of Medical Plant and Plant Medicine at the Botanical Society of China from 2003 to 2008. He has served as an editorial member for the *Journal of Asian Natural Products Research*, *Acta Pharmaceutica Sinica B*, the *Chinese Journal of Natural Medicines*, and *Natural Product Research and Development*. Professor Wang has dedicated his time to serve as a committee member for several key laboratories in China, including the Key Laboratory of Natural and Biomimetic Drugs at Peking University Health Science Center (1990-1996), the Key Laboratory of Phytochemistry and Sustainability of Western Plant Resources at the Kunming Institute of Botany, Chinese Academy of Sciences (1992-2007), at the Key Laboratory of Naturally Bioactive Chemicals and Their Functions, at the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (2008-2012), at currently at the Key Laboratory of Basic Substance and Resource Utilization of Chinese Herbal Medicine, Institute of Materia Medica and Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College (2012-present), and at

the Guangdong Higher Education Key Laboratory of Potential Chemical Components and Innovation of Traditional Chinese Medicines, College of Pharmacy, Jinan University (2010-present).

I have had the great pleasure of knowing Professor Wang for many years, indeed since he was with Professor Pelletier as a postdoctoral fellow, and worked with him on the chapters for *The Alkaloids* series, when I was the Editor. More personally though, I have been blessed to have had the great good fortune to have spent time in Chengdu with him on several occasions, and I have had the enormous pleasure to be the recipient of his tremendous generosity and hospitality. Together, we have traveled to many parts of Sichuan and Yunnan Provinces, as well as a highly memorable trip down the Yangtze River from Chongqing to Wuhan. We have visited many of the most spectacular sights in that part of China, including the giant panda sanctuaries in Wolong, the glorious Mt. Emei, one of the most revered mountains in Chinese Buddhism, the unparalleled scenery of Jiuzhaigou (twice!), several Tibetan autonomous areas, the ethnic communities in Lijiang and Shangri-la in Yunnan Province, and numerous wonderful temples and monasteries, as well as the remarkable water works of Dujiangyan. My life has been made significantly richer thanks to Professor Feng-Peng Wang, for which I am extremely grateful. So, at this time, it is truly and sincerely a very great pleasure to have the opportunity to honor him for his extensive contributions to natural products over the past 50 years, and to recognize the auspicious occasion of his 70<sup>th</sup> birthday.

Geoffrey A. Cordell,  
Natural Products Inc.  
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## Preface

It is my great privilege and pleasure to be able to write a preface for this special issue of the *Natural Product Communications (NPC)* in honor of the seventieth birthday of Dr. Feng-Peng Wang, a professor of Medicinal and Natural Product Chemistry of Sichuan University in P. R. China. Professor Wang has dedicated his 45-year professional life to the advancement of natural products research and education in China.

Professor Feng-Peng Wang was born in a family of a remote village located in the northwest part of China. His illiterate parents lived on farming a small piece of land, but their vision statement was to highly inspire their children to pursue knowledge through reading books. Professor Wang earned a B.S. degree in pharmacy in 1969 from Sichuan Medical College; he then was awarded with a M.S. degree and a P.D. degree in Medicinal Chemistry in 1981 and 1984, respectively, from the Institute of Material Medica, Peking Union Medical College & Chinese Academy of Medical Sciences. His lifetime love of natural products (especially diterpenoid alkaloids) was initiated by his master thesis and Ph.D. dissertation, especially under the guidance of Professor Xiao-Tian Liang who was a founder and pioneer of Chinese natural products chemistry. After graduation he worked for two years at the University of Georgia, USA, with Professor S. W. Pelletier as a postdoctoral associate in the field of diterpenoid alkaloids. In 1987, he returned back to his Alma mater---the West China University of Medical Sciences, where he persisted in his work on isolation, structural determination, chemical reactions, synthesis, and biological evaluation of diterpenoid alkaloids from *Aconitum* and *Delphinium* species. Since 1990, his research interests were extended to the conversional syntheses of anticancer taxane analogs from diterpenoid alkaloids, development of multi-drug resistance reversal agents for the combination therapy of cancer, and development of diterpenoid alkaloids for the potential treatment of cardiovascular diseases.

His 45-year affiliation with Sichuan University can be concluded as an excellent researcher and educator with productivity and high quality in both publications and training graduate students. Professor Wang has made numerous outstanding contributions to the field of Natural Products featuring the one in the field of diterpenoid alkaloids, a class of the largest and most complicated group of terpenoid alkaloids with an intriguing chemistry and numerous bioactivities. Professor Wang has 'dug out' over 200 new diterpenoid alkaloids from various *Aconitum* and *Delphinium* plants and prepared over 1000 new diterpenoid alkaloids. One diterpenoid alkaloid (bulleyaconitine A) that was first isolated by Dr. Wang *et. al.* has been developed into clinical use as non-narcotic analgesic and anti-inflammatory drug in China. Some diterpenoid alkaloids with novel skeletons (e.g. atropurpuran) discovered by his research group have become attracting and challenging synthetic targets of many excellent research groups due to their intrinsic beauty and structural complexity. Very recently, one compound designated as "WF" isolated from "Fuzi" (a famous traditional Chinese medicine) has been established by Dr. Wang's research group as a promising candidate that significantly enhance the survival rate of mice with chronic heart failure and to appreciably improve the cardiac structure and functions in mice, as compared to cedilanid. He has successfully developed the first third-generation multi-drug resistance reversal agent (XiuTaiJun) in China, which has entered human phase I clinical studies in China. During his productive and distinguished career spanning over 45 years, Professor Wang has authored or coauthored over 240 peer-reviewed scientific publications and 11 book chapters, and is an editor for 3 books. As an expert on diterpenoid alkaloids, he has persistently committed to the series collections of "The Alkaloids" edited by Professor Geoffrey A. Cordell and has compiled four chapters for volumes 42, 59, 67, and 69. Two books entitled "The Chemistry of Alkaloids" and "Modern Chemistry of Natural Products" compiled by Dr. Wang have been widely recognized by and have indeed benefited to the natural product community in China.

In addition to the high quality of his own research work, he has successfully trained 40 Master's level and 26 doctoral students, and 3 postdoctoral associates. Most of his students have highly successful careers in academies or pharmaceutical industry. His contributions continue to inspire his students and young scientists. In addition to his formal presentations in the scientific literature, Professor Wang has been and still is very active in helping and inspiring young scientists by serving as an academic committee member in a variety of universities and research institutes throughout China.

Throughout his highly rewarding tenure, Professor Wang has achieved numerous awards, including the first prize of Wu Jieping Medical Research Award – Paul Janssen Phytochemistry Research Award in 1995, two Natural Science Awards and one Technology Invention Award from the Ministry of Education in China (First Place, 2002, 2004, and 2015). Additionally, one doctoral dissertation supervised by him has achieved an Award for Top 100 National Excellent Doctoral Dissertations in China in 2003.

The author was fortunate enough to be associated with Professor Wang in the late 1990s as one of his Ph.D. students which inspired the author to be interested in natural products and initiated an over 15-year productive collaboration. The author has benefited much from his guidance.

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# Natural Product Communications

## 2015

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Petchienes A–E, Meroterpenoids from *Ganoderma petchii*Qin-Lei Gao<sup>a,c,1</sup>, Ping-Xia Guo<sup>d,1</sup>, Qi Luo<sup>a,c</sup>, Hui Yan<sup>a</sup> and Yong-Xian Cheng<sup>a,b,\*</sup><sup>a</sup>State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, People's Republic of China<sup>b</sup>College of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu 610075, People's Republic of China<sup>c</sup>University of Chinese Academy of Sciences, Yuquan Road 19, Beijing 100049, People's Republic of China<sup>d</sup>Shanxi University of Traditional Chinese Medicine, Taiyuan 030024, People's Republic of China

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Petchienes A–E (**1–5**), five new meroterpenoids, were isolated from the fruiting bodies of *Ganoderma petchii*. Their structures, including absolute configurations, were elucidated by means of spectroscopic and computational methods. Compound **4** was isolated as a racemic mixture, which was finally purified by chiral HPLC to yield individual (–) and (+)-antipodes. Biological evaluation showed that compounds **2** and (–)-**4** could increase intracellular free calcium concentration at 10  $\mu$ M in HEK-293 cells.

**Keywords:** *Ganoderma petchii*, Ganodermataceae, Meroterpenoids, Spiro-compound.

Meroterpenoids from the genus *Ganoderma* are characterized by a 1,4-dihydroxybenzene residue conjugated with a terpenoid moiety. Since the discovery from *G. pfeifferi* of ganomycins A and B in this class of meroterpenoids [1], few other investigations associated with this compound class from *Ganoderma* species have been reported. We isolated a novel meroterpenoid termed lingzhiol from *G. lucidum* [2a], and since then, several structurally diverse meroterpenoids with renal or neural protective effects were characterized by us [2b–2e]. In our continuing efforts focused on the search of bioactive meroterpenoids from *Ganoderma*, *G. petchii* became our research target. This fungus is distributed in China, Sri Lanka, Malaysia, Singapore and Indonesia. In China, it is used as a healthcare mushroom and seen in several markets of Chinese medical materials. Our efforts on this fungus led to the isolation of five new meroterpenoids, and here we describe their isolation, structure characterization and biological evaluation.

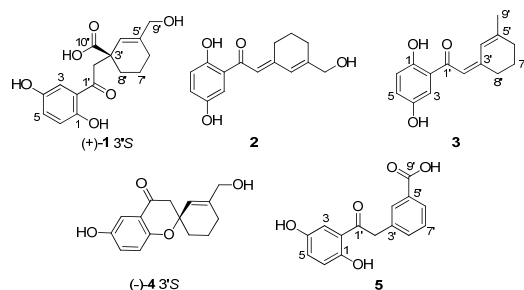


Figure 1: The chemical structures of compounds **1–5**.

Petchiene A (**1**) has the molecular formula  $C_{16}H_{18}O_6$  derived from its HRESIMS,  $^{13}C$  NMR and DEPT spectra. The  $^1H$  NMR spectrum (Table 1) of **1** contains a typical ABX spin system [ $\delta_H$  7.32 (1H, d,  $J$  = 2.7 Hz, H-3), 7.07 (1H, dd,  $J$  = 8.9, 2.7 Hz, H-5), 6.79 (1H, d,  $J$  = 8.9 Hz, H-6)], suggesting the presence of a 1,2,4-trisubstituted benzene ring, along with a resonance for an olefinic proton. The  $^{13}C$  NMR and DEPT spectra contain resonances for 16 carbons including five aliphatic methylenes (one oxygenated), four methines

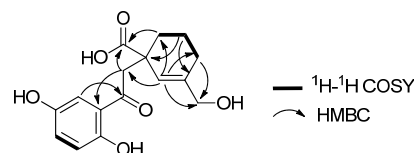


Figure 2: COSY and key HMBC correlations of **1**.

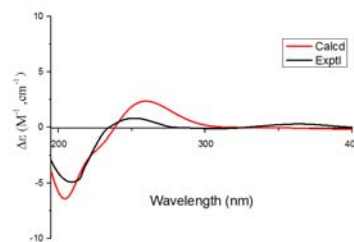


Figure 3: Calculated and experimental ECD spectra of (+)-**1** (red, at the B3LYP-SCRF (PCM)/6-311+G(2d,p)//B3LYP/6-311+G(2d,p) level in MeOH; black, experimentally observed in MeOH).

(all  $sp^2$ ), and seven quaternary carbons (one ketone, one carboxylic acid, four olefinic including two oxygenated). The  $^1H$ - $^1H$  COSY spectrum (Figure 2) showed correlations between H-5/H-6 and H-6'/H-7'/H-8'. In the HMBC spectrum, the correlation from H-3 to C-1' ( $\delta_C$  204.9) indicates that the phenyl unit is linked to C-1', and from H-2' ( $\delta_H$  3.49, 3.46) to C-2 ( $\delta_C$  120.1), C-1', C-3' ( $\delta_C$  44.8) indicate that C-2' is linked to C-1'. In addition, HMBC correlations between H-7'/C-3', C-5' and H-4'/C-6', C-8', in combination with COSY correlations of H-6'/H-7'/H-8' and degrees of unsaturation of **1**, indicate the presence of a six-membered ring. The positions of a carboxylic acid and a hydroxymethyl were assigned by the HMBC correlations between H-2', H-4'/C-10' and H-4', H-6'/C-9'. Hence, the planar structure of **1** was identified as shown. **1** was isolated as a non-racemic compound indicated by its optical rotation. The absolute configuration at the single chiral center of **1** was clarified by electronic circular dichroism (ECD) calculations. For this

purpose, DFT and TD-DFT calculations were carried out at 298 K in the gas phase with Gaussian 09 [3]. The ECD spectrum of (3'S)-**1**, correlates well with the experimental ECD spectrum of **1**, leading to the unambiguous assignment of the absolute configurations at the stereogenic centers in **1** as 3'S (Figure 3).

Petchiene B (**2**) was found to have the molecular formula  $C_{15}H_{16}O_4$  (8 degrees of unsaturation) deduced from its HRESIMS,  $^{13}C$  NMR and DEPT spectra. The  $^1H$  and  $^{13}C$  NMR spectra of **2** are similar to those of **1**. The only difference is that the  $\Delta^2$  double bond of **2** was formed by decarboxylation of **1** (Figure 4), which could be supported by HMBC correlations between H-2' ( $\delta_H$  6.77)/C-1', C-2, C-3' and H-7', H-8'/C-3' ( $\delta_C$  157.7). In addition, a ROESY correlation between H-2' ( $\delta_H$  6.77)/H-4' ( $\delta_H$  6.39) was observed, indicating a *trans* double bond between C-2' and C-3'.

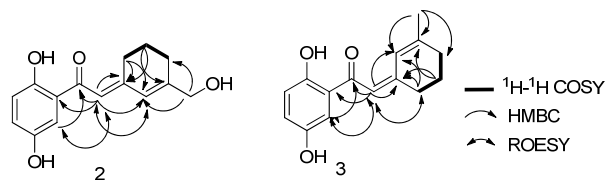


Figure 4: COSY and key HMBC correlations of **2** and **3**.

Table 1:  $^1H$  NMR data of **1–3** ( $\delta_H$  in ppm,  $J$  in Hz).

position	<b>1</b> <sup>a</sup>	<b>2</b> <sup>b</sup>	<b>3</b> <sup>c</sup>
3	7.32 d (2.7)	7.26 d (2.8)	7.26 overlap, 7.25d (2.6) <sup>d</sup>
5	7.07 dd (8.9, 2.7)	6.97 dd (8.8, 2.8)	6.98 dd (8.8, 2.8)
6	6.79 d (8.9)	6.77 overlap	6.86 d (8.8)
2'	a: 3.49 d (18.1) b: 3.46 d (18.1)	6.77 overlap	6.55s 6.56 s <sup>d</sup>
4'	5.82 s	6.39 s	6.06 s
6'	2.00 t-like (5.9)	2.20 t (5.9)	2.18t (5.8)
7'	a: 1.82 m b: 1.72 m	1.79 m	1.76 m
8'	a: 2.20 m b: 1.66 m	3.02 t (6.0)	2.98 t (6.3) 2.48 t (5.9) <sup>d</sup>
9'	3.96 s	4.14 s	1.91 s

<sup>a</sup>600 MHz in acetone- $d_6$ , <sup>b</sup>600 MHz in methanol- $d_4$ , <sup>c</sup>600 MHz in  $CDCl_3$ , <sup>d</sup> in methanol- $d_4$ .

Petchiene C (**3**), isolated as a yellow solid, has the molecular formula  $C_{15}H_{16}O_3$  (8 degrees of unsaturation), based on its HRESIMS,  $^{13}C$  NMR and DEPT spectra. The NMR data of **3** closely resemble those of **2**, indicating that they are analogues. The only difference is that a hydroxymethyl group in **2** was replaced by a methyl in **3** evidenced from HMBC correlations between H<sub>3</sub>-9' ( $\delta_H$  1.91)/C-4', C-5', C-6' (Figure 4). A ROESY correlation between H-2' ( $\delta_H$  6.56 in methanol- $d_4$ )/H-8' ( $\delta_H$  2.48) was observed, indicating the configuration of the double bond.

The molecular formula of racemic petchiene D (**4**) was determined to be  $C_{15}H_{16}O_4$  (8 degrees of unsaturation) by analysis of its HRESIMS,  $^{13}C$  NMR and DEPT spectra. Compound **4** could be generated from **2** via Michael addition reaction to construct a spiro compound. This conclusion is supported by the HMBC correlations between H-2'/C-1', C-2, C-3' ( $\delta_C$  79.1), C-4' and H-8'/C-2', C-3', C-4'. In addition, ROESY correlations between 4-OH ( $\delta_H$  9.43 in DMSO- $d_6$ )/H-3, H-5 were observed, indicating the position of the free hydroxyl group in the benzene ring. Thus far, the planar structure of **4** was identified as shown. Chiral HPLC analysis indicated that **4** is racemic. Subsequent separation by chiral HPLC yielded (+)- and (–)-**4** in a ratio of 1.3:1; the absolute configuration at the stereogenic center was assigned to be 3'S for (–)-**4** by the same methods as those of **1** (Figure 6).

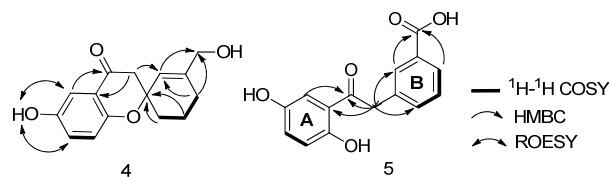


Figure 5: COSY and key HMBC correlations of **4** and **5**.

Table 2:  $^1H$  NMR data of **4** and **5** ( $\delta_H$  in ppm,  $J$  in Hz).

position	<b>4</b> <sup>a</sup>	<b>5</b> <sup>a</sup>
3	7.15 d (2.9)	7.36 d (2.9)
5	7.00 dd (8.9, 2.9)	7.10 dd (8.9, 2.9)
6	6.79 d (8.9)	6.80 d (8.9)
2'	a: 2.80d (16.5) b: 2.74 d (16.5)	4.39 s
4'	5.84 s	7.95bs
6'	a: 2.09overlap b: 1.99 m	7.92 d (7.7)
7'	a: 1.89 m b: 1.67 overlap	7.43t (7.6)
8'	a: 2.09overlap b: 1.67overlap	7.51 d (7.5)
9'	3.95s	

<sup>a</sup>600 MHz in methanol- $d_4$

Table 3:  $^{13}C$  NMR (150 MHz) data of **1–5** ( $\delta_C$  in ppm).

No.	<b>1</b> <sup>a</sup>	<b>2</b> <sup>b</sup>	<b>3</b> <sup>c</sup>	<b>4</b> <sup>b</sup>	<b>5</b> <sup>b</sup>
1	156.3	157.3	157.4	152.4	156.9
2	120.1	122.3	121.1	121.8	120.2
3	115.4	115.2	114.7	110.8	115.9
4	150.2	150.4	147.2	155.0	150.7
5	125.7	125.0	123.8	126.1	126.1
6	119.5	119.6	119.2	120.6	119.8
1'	204.9	197.1	195.5	194.7	204.8
2'	48.4	119.1	116.4	49.2	45.8
3'	44.8	157.7	157.7	79.1	136.4
4'	123.7	125.7	127.1	122.1	131.9
5'	141.4	154.3	151.6	145.6	132.4
6'	25.9	27.1	31.0	26.5	129.3
7'	19.9	23.2	22.3	19.4	129.7
8'	32.4	28.8	27.2	34.1	135.3
9'	66.6	66.1	24.9	66.1	170.0
10'	177.3				

<sup>a</sup>In acetone- $d_6$ , <sup>b</sup>In methanol- $d_4$ , <sup>c</sup>In  $CDCl_3$ .

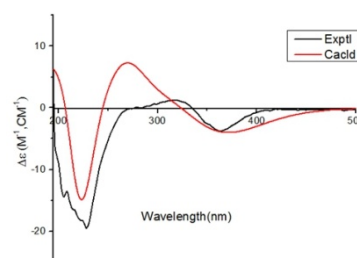


Figure 6: Calculated and experimental ECD spectra of (–)-**4** (red, at the B3LYP-SCRF (PCM)/6-311+G(2d,p)/B3LYP/6-311+G(2d,p) level in MeOH; black, experimentally observed in MeOH).

Petchiene E (**5**) was determined to have the molecular formula  $C_{15}H_{12}O_5$  (10 degrees of unsaturation) on the basis of HRESIMS analysis. The  $^1H$  NMR spectrum of **5** (Table 2) contains seven aromatic protons. The  $^{13}C$  NMR and DEPT spectra (Table 3) contain 15 signals ascribed to one  $sp^3$  methylene, seven methines (seven  $sp^2$ ), and seven quaternary carbon signals (one ketone, one carboxylic acid, and five  $sp^2$ ). The  $^1H$ - $^1H$  COSY spectrum shows the existence of fragments H-5/H-6, and H-6'/H-7'/H-8'. In the HMBC spectrum, correlations between H-3/C-1' and H-2'/C-2, C-1', C-3', C-4', C-8' indicate that the phenyl unit A is linked to C-1' and the phenyl unit B is linked to C-2' (Figure 5). The HMBC

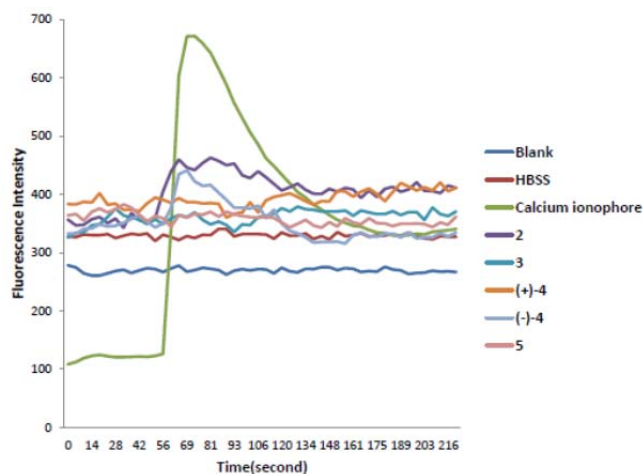


Figure 7: Effect of the isolates on intracellular  $\text{Ca}^{2+}$  concentration in HEK-293 cells.

correlations between H-4', H-6'/C-9' ( $\delta_{\text{C}}$  170.0) suggest that the carboxylic acid group is connected to C-5'. As a result, the structure of **5** was determined.

**Biological evaluation:** Calcium ( $\text{Ca}^{2+}$ ) is a fundamental second messenger that is involved in a wide range of cellular processes and, therefore, is of potential interest in drug discovery [4]. The intracellular free  $\text{Ca}^{2+}$  concentration is regulated through multiple mechanisms in neurons, and abnormalities in  $\text{Ca}^{2+}$  signaling have been implicated in many neurological disorders [5]. Considering that many species of *Ganoderma* are used for the treatment of central nervous system associated diseases, the isolates were evaluated for their potential on the changes in  $\text{Ca}^{2+}$  in HEK-293 cells. The results showed that compounds **2** and **(-)-4** could significantly elevate intracellular  $\text{Ca}^{2+}$  concentration at 10  $\mu\text{M}$  (Figure 7). Whether these two compounds are beneficial for neurological disorders needs further investigation.

## Experimental

**General:** Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Inc., People's Republic of China), C-18 silica gel (40–60  $\mu\text{m}$ ; Daiso Co., Japan), MCI gel CHP 20P (75–150  $\mu\text{m}$ ; Mitsubishi Chemical Industries, Tokyo, Japan) and Sephadex LH-20 (Amersham Pharmacia, Sweden). Optical rotations were recorded on a Horiba SEPA-300 polarimeter. UV spectra were recorded on a Shimadzu UV-2401PC spectrometer, IR spectra on a Bruker Tensor-27 spectrophotometer, and CD spectra on a Chirascan instrument. Semi-preparative HPLC was carried out using an Agilent 1200 liquid chromatography; the column used was a 250 mm  $\times$  9.4 mm, i.d., 5  $\mu\text{m}$ , Zorbax SB-C<sub>18</sub> and a 250 mm  $\times$  10 mm, i.d., 5  $\mu\text{m}$ , Daicel Chiralpak (IC), flow rate: 3 mL/min. NMR spectra were recorded on a Bruker AV-600 spectrometer, with TMS as an internal standard. EIMS and HREIMS were determined on an AutoSpec Premier P776 spectrometer. ESIMS and HRESIMS were measured on API QSTAR Pulsar 1 spectrometer.

**Fungal material:** The fruiting bodies of *G. petchii* (Lloyd) Steyaert were purchased from a market of Chinese medical materials located at Zhonghao-Luoshi-Wan of Kunming, People's Republic of China, in July 2014. A voucher specimen (CHYX-0588) was authorized by Prof. Zhu-Liang Yang at Kunming Institute of Botany and deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, People's Republic of China.

**Extraction and isolation:** The powdered fruiting bodies of *G. petchii* (60 kg) were extracted under reflux with 70% EtOH (150 L  $\times$  2 h  $\times$  3) to give a crude extract (3.39 kg), which was suspended in H<sub>2</sub>O followed by extraction with EtOAc to afford an EtOAc soluble extract. Separation of this extract (2.09 kg) using a MCI gel CHP 20P column eluted with a gradient of aqueous MeOH (10%–100%) produced 8 portions (Fr.1–Fr.8). Among them, fr.5 (410 g) was further separated via MCI gel CHP 20P washed with a gradient of aqueous MeOH (20%–100%) to yield 7 fractions (Fr.5.1–Fr.5.7). Fr.5.4 (3.48 g) was submitted to a RP-18 column with a gradient of aqueous MeOH (30%–80%) to afford 8 sub-fractions (Fr.5.4.1–Fr.5.4.8). Fr.5.4.4 (233 mg) was purified by preparative TLC (2 drops of formic acid in  $\text{CHCl}_3/\text{MeOH}$ , 9:1) followed by semi-preparative HPLC (MeOH/H<sub>2</sub>O, 43%) to yield compounds **1** (6.8 mg,  $t_{\text{R}}$  = 23 min), **2** (9.7 mg,  $t_{\text{R}}$  = 18 min) and **5** (1.0 mg,  $t_{\text{R}}$  = 25 min). Fr. 6 (50 g) was separated using MCI gel CHP 20P (MeOH/H<sub>2</sub>O, 10:90, 30:70, 50:50, 70:30, 80:20) to yield 4 fractions (Fr.6.1–Fr.6.4). Fr. 6.3 (12 g) was subjected to gel filtration on Sephadex LH-20 (MeOH) followed by chromatography on a RP-18 column (MeOH/H<sub>2</sub>O, 50%–70%) to yield **3** (5.3 mg,  $t_{\text{R}}$  = 28 min) and **4** (6.0 mg,  $t_{\text{R}}$  = 26 min). Racemic compound **4** was subjected to chiral HPLC to afford **(+)-4** (2.2 mg) and **(-)-4** (2.1 mg) (*n*-hexane/ethanol, 90:10).

### Petchiene A (1)

Pale yellow solid.

$[\alpha]_{\text{D}}^{23.4}$ : +12.3 (*c* 0.64, MeOH).

IR (KBr)  $\nu_{\text{max}}$ : 3423, 2935, 2872, 1707, 1644, 1624, 1590, 1486, 1449, 1369, 1280, 1222, 1179, 998, 869, 831, 785  $\text{cm}^{-1}$ .

UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 365 (3.57), 256 (3.87), 224 (4.17) nm.

CD (acetone):  $\Delta\epsilon_{209}$  -4.89,  $\Delta\epsilon_{347}$  -0.80.

<sup>1</sup>H and <sup>13</sup>C NMR: Tables 1 and 3.

ESIMS:  $m/z$  305 [M-H]<sup>-</sup>.

HRESIMS:  $m/z$  305.1043 [M-H]<sup>-</sup> (calcd for C<sub>16</sub>H<sub>17</sub>O<sub>6</sub>, 305.1031).

### PetchieneB (2)

Yellow solid.

UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 380 (3.59), 320 (4.07), 220 (3.99), 202 (4.12) nm.

<sup>1</sup>H and <sup>13</sup>C NMR: Tables 1 and 3.

ESIMS:  $m/z$  259 [M-H]<sup>-</sup>.

HRESIMS:  $m/z$  259.0974 [M-H]<sup>-</sup> (calcd for C<sub>15</sub>H<sub>15</sub>O<sub>4</sub>, 259.0976).

### Petchiene C (3)

Yellow solid.

UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 384 (1.50), 327 (3.66), 226 (3.99) nm.

<sup>1</sup>H and <sup>13</sup>C NMR: Tables 1 and 3.

ESIMS:  $m/z$  243 [M-H]<sup>-</sup>.

HRESIMS:  $m/z$  243.1023 [M-H]<sup>-</sup> (calcd for C<sub>15</sub>H<sub>15</sub>O<sub>3</sub>, 243.1021).

### (±)-Petchiene D (4)

Pale yellow solid.

$[\alpha]_{\text{D}}^{24}$ : +102.2 (*c* 0.27, MeOH); **(+)-4**;  $[\alpha]_{\text{D}}^{24}$ : -123.3 (*c* 0.17, MeOH); **(-)-4**.

UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 362 (0.81), 255 (1.47), 228 (4.15) nm; CD (MeOH) **(+)-4**:  $\Delta\epsilon_{230}$  +18.89,  $\Delta\epsilon_{364}$  +2.99; CD (MeOH) **(-)-4**:  $\Delta\epsilon_{230}$  -18.89,  $\Delta\epsilon_{364}$  -2.99.

<sup>1</sup>H and <sup>13</sup>C NMR: Tables 2 and 3.

ESIMS:  $m/z$  259 [M-H]<sup>-</sup>.

HRESIMS:  $m/z$  259.0974 [M-H]<sup>-</sup> (calcd for C<sub>15</sub>H<sub>15</sub>O<sub>4</sub>, 259.0970).

### Petchiene E (5)

Pale yellow solid.



UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 368 (3.51), 256 (3.76), 223 (4.20), 202 (4.34) nm.

IR (KBr)  $\nu_{\max}$ : 3395, 3076, 2927, 1701, 1634, 1612, 1487, 1446, 1380, 1351, 1324, 1267, 1205, 1182, 784  $\text{cm}^{-1}$ .

$^1\text{H}$  and  $^{13}\text{C}$  NMR: Tables 2 and 3.

ESIMS:  $m/z$  271  $[\text{M}-\text{H}]^-$ .

HRESIMS:  $m/z$  271.0614  $[\text{M}-\text{H}]^-$  (calcd for  $\text{C}_{15}\text{H}_{11}\text{O}_5$ , 271.0612).

**Calcium imaging assay:** The calcium imaging assay was performed by an automated, cell-based fluorescence-imaging system (Array scan), as previously reported, with slight modification [6,7]. The HEK293 cells were seeded at a density of  $2 \times 10^4$  cells/well in 96-well plate coated with poly-L-lysine and incubated for 12 h. Then, the HEK-293 cells were washed 3 times by HBSS (1.26 mM  $\text{CaCl}_2$ , 0.493 mM  $\text{MgCl}_2$ , 0.407 mM  $\text{MgSO}_4$ , 5.33 mM  $\text{KCl}$ , 0.441 mM  $\text{KH}_2\text{PO}_4$ , 4.17 mM  $\text{NaHCO}_3$ , 137.93 mM  $\text{NaCl}$ , 0.338 mM  $\text{NaH}_2\text{PO}_4$ , 5.56 mM D-Glucose, pH 7.2-7.4) and loaded finally with

2  $\mu\text{M}$  Fluo-4 AM (DOJINDO) for 80 min at  $37^\circ\text{C}$  in HBSS. After incubation, the cells were washed mildly 3 times by HBSS. After the loading, calcium imaging was analyzed using Array scan VTI HCS Reader (Cellomics, The Thermo Scientific, Pittsburgh, PA). Fluorescence of Fluo-4 AM was excited at 488 nm, and emitted at 543 nm and recorded as times-series mode per 5 sec. The value of the changed fluorescence was expressed as the relative intensity compared with the initial value.

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Megastigmane Glycosides from the Leaves of *Tripterygium wilfordii*Lin Ni<sup>a,b</sup>, Xiao-mei Zhang<sup>c</sup>, Xing Zhou<sup>c</sup>, Jie Ma<sup>a</sup>, Chuang-jun Li<sup>a</sup>, Li Li<sup>a</sup>, Tian-tai Zhang<sup>a</sup> and Dong-Ming Zhang<sup>a,\*</sup><sup>a</sup>State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China<sup>b</sup>College of Plant Protection, Fujian Agriculture and Forestry University, Fuzhou 350002, China<sup>c</sup>Chongqing Academy of Chinese Materia Medica, Chongqing 400065, China

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Two new megastigmane glycosides, named wilfordonisides A and B (**1** and **2**), and four known compounds (**3-6**) were isolated from the leaves of *Tripterygium wilfordii*, and one new aglycon, named wilfordoninol A (**2a**), was acquired by enzymatic hydrolysis of **2**. The absolute stereostructures of the compounds were determined by Mosher's method and by CD. At a concentration of 10  $\mu$ M, compounds **1**, **3**, and **5** inhibited STAT1 translocation by  $38.1 \pm 0.9\%$ ,  $55.8 \pm 0.8\%$ , and  $53.9 \pm 1.0\%$ , respectively.

**Keywords:** *Tripterygium wilfordii*, Megastigmane glycosides, Wilfordoniside, Wilfordoninol, STAT1.

*Tripterygium wilfordii* Hook. f., family Celastraceae, has been used in traditional Chinese medicine for the treatment of cancer, rheumatoid arthritis, and skin disorders for hundreds of years [1]. A number of studies have been focused in China on the chemistry and pharmacology of this species and hundreds of compounds have been reported [2], included triterpenoids [3], diterpenoids [4], lignins [5], and alkaloids [6]. In the course of chemical investigations of the leaves of *T. wilfordii*, we have isolated two new megastigmane glycosides, named wilfordonisides A and B (**1** and **2**), along with four known compounds (**3-6**) (Figure 1), and one new aglycon, named wilfordoninol A (**2a**), which was acquired by enzymatic hydrolysis of **2**. In this paper, we report the structure elucidation of the new compounds and their biological activities.

Wilfordoniside A (**1**) was obtained as a colorless amorphous powder with a molecular formula of  $C_{19}H_{34}O_7$ , based on HRESIMS analysis ( $m/z$  397.2207 [ $M + Na$ ]<sup>+</sup>, calcd 397.2197). The molecular formula accounted for 3 degrees of hydrogen deficiency. The IR spectrum of **1** showed absorption bands at 3383 and 1666  $cm^{-1}$  ascribable to hydroxyl and double bond functions, respectively. The <sup>1</sup>H and <sup>13</sup>C NMR (Tables 1 and 2) spectra of **1**, which were assigned by various NMR experiment, showed signals assignable to 1  $\beta$ -glucose and 13 carbons of the aglycone moiety. The aglycone contained four methyls [ $\delta_H$  0.87, 0.90 (3H each, both s, H<sub>3</sub>-11, 12; 1.24 (3H, d,  $J = 6.4$  Hz, H<sub>3</sub>-10), and 0.88 (3H, overlap, H<sub>3</sub>-13)], and two methines [ $\delta_H$  3.90 (1H, m, H-3) and 4.25 (1H, m, H-9)] bearing an oxygen function and one *trans*-configuration double bond [ $\delta_H$  5.32 (1H, dd,  $J = 15.4$  and 10.0 Hz, H-7) and 5.48 (1H, dd,  $J = 15.4$  and 6.3 Hz, H-8)], together with two methylenes, two methines and a quaternary carbon. As shown in Figure 2, the <sup>1</sup>H-<sup>1</sup>H COSY experiment on **1** indicated the presence of a partial structure, written in bold lines, and in the HMBC experiment, long range correlations were observed between the following: H<sub>3</sub>-11 and C-1 ( $\delta_C$  36.1), C-12 ( $\delta_C$  32.2), C-6 ( $\delta_C$  58.9); H-8 and C-6, C-7 ( $\delta_C$  131.4), C-9 ( $\delta_C$  69.6), C-10 ( $\delta_C$  24.4); H<sub>3</sub>-10 and C-8 ( $\delta_C$  138.8) and C-9; H<sub>3</sub>-13 and C-4 ( $\delta_C$  44.2) and C-5 ( $\delta_C$  32.5). HMBC correlations from H-3 ( $\delta_H$  3.90, m) to C-1' ( $\delta_C$  103.0); and from H-1' [ $\delta_H$  4.37 (1H, d,  $J = 7.8$  Hz)] to C-3 ( $\delta_C$  76.0) suggested that the glucose was connected to C-3. The relative stereostructure of **1**, except for the 9-position, was

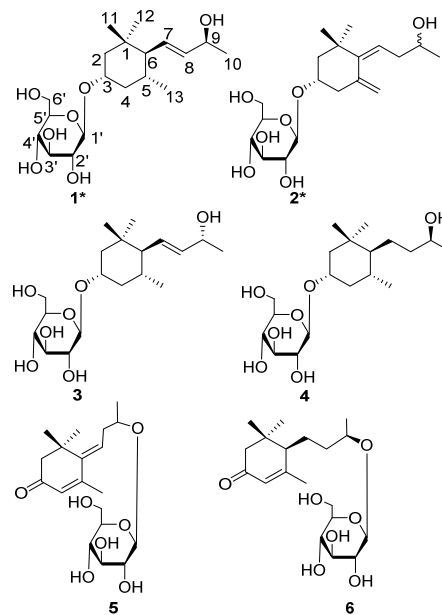
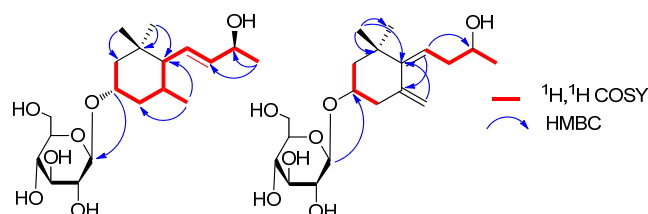
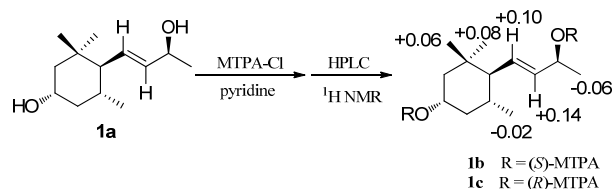


Figure 1 : Structures of compounds 1-6.

characterized by the NOESY experiment, which showed NOE correlations between H-2 $\beta$  and H-3; H-3 and H-5; H-5 and H-4 $\beta$ ; H-4 $\alpha$  and H-6; and H-6 and H-2 $\alpha$ , as shown in Figure 4.

Hydrolysis of **1** with snailase liberated its aglycon (**1a**), and glucose. The glucose was identified as D-(+)-glucose from its optical rotation {D-(+)-glucose had a positive optical rotation ( $[\alpha]_D^{25} +45.7$ ), and the optical rotation of glucose is  $[\alpha]_D^{25} +39.8$ }. Finally, the absolute configuration of **1a** was characterized by application of the modified Mosher's method [7, 8].

Compound **1a** was treated with (*R*)- and (*S*)- $\alpha$ -methoxy- $\alpha$ -(trifluoromethyl)phenylacetyl chloride and gave the 3,9-di-(*S*)-MTPA ester (**1b**) and 3,9-di-(*R*)-MTPA (**1c**) ester, respectively. As

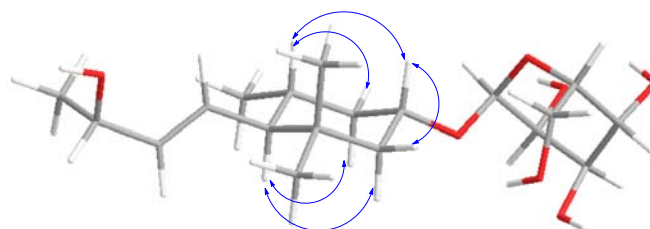
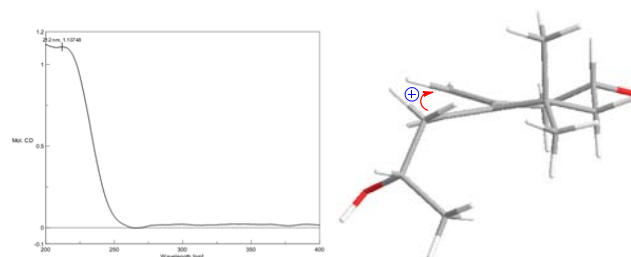
Figure 2: Key  $^1\text{H}$ - $^1\text{H}$  COSY and HMBC correlations of **1** and **2**.Figure 3: Treatment of **1a** by Mosher's method

shown in Figure 3, the signals due to protons attached to the 10- and 13-position in the 3,9-di-(*S*)-MTPA ester were observed at higher fields compared with those of the 3,9-di-(*R*)-MTPA ester [ $\Delta\delta$ : negative], while the signals due to protons of the 7-, 8-, 11- and 12-positions in the 3,9-di-(*S*)-MTPA ester were lower field compared with those of 3,9-di-(*R*)-MTPA ester [ $\Delta\delta$ : positive]. Thus, the absolute configurations at the 3 and 9-positions of **1a** were determined to be 3*S* and 9*R*. Accordingly, the absolute configuration of 3*S*, 5*R*, 6*R*, and 9*R* was established. Meanwhile, by comparison of spectroscopic data [8], compound **1a** had the same NMR data as sarmentol F.

Wilfordonaside B (**2**) was obtained as colorless oil with a molecular formula of  $\text{C}_{19}\text{H}_{32}\text{O}_7$ , based on HRESIMS analysis ( $m/z$  395.2046 [ $\text{M} + \text{Na}$ ] $^+$ , calcd 395.2040). The molecular formula accounted for 4 indices of hydrogen deficiency. The IR spectrum of **2** showed absorption bands at 3395 and 1646  $\text{cm}^{-1}$  ascribable to hydroxyl and double bond functions, respectively. The characteristic  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopic data (Tables 1 and 2) indicated that compound **2** was also a glycoside of a megastigmane derivative. As shown in Figure 2, the  $^1\text{H}$ - $^1\text{H}$  COSY experiment on **2** indicated the presence of a partial structure, written in bold lines, and in the HMBC experiment, long range correlations were observed between the following:  $\text{H}_3$ -11 [ $\delta_{\text{H}}$  0.92 (3H, s)] and C-1 ( $\delta_{\text{C}}$  36.8), C-12 ( $\delta_{\text{C}}$  28.4), C-6 ( $\delta_{\text{C}}$  147.6); H-7 [ $\delta_{\text{H}}$  5.30 (1H, m)] and C-1, C-5 ( $\delta_{\text{C}}$  142.6), C-9 ( $\delta_{\text{C}}$  66.2); H-13 [ $\delta_{\text{H}}$  4.61 (1H, m) and 5.11 (1H, m)] and C-6. HMBC correlations from H-3 ( $\delta_{\text{H}}$  3.90, m) to C-1' ( $\delta_{\text{C}}$  100.8) suggested that the glucose was connected to C-3. The relative configuration of the C=C double bond between C-6 and C-7 was deduced as *Z* by NOE difference experiment showing strong enhancements of  $\text{H}_3$ -11 and  $\text{H}_3$ -12 on irradiation of H-7. Hydrolysis of **2** with snailase liberated its aglycon, a new norsesquiterpene, named wilfordoninol A (**2a**), and glucose. The glucose was also identified D-(+)-glucose by the similar and positive optical rotation. The absolute configuration of C-3 was determined by application of the CD helicity rule [9, 10]. Thus, since the CD spectrum of **2a** (Figure 5) showed a positive Cotton effect at 212 nm, the 3-position was determined to have the *S* configuration.

The known compounds were identified by comparison of spectroscopic data with those reported in the literature as crotonionoside H (**3**) [11], alangioside J (**4**) [12], (*E*)-4-[3'-( $\beta$ -D glucopyranosyloxy)butylidene]-3,5,5-trimethyl-2-cyclohexen-1-one (**5**) [13], and blumenol C glucoside (**6**) [14].

STAT1 (signal transducer and activator of transcription 1) plays a crucial role in signaling by interferons (IFNs), thereby regulating

Figure 4: Key NOESY correlations of **1**.Figure 5: The CD curve of compound **2a**.Table 1:  $^{13}\text{C}$  NMR spectral data of compounds **1-2** and **1a-2a**

NO.	<b>1</b> <sup>a</sup>	<b>1a</b> <sup>b</sup>	<b>2</b> <sup>c</sup>	<b>2a</b> <sup>b</sup>
1	36.1	35.8	36.8	39.2
2	48.1	51.1	47.6	50.8
3	76.0	67.4	72.9	69.6
4	44.2	45.6	42.6	47.3
5	32.5	32.2	142.6	144.7
6	58.9	58.5	147.6	150.0
7	131.4	131.2	117.5	118.6
8	138.8	138.5	38.0	38.1
9	69.6	69.3	66.2	69.1
10	24.4	24.1	23.1	23.1
11	22.0	21.8	27.3	27.9
12	32.2	31.9	28.4	29.0
13	22.1	21.8	113.9	114.5
1'	103.0		100.8	
2'	75.4		73.4	
3'	78.4		76.7	
4'	72.0		70.0	
5'	78.1		76.7	
6'	63.1		61.0	

<sup>a</sup> In  $\text{MeOD}-d_4$  (125 MHz). <sup>b</sup> In  $\text{MeOD}-d_4$  (150 MHz). <sup>c</sup> In  $\text{DMSO}-d_6$  (125 MHz).

antiviral responses, cell proliferation, apoptosis, immune surveillance, and tumor suppression. The inhibitory effects of compounds **1-6** were evaluated *in vitro* for STAT1 translocation by immunofluorescence assay. Compounds **1**, **3**, and **5** inhibited STAT1 translocation by  $38.1 \pm 0.9\%$ ,  $55.8 \pm 0.8\%$ , and  $53.9 \pm 1.0\%$ , respectively, at a concentration of 10  $\mu\text{M}$ ; the other compounds showed weak activity.

## Experimental

**General experimental procedures:** Optical rotations were measured on a JASCO P2000 automatic digital polarimeter, UV spectra on a JASCO V-650 spectrophotometer, and IR spectra on a Nicolet 5700 spectrometer using a FT-IR microscope transmission method. CD spectra were measured on a JASCO J-815 CD spectrometer. NMR spectra were acquired with an INOVA-500 spectrometer. HRESIMS were collected on an Agilent 1100 series LC/MSD ion trap mass spectrometer. Preparative HPLC was conducted using a Shimadzu LC-6AD instrument with a SPD-20A detector and a YMC-Pack ODS-A column (250 $\times$ 20 mm, 5  $\mu\text{m}$ ). Column chromatography (CC) was performed with silica gel (200-300 mesh, Qingdao Marine Chemical Inc., Qingdao, People's Republic of China), polyamide (60-100 mesh, Changfeng Chemical Inc., Jiangsu, People's Republic of China), D101 macroporous (Huangguang Chemical Inc., Tianjin, People's Republic of China),

**Table 2:** <sup>1</sup>H NMR spectral data of compounds **1-3** and **1a-3a**.

NO.	<b>1</b> <sup>a</sup>	<b>1a</b> <sup>b</sup>	<b>2</b> <sup>c</sup>	<b>2a</b> <sup>b</sup>
2α	1.18, t, (12.4)	1.11, t, (12.4)	1.31, m	1.28, m
2β	1.87, br d (12.4)	1.69, br d (12.4)	1.83, m	1.95, m
3	3.90 (overlap)	3.73, m	3.89, m	3.88, m
4α	1.05, q (12.4)	0.90, q (12.4)	1.83 (overlap)	1.80, m
4β	2.13, br d (12.4)	1.98, br d (12.4)	2.75, dd (12.3, 3.4)	2.65, m
5	1.58, m	1.55, m		
6	1.33, t (10.0)	1.29, t (10.0)		
7	5.32, dd (15.4, 10.0)	5.30, dd (15.4, 10.0)	5.30, m	5.40, dd (8.0, 6.4)
8	5.48, dd (15.4, 6.3)	5.46, dd (15.4, 6.3)	2.13, m; 2.22, m	2.26, m; 2.38, m
9	4.25, m	4.23, m	3.60, dd (10.7, 5.9)	3.75, m
10	1.24, d (6.4)	1.23, d (6.5)	1.01, d (6.2)	1.15, d (6.2)
11	0.87, s	0.85, s	0.92, s	1.00, s
12	0.90, s	0.87, s	1.09, s	1.16, s
13	0.88 (overlap)	0.87 (overlap)	4.61, br s; 5.11, br s	4.68, d (2.2); 5.12, d (2.2)
1'	4.37, d (7.8)		4.25, d (7.8)	
2'	3.16, m		2.87, m	
3'	3.36, m		3.17, m	
4'	3.30, m		3.03, m	
5'	3.30, m		3.03, m	
6'	3.89, m; 3.68, m		3.65, m; 3.43, m	

<sup>a</sup> In MeOD-*d*<sub>4</sub> (500 MHz). <sup>b</sup> In MeOD-*d*<sub>4</sub> (600 MHz). <sup>c</sup> In DMSO-*d*<sub>6</sub> (500 MHz).

and ODS (50 μm, YMC, Japan). TLC was carried out on glass precoated silica gel GF<sub>254</sub> plates. Compounds were visualized either under UV light or by spraying with 10% sulfuric acid in EtOH followed by heating.

**Plant material:** The leaves of *Tripterygium wilfordii* (TWHF) were collected in Taining, Fujian, China, in September 2009 and identified by Professor Lin Ma from the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher specimen (No. 20090034) is deposited at the herbarium of the Institute of Material Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, China.

**Extraction and isolation:** Air-dried leaves of TWHF (100 kg) were reduced to a coarse powder and refluxed with 80% EtOH (400 L×2h×3). After evaporation of ethanol *in vacuo*, the aqueous residue was diluted with water, and then partitioned with EtOAc (30L×3). The water layer was subjected to passage over polyamide by elution with water and 50% EtOH-water. The water extract was subjected to passage over D101 macroporous adsorption resin by elution with water and 30%, 60%, 95% EtOH-water in sequence to give fractions A1, A2 (651.0 g), A3 (338.5 g), and A4 (12.4g). Fraction A3, with an equal weight of diatomite, was successively refluxed with EtOAc, EtOH, MeOH to obtain fractions B1 (13.62 g), B2 (151.38 g) and B3 (68.87 g). Fraction B2 was subjected to CC on silica gel (200-300 mesh) with CHCl<sub>3</sub>-MeOH (15: 1 – 1: 1) to afford 7 fractions (C1 - C7). Fraction C6 (8.625 g) was passed over an RP-18 column with MeOH-water (15%-75%), and finally purified by preparative HPLC (detected at 210 nm, 8 mL/min) to give **1** (43 mg), **2** (3 mg), **3** (8 mg), **4** (7 mg), **5** (5 mg), and **6** (14 mg).

**Enzymatic hydrolysis of 1 and 2 with snailase:** A solution of **1** and **2** (8.0 and 3.0 mg, respectively) in H<sub>2</sub>O (1.0 mL) was treated with snailase (10.0 and 5.0 mg, respectively). The solution was stirred at 37°C for 5 h; after cooling, the reaction mixture was extracted with EtOAc. The aqueous layer was subjected to CC on silica gel (200-300 mesh) with CH<sub>3</sub>CN-H<sub>2</sub>O (8:1) to afford glucose (1.8 mg). The EtOAc layer was purified by HPLC (25% CH<sub>3</sub>CN-H<sub>2</sub>O, detected at 210 nm, 8 mL/min) to furnish **1a** (2.3 mg) and **2a** (0.5 mg).

**Wilfordoninol A (2a)**  
Colorless oil (MeOH).

<sup>1</sup>H and <sup>13</sup>C NMR: Tables 1 and 2.

HRESIMS: *m/z* 233.1509 [M + Na]<sup>+</sup> (calcd for C<sub>13</sub>H<sub>22</sub>NaO<sub>2</sub>, 233.1522).

**Preparation of (R)- and (S)-MTPA esters of 1a:** A solution of compound **1a** (1.0 mg) in dry CH<sub>2</sub>Cl<sub>2</sub> (2.0 mL) was treated with (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*R*)-MTPA-Cl, 10 mg] in the presence of anhydrous pyridine, and the mixture was stirred under reflux at 40°C for 6 h. After cooling, the reaction mixture was poured into ice-water and extracted with EtOAc. The EtOAc extract was washed successively with 5% HCl, NaHCO<sub>3</sub>-saturated H<sub>2</sub>O and brine, dried over Na<sub>2</sub>SO<sub>4</sub> and filtered. Removal of the solvent from the filtrate under reduced pressure afforded a residue, which was purified by preparative HPLC (90% CH<sub>3</sub>CN-H<sub>2</sub>O, detected at 210 nm, 8 mL/min) to acquire the (*S*)-MTPA ester of **1a** (compound **1b**, 0.85 mg). Using a similar procedure, (*R*)-MTPA ester of **1a** (compound **1c**, 0.73 mg) was obtained from compound **1a** (1.0 mg) with (*S*)-MTPA-Cl (8 mg).

#### Compound 1b

Colorless oil.

<sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ: 0.85, 0.91 (3H, each, both s, H<sub>3</sub>-11, 12), 0.85 (3H, d, *J* = 6.0 Hz, H<sub>3</sub>-13), 1.43 (3H, d, *J* = 6.0 Hz, H<sub>3</sub>-10), 5.50 (1H, dd, *J* = 15.4, 10.0 Hz, H-7), 5.44 (1H, dd, *J* = 15.4, 7.8 Hz, H-8).

#### Compound 1c

Colorless oil.

<sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ: 0.93, 0.97 (3H, each, both s, H<sub>3</sub>-11, 12), 0.83 (3H, d, *J* = 6.0 Hz, H<sub>3</sub>-13), 1.37 (3H, d, *J* = 6.0 Hz, H<sub>3</sub>-10), 5.60 (1H, dd, *J* = 15.4, 10.0 Hz, H-7), 5.58 (1H, dd, *J* = 15.4, 7.8 Hz, H-8).

**Biological activities:** Recombinant U2OS cells (Thermo Scientific, Rorford IL, USA) that stably express human STAT1-EGFP fusion protein were cultured in DMEM supplemented with 10% FBS and G418 (0.5 mg/mL) in a 37°C incubator with 5% CO<sub>2</sub>. Cells were plated in 96-well plates (2.5 × 10<sup>3</sup> cell mL<sup>-1</sup>) and preincubated with samples for 30 min, followed by a further 1 h treatment with IFN-γ to evaluate the nuclear translocation of STAT1-EGFP. The capacity of translocation was detected with high content analysis by Cellomics ArrayScan V<sup>TI</sup> HCS Reader (Thermo Fisher Scientific Cellomics, Pittsburgh, PA, USA).

**Wilfordoside A (1)**

Colorless oil (MeOH).

 $[\alpha]_{\text{D}}^{25}$ :  $-52.3$  ( $c$  0.1, MeOH).IR (microscope)  $\nu_{\text{max}}$ : 3383, 2926, 1666, 1367, 1075, and 944  $\text{cm}^{-1}$ . $^1\text{H}$  and  $^{13}\text{C}$  NMR: Tables 1 and 2.HREIMS:  $m/z$  397.2207 ( $[\text{M} + \text{Na}]^+$ , calc.  $\text{C}_{19}\text{H}_{34}\text{NaO}_7$ , 397.2197).**Wilfordoside B (2)**

Colorless oil (MeOH).

 $[\alpha]_{\text{D}}^{25}$ :  $-47.3$  ( $c$  0.1 MeOH).IR (microscope)  $\nu_{\text{max}}$ : 3395, 2921, 1646, 1468, 1419, and 1181  $\text{cm}^{-1}$ . $^1\text{H}$  and  $^{13}\text{C}$  NMR: Tables 1 and 2.HRESIMS:  $m/z$  395.2046 ( $[\text{M} + \text{Na}]^+$ , calc.  $\text{C}_{19}\text{H}_{32}\text{NaO}_7$ , 395.2040).**Supplementary data:** NMR, IR, and CD data are available.**Acknowledgments** - This work was supported by the National Key Technology R & D program of the Ministry of Science and Technology of China (No. 2011BAI01B05), the National Natural Science Foundation of China (NO. 21572275), and the National Mega-project for Innovative Drugs (No. 2012ZX09301002-002).**References**

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Cytochalasans and Sesquiterpenes from *Eutypella scoparia* 1-15Shuang Qi<sup>a,b</sup>, Yue Wang<sup>a,b</sup>, Zhonghui Zheng<sup>a,b</sup>, Qingyan Xu<sup>a,b,\*</sup> and Xianming Deng<sup>a,b,\*</sup><sup>a</sup>State Key Laboratory of Cellular Stress Biology, Innovation Center for Cell Signaling Network, School of Life Sciences, Xiamen University, Xiamen, Fujian, 361102, China<sup>b</sup>State-Province Joint Engineering Laboratory of Targeted Drugs from Natural Products, Xiamen University, Xiamen, Fujian, 361102, China

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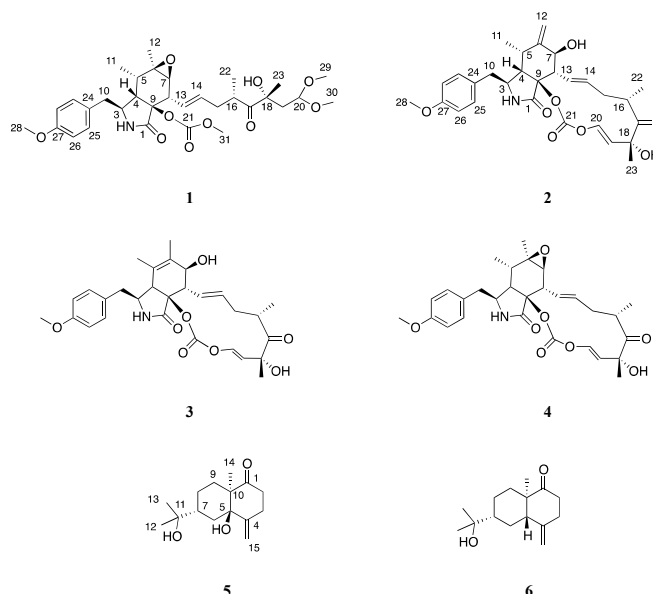
Three new compounds, an open-chain cytochalasan scoparasin C (**1**), a pyrichalasan scoparasin D (**2**), and a  $\beta$ -eudesmol type sesquiterpene scopararane C (**5**), along with three known compounds (**3**, **4** and **6**), were isolated from the marine fungus *Eutypella scoparia* 1-15. Their structures were determined on the basis of comprehensive NMR and MS analysis. Compound **2** exhibited potent cytotoxicities with very low IC<sub>50</sub> values against several cancer cell lines, including A375, A549, HepG2 and MCF-7.

**Keywords:** Cytochalasan, Scoparasins, Scopararanes, Cytotoxic activity.

Cytochalasans comprise a diverse group of fungal secondary metabolites with a characteristic tricyclic core structure in which an 11 to 14-membered macrocycle ring bearing either a carbocycle, a lactone or a cyclic carbonate, is fused to a highly substituted perhydroisoindol-1-one [1]. Based on the L-amino acids incorporated into the hydrogenated isoindolone, the canonical cytochalasans are divided into groups of cytochalasins (Phe), pyrichalasins (Tyr), chaetoglobosins (Trp), aspochalasins (Leu), and alachalasins (Ala) [2]. Cytochalasans exhibit a wide range of bioactivities, e.g., interfering with cellular mitosis, anti-parasite, inhibition of TNF- $\alpha$  and anticancer [3].

In our ongoing search for bioactive compounds from marine microbes, four cytochalasans (**1-4**) and two sesquiterpenes (**5-6**) were isolated from *Eutypella scoparia* 1-15, a fungal strain from mangrove rhizosphere soil of Jimei, Fujian Province, China. Compounds **1**, **2** and **5** were proved to be new natural products named scoparasin C, scoparasin D, and scopararane C, respectively.

Scoparasin C (**1**) was obtained as colorless oil. The molecular formula was established as C<sub>32</sub>H<sub>45</sub>NO<sub>10</sub> on the basis of HR-ESI-MS ( $m/z$  626.2926 [M+Na]<sup>+</sup>, calcd. for C<sub>32</sub>H<sub>45</sub>NNaO<sub>10</sub><sup>+</sup>: 626.2936), with 11 degrees of unsaturation. A strong absorption band at 1716 cm<sup>-1</sup> in the IR spectrum indicated the presence of a ketone and/or a lactam carbonyl functional group, corresponding to the signal at  $\delta$  5.71 (1H, s, -NH-CO-) in the <sup>1</sup>H NMR (CDCl<sub>3</sub>) spectrum. The <sup>13</sup>C NMR and DEPT spectra showed 30 resolved peaks corresponding to 32 carbons, which were classified into eight methyls (four *O*-methyl,  $\delta_c$  55.3, 54.9, 53.9, and 54.4), three methylenes ( $\delta_c$  43.1, 42.0, and 36.0), eleven methines (four *sp*<sup>2</sup> methines,  $\delta_c$  131.9, 130.1, 127.3, and 114.5), eight quaternary carbons (two olefinic, with  $\delta_c$  129.4 and 158.8, two carbonyl with  $\delta_c$  171.0 and 218.1, and three *sp*<sup>3</sup> carbon with  $\delta_c$  57.4, 77.7, and 84.9). The remaining quaternary carbon of  $\delta_c$  153.9 indicated a carbonate moiety. The <sup>1</sup>H spectrum indicated a *para*-methoxybenzene ring according to the presence of the characteristic proton resonances,  $\delta$  7.14 (d,  $J$  = 8.5 Hz, 2H), 6.90 (d,  $J$  = 8.6 Hz, 2H), and 3.82 (s, 3H). Apart from the eight degrees of unsaturation due to the benzene ring, three carbonyl groups, and one olefinic bond, the remaining three degrees of unsaturation indicated that **1** possessed a tricyclic framework. By extensive

**Figure 1:** Structures of compounds **1-6** isolated from *Eutypella scoparia* 1-15.

analysis of the 2D NMR spectra (HMBC and <sup>1</sup>H-<sup>1</sup>H COSY), the correlations from H-2 to C-3, C-4, and C-9, H-5 to C-3, C-4 and C-6, H-7 to C-8, C-9, and C-13, indicated the presence of a 10-phenyl-substituted perhydro-isoindol-1-one skeleton, the structural feature of cytochalasans. Further analysis confirmed the epoxyp propane moiety, and the positions of the eight-carbon chain system and the methoxycarbonate group at C-8 and C-9, respectively. Therefore, the structure of scoparasin C (**1**) was elucidated (Figure 1) as a new open-chain cytochalasan, a methoxyl derivative of cytochalasin Z<sub>18</sub> [4].

Scoparasin D (**2**) was obtained as white powder. The <sup>1</sup>H and <sup>13</sup>C NMR spectra of **2** were similar to those of **1** with the exception of the absence of three methoxy groups ( $\delta_c$  53.9, 54.4, and 54.9) and the presence of two olefinic carbons ( $\delta_c$  120.3 and 142.2) (Table 1). The signal of H<sub>3</sub>-12 ( $\delta_H$  1.34) in **1** was replaced with two signals of

**Table 1:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data of compounds **1** and **2**.

Position	<b>1</b>		<b>2</b>		Position	<b>1</b>		<b>2</b>	
	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$		$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$
1	/	171.0s	/	169.0s	17	/	218.1s	/	211.8s
2-NH	5.71 (1H,s)	/	4.49 (1H,bs)	/	18	/	77.7s	/	77.2s
3	3.63 (1H, d, 4.1, 9.1)	54.3d	3.26 (1H,dd, 4.2, 9.4)	53.6d	19	2.24 (1H,dd, 5.7,14.5) 1.93 (1H,dd, 5.3,14.5)	42.0t	5.66 (1H,d, 11.8)	120.3d
4	2.61 (1H,t, 4.4)	49.9d	2.96 (1H,m)	48.1d	20	4.47 (1H,t, 5.5)	102.9d	5.68 (1H,bs)	142.3d
5	2.20 (1H)	35.5d	3.37 (1H,m)	32.1d	21	/	153.9s	/	149.6s
6	/	57.4s	/	148.5s	22	1.10 (3H,d, 6.8)	17.1q	1.20 (3H,d)	20.2q
7	2.81 (1H, d, 4.7)	60.1d	3.84 (1H,d, 11.3)	69.3d	23	1.32 (3H,s)	26.5q	1.54 (3H,s)	24.1q
8	2.83 (1H,d, 5.3)	47.2d	2.97 (1H,m)	40.9d	24	/	129.4s	/	128.6s
9	/	84.9s	/	86.2s	25	7.14 (2H, d, 8.7)	130.1d	7.06 (2H,d, 8.5)	130.3d
10	2.93 (1H,dd, 9.7, 13.6) 2.85 (1H,dd, 4.5, 13.9)	43.1t	2.88 (1H,dd, 9.4, 13.6) 2.61 (1H,dd, 9.4, 13.7)	43.8t	26	6.90 (2H,d, 8.7)	114.5d	6.87 (2H,d, 8.5)	114.4d
11	1.17 (3H,d, 7.4)	14.0q	1.18 (3H,d, 6.8)	14.6q	27	/	158.8s	/	158.9s
12	1.34 (3H,s)	20.4q	5.42 (1H,bs) 5.20 (1H,bs)	114.5t	28	3.82 (3H,s)	55.3q	3.82 (3H, s)	55.3q
13	5.99 (1H,dd, 7.9, 15.2)	127.3d	5.76 (1H,dd, 11.7, 15.1)	128.2d	29	3.32 (3H,s)	54.4q		
14	5.60 (1H,dd, 7.7, 15.2)	131.9d	5.39 (1H,m)	133.8d	30	3.34 (3H,s)	53.9q		
15	2.50 (1H,dd, 7.5, 14.0) 2.18 (1H,m)	36.0t	2.74 (1H,m) 2.18 (1H,m)	38.8t	31	3.81 (3H, s)	54.9q		
16	3.19 (1H,dd, 5.8, 6.8)	39.6d	2.99 (1H,m)	40.9d					

Spectra were recorded at 600 MHz for  $^1\text{H}$  using TMS as internal standard in  $\text{CDCl}_3$ .

$\delta_{\text{H}}$  (5.20, bs, 1H) and  $\delta_{\text{H}}$  (5.42, bs, 1H), characteristic of an exocyclic double bond. Further analysis of the COSY and HMBC spectra of **2** revealed the presence of a macrocyclic structure (Figure 1). This deduction was also confirmed by the molecule formula of **2**,  $\text{C}_{29}\text{H}_{35}\text{NO}_8$ , established on the basis of the HR-ESI-MS ( $m/z$  548.2240  $[\text{M}+\text{Na}]^+$ , calcd. for  $\text{C}_{29}\text{H}_{35}\text{NNaO}_8^+$ : 548.2255).

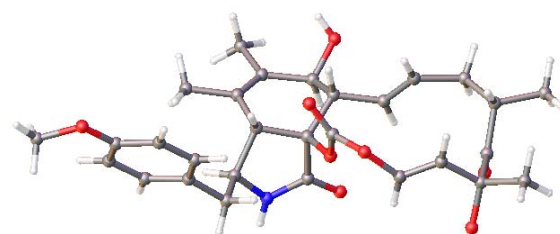
Compound **2** was previously obtained via semi-synthesis from phenochalasin B (**4**) [5], but it is the first time that it has been isolated from a natural source.

Previous isotope labeling experiments have proved that the skeletons of cytochalasans share the same stereochemistry, viz., *cis*-conjunction across the C-4/C-9, *trans*-conjunction across the C-8/C-9, and *S*-configuration at C-16. The cross-peaks at H-3/H-11 and H-5/H-8/H-10 in NOE correlations of compounds **1** and **2** suggested that both compounds had the same relative stereochemistry as previously isolated cytochalasans. The cross peak of H-16/H<sub>3</sub>-23 indicated the *R*-configuration at C-18 in both compounds, which is consistent with known cytochalasans [6].

Scoparane C (**5**) had the molecular formula of  $\text{C}_{15}\text{H}_{24}\text{O}_3$ , deduced from HR-ESI-MS ( $m/z$  253.1796  $[\text{M}+\text{H}]^+$ , calcd. for  $\text{C}_{15}\text{H}_{25}\text{O}_3^+$ : 253.1798), which indicated four degrees of unsaturation in the molecule. The  $^{13}\text{C}$  NMR and DEPT spectra ( $\text{CDCl}_3$ ) displayed two  $\text{sp}^2$  carbons ( $\delta_{\text{C}}$  148.4 and 110.8) and a carbonyl carbon ( $\delta_{\text{C}}$  214.2). The remaining two degrees of unsaturation were attributed to two rings. The  $^{13}\text{C}$  NMR spectrum of **5** was similar to that of **6** except that  $\delta_{\text{C}}$  47.8 (d) in **6** was changed to  $\delta_{\text{C}}$  77.9 (s). Extensive analysis of  $^1\text{H}$  NMR and HMBC spectra revealed that scoparane C (**5**) is a new  $\beta$ -eudesmol sesquiterpene.

Compound **3** and compound **4** had  $^1\text{H}$  and  $^{13}\text{C}$  NMR data that were identical to those of scoparasin A [7] and phenochalasin B [8], respectively. We further confirmed the structure of **3** by X-ray diffraction (Figure 2), which also demonstrated the absolute configurations of C-16 and C-18. Compound **6** was confirmed to be ent-4(15)-eudesmen-11-ol-1-one, a known  $\beta$ -eudesmol sesquiterpene.

All compounds were evaluated for their cytotoxic activities against A375, A549, HepG2 and MCF-7 cell lines by the MTT method [9].

**Figure 2:** X-ray structure of **3**.**Table 2:**  $\text{IC}_{50}$  values of **2-4** against four cancer cell lines ( $\mu\text{M}$ ).

Comps	A375	A549	HepG2	MCF-7
<b>2</b>	1.08	2.25	3.51	3.40
<b>3</b>	5.74	10.85	9.44	> 50
<b>4</b>	0.47	1.80	0.80	0.63

Compounds **1**, **5**, and **6** did not show any activity ( $\text{IC}_{50} > 50 \mu\text{M}$ ), while compound **4** had potent inhibitory activities against all four cancer cell lines (Table 2). These results implied that the macrocycle might be essential for the bioactivity of cytochalasans.

## Experimental

**General experimental procedures:** Infrared spectra (IR) were determined on a Thermo Nicolet iS5 FT-IR spectrometer, ultraviolet (UV) absorption spectra, in MeOH, on a Beckman Coulter DU 800 spectrophotometer, and optical rotations on a Perkin-Elmer 341 polarimeter.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded in  $\text{CDCl}_3$  on a 600 MHz Bruker Avance 600 spectrometer. HR-FT-MS were measured with a Bruker autoflex speed Maldi-Tof mass spectrometer. Silica GF<sub>254</sub> for thin layer chromatography (TLC) was produced by the Qingdao Marine Chemical Company, and Sephadex LH-20 by Pharmacia Biotech.

**Fungal material:** The fungal strain 1-15 was isolated from mangrove rhizosphere soil from Jimei, Fujian Province, China, in September, 2011. The fungus was identified as *Eutypella scoparia* by comparing the internal transcribed spaces (ITS) gene sequence with *Eutypella scoparia* PanM2024-1-P15 in the GenBank database, with 99% similarity (ITS1: 5'-TCCGTAGGTGAACCTGCGG -3', ITS4: 5'-TCCTCCGCTTATTGATATGC-3').

**Fermentation:** *Eutypella scoparia* 1-15 was cultured on potato dextrose agar (PDA) medium at 28°C for 35 days. A total of 20 L of fungal solid culture was prepared.

**Extraction and isolation:** The fermented material was diced and extracted 5 times with EtOAc/MeOH/AcOH (80:15:5, v/v/v) at room temperature. After removing the solvent under vacuum, the extract was partitioned between H<sub>2</sub>O and EtOAc. Then the EtOAc layer was concentrated and resuspended in 95% methanol (70 mL), and washed with light petroleum (PE) 5 times. The MeOH layer was concentrated to afford the defatted extract (8 g). This was fractionated by medium-pressure liquid chromatography over an RP-18 column (170 g) eluting with 2 L each of H<sub>2</sub>O-MeOH (100:0, 70:30, 50:50, 30:70, 0:100, v/v) to give fractions A-N. Compound **5** (6.0 mg) and **6** (9.5 mg) were obtained from fraction A (1.4 g) by silica gel CC (30 × 1.5 cm) eluting with EtOAc/PE (1:25, 1:20, 1:10, 1:6, v/v). Fraction E (1.8 g) was further purified by CC over silica gel (200-300 mesh) eluted with a CH<sub>2</sub>Cl<sub>2</sub>-MeOH gradient (from 200:1 to 0:100, v/v) to give compounds **1** (3 mg) and **4** (20 mg). Fraction I (2.0 g) was further purified over Sephadex LH-20 eluting with MeOH to give compounds **2** (2.3 mg) and **3** (5 mg).

#### Scoparasin C (1)

[ $\alpha$ ]<sub>D</sub><sup>25</sup>: +20 (c 0.1, MeOH).

IR (KBr): 2967.5, 1747.2, 1716.3, 1513.2, 1263.9 cm<sup>-1</sup>.

UV/Vis  $\lambda_{\max}$  (MeOH) nm: 274, 225, 205.

MS (EI, 70 eV):  $m/z$  (%) = 626.44 [M+Na<sup>+</sup>] (100).

HRMS-FAB:  $m/z$  [M+Na<sup>+</sup>] calcd. for C<sub>32</sub>H<sub>45</sub>NNaO<sub>10</sub>: 626.2936; found: 626.2926.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

#### Scoparasin D (2)

[ $\alpha$ ]<sub>D</sub><sup>25</sup>: +88.0 (c 0.10, MeOH).

Rf: 0.58 (CHCl<sub>3</sub>-MeOH, 10:1).

IR (KBr): 2967.5, 1767.5, 1716.0, 1512.7, 1248.3, 1032.9 cm<sup>-1</sup>.

UV/Vis  $\lambda_{\max}$  (MeOH) nm: 274, 225, 209.

MS (EI, 70 eV):  $m/z$  (%) = 548.26 [M+Na<sup>+</sup>] (100).

HRMS-FAB:  $m/z$  [M+Na<sup>+</sup>] calcd. for C<sub>29</sub>H<sub>35</sub>NO<sub>8</sub>: 548.2255; found: 548.2240.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

#### Scoparasin A (3)

Rf: 0.58 (CHCl<sub>3</sub>-MeOH, 10:1).

<sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): 1.21 (3H, s, Me-22), 1.53 (3H, s, Me-23), 1.55 (3H, s, Me-11), 1.70 (3H, s, Me-12), 2.17 (1H, m, Ha-15), 2.75 (1H, m, Hb-15), 2.78 (1H, m, Ha-10), 2.81 (1H, m, Hb-10), 2.85 (1H, m, H-8), 2.99 (1H, m, H-16), 3.44 (1H, t,  $J$  = 6.8 Hz, H-3), 3.81 (3H, s, Me-28), 3.90 (1H, m, H-7), 3.91 (1H, m, H-4), 5.41 (1H, ddd,  $J$  = 3.9 Hz, 10.6 Hz, 15.3 Hz, H-14), 5.69 (1H, d,  $J$  = 11.6 Hz, H-19), 5.76 (1H, s, NH), 6.24 (1H, dd,  $J$  = 11.3 Hz, 16.0 Hz, H-13), 6.70 (1H, d,  $J$  = 11.6 Hz, H-20), 6.89 (2H, d,  $J$  = 8.6 Hz, H-26), 7.10 (2H, d,  $J$  = 8.5 Hz, H-25).

<sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>): 14.0 (CH<sub>3</sub>), 17.7 (CH<sub>3</sub>), 20.3 (CH<sub>3</sub>), 24.8 (CH<sub>3</sub>), 39.0 (CH<sub>2</sub>), 40.9 (CH<sub>2</sub>), 43.2 (CH<sub>2</sub>), 48.3 (CH), 50.0 (CH), 55.3 (CH<sub>3</sub>), 59.2 (CH), 70.1 (CH), 76.6 (C), 86.2 (C), 114.3 (CH), 120.4 (CH), 128.6 (C), 129.4 (CH), 125.3 (C), 130.3 (CH), 131.7 (C), 133.5 (CH), 142.5 (CH), 149.0 (C), 158.7 (C), 169.9 (C), 211.5 (C).

MS (EI, 70 eV):  $m/z$  (%) = 548.36 [M+Na<sup>+</sup>] (100).

Crystallographic data of scoparasin A (**3**): White crystal obtained from MeOH-H<sub>2</sub>O (1:1), orthorhombic, space group: P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>,  $a$  = 11.6982(3) Å,  $b$  = 12.8520(5) Å,  $c$  = 17.2917(6) Å,  $V$  = 2599.73 (15) Å<sup>3</sup>,  $Z$  = 4,  $D_c$  = 1.343 g/cm<sup>3</sup>,  $\mu$ (Cu K $\alpha$ ) 0.805 mm<sup>-1</sup>,  $F(000)$  = 1120.0, crystal size 0.45 × 0.2 × 0.2 mm, 26153 reflections collected,

5356 independent reflections ( $R_{\text{int}}$  = 0.0769), final  $R$  0.0417 [ $I > 2\sigma(I)$ ] and  $wR_2$  0.1234 (all data), Flack parameter 0.10 (17).

#### Phenochalasin B (4)

Rf: 0.57 (CHCl<sub>3</sub>-MeOH, 10:1).

<sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): 1.19 (3H, s, Me-22), 1.53 (3H, s, Me-23), 1.13 (3H, d,  $J$  = 7.1 Hz, H-11), 1.26 (3H, s, Me-12), 2.17 (1H, m, Ha-15), 2.31 (1H, m, H-5), 2.68 (1H, m, Hb-15), 2.63 (1H, m, Ha-10), 2.84 (1H, m, Hb-10), 2.66 (1H, m, H-8), 2.96 (1H, m, H-16), 3.69 (1H, brs, H-3), 3.81 (3H, s, Me-28), 2.65 (1H, m, H-7), 3.01 (1H, m, H-4), 5.25 (1H, m, H-14), 5.63 (1H, d,  $J$  = 11.6 Hz, H-19), 6.14 (1H, s, NH), 5.91 (1H, dd,  $J$  = 9.3 Hz, 14.6 Hz, H-13), 6.53 (1H, d,  $J$  = 11.7 Hz, H-20), 6.89 (2H, d,  $J$  = 7.3 Hz, H-26), 7.08 (2H, d,  $J$  = 7.3 Hz, H-25).

<sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>): 13.2 (CH<sub>3</sub>), 19.7 (CH<sub>3</sub>), 20.3 (CH<sub>3</sub>), 24.3 (CH<sub>3</sub>), 35.8 (CH), 39.1 (CH<sub>2</sub>), 44.1 (CH<sub>2</sub>), 45.9 (CH), 60.4 (CH), 48.0 (CH), 53.7 (CH), 55.3 (CH<sub>3</sub>), 57.3 (C), 76.7 (C), 87.0 (C), 114.3 (CH), 120.4 (CH), 127.9 (C), 128.4 (CH), 130.5 (CH), 131.6 (CH), 142.1 (CH), 149.4 (C), 158.9 (C), 170.0 (C), 211.8 (C). MS (EI, 70 eV):  $m/z$  (%) = 548.26 [M+Na<sup>+</sup>] (100).

#### Scoparane C (5)

[ $\alpha$ ]<sub>D</sub><sup>25</sup>: -35.5 (c 0.24, MeOH).

Rf: 0.58 (CHCl<sub>3</sub>-MeOH, 10:1).

IR (KBr): 2924.8, 1699.9 cm<sup>-1</sup>.

UV/Vis  $\lambda_{\max}$  (MeOH) nm: 234, 220, 204.

<sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): 2.70 (1H, dd,  $J$  = 8.2 Hz, Ha-2), 2.42 (1H, dd,  $J$  = 1.7 Hz, 8.2 Hz, Hb-2), 2.51 (1H, dd,  $J$  = 7.3 Hz, Ha-3), 3.02 (1H, dd,  $J$  = 2.0 Hz, 7.3 Hz, Hb-3), 1.81 (2H, m, H-6), 1.80 (1H, m, H-7), 1.77 (2H, m, H-8), 1.63 (2H, m, H-9), 1.27 (3H, s, H-12), 2.07 (3H, s, H-13), 1.08 (3H, s, H-14), 5.04 (1H, m, Ha-15), 5.11 (1H, dd,  $J$  = 0.8 Hz, 2.0 Hz, Hb-15).

<sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>): 20.1 (CH<sub>3</sub>), 21.3 (CH<sub>2</sub>), 26.7 (CH<sub>3</sub>), 26.8 (CH<sub>2</sub>), 28.1 (CH<sub>3</sub>), 30.0 (CH<sub>2</sub>), 30.9 (CH<sub>2</sub>), 36.4 (CH<sub>2</sub>), 42.9 (CH), 51.3 (C), 72.5 (C), 77.9 (C), 110.8 (CH<sub>2</sub>), 148.4 (C), 214.2 (C).

MS (EI, 70 eV):  $m/z$  (%) = 253.18 [M+H<sup>+</sup>] (100).

HRMS-FAB:  $m/z$  [M+H<sup>+</sup>] calcd. for C<sub>15</sub>H<sub>25</sub>O<sub>3</sub><sup>+</sup>: 253.1798; found: 253.1796.

#### Ent-4(15)-eudesmen-11-ol-1-one (6)

Rf: 0.77 (CHCl<sub>3</sub>-MeOH, 1:1).

<sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): 2.71 (1H, m, Ha-2), 2.39 (1H, dd,  $J$  = 2.0 Hz, 6.2 Hz, Hb-2), 2.45 (1H, m, Ha-3), 2.62 (1H, dd,  $J$  = 2.2 Hz, 7.6 Hz, Hb-3), 2.15 (1H, dd,  $J$  = 1.4 Hz, 11.5 Hz, H-5), 1.60 (1H, m, Ha-6), 1.85 (1H, m, Hb-7), 1.33 (1H, m, H-7), 1.24 (1H, m, Ha-8), 1.77 (1H, m, Hb-8), 1.37 (1H, m, Ha-9), 1.84 (1H, m, dd,  $J$  = 2.5 Hz, 5.6 Hz, Hb-9), 1.24 (3H, s, H-12), 1.25 (3H, s, H-13), 1.01 (3H, s, H-14), 4.80 (1H, d,  $J$  = 1.4 Hz, Ha-15), 5.11 (1H, d,  $J$  = 1.3 Hz, Hb-15).

<sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>): 16.6 (CH<sub>3</sub>), 21.9 (CH<sub>2</sub>), 26.9 (CH<sub>3</sub>), 24.4 (CH<sub>2</sub>), 27.5 (CH<sub>3</sub>), 32.1 (CH<sub>2</sub>), 34.6 (CH<sub>2</sub>), 38.1 (CH<sub>2</sub>), 48.4 (CH), 47.8 (CH), 48.3 (C), 72.6 (C), 109.1 (CH<sub>2</sub>), 146.5 (C), 215.1 (C).

MS (EI, 70 eV):  $m/z$  (%) = 237.40 [M+H<sup>+</sup>] (100).

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Concise Synthesis of Taiwaniaquinol B and 5-*epi*-Taiwaniaquinone G

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The natural product taiwaniaquinol B and the unnatural 5-*epi*-taiwaniaquinone G were synthesized based on a highly efficient tandem acylation–Nazarov cyclization approach to build the tricyclic skeleton.

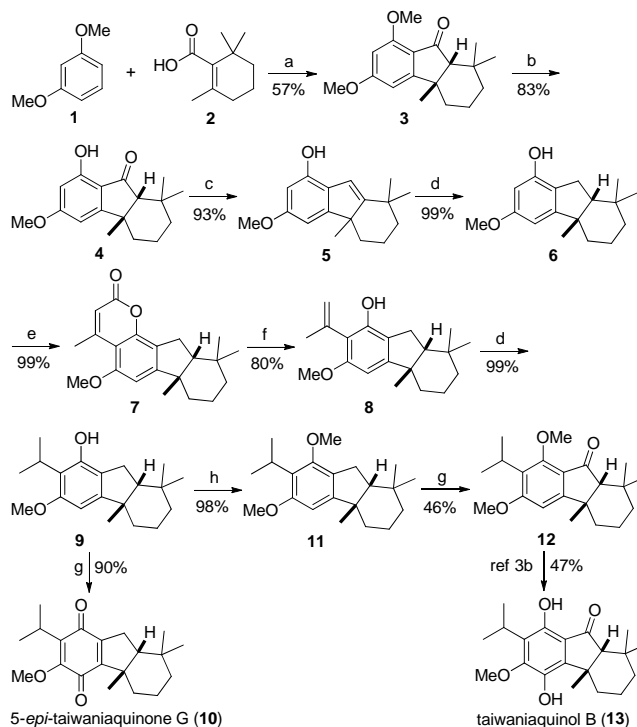
**Keywords:** Taiwaniaquinol B, 5-*Epi*-taiwaniaquinone G, Tandem reaction, Nazarov cyclization.

The taiwaniaquinoids are a family of tricyclic diterpenoids possessing a [6,5,6]-*abeo*-abietane skeleton isolated from *Taiwania cryptomerioides*, *Salvia dichroantha*, *Thuja standishii* and other related plants [1]. The unusual structural features and the promising biological activities [1d,2] of these compounds have attracted great interest for their chemical synthesis [3,4].

The tandem acylation–Nazarov cyclization approach for the construction of fused cyclopentenones by treatment of aromatics with  $\alpha,\beta$ -unsaturated carboxylic acids in the presence of TFAA and a suitable Lewis acid catalyst [5] has been proved highly efficient in natural product synthesis [5b,6]. Herein, we report our synthetic route towards taiwaniaquinol B and the unnatural 5-*epi*-taiwaniaquinone G using this strategy as a key step.

As shown in Scheme 1, treatment of resorcinol dimethyl ether (1) with  $\beta$ -cyclogeranic acid (2) in the presence of TFAA and  $\text{ZnCl}_2$  resulted in the formation of the desired acylation–Nazarov cyclization product 3 in 57% isolated yield [6b]. The relative stereochemistry of 3 was assigned as *cis* on the basis of NOE correlations which was the thermodynamically more stable product. Selective deprotection of the methoxy group adjacent to the carbonyl group in 3 by treatment with  $\text{BBr}_3$  gave 4, which was converted into hexahydrofluorene derivative 6 in excellent yield via hydrogenation of the intermediate tetrahydrofluorene 5. It needs to be indicated that all attempts to transform 4 directly into 6 via Wolff–Kishner or Clemmensen reduction failed to deliver the latter in any respectable yield. Next, the requisite isopropyl group was installed through a three-step sequence including Pechmann condensation of 6 with ethyl acetoacetate (EAA) to provide coumarin derivative 7, hydrolysis and hydrogenation, which led to the formation of 9 in excellent combined yield. Oxidation of 9 with  $\text{CrO}_3$  gave 5-*epi*-taiwaniaquinone G 10, the spectroscopic data of which were identical to those previously reported in the literature [4c]. On the other hand, protection of the phenolic hydroxyl group in 9 gave 11, which on treatment with  $\text{CrO}_3$  was oxidized to tetrahydrofluorenone 12. Finally, compound 12 could be converted into taiwaniaquinol B 13 following known literature procedures through selective demethylation, CAN oxidation, and sodium dithionite reduction [7].

In summary, we have developed a facile route to the synthesis of taiwaniaquinol B and the unnatural 5-*epi*-taiwaniaquinone G. The



**Scheme 1:** Reagents and conditions: (a) TFAA,  $\text{ZnCl}_2$ , toluene, reflux; (b)  $\text{BBr}_3$ , DCM,  $-60 - 0^\circ\text{C}$ ; (c)  $\text{LiAlH}_4$ , THF,  $0^\circ\text{C} - \text{rt}$ , then 6M HCl; (d) 10% Pd/C,  $\text{H}_2$ , MeOH,  $40^\circ\text{C}$ ; (e) MsOH, EAA,  $\text{Al}_2\text{O}_3$ , rt; (f) KOH, DMSO/ $\text{H}_2\text{O}$ , reflux; (g)  $\text{CrO}_3$ , 3,5-dimethylpyrazole, DCM,  $-15 - -10^\circ\text{C}$ ; (h) MeI,  $\text{K}_2\text{CO}_3$ , acetone, reflux.

key steps involve a tandem acylation–Nazarov cyclization reaction to build the tricyclic skeleton and a Pechmann condensation strategy to install the isopropyl group onto the aromatic ring.

## Experimental

**General:** MP, XT4A hot-stage apparatus; IR, IFS25 FT-IR spectrometer; NMR, Agilent 400 MHz spectrometer; MS, Q-TOF micro spectrometer. Flash column chromatography was performed over silica gel 200-300 mesh.

**5,6,7,8,8a,9-Hexahydro-2-isopropyl-3-methoxy-4b,8,8-trimethyl-1H-fluorene-1,4(4bH)-dione (10) [4c]:** A mixture of  $\text{CrO}_3$  (238 mg,

2.38 mmol) and 3,5-dimethylpyrazole (229 mg, 2.38 mmol) in DCM (6 mL) was stirred at -15°C for 10 min. A solution of **9** (40 mg, 0.13 mmol) in DCM (6 mL) was added. The reaction mixture was allowed to warm to -10°C and stirred for a further 1 h before being quenched with H<sub>2</sub>O (10 mL). The separated aqueous phase was extracted with DCM (3 x 10 mL). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, then filtered and evaporated *in vacuo*. The residue was purified by column chromatography (CC) on silica gel (3% ethyl acetate in light petroleum) to give 5-*epi*-taiwaniaquinone G **10** (38 mg, 90%) as a yellow oil.

IR (neat): 1648, 1592, 1460 cm<sup>-1</sup>.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 3.92 (s, 3H), 3.20 (sept, 1H, *J* = 7.0 Hz), 2.65 (dd, 1H, *J* = 18.0, 8.2 Hz), 2.36 (dd, 1H, *J* = 18.0, 11.4 Hz), 1.89 (m, 1H), 1.74 (dd, 1H, *J* = 11.4, 8.2 Hz), 1.52 (s, 3H), 1.46-1.40 (m, 2H), 1.30-1.28 (m, 3H), 1.21 (d, 3H, *J* = 7.0 Hz), 1.19 (d, 3H, *J* = 7.0 Hz), 1.08 (s, 3H), 0.93 (s, 3H).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): 187.5, 182.8, 156.7, 152.6, 146.3, 136.9, 61.2, 55.1, 48.1, 35.1, 34.4, 31.9, 31.2, 31.1, 29.6, 24.6, 24.4, 20.8, 20.7, 18.1.

HRMS-ESI: *m/z* [M + H<sup>+</sup>] calcd for C<sub>20</sub>H<sub>29</sub>O<sub>3</sub>: 317.2117; found 317.2113.

**2,3,4,4a,9,9a-Hexahydro-7-isopropyl-6,8-dimethoxy-1,1,4a-trimethyl-1H-fluorene (11)**: Methyl iodide (68 μL, 1.1 mmol) was added dropwise to a mixture of **9** (58 mg, 0.19 mmol) and K<sub>2</sub>CO<sub>3</sub> (67 mg, 0.48 mmol) in acetone (3 mL). After addition, the mixture was heated at reflux for 3 h, then cooled and evaporated *in vacuo*. The residue was purified by CC on silica gel (3% ethyl acetate in light petroleum) to give **11** (60 mg, 98%) as a colorless solid. MP: 115-116°C.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 6.42 (s, 1H), 3.79 (s, 3H), 3.75 (s, 3H), 3.48 (sept, 1H, *J* = 7.1 Hz), 2.82 (dd, 1H, *J* = 15.1, 7.8 Hz), 2.66 (dd, 1H, *J* = 15.1, 11.1 Hz), 1.83 (dd, 1H, *J* = 11.1, 7.8 Hz), 1.66-1.55 (m, 2H), 1.44-1.36 (m, 2H), 1.41 (s, 3H), 1.30 (d, 3H, *J* =

7.1 Hz), 1.29 (d, 3H, *J* = 7.1 Hz), 1.27-1.19 (m, 2H), 1.11 (s, 3H), 0.95 (s, 3H).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): 158.4, 155.0, 154.1, 126.4, 124.7, 101.1, 60.6, 57.6, 55.9, 45.6, 36.4, 35.3, 32.3, 31.2, 30.8, 29.6, 25.6, 25.1, 21.6, 21.5, 19.1.

HRMS-ESI: *m/z* [M + H<sup>+</sup>] calcd for C<sub>21</sub>H<sub>33</sub>O<sub>2</sub>: 317.2481; found 317.2459.

**2,3,4,4a-Tetrahydro-7-isopropyl-6,8-dimethoxy-1,1,4a-trimethyl-1H-fluorene-9(9aH)-one (12)** [7]: A mixture of CrO<sub>3</sub> (326 mg, 3.27 mmol) and 3,5-dimethylpyrazole (317 mg, 3.27 mmol) in DCM (7 mL) was stirred at -15°C for 10 min. A solution of **11** (58 mg, 0.18 mmol) in DCM (7 mL) was added. The reaction mixture was allowed to warm to -10°C and stirred for 10 h before being quenched with H<sub>2</sub>O (15 mL). The separated aqueous phase was extracted with DCM (3 x 10 mL). The combined organic extracts were dried over Na<sub>2</sub>SO<sub>4</sub>, then filtered and evaporated *in vacuo*. The residue was purified by preparative TLC (3% ethyl acetate in light petroleum) to give **12** (21 mg, 46%, 71% based on recovered starting material) as a yellow oil, together with 20 mg of unreacted starting material recovered.

MP: 136-137°C.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 6.57 (s, 1H), 3.91 (s, 3H), 3.90 (s, 3H), 3.57 (sept, 1H, *J* = 7.1 Hz), 2.11 (s, 1H), 2.07 (m, 1H), 1.65-1.56 (m, 2H), 1.51-1.32 (m, 3H), 1.28 (d, 6H, *J* = 7.1 Hz), 1.25 (s, 3H), 1.23 (s, 3H), 0.70 (s, 3H).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): 204.5, 164.8, 164.0, 157.1, 128.1, 121.7, 99.8, 65.7, 62.1, 55.8, 41.4, 38.6, 34.5, 34.0, 33.4, 32.6, 24.7, 24.5, 21.2, 18.4.

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## Effect of Enzyme Inhibitors on Terpene Trilactones Biosynthesis and Gene Expression Profiling in *Ginkgo biloba* Cultured Cells

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The biosynthetic pathway of terpene trilactones of *Ginkgo biloba* is unclear. In this present study, suspension cultured cells of *G. biloba* were used to explore the regulation of the mevalonic acid (MVA) and methylerythritol 4-phosphate (MEP) pathways in response to specific enzyme inhibitors (lovastatin and clomazone). The results showed that the biosynthesis of bilobalide was more highly correlated with the MVA pathway, and the biosynthesis of ginkgolides was more highly correlated with the MEP pathway. Meanwhile, according to the results, it could be speculated that bilobalide might be a product of ginkgolide metabolism.

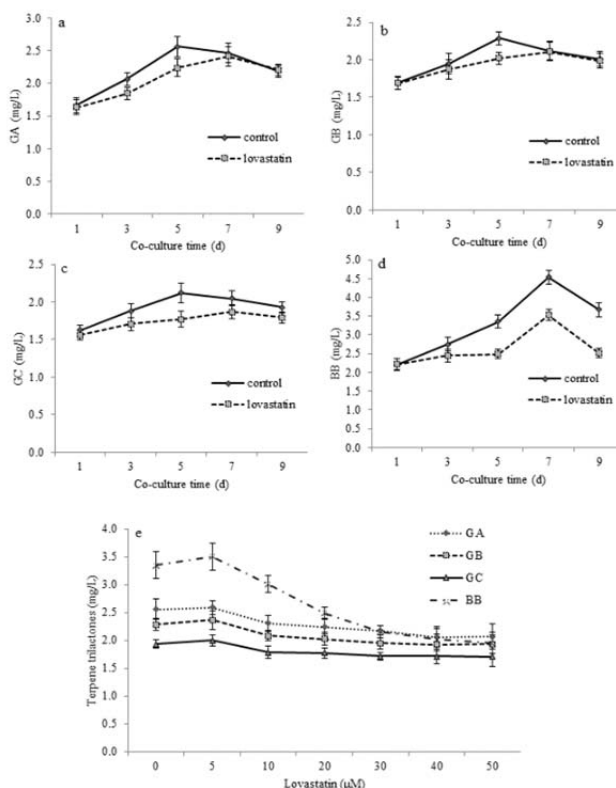
**Keywords:** *Ginkgo biloba*, Terpene trilactones, Biosynthesis, Enzyme inhibitors, Gene expression.

*Ginkgo biloba* L., a traditional herb in China, is one of the oldest living plants [1]. The leaf extract of ginkgo has been employed for treating cerebrovascular and cardiovascular diseases for centuries [2]. Modern pharmacological studies showed that *G. biloba* extract could be also used for the treatment of chronic schizophrenia and Alzheimer's disease, as well as possessing antitumor activity [3-5]. Terpene trilactones, which include ginkgolides and bilobalide, are surmised to be responsible for most of the pharmacological properties of *G. biloba* extracts.

Ginkgolides are commonly supposed to be acquired via the methylerythritol 4-phosphate (MEP) pathway [6], but several experiments indicated that mevalonic acid (MVA) pathway is dominant in undifferentiated *G. biloba* cells [7]. On the other hand, bilobalide (BB) is deemed to be synthesised through the MVA pathway [8], but some authors suggest that BB is a product of ginkgolides metabolism [9]. It has been reported that 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) are the rate-limiting enzymes for the MEP pathway. Similarly, HMG-CoA reductase (HMGR) catalyzes the major rate determining steps of the MVA pathway [10].

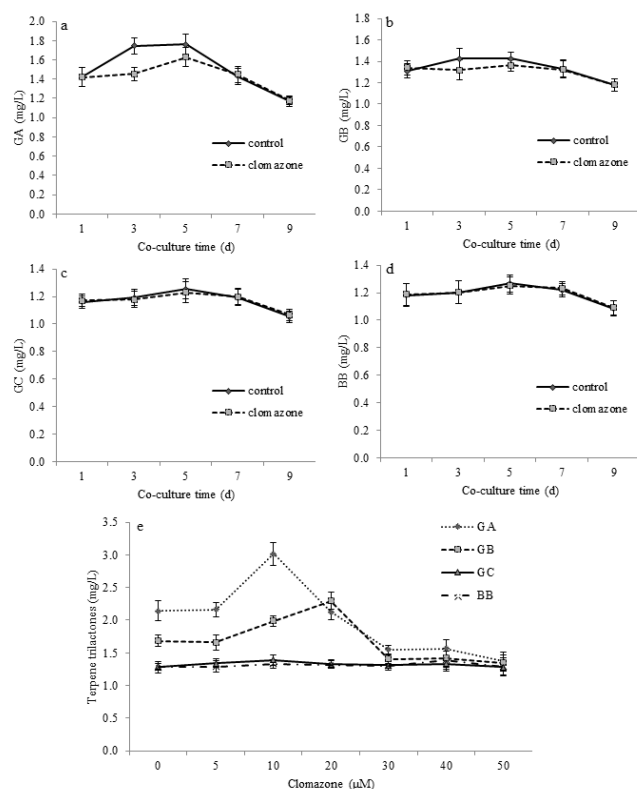
Inhibitors of key enzymes involved in the biosynthesis pathway have been used as an additional tool to study the regulation of secondary metabolite production in plants. Lovastatin (mevinolin) can induce a specific inhibition of HMGR in the MVA pathway [11]. Clomazone is a soil-applied herbicide, and is the precursor of 5-keto clomazone, an inhibitor of DXS in the MEP pathway [12]. In the present study, we investigated the yields of terpene trilactones and the expression of key biosynthetic genes in *G. biloba* cell cultures by feeding lovastatin and clomazone to gain a better understanding of the regulation of terpene trilactones biosynthesis.

Compared with control groups, the yields of ginkgolide A (GA), ginkgolide (GB) and ginkgolide (GC) in 20  $\mu$ M lovastatin-treated groups had a reduction of 12.6%, 11.4% and 16.4% at the 5<sup>th</sup> day, respectively, and that of BB had a reduction of 31.7% at the 9<sup>th</sup> day.



**Figure 1:** Effect of 20  $\mu$ M lovastatin on GA (a), GB (b), GC (c), and BB (d) production in *G. biloba* suspension cultured cells, and effect of lovastatin on terpene trilactones (e) production in *G. biloba* suspension cultured cells harvested at day 5 after treatment. Lovastatin was supplied to 14-day-old *G. biloba* suspension cultured cells.

Interestingly, the yields of GA, GB and GC in lovastatin-treated groups did not have an apparent reduction on the 9<sup>th</sup> day compared with those of control groups (Figure 1). In 50  $\mu$ M lovastatin-treated group, the yields of GA, GB, GC and BB had a reduction of 19.4%,



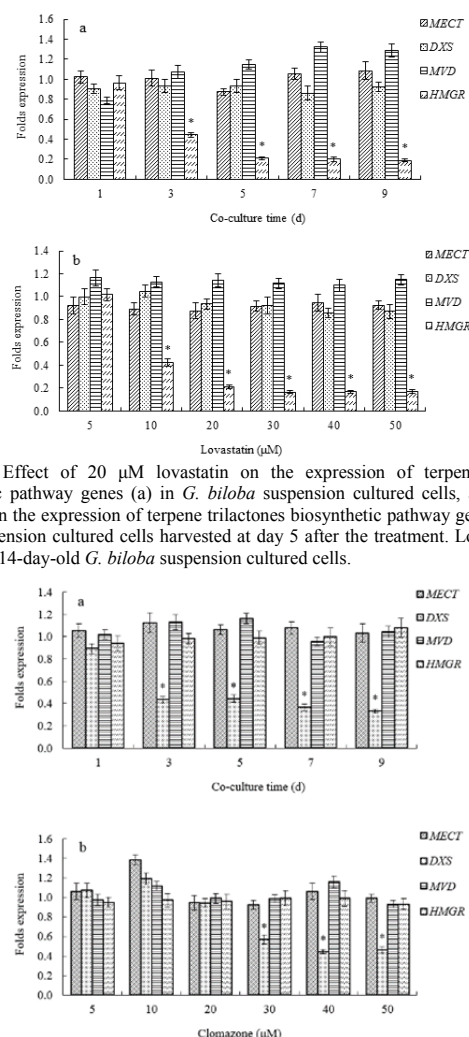
**Figure 2:** Effect of 40  $\mu\text{M}$  clomazone on GA (a), GB (b), GC (c), and BB (d) production in *G. biloba* suspension cultured cells, and effect of clomazone on terpene trilactones (e) production in *G. biloba* suspension cultured cells harvested at day 5 after the treatment. Clomazone was supplied to 14-day-old *G. biloba* suspension cultured cells.

15.6%, 11.7% and 41.9% compared with the control group, respectively (Figure 1). These results indicated that BB was mainly synthesized from the MVA pathway, and parts of ginkgolides might be transformed into BB.

Compared with control groups, the yields of GA and GB in the 40  $\mu\text{M}$  clomazone-treated groups had a reduction of 16.8% and 7.5% at the 3<sup>rd</sup> day, and that of GC and BB of 1.9% and 1.3% at the 5<sup>th</sup> day, respectively (Figure 2). In the 50  $\mu\text{M}$  clomazone-treated group, the yields of GA and GB had a reduction of 36.3% and 20.0% (1.37 and 1.35 mg/L), while the yields of GC and BB were basically unchanged comparing with the control group (Figure 2). These results demonstrated that GA and GB were mainly obtained from the MEP pathway, and further indicated that the MVA pathway was the predominant one for BB.

After treatment with lovastatin (20  $\mu\text{M}$ ), the mRNA level of *HMGR* decreased significantly, and the transcript level of *DXS* decreased mildly. Conversely, the transcript level of *MECT* and *MVD* increased slightly. The trend in gene expressions was similar for the groups treated with different concentration of lovastatin (Figure 3). After treatment with 40  $\mu\text{M}$  of clomazone, the mRNA level of *MECT* and *MVD* increased moderately. On the other hand, the *DXS* transcript level decreased slightly at the 1<sup>st</sup> day and then decreased rapidly with the increasing culture time, and *HMGR* remained stable. For the groups treated with different concentrations of clomazone, the results were similar (Figure 4). The qRT-PCR results of gene expression were consistent with the change in terpene trilactone concentrations in *G. biloba* cultured cells.

The present study provided a first attempt towards a better understanding of the regulation of the biosynthesis of terpene



**Figure 3:** Effect of 20  $\mu\text{M}$  lovastatin on the expression of terpene trilactones biosynthetic pathway genes (a) in *G. biloba* suspension cultured cells, and effect of lovastatin on the expression of terpene trilactones biosynthetic pathway genes (b) in *G. biloba* suspension cultured cells harvested at day 5 after the treatment. Lovastatin was supplied to 14-day-old *G. biloba* suspension cultured cells.

**Figure 4:** Effect of 40  $\mu\text{M}$  clomazone on the expression of terpene trilactones biosynthetic pathway genes (a) in *G. biloba* suspension cultured cells, and effect of clomazone on the expression of terpene trilactones biosynthetic pathway genes (b) in *G. biloba* suspension cultured cells harvested at day 5 after the treatment. Clomazone was supplied to 14-day-old *G. biloba* suspension cultured cells.

trilactones in *G. biloba* cultured cells. The *in vivo* feeding experiments with two specific inhibitors, lovastatin and clomazone, showed that the biosynthesis of BB was more highly correlated with the MVA pathway, while the biosynthesis of ginkgolides was more highly correlated with the MEP pathway. Meanwhile, it could be speculated that BB might be a product of ginkgolides metabolism according to the results. This proposal requires further research by incorporation of labeled precursors and analyses of transgenic lines and mutants.

## Experimental

**Plant materials and methods:** The cultured cells of *G. biloba* used in this research were established by our research group. Prior to the experiments, fresh 20-day-old cells (5.0 g) were transferred into 250 mL Erlenmeyer flasks and suspended in 100 mL of MS medium [13] supplemented with 30 g/L sucrose, 2.0 mg/L  $\alpha$ -naphthalene acetic acid (NAA), 2.0 mg/L indolebutyric acid (IBA), and 5.0 mg/L ascorbic acid without agar, maintained in a rotary shaker (110 rpm) at 25°C in darkness.

**Inhibitors treatment:** After being filter-sterilized, the solutions of lovastatin and clomazone [14] were supplied individually to 14-day-

old suspension cultures to give final concentrations of 5, 10, 20, 30, 40 and 50  $\mu\text{M}$ , respectively. Experiments for control group were made with corresponding blank solutions. 1, 3, 5, 7 and 9 days after the addition of the inhibitors, the cultured cells were harvested. The cultures were separated from liquid media by vacuum filtration, washed with distilled water, and dried at 50°C. The spent culture medium was used for analyzing terpene trilactones production. Elicitation experiments were made in triplicate.

**Terpene trilactones extraction and determination:** The cultured cells were separated from the culture medium by vacuum filtration and the spent medium was partitioned into ethyl acetate (v/v). The ethyl acetate extracts were combined and concentrated to dryness. The residue was dissolved in 1.0 mL of methanol and then analyzed by HPLC-ELSD. Dry cultured cells were extracted sonically with methanol 3 times. After concentration of the combined extract solutions under vacuum, the residue was dissolved in a mixture of water and ethyl acetate (1:1, v/v), and partitioning with ethyl acetate (v/v) 3 times. The ethyl acetate extracts were combined and concentrated to dryness. The residue was dissolved in 1.0 mL of methanol and then analyzed by HPLC-ELSD. For determination of ginkgolides and bilobalide, HPLC analysis was performed using a 1260 HPLC instrument (Agilent Technologies, US) equipped with an evaporative light-scattering detector (ELSD, Alltech Technologies, US). Chromatographic separations were performed

on an Agilent Hypersil C18 column (5  $\mu\text{m}$ , 250  $\times$  4.0 mm) (Agilent Technologies, US) at 25°C with gradient elution consisting of methanol and water.

**Monitoring gene expression by qPCR:** The extraction of total RNA from cultured cells was performed using Trizol according to reference [15]. In the present study, transcript levels of GAPDH, the genes involved in the terpene trilactones biosynthesis pathway (HMGR, MVD, DXS and MECT), were monitored in cultured cells of *G. biloba* treated with either lovastatin or clomazone. The qPCR reference manuals of PrimeScript™ reagent kit and SYBR® Premix Ex Taq™ were used.

**Statistical analysis:** All values are mean  $\pm$  SD. Statistical analyses were performed using an unpaired, two-tailed Student's t-test. All comparisons were made relative to untreated controls and significant difference is indicated as \*  $p < 0.05$ .

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Macrocyclic Diterpenoids from the Latex of *Euphorbia helioscopia*Juan Hua<sup>a,b</sup>, Yan-Chun Liu<sup>a</sup>, Shu-Xi Jing<sup>a</sup>, Shi-Hong Luo<sup>a,\*</sup> and Sheng-Hong Li<sup>a,\*</sup><sup>a</sup>State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China<sup>b</sup>University of Chinese Academy of Sciences, Beijing 100049, China

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One new jatrophane diterpenoid, 7 $\alpha$ ,9 $\beta$ ,15 $\beta$ -triacetox-3 $\beta$ -benzoyloxy-14 $\beta$ -hydroxyjatropha-5*E*,11*E*-diene (**3**), together with four known macrocyclic diterpenoids, euphoheliosnoid A (**1**), epieuphoscopin B (**2**), euphohelioscopin A (**4**) and euphoscopin C (**5**), were isolated from the stem latex of *Euphorbia helioscopia*. Their structures were established by spectroscopic analyses. In the anti-inflammatory assay, euphohelioscopin A (**4**) exhibited moderate inhibitory activity on the release of cytokine TNF- $\alpha$  ( $IC_{50} = 23.7 \pm 1.7 \mu M$ ), IL-6 ( $IC_{50} = 46.1 \pm 1.1 \mu M$ ) and chemokine MCP-1 ( $IC_{50} = 33.7 \pm 3.8 \mu M$ ) in lipopolysaccharide (LPS) induced RAW 264.7 macrophages without notable cytotoxicity ( $IC_{50} > 80 \mu M$ ).

**Keywords:** *Euphorbia helioscopia*, Latex, Jatrophane diterpenoid, Lathyrane diterpenoid, Anti-inflammatory activity.

Plant latex is a sticky emulsion stored in laticifers and usually released immediately from damaged tissue in over 20,000 species from about 40 families of flowering plants [1]. Besides various proteins, plant latex contains a wide range of secondary metabolites, including alkaloids, terpenoids, cardenolides and phenolics [1]. These metabolites have been widely accepted to play a significant defensive role against herbivores and pathogens [1, 2], and also possess significant pharmacological activities, such as anticancer, antiviral, and anti-inflammatory effects [3, 4].

*Euphorbia* is the largest genus in the family Euphorbiaceae that has been extensively distributed worldwide. All species of *Euphorbia* are characteristic for the production of a white latex that is rich in secondary metabolites especially terpenoids responsible for diverse biological activities [5, 6]. For example, tirucallol, a tetracyclic triterpenoid isolated from the latex of *E. lactea*, was reported to show anti-inflammatory activity *in vivo* [7]. Diterpenoid esters that were isolated from the latex of *E. poissonii* exhibited strong and selective cytotoxic effect against human kidney carcinoma (A-498) cell line [8].

*E. helioscopia* L., a biennial herb with milky latex widely distributed in most parts of China, has been used as a traditional Chinese medicine to treat malaria, osteomyelitis, and bacillary dysentery [9, 10]. Previous phytochemical investigations of *E. helioscopia*, mainly focused on the whole plant, have uncovered diverse secondary metabolites, including phenols, triterpenoids and a series of structurally interesting and potentially bioactive macrocyclic diterpenoids [11-15]. However, detailed investigation of the secondary metabolites in the latex is still lacking.

To characterize the lactic metabolites, the stem latex of *E. helioscopia* was suspended in methanol to remove the macromolecular proteins and polysaccharides, and was then analyzed by reversed-phase HPLC equipped with a diode array detector and recorded at 238 nm. Five major peaks with retention times of 6.90, 7.59, 7.99, 10.38 and 12.58 min, respectively, were detected in the chromatogram (Figure 1). Subsequently, using column chromatography and reversed-phase semi-preparative HPLC, the five compounds were isolated and identified as a new (**3**)

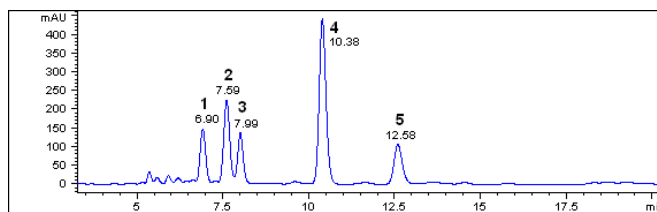


Figure 1: HPLC analysis of the secondary metabolites in stem latex of *Euphorbia helioscopia* (238 nm).

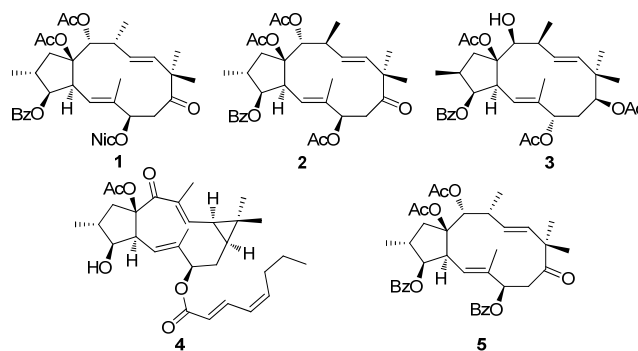


Figure 2: Chemical structures of compounds 1-5.

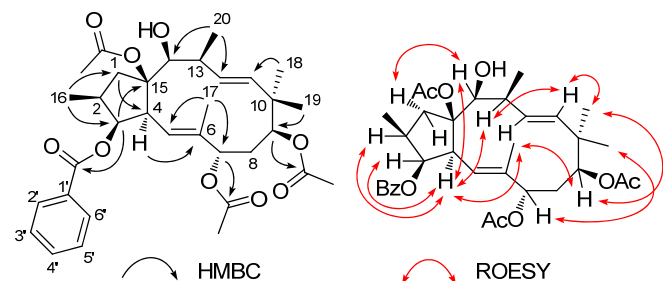
and three known jatrophane diterpenoids (**1**, **2**, and **5**), and a known lathyrane diterpenoid (**4**) (Figure 2).

Compound **3** was obtained as a white solid. Its molecular formula was  $C_{33}H_{44}O_9$  according to the  $^{13}C$  NMR spectroscopic and HREIMS data ( $[M]^+$ , found:  $m/z$  584.2977; calcd. for 584.2985). The  $^1H$  NMR spectrum showed the presence of eight methyl signals [ $\delta_H$  0.89, 0.91, 0.96, 1.05, 1.09, 1.76, 1.94, and 2.38 (each 3H)], and five aromatic protons [ $\delta_H$  8.05 (2H), 7.63 (1H) and 7.54 (2H)]. The  $^{13}C$  NMR spectrum of **3** exhibited 33 carbon resonances. The carbonyl resonances ( $\delta_C$  169.1, 169.7, 174.6 and 165.6) together with the aforementioned proton signals revealed the presence of three acetoxyl groups and a benzoyloxy group. Apart from the above 13 signals responsible for substituents, 20 carbons including five



**Table 1:** NMR spectral data of **3** in acetone-*d*<sub>6</sub><sup>a</sup>

Position	$\delta_{\text{H}}$ ( $J$ [Hz])	$\delta_{\text{C}}$	HMBC
1 $\alpha$	2.86 (1H, dd, 13.4, 6.8)	45.1, t	C-2, 3, 4, 15, 16
1 $\beta$	2.19 (1H, t, 13.4)		
2	2.29 (1H, m)	37.9, d	C-1
3	5.39 (1H, t, 3.7)	81.3, d	C-1, 15, -C=O (3-OBz)
4	3.32 (1H, dd, 4.5, 10.2)	49.0, d	C-3, 5, 6
5	5.76 (1H, d, 10.2)	120.7, d	C-6, 7, 15, 17
6	-	133.8, s	-
7	4.89 (1H, d, 7.0)	73.8, d	C-5, 6, 8, 9, 17, -C=O (7-OAc)
8 $\alpha$	2.11 (1H, dd, 3.3, 16.0)	33.1, t	C-7, 9, 10
8 $\beta$	1.83 (1H, ddd, 3.3, 7.0, 16.0)		
9	4.91 (1H, t, 3.2)	74.6, d	C-10, 18, -C=O (9-OAc)
10	-	40.1, s	-
11	5.09 (1H, d, 15.7)	136.1 d	C-9, 10, 18, 19
12	5.52 (1H, dd, 9.0, 15.7)	130.5, d	C-13, 14
13	2.54 (1H, m)	40.4, d	C-11, 12, 20
14	3.58 (1H, d, 5.6)	81.2, d	C-1, 12, 13, 15, 20
15	-	95.8, s	-
16	0.96 (3H, d, 6.6)	13.7, q	C-1, 2, 3
17	1.76 (3H, s)	15.9, q	C-4, 5, 6, 7
18	0.89 (3H, s)	20.4, q	C-9, 10, 11, 19
19	0.91 (3H, s)	22.9, q	C-9, 10, 11, 18
20	1.05 (3H, d, 7.0)	21.0, q	C-12, 13, 14
3-OBz C=O	-	165.6, s	-
1'	-	131.2, s	-
2'/6'	8.05 (2H, m)	130.3, d	C-2'/6', 4', -C=O (3-OBz)
3'/5'	7.54 (2H, t, 7.6)	129.4, d	C-1', 3'/5'
4'	7.63 (1H, t, 7.4)	133.8, d	C-2'/6'
7-OAc	1.09 (3H, s)	19.9, q	-
		169.1, s	
9-OAc	1.94 (3H, s)	21.1, q	-
		169.7, s	
15-OAc	2.38 (3H, s)	22.4, q	-
		174.6, s	
14-OH	4.86 (1H, d, 4.2)	-	-

<sup>a</sup> <sup>1</sup>H NMR was recorded at 600 MHz; <sup>13</sup>C NMR was recorded at 150 MHz.**Figure 3:** Key HMBC (black arrows, left), and ROESY (red arrows, right) correlations for the structure elucidation of **3**.

methyls, two methylenes, ten methines and three quaternary carbons were also observed in the <sup>13</sup>C NMR spectrum, suggesting that **3** might be a jatrophone diterpenoid [9, 11]. The jatrophone skeleton was further confirmed by 2D NMR experiments, in which a disubstituted double bond ascribable to C-11 and C-12, and a trisubstituted double bond attributable to C-5 and C-6 were observed (Figure 3). The simultaneous HMBC correlations of the proton signals at  $\delta_{\text{H}}$  5.39 (H-3) and 8.05 (2H, H-2' and H-6') with the carbonyl carbon signal at  $\delta_{\text{C}}$  165.6 disclosed that the benzoyloxy group was attached to C-3. Similarly, the <sup>1</sup>H-<sup>13</sup>C long-range couplings from the proton signals at  $\delta_{\text{H}}$  4.89 (H-7) and 4.91 (H-9) with the carbonyl carbon signals at  $\delta_{\text{C}}$  169.1 and 169.7 demonstrated the presence of two acetoxy groups at C-7 and C-9, respectively. The last acetoxy group could be assigned to C-15 because no HMBC correlations were observed between the carbonyl carbon ( $\delta_{\text{C}}$  174.6) and any protons from the jatrophone skeleton, and further supported by the dramatically downfield

shifted C-15 ( $\delta_{\text{C}}$  95.8) compared with the chemical shift of the same carbon of similar jatrophone diterpenoids [9]. The <sup>1</sup>H-<sup>1</sup>H COSY spectrum showed clear correlation between the proton at  $\delta_{\text{H}}$  3.58 (H-14) and the hydroxy resonance at  $\delta_{\text{H}}$  4.86, thus the hydroxy group was assignable to C-14.

The relative stereochemistry of **3** was established by analysis of the coupling constant pattern and ROESY spectrum. For the reported jatrophone diterpenoids, the angular proton H-4 is exclusively  $\alpha$ -oriented and the C-15 acetoxy group  $\beta$ -oriented [5, 6]. The ROESY cross-peaks of H-4/H-3, H-4/H-13, H-4/H-14, H-1 $\alpha$ /H-2, H-1 $\alpha$ /H-14, and of Me-18/H-9 indicated  $\alpha$ -orientation of these protons. The ROESY cross-peak of Me-19/H-7 revealed their  $\beta$ -orientation. Therefore, the structure of **3** was elucidated as 7 $\alpha$ ,9 $\beta$ ,15 $\beta$ -triaceoxy-3 $\beta$ -benzoyloxy-14 $\beta$ -hydroxyjatropha-5*E*,11*E*-diene.

The four known compounds were identified as euphohelioscoid A (**1**) [13], epiuphoscopin B (**2**) [14, 15], euphohelioscopin A (**4**) [15] and euphoscopin C (**5**) [14] by comparison of their <sup>1</sup>H and <sup>13</sup>C NMR data with those previously reported in the literatures.

The anti-inflammatory activity of euphohelioscopin A (**4**) was assayed as described previously [16]. The compound showed moderate inhibitory activity on the production of the pro-inflammatory cytokines TNF- $\alpha$  ( $\text{IC}_{50} = 23.7 \pm 1.7 \mu\text{M}$ ), IL-6 ( $\text{IC}_{50} = 46.1 \pm 1.1 \mu\text{M}$ ) and chemokine MCP-1 ( $\text{IC}_{50} = 33.7 \pm 3.8 \mu\text{M}$ ) in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages, without notable cytotoxicity ( $\text{IC}_{50} > 80 \mu\text{M}$ ).

## Experimental

**General:** Column chromatography (CC) was performed on 200-300 mesh silica gel (Qingdao Marine Chemical Factory, China), Sephadex LH-20 (Amersham Pharmacia Biotech, Sweden) and MCI gel CHP-20P (75-150  $\mu\text{m}$ , Mitsubishi Chemical Corp., Tokyo, Japan). Optical rotations were obtained on a Horiba-SEAP-300 spectropolarimeter, UV spectra were measured on a Shimadzu-210A double-beam spectrophotometer, and IR spectra in KBr discs were recorded on a Bruker-Tensor-27 spectrometer. NMR spectra were carried out on a Bruker Avance III 600 spectrometer (Bruker, Karlsruhe, Germany) with TMS as internal standard. MS were recorded on a VG-Auto-Spec-3000 spectrometer. Centrifugation was performed with an Eppendorf 5810R (Eppendorf, Hamburg, Germany). HPLC analysis was performed on an Agilent 1200 (Agilent, USA) series instrument equipped with a quaternary pump, a vacuum degasser, an autosampler, a thermostated column compartment and a diode array detector. Semi-preparative HPLC was also performed on the same system with a Zorbax SB-C<sub>18</sub>, 9.4  $\times$  250 mm column at 30°C. TLC spots were visualized under UV light and by dipping into 5% H<sub>2</sub>SO<sub>4</sub> in ethanol followed by heating.

**Plant material:** Latex of *E. helioscopia* was collected from plants growing in Kunming Botanical Garden in February 2014.

**Metabolite analysis of latex by HPLC:** One hundred  $\mu\text{L}$  stem latex of *E. helioscopia* was suspended in 200  $\mu\text{L}$  methanol in an ultrasonic bath for 10 min and then centrifuged at 12,000 rpm for 5 min. After centrifugation, the supernatant was analyzed by HPLC. At a flow rate of 1 mL/min, 10  $\mu\text{L}$  of the sample was injected into a Zorbax SB-C<sub>18</sub> column (5  $\mu\text{m}$ , 4.6  $\times$  250 mm), and the column temperature was maintained at 30°C. A mobile phase composed of (A) water and (B) acetonitrile was used (0-20 min: isocratic 80% B, 20-25 min: linear gradient of 80-95% of B). The eluent was monitored at 200-400 nm.



**Extraction and isolation:** Ten mL stem latex was suspended in 50 mL methanol and centrifuged as described above. The supernatant was concentrated *in vacuo* to remove the organic solvent, and was then partitioned with EtOAc. The EtOAc fraction (205 mg) was chromatographed on a silica gel column, eluting successively with light petroleum/ethyl acetate (10: 0, 9: 1, 4: 1, 1: 1) and acetone to give 5 fractions (A-E). Fraction B was further subjected to MCI gel CC eluting with methanol/water (60-100%) to give 4 sub-fractions (2a-2d). Sub-fraction 2c (77 mg) (methanol/water, 80%) was purified by reversed-phase semi-preparative HPLC using 75% acetonitrile in water as eluent (flow rate: 3 mL/min; column: Zorbax SB-C<sub>18</sub>, 5  $\mu$ m, 9.4  $\times$  250 mm; detection: UV 238 nm; retention times: *t* = 8.1, 9.4, 10.8, 12.1, 13.6, and 16.3 min) to yield **1** (3 mg), **2** (5 mg), **3** (4 mg), **4** (13 mg) and **5** (8 mg), respectively.

**7 $\alpha$ ,9 $\beta$ ,15 $\beta$ -Triacetox-3 $\beta$ -benzoyloxy-14 $\beta$ -hydroxyjatroph-5E,11E-diene (3)**

White solid.

$[\alpha]_D^{20}$ : -15.6 (*c* 0.06, MeOH).

UV  $\lambda_{max}$  (MeOH) nm (log  $\epsilon$ ): 201 (2.57), 228 (2.07), 271 (0.15).

IR (KBr)  $\nu_{max}$ : 3423, 2926, 1738, 1628, 1246, 1026, 713  $cm^{-1}$ .

<sup>1</sup>H NMR (600 MHz, acetone-*d*<sub>6</sub>) and <sup>13</sup>C NMR (150 MHz, acetone-*d*<sub>6</sub>): Table 1.

HR-EI-MS *m/z*: 584.2977 [*M*]<sup>+</sup> (calcd for C<sub>33</sub>H<sub>44</sub>O<sub>9</sub>, 584.2985).

**Anti-inflammatory assay:** The murine macrophage RAW 264.7 cell line was cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum in a 37°C, 5% CO<sub>2</sub> incubator. Before anti-inflammatory assay, test compounds were assessed for their cytotoxicity against the RAW 264.7 cell line and were found to be non-toxic at the tested concentrations (80, 40, 20, 10, 5, and 0  $\mu$ M). Anti-inflammatory activity was assessed by enzyme-linked immunosorbent assay (ELISA) using commercial tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and monocyte chemoattractant protein 1 (MCP-1) detecting kits (BD Biosciences, Mountain View, CA, USA), as previously described [16].

**Supplementary data:** <sup>1</sup>H and <sup>13</sup>C NMR, HSQC, HMBC, ROESY and <sup>1</sup>H-<sup>1</sup>H COSY spectra of the new compound **3**.

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A New Triterpenoid from the Aerial Parts of *Agrimonia pilosa*Jiang-Hao Ma<sup>a,b,†</sup>, Qing-Hua Jiang<sup>c,†</sup>, Ying Chen<sup>a,b</sup>, Xiu-Fang Nie<sup>b</sup>, Tie Yao<sup>b</sup>, Li-Qin Ding<sup>a</sup>, Feng Zhao<sup>d</sup>, Li-Xia Chen<sup>b,\*</sup> and Qiu Feng<sup>a,\*</sup><sup>a</sup>School of Chinese Materia Medica and Tianjin State Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China<sup>b</sup>Department of Natural Products Chemistry, School of Traditional Chinese Materia Medica, Key Laboratory of Structure-Based Drug Design & Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, China<sup>c</sup>Department of Pharmacy, Shengjing Hospital of China Medical University, Shenyang 110004, China<sup>d</sup>School of Pharmacy, Yantai University, Yantai 264005, China

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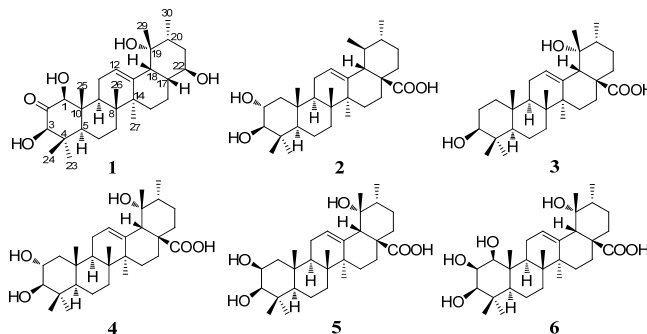
(1S,3R,17R,18R,19R,20R,22R)-1,3,19,22-tetrahydroxy-28-norurs-12-en-2-one (**1**), along with 5 known triterpenoids (**2-6**), were isolated from the aerial parts of *Agrimonia pilosa* Ledeb. Their structures were established based on extensive spectroscopic and MS analysis. The absolute configuration of compound **1** was deduced by circular dichroism (CD). Compound **1** was the first example of a 28-norursene backbone isolated from the genus *Agrimonia*. Compounds **2-6** were tested for anti-inflammatory activities against RAW 264.7 macrophages. A plausible biosynthetic pathway for compound **1** in *A. pilosa* was also proposed.

**Keywords:** *Agrimonia pilosa*, Ursane 28-nortriterpene, Anti-inflammatory, Biogenetic pathway.

*Agrimonia*, family Rosaceae, includes about a dozen species, which are perennial herbaceous flowering plants, mainly distributed in the temperate regions of the Northern Hemisphere. Some species have been used in traditional medicine. *A. pilosa* Ledeb, common name Herba Agrimoniae, (*Xianhecao* in Chinese), is a traditional Chinese medicine (TCM), and is derived from the dried aerial parts of *A. pilosa* according to the Chinese Pharmacopoeia [1]. It is used for the treatment of abdominal pain, sore throat, headaches, bloody and mucoid dysentery and heat stroke. Pharmacological studies on the extracts prepared from the aerial parts of *A. pilosa* demonstrated broad biological properties, such as anti-inflammatory [2-3], anti-adipogenic [4], antioxidant [5] and antiviral [6] effects. Chemical studies on the aerial parts of *A. pilosa* showed the presence of flavonoids [7-9], 3,4-dihydroisocoumarins [2], and triterpenoids [10-11].

Recently, our research group has been examining the constituents of *A. pilosa* and has hitherto reported the isolation and identification of several flavonoids [12] and phenolic constituents [13]. Our continuous interest in bioactive constituents of this plant thus prompted us to investigate the other major chemical group in this plant - triterpenoids, and their inhibitory activities on NO and interleukin-6 (IL-6) production in RAW 264.7 macrophages induced by lipopolysaccharide (LPS). In this paper, we describe the isolation and structural elucidation of one new triterpenoid (**1**), together with five known ones (**2-6**) (Figure 1), and their *in vitro* anti-inflammatory evaluation on inhibitory activities in RAW 264.7 macrophages. The biogenetic pathway for compound **1** is also proposed (Scheme 1).

Compound **1** was obtained as a white amorphous powder. Its molecular formula was determined to be C<sub>29</sub>H<sub>46</sub>O<sub>5</sub>, with seven degrees of unsaturation according to the positive-ion ESI-MS data

**Figure 1:** Structures of compounds **1-6** isolated from *Agrimonia pilosa*

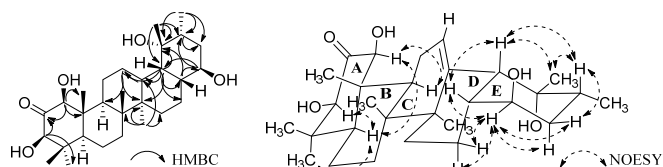
at  $m/z$  497.4  $[M+Na]^+$  and 473.2  $[M-H]^-$ , confirmed unambiguously from the pseudo-molecular ion peak at  $m/z$  519.3340  $[M+HCOO]^-$  obtained by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). The <sup>1</sup>H NMR spectrum (Table 1) of **1**, displayed the characteristic signals of six tertiary methyl groups at  $\delta_H$  1.21 (s, H-23), 0.69 (s, H-24), 0.80 (s, H-25), 0.86 (s, H-26), 1.31 (s, H-27) and 1.19 (s, H-29), one secondary methyl group at  $\delta_H$  0.92 (d,  $J = 5.8$  Hz, H-30), three oxymethine protons at  $\delta_H$  4.08 (s, H-1), 3.99 (d,  $J = 1.4$  Hz, H-3) and 3.84 (like-dd,  $J = 2.0$  Hz, H-22), and one tri-substituted olefinic proton at  $\delta_H$  5.35 (t,  $J = 3.6$  Hz, H-12). In addition, the <sup>13</sup>C NMR spectrum (Table 1) exhibited 29 carbon signals, including characteristic signals of seven methyls at  $\delta_C$  16.3 (C-23), 29.3 (C-24), 11.8 (C-25), 17.3 (C-26), 23.4 (C-27), 27.2 (C-29) and 15.6 (C-30), three oxygenated methines at  $\delta_C$  84.2 (C-1),  $\delta_C$  81.3 (C-3) and 72.3 (C-22), one quaternary oxygen-bearing carbon at  $\delta_C$  74.0 (C-19), two olefinic carbons at  $\delta_C$  128.7 (C-12) and 138.4 (C-13), and a ketone carbonyl carbon at  $\delta_C$  211.4 (C-2). The above NMR spectroscopic data were similar to those of 1 $\beta$ -hydroxy-2-oxopomolic acid [14], except for the appearance of a

**Table 1:** The  $^1\text{H}$  and  $^{13}\text{C}$ -NMR data<sup>a,b</sup> of **1** in  $\text{CDCl}_3$ .

No.	Carbon signals	Proton signals
1	84.2	4.08 (1H, s)
2	211.4	—
3	81.3	3.99 (1H, d, $J = 1.4$ Hz)
4	45.9	—
5	51.1	1.38 (1H, m)
6	18.2	1.75 (1H, d, $J = 10.1$ Hz) 1.58 (1H, m)
7	32.9	1.59 (1H, m) 1.44 (1H, m)
8	40.5	—
9	48.6	2.15 (1H, m)
10	49.7	—
11	26.3	2.48 (1H, ddd, $J = 21.0, 11.0, 3.6$ Hz) 2.16 (1H, m)
12	128.7	5.35 (1H, t, $J = 3.6$ Hz)
13	138.4	—
14	42.7	—
15	31.8	1.59 (1H, m) 1.23 (1H, m)
16	23.7	2.14 (1H, m) 1.23 (1H, m)
17	45.0	1.57 (1H, m)
18	47.9	2.38 (1H, d, $J = 5.6$ Hz)
19	74.0	—
20	34.3	1.93 (1H, m) 1.84 (1H, td, $J = 14.0, 2.4$ Hz)
21	33.9	1.40 (1H, m)
22	72.3	3.84 (1H, like-dd, $J = 2.0, 2.0$ Hz)
23	16.3	1.21 (3H, s)
24	29.3	0.69 (3H, s)
25	11.8	0.80 (3H, s)
26	17.3	0.86 (3H, s)
27	23.4	1.31 (3H, s)
29	27.2	1.19 (3H, s)
30	15.6	0.92 (3H, d, $J = 5.8$ Hz)

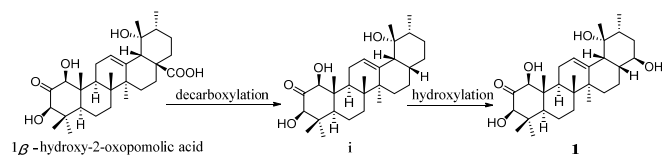
<sup>a)</sup> 600 MHz for  $^1\text{H}$  NMR; the coupling constants ( $J$ ) are in parentheses and reported in Hz; chemical shifts are given in ppm. <sup>b)</sup> 100 MHz for  $^{13}\text{C}$  NMR.

hydroxyl-carrying carbon and a methine instead of a methylene and a carboxyl group, which implied that compound **1** has a 28-norursene skeleton. This deduction was further supported by the HMBC correlations from  $\delta_{\text{H}}$  3.84 (H-22) to  $\delta_{\text{C}}$  47.9 (C-18) and 34.3 (C-20), and from  $\delta_{\text{H}}$  2.38 (H-18) to  $\delta_{\text{C}}$  128.7 (C-12), 138.4 (C-13), 42.7 (C-14), 23.7 (C-16), 45.0 (C-17) and 74.0 (C-19), and from  $\delta_{\text{H}}$  1.57 (H-17) to  $\delta_{\text{C}}$  72.3 (C-22) and 74.0 (C-19), and from  $\delta_{\text{H}}$  0.92 (Me-30) to  $\delta_{\text{C}}$  34.3 (C-20), 74.0 (C-19) and 33.9 (C-21), which established that a hydroxyl group was located at C-22 and a hydrogen atom instead of a carboxyl group located at C-17 of ring E (Figure 2). The location of the other functional groups and NMR data assignments of **1** were determined by HSQC and HMBC spectroscopic analysis. The strong NOESY correlations of  $\delta_{\text{H}}$  4.08 (H-1) with  $\delta_{\text{H}}$  1.38 (H-5) and 2.15 (H-9),  $\delta_{\text{H}}$  3.99 (H-3) with  $\delta_{\text{H}}$  1.38 (H-5) and 1.21 (Me-23) suggested that H-1 and H-3 were  $\alpha$ -oriented on ring A, the same as for 1 $\beta$ -hydroxy-2-oxopomolic acid (Figure 2). Furthermore, the NOESY correlations of  $\delta_{\text{H}}$  2.38 (H-18) with  $\delta_{\text{H}}$  1.57 (H-17), 1.19 (Me-29) and 1.93 (H-20) suggested that ring D and E had a *cis*-configuration, and H-18 and H-20 were in the same orientation. The NOESY correlations of  $\delta_{\text{H}}$  1.93 (H-20) with  $\delta_{\text{H}}$  1.19 (Me-29) and 1.40 (H-21 $\beta$ ), and no correlations of H-20 with H-17 and H-22 indicated that ring E possessed a chair instead of a boat conformation [15]. H-21 $\alpha$  was axial-oriented, deduced by the triplet of doublets and vicinal coupling constant ( $J = 14.0, 2.4$  Hz). The NOESY correlations of  $\delta_{\text{H}}$  3.84 (H-22) with  $\delta_{\text{H}}$  2.14 (H-16 $\alpha$ ), 1.84 (H-21 $\alpha$ ), 1.40 (H-21 $\beta$ ) and 1.23 (H-16 $\beta$ ) suggested that H-22 is equatorially-oriented (Figure 2). This is confirmed by the dd-like signal and vicinal coupling constant ( $J = 2.0, 2.0$  Hz) that corresponds to a dihedral angle of ca.  $60^\circ$  from H-22 to H-17, H-21 $\alpha$  and H-21 $\beta$ , respectively. The absolute configuration of **1** was established by the CD octant rule [16-17] due to the presence of a cyclohexone ring A moiety. In the CD spectrum of **1**, a positive Cotton effect at 284 nm ( $\Delta\epsilon = +1.17$ ) due to the  $n-\pi^*$  electronic transition suggested 1*S*,3*R*,17*R*,18*R*,19*R*,20*R*,22*R* configurations of

**Figure 2:** Selected HMBC and NOESY correlations of compound **1**.

**1**. Therefore, compound **1** was established to be (1*S*,3*R*,17*R*,18*R*,19*R*,20*R*,22*R*)-1,3,19,22-tetrahydroxy-28-norurs-12-en-2-one.

The 28-norursene skeleton is unprecedented in the genus *Agrimonia* from the view point of chemotaxonomy. Compound **1** could be produced from 1 $\beta$ -hydroxy-2-oxopomolic acid, a key biosynthetic precursor isolated from *Rosa woodsii* (Rosaceae family), through decarboxylation [18] and hydroxylation reactions (Scheme 1).

**Scheme 1:** Plausible biogenetic pathway for compound **1**.

Five known triterpenoid derivatives were identified as corosolic acid (**2**) [19], pomolic acid (**3**) [20], tormentic acid (**4**) [19], *epi*-tormentic acid (**5**) [21], and 1 $\beta$ ,2 $\beta$ ,3 $\beta$ ,19 $\alpha$ -tetrahydroxyurs-12-en-28-oic acid (**6**) [11], respectively, by analysis of their spectroscopic (NMR) and MS data with those reported in the literature.

**Table 2:** Inhibitory effects of compounds **2-6** on NO and IL-6 production induced by LPS in mouse monocyte-macrophage RAW 264.7 cells. (n=3).

Comp.	Anti-inflammatory activity (IC <sub>50</sub> [μM])	
	NO	IL-6
<b>2</b>	33.2±2.8	42.5±3.2
<b>3</b>	25.6±1.9	42.2±2.7
<b>4</b>	52.6±4.8	76.7±5.5
<b>5</b>	55.6±4.2	85.1±6.9
<b>6</b>	59.1±4.0	>100
hydrocortisone <sup>a</sup>	56.5±4.6	63.1±5.2

<sup>a)</sup> Positive control

Compounds **2-6** were tested for their inhibitory activities on NO and IL-6 production in mouse monocyte-macrophage RAW 264.7 cells stimulated by LPS (Table 2). Compounds **2** and **3** showed mild inhibitory effects, while the activities of **4** and **5** were very weak. Compound **6** exhibited no significant inhibitory activity with an IC<sub>50</sub> value higher than 100  $\mu\text{M}$  concentration. The cytotoxic activities of compounds **2-6** against RAW 264.7 macrophages were also tested by the MTT assay, but none of the compounds exhibited significant cytotoxicity at their effective concentrations. The IC<sub>50</sub> value of **1** was not acquired due to paucity of sample.

## Experimental

**Apparatus and reagents:** Optical rotation was measured with a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Bruker IFS 55 spectrometer. The CD spectrum was determined with a Bio-Logic Science MOS-450 spectrometer. NMR experiments were performed on Bruker ARX-400 and AV-600 spectrometers. The chemical shifts are stated relative to TMS and expressed in  $\delta$  values (ppm), with coupling constants are reported in Hz. The HRESIMS was obtained on an Agilent 6210 TOF mass spectrometer, and ESI-MS were recorded on an Agilent Series 1100 SL mass spectrometer. Silica gel GF254 prepared for TLC and

silica gel (200-300 mesh) for column chromatography (CC) were obtained from Qingdao Marine Chemical Factory (Qingdao, China). Sephadex LH-20 was a product of Pharmacia. Octadecyl silica gel was purchased from Merck Chemical Company Ltd. RP-HPLC separations were conducted using an LC-6AD liquid chromatograph with a YMC Pack ODS-A column (250 × 20mm, 5 μm, 120 Å) and SPD-10A VP UV/VIS detector. All reagents were either HPLC or analytical grade and were purchased from Tianjin Damao Chemical Company. Compounds were detected on TLC plates either under UV light or by heating after spraying with anisaldehyde-H<sub>2</sub>SO<sub>4</sub> reagent.

**Plant material:** Dried aerial parts of *A. pilosa* were purchased from Shenyang Northeast Pharmacy, China, and identified by Professor Qishi Sun, Department of Pharmaceutical Botany, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University. A voucher specimen (AP-20061010) has been deposited in the herbarium of the Department of Natural Products Chemistry, Shenyang Pharmaceutical University.

**Extraction and isolation:** The dried aerial parts of *A. pilosa* (10 kg) were cut into approximately 2 cm pieces and extracted with 70% EtOH (100 L × 2 h × 2). The resulting extract (1.0 kg) was concentrated *in vacuo*, suspended in H<sub>2</sub>O (3 L), and partitioned successively with cyclohexane, EtOAc, and *n*-BuOH (3 L × 3). The EtOAc extract (110 g) was subjected to silicagel CC (10 × 80 cm) and eluted with chloroform/MeOH (100:1, 30:1, 20:1, 10:1, 5:1, 2:1, 1:1 and 0:1 v/v) to obtain 7 fractions (EA-EG). Fraction EA (23 g) was subjected to a silica gel column (6 × 80 cm) and eluted with CH<sub>2</sub>Cl<sub>2</sub>/EtOAc (from 40:1 to 0:1) to produce 7 fractions (EA1-EA7). Fraction EA4 (180 mg) was chromatographed over Sephadex LH-20 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 1:1; 1.5 × 30 cm) to give compound **1** (3.5 mg). Fraction ED (20.6 g) was subjected to a silica gel column (6 × 80 cm) and eluted with CH<sub>2</sub>Cl<sub>2</sub>/acetone (from 40:1 to 0:1) to yield ED1-ED6. Separation of ED4 (1.4 g) on a reversed-phase C<sub>18</sub> silica gel column (2.5 × 30 cm) by elution with MeOH/H<sub>2</sub>O (30:70, 50:50, 70:30 and 100:0 v/v) yielded fractions ED4-1 to ED4-5. ED4-5 (380 mg) was purified by preparative TLC (CH<sub>2</sub>Cl<sub>2</sub>/acetone, 3:1) to obtain **2** (11.5 mg) and **4** (22.3mg). ED5 (4.3 g) was subjected to RP-C<sub>18</sub> silica gel CC (2.5 × 30 cm) and eluted with MeOH/H<sub>2</sub>O (1:9 to 8:2) to yield ED5-1 and ED5-2. ED5-1 (100 mg) was separated by HPLC (50%MeOH/H<sub>2</sub>O, 6mL/min) to afford compound **3** (56.2 mg, *t<sub>R</sub>* = 39 min) and compound **5** (28.8 mg, *t<sub>R</sub>* = 25 min). ED5-2 (120 mg) was purified by preparative TLC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 7:1) to obtain **6** (13.6 mg).

**(1S,3R,17R,18R,19R,20R,22R)-1,3,19,22-tetrahydroxy-28-norurs-12-en-2-one (3)**

Amorphous white powder (MeOH).

[α]<sub>D</sub><sup>25</sup>: +46.0 (*c* 0.10, MeOH).

CD (MeOH): 284 (Δε = +1.17) nm.

IR ν<sub>max</sub> (KBr): 3448, 2928, 1715, 1618, 1459, 1384, 1021 cm<sup>-1</sup>.

<sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>): Table 1.

<sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

ESI-MS (*m/z*): 497.4 [M + Na]<sup>+</sup>, 473.2 [M - H]<sup>-</sup>.

HRESIMS (*m/z*): 519.3340 [M + HCOO]<sup>-</sup> (calcd. for C<sub>30</sub>H<sub>47</sub>O<sub>7</sub>: 519.3322).

**Inhibition of NO and IL-6 production bioassay:** The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction. Briefly, RAW 264.7 cells were seeded into 96-well tissue culture plates at a density of 1×10<sup>5</sup> cells/well, and stimulated with 1 μg/mL of LPS in the presence or absence of test compounds. After incubation at 37°C for 24 h, 100 μL of cell-free supernatant was mixed with 100 μL of Griess reagent (mixture of equal volumes of reagent A and reagent B, A: 1%, w/v, sulfanilamide in 5%, w/v, phosphoric acid, B: 0.1%, w/v, of *N*-(1-naphthyl)ethylenediamine). The test groups, LPS groups, and control groups were treated in the same way as the experiment for NO analysis, respectively. Absorbance was measured in a microplate reader at 540 nm. Nitrite concentrations and the inhibitory rates were calculated by a calibration curve prepared with sodium nitrite standards. Cytotoxicity was determined by the mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay, after 24 h incubation with test compound. The cells in the 96-well plates were cultured for 6 h, and the levels of IL-6 in the cell culture supernatant were detected by using a mouse IL-6 ELISA kit according to the manufacturer's recommendations [22]. Data are presented as the mean ± standard deviation. Each experiment was performed at least 3 times.

**Supplementary data:** Spectral data relating to this article are available online.

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## Two New 18-Norschiartane-type Schinortriterpenoids from *Schisandra lancifolia*

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Two new 18-norschiartane-type schinortriterpenoids, namely wuwezidilactones Q (1) and R (2) were isolated from the stems of *Schisandra lancifolia*. Their structures were determined on the basis of extensive spectroscopic analysis. The absolute configurations of the new compounds were further determined by ROESY and an empirical comparison of their experimental ECD spectra with literature.

**Keywords:** *Schisandra lancifolia*, Schinortriterpenoids, Absolute configuration.

Plants of Schisandraceae family, contains the genera *Schisandra* and *Kadsura*, represent an important group of plants. Some of these plants were important Traditional Chinese Medicines (TCMs), and used in Chinese folk for more than two thousand years. Chemical investigation on this family was mostly focused on lignans and triterpenoids since 1970's. In 2003, Sun and co-workers reported a novel highly oxygenated nortriterpenoid, micrandilactone A, from *Schisandra micrantha* [1]. This discovery revealed a new kind of natural triterpenoid, called as schinortriterpenoid (SNT). In the past decades, a series of SNTs with different novel skeletons were isolated and identified from this family [2,3], especially from *Schisandra* genus, attracting more and more attention from natural product chemists, as well as organic chemists.

*Schisandra lancifolia* (Rehd. et. Wils.) A. C. Smith, a species of *Schisandra* genus distributed in the southwest of China, was used to staunch, treat fractures, and eliminate stasis to reduce swelling [4]. Phytochemistry research of this species has led to the discovery of a series of SNTs with different new skeletons, such as 18-Norschiartanes [5], Norcycloartanes [6], Schisanartanes [7], 1,2,3-trinorlancischiartanes [8], 2,3-dinorlancischiartanes [9], lancifoartanes [10], 12,22-cyclopreschisanartanes [11]. Our further investigation into this plant aimed at finding more SNTs with diverse skeletons led to the isolation of two new 18-norschiartane-type schinortriterpenoids. This article deals with the isolation, structure elucidation of the new compounds.

Wuwezidilactone Q (1) was obtained as a white amorphous powder. Its molecular formula  $C_{28}H_{36}O_8$  was determined by HRESIMS at  $m/z$  523.2298  $[M+Na]^+$  (calcd for  $C_{28}H_{36}O_8Na$  523.2302) with 11 indices of hydrogen deficiency.  $^1H$ ,  $^{13}C$  NMR and DEPT spectra of 1 displayed three methyl singlets and one methyl doublet, six methylenes, four  $sp^3$  methines, four oxygenated  $sp^3$  methines, two trisubstituted double bonds, four  $sp^3$  quaternary carbons and two ester groups. All of the above data of 1 suggested that 1 was a 18-norschiartane-type nortriterpenoid with 28 carbons. Comparison of NMR data of 1 with those of lancifodilactone A indicated that the acetoxy group at C-12 in lancifodilactone A was replaced by a hydroxyl group in 1 without causing significant chemical

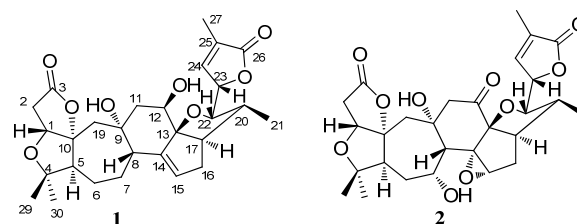


Figure 1: The structures of compounds 1 and 2.

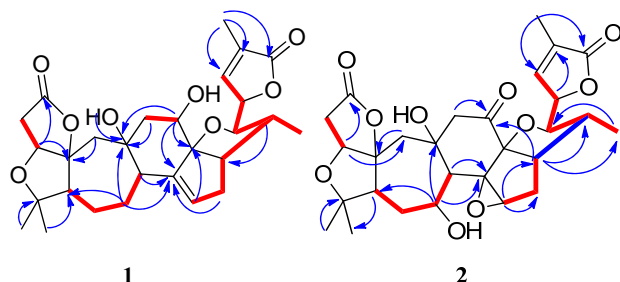
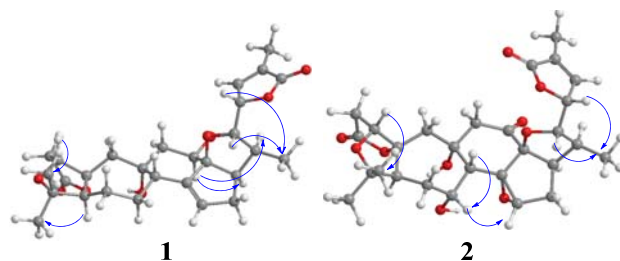
shifts at C-11, C-12, and C-13. This was confirmed by  $^1H$ - $^1H$  COSY correlation of H-11 with H-12 and HMBC correlations from H-12 to C-9, C-13, and C-14. Furthermore, compound 1 differed from lancifodilactone A at C-7 ( $\delta_C$  69.8) in ring C, the hydroxyl group at C-7 in lancifodilactone A was replaced by a methylene ( $\delta_C$  26.4) in 1. In addition, a downfield shift of C-5 in 1 ( $\delta_C$  60.7) compared with that in lancifodilactone A ( $\delta_C$  52.3) could ascribe to the absence of  $\gamma$ -steric compression between the oxygen atom at C-7 and H-5 in 1 [5,12]. The change at C-7 was further proved by  $^1H$ - $^1H$  COSY correlations of H-7 with H-6, and H-8 and HMBC correlations from H-7 to C-5, C-8, C-9, and C-14. The relative configuration of 1 was deduced to be the same as that of lancifodilactone A from the similar carbon chemical shifts and ROESY correlations. The ROESY correlations of H-23 with Me-21, H-12 with H-17, and H-20 suggested that H-12 was  $\alpha$ -oriented. The absolute configuration of C-23 could be determined by intense positive or negative cotton effects (CEs) around 210nm. The experimental ECD spectrum of 1 showed Negative CE at 212 nm, indicated that the absolute configuration of C-23 was “S” [12]. Thus, the absolute configuration of 1 was finally determined as shown in Figure 1 by an empirical comparison the experimental ECD with that of wuwezidilactones J-P.

Wuwezidilactone R (2) was obtained as a white amorphous powder. Its molecular formula  $C_{28}H_{34}O_{10}$  was deduced by HRESIMS at  $m/z$  553.2047  $[M+Na]^+$  (calcd for  $C_{28}H_{34}O_{10}Na$  553.2044) with 12 indices of hydrogen deficiency. Careful Analysis of the  $^1H$ ,  $^{13}C$  NMR and DEPT spectra of 2 disclosed that 2 was also a 18-norschiartane-type nortriterpenoid. Structure of 2 was almost the

**Table 1:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data (acetone- $d_6$ ) of compounds **1** and **2**.

Position	<b>1</b>		<b>2</b>	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$
1	4.29, d (4.8)	81.8 d	4.28, d (5.4)	82.1 d
2 $\alpha$	2.44, d (18.0)	36.0 t	2.95, overlapped	35.6 t
2 $\beta$	2.92, overlapped		2.44, overlapped	
3		174.7 s		174.8 s
4		85.1 s		84.8 s
5	2.29, overlapped	60.7 d	2.76, dd (13.2, 4.8)	52.9 d
6 $\alpha$	1.47, overlapped	27.6 t	2.03, overlapped	32.7 t
6 $\beta$	1.81, overlapped		1.76, overlapped	
7 $\alpha$	1.81, overlapped	26.4 t		69.6 d
7 $\beta$	2.00, m		4.32, br t	
8	2.25, overlapped	49.3 d	2.73, br s	46.4 d
9		77.2 s		80.1 s
10		99.9 s		98.7 s
11 $\alpha$	1.81, m	43.1 t	2.21, d (13.2)	54.0 t
11 $\beta$	1.91, dd (14.4, 2.4)		2.92, overlapped	
12	3.57, br d	72.9 d		202.4 s
13		97.8 s		93.7 s
14		143.4 s		74.3 s
15	5.58, m	128.0 d	3.96, br s	57.5 d
16 $\alpha$	2.26, overlapped	31.9 t	2.09, overlapped	26.6 t
16 $\beta$	2.37, m		1.79, overlapped	
17	3.04, dt (9.0, 4.2)	45.7 d	2.59, m	41.6 d
19 $\alpha$	2.09, overlapped	44.6 t	2.11, overlapped	45.2 t
19 $\beta$	2.18, d (15.6)		2.43, overlapped	
20	2.31, overlapped	37.9 d	2.51, m	38.2 d
21	1.01, d (6.6)	12.6 q	1.06, d (7.2)	11.9 q
22	3.52, dd (10.2, 3.6)	83.1 d	3.91, dd (9.6, 3.0)	85.3 d
23	5.01, m	81.0 d	4.88, m	81.0 d
24	7.28, m	147.8 d	7.23, m	147.2 d
25		130.3 s		130.9 s
26		174.4 s		174.0 s
27	1.84, br s	10.7 q	1.83, br s	10.8 q
29	1.14, s	22.4 q	1.14, s	22.3 q
30	1.29, s	29.2 q	1.27, s	28.7 q

$\delta_{\text{H}}$  were recorded at 600 MHz,  $\delta_{\text{C}}$  were recorded at 150 MHz.

**Figure 2:** Selected HMBC (blue arrows H→C) and  $^1\text{H}$ - $^1\text{H}$  COSY (red lines) correlations of **1** and **2**.**Figure 3:** Key ROESY correlations (blue arrows) of **1** and **2**.

same as that of wuweizidilactone **M** except for a hydroxyl group at C-7 in **2** rather than an acetoxy group. This could be confirmed by the chemical shift at C-7 ( $\delta_{\text{C}}$  69.6), and the  $^1\text{H}$ - $^1\text{H}$  COSY correlations of H-7 with H-6, and H-8 and HMBC correlations from H-5, H-6, and H-8 to C-7. The ROESY experiment of **2** was similar to wuweizidilactone **M**, revealed that they had a same relative configuration. The ROESY correlation of H-7 with H-15 suggested that H-7 was  $\beta$ -oriented. The absolute configuration of **23S** was proved by Negative CE at 209 nm in ECD spectrum. At last, the absolute configuration of **2** was finally determined as shown in Figure 1 by the same way as that of **1**.

## Experimental

**General:** 1D and 2D NMR spectra were recorded on Bruker DRX 600 and DRX 500 spectrometers using TMS as the internal standard. Chemical shifts ( $\delta$ ) are expressed in ppm relative to the TMS signals. HRESIMS was performed on an API QSTAR Pulsar i spectrometer. UV spectra were obtained on a Shimadzu UV-2401PC spectrophotometer. IR spectra were obtained on a Bruker Tensor-27 FT-IR spectrometer using KBr pellets. Optical rotations were measured in MeOH with Horiba SEPA-300 and JASCO P-1020 polarimeters. Experimental ECD spectra were measured on a Chirascan instrument. Column chromatography (CC) was performed with silica gel (100–200 mesh; Qingdao Marine Chemical, Inc., Qingdao, People's Republic of China), MCI gel (CHP20P, 75–150  $\mu\text{m}$ , Mitsubishi Chemical Corporation, Tokyo, Japan). Preparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with a Zorbax SB-C18 (21.2 mm  $\times$  250 mm) column. Semipreparative HPLC was performed on an Agilent 1200 liquid chromatograph with a Zorbax SB-C18 (9.4 mm  $\times$  250 mm) column. Fractions were monitored by thin layer chromatography, spots were visualized by UV light (254 nm and 365 nm) and by heating silica gel plates sprayed with 10%  $\text{H}_2\text{SO}_4$  in EtOH. All solvents used in column chromatography were distilled including petroleum ether (60–90°C).

**Plant Material:** The stems of *S. lancifolia* were collected in Shangri-La of Yunnan Province, People's Republic of China, in October 2013 and identified by Prof. Xi-Wen Li at Kunming Institute of Botany. A voucher specimen has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

**Extraction and isolation:** The air-dried and powdered stems of *S. lancifolia* (7.0 Kg) were extracted with aqueous acetone (4  $\times$  8L, 3 days each) at room temperature. The extracted solution was distilled under reduced pressure to remove acetone, and then was partitioned between EtOAc and  $\text{H}_2\text{O}$  to afford EtOAc part. The EtOAc extract (250 g) was eluted with  $\text{CHCl}_3/\text{Me}_2\text{CO}$  (1:0, 9:1 8:2, 6:4, 1:1, 0:1) on silica gel column chromatography to give 6 fractions A-F. Fraction C (8:2) was decolorized on MCI gel with 90% MeOH/ $\text{H}_2\text{O}$  and was separated on Rp-18 with MeOH/ $\text{H}_2\text{O}$  (3:7, 4:6, 6:4, 7:3, 8:2, 1:0) to afford fractions C1-C6. Fraction C3 was separated on silica gel CC with petroleum ether/acetone (5:1, 3:1, 2:1, 1:1, 0:1) to obtain C31-C38. C33 was purified by Sephadex LH-20 CC, eluting with  $\text{CHCl}_3/\text{MeOH}$  (1:1), and by preparative HPLC to afford **1** (1.9 mg).

Fraction D (6:4) was successfully separated on MCI gel with 90% MeOH/ $\text{H}_2\text{O}$  to remove pigments and then on Rp-18 with MeOH/ $\text{H}_2\text{O}$  (3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, 1:0) to afford fractions D1-D8. D3 was separated over silica gel CC with petroleum ether/acetone (5:1, 3:1, 2:1, 1:1, 0:1) to afford D31-D36. D36 was purified by repeated Sephadex LH-20 CC, eluting with  $\text{CHCl}_3/\text{MeOH}$  (1:1), and by Semipreparative HPLC to afford **2** (4 mg).

## Wuweizidilactone **Q** (**1**)

$[\alpha]_{\text{D}}^{23}$ : +23 ( $c$  0.12, MeOH).

IR (KBr)  $\nu_{\text{max}}$ : 3343, 2924, 1760, 1629, 1434, 1385, 1326, 1197, 1109, 1082, 1037.

UV (MeOH)  $\lambda_{\text{max}}$  nm (log  $\epsilon$ ): 204 (3.88).

ECD ( $c$  0.05 MeOH)  $\lambda_{\text{max}}$  nm: 209, 251.

$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR: Table 1.

HRESIMS:  $m/z$  523.2298  $[\text{M}+\text{Na}]^+$  (calcd for  $\text{C}_{28}\text{H}_{36}\text{O}_8\text{Na}$ , 523.2302).



**Wuweizidilactone Q (2)** $[\alpha]_D^{23}$ : +34 (*c* 0.24, MeOH).IR (KBr)  $\nu_{\max}$ : 3442, 2925, 1762, 1629, 1384, 1221, 1175, 1106, 1067, 957  $\text{cm}^{-1}$ .UV (MeOH)  $\lambda_{\max}$  nm (log  $\epsilon$ ): 208 (3.95), 275 (2.79).ECD (*c* 0.07 MeOH)  $\lambda_{\max}$  nm: 212, 302. $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR: Table 1.HRESIMS:  $m/z$  553.2047  $[\text{M}+\text{Na}]^+$  (calcd for  $\text{C}_{28}\text{H}_{34}\text{O}_{10}\text{Na}$  553.2044).**Supplementary data:** 1D and 2D NMR, HRESIMS, UV, IR, and CD spectra of **1** and **2** are available.**Acknowledgments** - This project was supported financially by the National Natural Science Foundation of China (81373290 and 21322204).**References**

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Terpenoids and Steroids from *Euphorbia hypericifolia*

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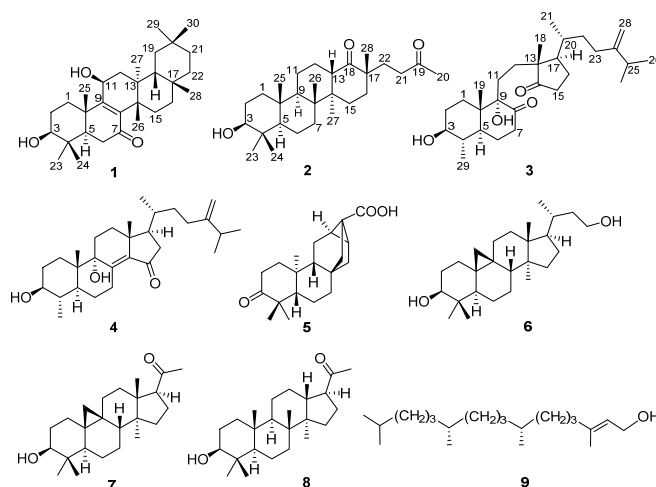
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Two new triterpenoids and two new sterols, named euphyperins A–D (1–4), including an oleanane-type triterpenoid (1), a lupane-type nortriterpenoid (2), and two cholestane-type steroids (3 and 4), along with five known compounds (5–9) were isolated from the twigs and leaves of *Euphorbia hypericifolia*. Euphyperin B (2) represents a rare lupane-type nortriterpenoid, and euphyperin C (3) is a novel 8,14-secocholestane-type steroid. Euphyperin A (1) exhibited moderate PTP1B inhibitory activity with an  $IC_{50} = 17.05 \pm 1.12 \mu\text{g/mL}$ .

**Keywords:** *Euphorbia hypericifolia*, Triterpenoid, Steroid, PTP1B inhibitor.

The genus *Euphorbia*, with more than 2000 species, is one of the largest genera of angiosperms [1]. Diterpenoids in this genus are the focus of natural product research, because of their wide range of potentially valuable bioactivities [2]. Our great interest in structural and biological diversities of this genus resulted in some exciting research findings, such as the first secolathyrane diterpenoid with an unprecedented skeleton [3], ingol-type diterpenoids with 11 $\beta$ -HSD1 inhibitory activities [4], and abietane-type diterpenoids with anti-HIV activities [5]. *E. hypericifolia* Linn. (Euphorbiaceae) has long been used as a traditional herbal medicine in China for promoting lactation [6]. Previous chemical studies on this plant only afforded some flavonoids and ellagic acid [7]. In the continuing search for structurally diverse and bioactive compounds from the genus *Euphorbia*, four new compounds, named euphyperins A–D, including one oleanane-type triterpenoid, one degraded lupane-type triterpenoid, and two sterols, along with five known compounds were isolated from the twigs of *E. hypericifolia*. Among them, euphyperin B (2) represents a rare class of lupane-type nortriterpenoid [8], and euphyperin C (3) possesses a rare 8,14-secocholestane-type steroid skeleton, which has only been found previously in a marine sponge [9]. Euphyperin A (1) exhibited PTP1B inhibitory activity with an  $IC_{50} = 17.05 \mu\text{g/mL}$ . Herein we describe the isolation, structure characterization, and biological activities of these compounds.

Euphyperin A (1) was isolated as a white, amorphous powder, and its molecular formula was determined as  $C_{30}H_{48}O_3$  (seven indices of hydrogen deficiency) based on an HR-EIMS ion at  $m/z$  456.3606 [ $M^+$ ] (calcd 456.3603), as well as comprehensive analysis of its  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra. The UV absorption band at 253 nm exhibited a conjugated unsaturated carbonyl system, consistent with the IR absorption (Supporting Information Figure S10) at  $1649\text{ cm}^{-1}$ . The  $^{13}\text{C}$  and DEPT spectra (Table 1) displayed 30 signals of carbons including eight methyls, nine methylenes, four methines (two oxygenated at  $\delta_C$  67.3 and 78.5) and nine quaternary. Three overlapped signals at  $\delta_C$  30.9 (C-17) and 39.9 (C-10 and C-14) were distinguished by HMBC correlations. The signals at  $\delta_C$  144.7, 158.8, and 201.1 confirmed the existence of the aforementioned enone system, which accounted for two indices of hydrogen deficiency, and the remaining five thus required 1 being pentacyclic. The above data with the aid of analysis of its 2D NMR spectra suggested that it was an oleanane-type triterpenoid bearing



an  $\alpha,\beta$ -enone system and two hydroxyls. The attachments of these two hydroxyls were at C-3 and C-11, as determined by the chemical shifts of the relevant protons and carbons as well as the HMBC correlations (Figure 1A) from H-3 to C-1, C-4, C-23, and C-24, and from H-11 to C-8, C-9, and C-12. The tetrasubstituted  $\Delta^8$  double bond in the  $\alpha,\beta$ -enone system was located by the multiple correlations from H-11, H<sub>2</sub>-15, and H<sub>3</sub>-26 to C-8 at  $\delta_C$  144.7, and from H-5, H-11, H<sub>2</sub>-12, and H<sub>3</sub>-25 to C-9 at  $\delta_C$  158.8, respectively. The location of the carbonyl group was assigned at C-7 as deduced from the HMBC correlations from H<sub>2</sub>-6 to C-7 at  $\delta_C$  201.1. The above deduction unambiguously suggested that 1 was an analogue of 7-oxoisomultiflorenol [10], and the only difference was the presence of a hydroxy group at C-11 ( $\delta_C$  67.3).

The relative configuration of 1 was almost identical to that of 7-oxoisomultiflorenol by comparing their  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra, as well as analysis of the ROESY spectrum (Figure 1B) of 1, in which the cross-peaks of H-1 $\alpha$ /H-3, H-3/H-5, and H-3/H<sub>3</sub>-23 revealed that they were cofacial and assigned to be  $\alpha$ -oriented randomly. In consequence, the ROESY cross-peaks of H-11/H-1 $\alpha$ , H-11/H-12 $\alpha$ , and H-11/H<sub>3</sub>-27 suggested H-11 was also  $\alpha$ -oriented. Thus, the structure of 1 was assigned.

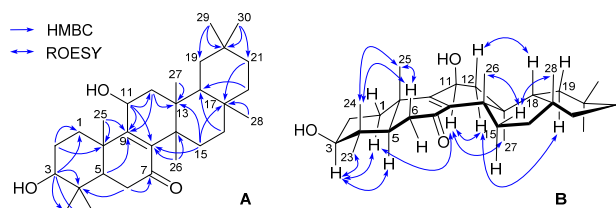


Figure 1: Selected HMBC (A) and ROESY (B) correlations of **1**.

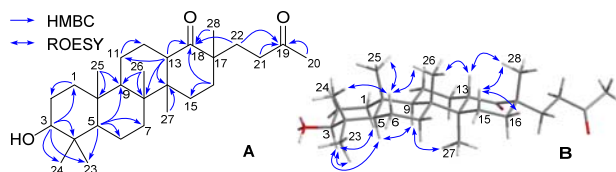


Figure 2: Selected HMBC (A) and ROESY (B) correlations of **2**.

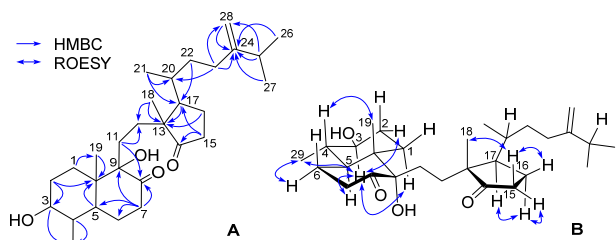


Figure 3: Selected HMBC (A) and ROESY (B) correlations of **3**.

Euphyperin B (**2**), a white, amorphous powder, gave a molecular formula  $C_{28}H_{46}O_3$  as determined by the  $^{13}C$  NMR data and an HR-ESI(+)MS ion at  $m/z$  453.3351  $[M + Na]^+$  (calcd 453.3345), requiring six indices of hydrogen deficiency. The  $^1H$  NMR spectrum (Table 1) of **2** showed signals of six singlet methyl groups ( $\delta_H$  0.76, 0.76, 0.86, 0.96, 1.11, and 1.14), one acetyl group ( $\delta_H$  2.15, s, H-20) and one oxygenated methine ( $\delta_H$  3.20, dd,  $J = 11.3$ , 4.9 Hz, H-3). The  $^{13}C$  NMR and DEPT spectra (Table 1) displayed 28 carbon signals for seven methyls, ten methenes, four methines and seven quaternaries, including two carbonyls ( $\delta_C$  209.5 and 217.9). The two keto groups accounted for two indices of hydrogen deficiency, and the remaining four thus indicated **2** being tetracyclic. The aforementioned analysis obviously suggested that the structure of **2** resembled that of 29,30-dinor-3 $\beta$ -acetoxy-18,19-dioxo-18,19-secolupane [8], and the only difference was the presence of a 3-OH in **2** replacing the 3-OAc in the latter as judged by the shielded H-3 ( $\Delta\delta_H$  1.25). This conclusion was confirmed by the HMBC correlations (Figure 2A) from H-3 to C-1, C-4, C-23, and C-24. The locations of the two keto groups at C-18 and C-19 were confirmed by the HMBC correlations from H-13, H<sub>2</sub>-16, H<sub>2</sub>-22, and H<sub>3</sub>-28 to C-18 ( $\delta_C$  217.9), and from H<sub>3</sub>-20, H<sub>2</sub>-21, and H<sub>2</sub>-22 to C-19 ( $\delta_C$  209.5).

The relative configuration of **2** was assigned in the same way as that of 29,30-dinor-3 $\beta$ -acetoxy-18,19-dioxo-18,19-secolupane, mainly by comparing their NMR data, which were corroborated by the ROESY spectrum (Figure 2B), in which the  $\alpha$ -axial orientation of H-3 was determined by the cross-peaks of H-3/H-1 $\alpha$ , H-5, and H<sub>3</sub>-23. The correlations for H-6 $\beta$ /H<sub>3</sub>-24, H-6 $\beta$ /H<sub>3</sub>-25, H-6 $\beta$ /H<sub>3</sub>-26, H-13/H<sub>3</sub>-26, H-13/H<sub>3</sub>-28, H-15 $\beta$ /H-16 $\beta$ , and H-15 $\beta$ /H<sub>3</sub>-28 revealed that they were all  $\beta$ -oriented. The structure of **2** was therefore determined.

Euphyperin C (**3**) gave a molecular formula  $C_{29}H_{48}O_4$  with six indices of hydrogen deficiency based on the  $^{13}C$  NMR data and an HR-ESI(+)MS ion at  $m/z$  483.3436  $[M + Na]^+$  (calcd 483.3450).

Table 1:  $^1H$  and  $^{13}C$  NMR data of compounds **1** and **2** in  $CDCl_3$ .

No.	<b>1</b>		<b>2</b>	
	$\delta_H$ (mult., $J$ in Hz) <sup>a</sup>	$\delta_C$ <sup>b</sup>	$\delta_H$ (mult., $J$ in Hz) <sup>a</sup>	$\delta_C$ <sup>b</sup>
1	$\alpha$ 1.58, m $\beta$ 2.46, dd (13.2, 3.2)	33.8	$\alpha$ 0.95 td (12.4, 4.3) $\beta$ 1.73 m	39.0
2	1.75, m (2H)	27.5	1.62 m (2H)	27.5
3	3.32, m	78.5	3.20 dd (11.3, 4.9)	78.9
4		39.1		39.0
5	1.64, dd (13.2, 5.9)	48.7	0.69 dd (11.4, 2.1)	55.6
6	$\alpha$ 2.41, dd (19.1, 5.9) $\beta$ 2.45, dd (19.1, 13.2)	36.9	$\alpha$ 1.37 m $\beta$ 1.55 m	18.4
7		201.1	1.31 m 1.48 m	34.2
8		144.7		41.1
9		158.8	1.21 m	51.0
10		39.9		37.4
11	4.60, q (8.3)	67.3	1.20 m 1.56 m	20.1
12	$\beta$ 1.39, m $\alpha$ 2.15, dd (12.7, 8.0)	42.5	1.30 m 1.64 m	22.3
13		39.7	2.70 dd (11.8, 3.6)	48.1
14		39.9		46.6
15	$\alpha$ 1.41, m $\beta$ 2.29, dt (15.9, 9.0)	29.5	$\alpha$ 1.30 m $\beta$ 2.04 td (13.4, 4.5)	27.0
16	1.35, m 1.52, m	35.6	$\beta$ 1.52 m $\alpha$ 1.69 m	34.2
17		30.9		46.4
18	1.59, m	42.1		217.9
19	$\alpha$ 1.19, dd (13.9, 5.6) $\beta$ 1.35, m	35.5		209.5
20		28.6	2.15 s	30.0
21	1.27, m 1.45, m	33.0	2.47 m (2H)	39.1
22	0.91, m 1.46, m	38.4	1.67 m (2H)	32.3
23	0.99, s	27.7	0.96 s	28.1
24	0.91, s	15.3	0.76 s	15.5
25	1.21, s	19.7	0.86 s	16.8
26	1.51, s	27.4	1.11 s	16.1
27	0.96, s	19.1	0.76 s	16.2
28	1.14, s	30.9	1.14 s	24.4
29	0.96, s	34.3		
30	0.98, s	32.0		

<sup>a,b</sup> Data were measured at 400 and 125 MHz, respectively.

Interpretation of its  $^1H$  NMR spectrum (Table 2) showed the presence of two methyl singlets ( $\delta_H$  0.74 and 0.86, each 3H), four methyl doublets ( $\delta_H$  1.00, 3H, d,  $J = 6.6$  Hz; and three overlapped  $\delta_H$  1.03, each 3H, d,  $J = 6.7$  Hz), one oxygenated methine ( $\delta_H$  3.16, 1H, td,  $J = 10.4$ , 5.1 Hz), and one terminal methine group ( $\delta_H$  4.68 and 4.74, each 1H, s). The  $^{13}C$  NMR data (Table 2), with the aid of the HMBC spectrum (Figure 3A), displayed signals of 29 carbons, suggesting the existence of several characteristic functional groups including two keto groups ( $\delta_C$  215.4 and 224.5), one oxygenated methine ( $\delta_C$  75.9), one oxygenated quaternary carbon ( $\delta_C$  83.5), and one terminal methene group ( $\delta_C$  106.5 and 156.5). The above data indicated that **3** likely possessed a steroidal structure which was highly similar to that of (24R)-3 $\beta$ -hydroxy-24-methyl-4-methylene-5 $\alpha$ -8,14-secocholestane-8,14-dione [9], except for the presence of a  $\Delta^{24(28)}$  double bond and in the concomitant absence of a  $\Delta^{4(29)}$  double bond. This deduction was further verified by the HMBC spectrum, in which the  $\Delta^{24(28)}$  double bond was verified by the key correlations from H<sub>2</sub>-23, H-25, H<sub>3</sub>-26, H<sub>3</sub>-27, and H<sub>2</sub>-28 to C-24 at  $\delta_C$  156.5, and from H<sub>2</sub>-23 and H-25 to C-28 at  $\delta_C$  106.5. In addition, CH<sub>3</sub>-29 was located by the correlations from H-3 and H-4 to C-29 at  $\delta_C$  15.6.

The relative configuration of **3** was assigned by comparison of NMR data with those of (24R)-3 $\beta$ -hydroxy-24-methyl-4-methylene-5 $\alpha$ -8,14-secocholestane-8,14-dione [9] and the ROESY experiment (Figure 3B). In particular, H-3 and CH<sub>3</sub>-29 were assigned to be  $\alpha$ -oriented on the basis of the ROESY cross-peaks of H-1/H-3, H-2 $\alpha$ /H-3, H-3/H-5, H-3/H<sub>3</sub>-29, and H-6 $\alpha$ /H<sub>3</sub>-29. The structure of **3** possessing a rare 8,14-seco steroid skeleton was thus elucidated as depicted.

**Table 2:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of compounds **3** and **4** in  $\text{CDCl}_3$ .

No.	<b>3</b>		<b>4</b>	
	$\delta_{\text{H}}$ (mult., $J$ in Hz) <sup>a</sup>	$\delta_{\text{C}}$ <sup>c</sup>	$\delta_{\text{H}}$ (mult., $J$ in Hz) <sup>b</sup>	$\delta_{\text{C}}$ <sup>c</sup>
1	$\alpha$ 1.27 m	29.6	$\alpha$ 1.47 m	29.7
	$\beta$ 1.58 m		$\beta$ 1.75 m	
2	$\beta$ 1.51 m	30.4	$\beta$ 1.47 m	30.7
	$\alpha$ 1.89 m		$\alpha$ 1.91 m	
3	3.16 td (10.4, 5.1)	75.9	3.16 m	75.9
4	1.35 m	40.2	1.28 m	40.0
5	1.51 m	44.1	1.69 m	41.8
6	$\beta$ 1.39 m	26.4	$\beta$ 1.12 m	25.0
	$\alpha$ 2.08 m		$\alpha$ 1.81 m	
7	2.48 m	36.8	$\alpha$ 1.96 m	22.8
	2.52 m		$\beta$ 3.96 m	
8		215.4		148.5
9		83.5		74.7
10		45.7		42.2
11	1.47 m	27.5	$\alpha$ 1.62 m	28.0
	1.71 m		$\beta$ 1.96 m	
12	1.14 td (13.3, 3.4)	30.2	$\alpha$ 1.51 m	33.9
	1.51 m		$\beta$ 2.02 m	
13		52.1		43.3
14		224.5		141.3
15	$\alpha$ 2.08 m	37.5		208.5
	$\beta$ 2.40 dd (18.7, 8.7)			
16	$\beta$ 1.51 m	23.8	$\beta$ 2.07 m	42.7
	$\alpha$ 2.16 m		$\alpha$ 2.43 dd (18.9, 7.5)	
17	1.85 m	46.3	1.57 m	50.4
18	0.86 s	18.7	0.97 s	17.4
19	0.74 s	14.2	0.84 s	16.9
20	1.55 m	34.2	1.57 m	34.5
21	1.00 d (6.6)	18.3	1.04 d (6.6)	19.3
22	1.26 m	33.3	1.23 m	34.3
	1.65 m		1.64 m	
23	1.94 m	31.4	1.91 m	30.6
	2.17 m		2.09 m	
24		156.5		156.3
25	2.23 m	33.9	2.21 m	33.9
26	1.03 d (6.7)	22.0	1.02 d (6.7)	22.0
27	1.03 d (6.7)	22.1	1.02 d (6.7)	22.1
28	4.68 s	106.5	4.65 s	106.6
	4.74 s		4.73 s	
29	1.03 d (6.7)	15.6	1.02 d (6.7)	15.5

<sup>a-c</sup> Data were measured at 400, 500, and 125 MHz, respectively.

Euphyperin D (**4**) was determined to possess a molecular formula  $\text{C}_{29}\text{H}_{46}\text{O}_3$ , as deduced from the  $^{13}\text{C}$  NMR data and an HREIMS ion at  $m/z$  442.3440  $[\text{M}]^+$  (calcd 442.3447). The  $^{13}\text{C}$  NMR spectrum (Table 2) showed the presence of two double bonds ( $\delta_{\text{C}}$  106.6, 141.3, 148.5, and 156.3) and two oxygenated carbons ( $\delta_{\text{C}}$  74.7 and 75.9), but no carbonyl groups. Further analysis of its  $^1\text{H}$  and 2D NMR spectra indicated that **4** was a C<sub>29</sub> steroid, structurally similar to **3** and 9 $\alpha$ -hydroxy-15-oxoconicasterol [11] and shared the same A ring and side chain from C-20 to C-28 with **3** and the same B, C, and D rings with 9 $\alpha$ -hydroxy-15-oxoconicasterol based on the similar NMR patterns of the involved protons and carbons, which were reaffirmed by the HMBC spectrum (Supporting Information Figure S30). The relative stereochemistry of **4** was established to be the same as that of **3** by NMR comparison and the biosynthetic reasoning that **4** undergoes reduction of the carbonyl at C-15 and subsequently oxidative cleavage of the  $\Delta^{8(14)}$  double bond to form compound **3**. Accordingly, the structure of **4** was unequivocally characterized.

Five known compounds, 3-oxo-*ent*-trachyloban-17-oic acid (**5**) [12], 9,19-cyclo-24-nor-5 $\alpha$ ,9 $\beta$ -cholane-3 $\beta$ ,23-diol (**6**) [13], 9 $\beta$ ,19-cyclo-4,4,14 $\alpha$ -trimethyl-3 $\beta$ -hydroxy-5 $\alpha$ -pregnan-20-one (**7**) [14], 3 $\beta$ -hydroxy-22,23,24,25,26,27-hexanordammarane-20-one (**8**) [15], and phytol (**9**) [16] were also isolated. Their structures were identified on the basis of the NMR and ESIMS data.

Protein tyrosine phosphatase 1B (PTP1B) plays a major role in metabolic signaling pathways, making it ideal as a therapeutic drug target for type 2 diabetes and obesity. In addition, triterpenoids as PTP1B inhibitors have recently been reported as potential agents for

the chemoprevention and therapy of breast cancer [17]. Euphyperins A (**1**) and B (**2**) were tested *in vitro* for their inhibitory effect on PTP1B activity [18], using oleanolic acid (purity > 97%; Sigma-Aldrich Co., St Louis, MO, USA) as the positive control ( $\text{IC}_{50}$  value: 1.05  $\mu\text{g/mL}$ ). Euphyperin A (**1**) displayed moderate PTP1B inhibitory activity with an  $\text{IC}_{50}$  value of  $17.05 \pm 1.12$   $\mu\text{g/mL}$ . Euphyperins A (**1**) and B (**2**) were also evaluated for their *in vitro* inhibition against XBP1 mRNA splicing, which is an important therapeutic target in cancer treatment, but neither was active.

## Experimental

**General experimental procedures:** Optical rotations, Perkin-Elmer 341 polarimeter; UV, Shimadzu UV-2550 spectrophotometer; IR, Perkin Elmer 577 IR spectrometer; NMR, Bruker AM-400 or AM-500 NMR spectrometers; ESIMS and HR-ESIMS were performed on a Bruker Daltonics Esquire 3000 plus and a Waters-Micromass Q-TOF Ultima Global mass spectrometer, respectively. EIMS and HR-EIMS were obtained on a Finnigan MAT-95 mass spectrometer. Semi-preparative HPLC was carried out on a Waters 1525 binary pump system with a Waters 2489 UV detector and a YMC-Pack ODS-A column (250  $\times$  10 mm, S-5  $\mu\text{m}$ ). Silica gel (300  $\times$  400 mesh, Qingdao Marine Chemical Co. Ltd), C<sub>18</sub> reversed-phase silica gel (150  $\times$  200 mesh, Merck), CHP20P MCI gel (75–150  $\mu\text{m}$ , Mitsubishi Chemical Industries, Ltd.), D101-macroporous absorption resin (Sinopharm Chemical Reagent Co. Ltd, Shanghai, People's Republic of China), and Sephadex LH-20 gel (Amersham Biosciences) were used for column chromatography (CC), and precoated silica gel GF<sub>254</sub> plates (Qingdao Marine Chemical Co. Ltd) for TLC. All solvents used for CC were of analytical grade (Shanghai Chemical Reagents Co. Ltd), and all solvents used for HPLC were of HPLC grade (J&K Scientific Ltd.).

**Plant material:** The twigs and leaves of *Euphorbia hypericifolia* were collected in September 2011 from Guangxi Province, People's Republic of China, and were authenticated by Professor Shao-Qing Tang of Guangxi Normal University. A voucher specimen (accession number: EuH-2011-1Y) has been deposited in Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

**Extraction and isolation:** Air-dried powder of *E. hypericifolia* (5 kg) was extracted with 95% EtOH, 3 times, and the resulting residue was suspended in H<sub>2</sub>O and partitioned between H<sub>2</sub>O and EtOAc to give a crude extract (630 g). This was subjected to CC (D101-macroporous absorption resin, eluted with 30%, 80%, and 90% EtOH in H<sub>2</sub>O) to obtain the major part (240 g) as monitored by TLC. This part was subjected to an MCI gel column (MeOH/H<sub>2</sub>O, 50 to 100%) to give 5 major fractions, A–E. Fraction D was subjected to silica gel CC eluted with light petroleum/acetone (20:1 to 1:1, v/v) to afford 8 fractions (D1–D8). Fraction D4 (10:1 to 8:1) was separated over a column of C<sub>18</sub> reversed-phase silica gel (MeOH/H<sub>2</sub>O, 60% to 85%) to give 5 sub-fractions (D4a–D4e). Fraction D4e was purified over a silica gel column eluted with light petroleum/ethyl acetate (5:1 to 3:1) to give 2 fractions D4e1 and D4e2, each of which was further purified on a column of Sephadex LH-20 eluted with ethanol to produce compounds **2** (10 mg) and **3** (3 mg). In similar procedures, fraction D4b yielded compound **5** (3 mg). Of fraction D5 (8:1 to 5:1), sub-fraction D5c2 was purified over a column of Sephadex LH-20 to yield compound **4** (4 mg), and sub-fraction D5c3 by semi-preparative HPLC with a mobile phase of 80% CH<sub>3</sub>CN in H<sub>2</sub>O to yield compound **1** (5 mg). Fraction D2 (16:1 to 12:1) was chromatographed on a column of C<sub>18</sub> reversed-phase silica gel (MeOH/H<sub>2</sub>O, 65% to 85%) to give 3 major fractions, each of which was separated by semi-preparative HPLC

(75% CH<sub>3</sub>CN in H<sub>2</sub>O) to produce compounds **6** (12 mg), **7** (5 mg), and **8** (4 mg). Fraction E was purified over a column of silica gel (light petroleum/ethyl acetate, 100:1 to 20:1) to afford the major compound **9** (4.2 g).

### Euphyperin A (1)

White amorphous powder.

$[\alpha]_D^{27}$ : +1.5 (*c* 0.14, MeOH).

IR (KBr)  $\nu_{\max}$ : 3423, 2952, 2925, 2866, 1649, 1458, 1381, 1308, 1269, 1038 cm<sup>-1</sup>.

UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 253 (3.74) nm.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

ESI(+)-MS *m/z*: 457.5 [M + H]<sup>+</sup>, 936.1 [2M + Na]<sup>+</sup>; ESI(-)-MS *m/z* 501.8 [M + HCO<sub>2</sub>]<sup>-</sup>; EIMS *m/z*: 456 [M]<sup>+</sup> (52), 438 (28), 423 (44), 302 (30), 387 (37), 252 (93), 191 (100), 95 (30).

HR-EIMS *m/z*: 456.3606 [M]<sup>+</sup> (calcd for C<sub>30</sub>H<sub>48</sub>O<sub>3</sub>, 456.3603).

### Euphyperin B (2)

White amorphous powder.

$[\alpha]_D^{27}$ : +24.9 (*c* 0.33, MeOH).

IR (KBr)  $\nu_{\max}$ : 3500, 2937, 2864, 1701, 1448, 1381, 1362, 1286, 1047, 1003 cm<sup>-1</sup>.

UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 208 (4.10) nm.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

ESI(+)-MS *m/z*: 431.2 [M + H]<sup>+</sup>, 883.5 [2M + Na]<sup>+</sup>.

HR-ESI(+)-MS *m/z*: 453.3351 [M + Na]<sup>+</sup> (calcd for C<sub>28</sub>H<sub>46</sub>O<sub>3</sub>Na, 453.3345).

### Euphyperin C (3)

White amorphous powder.

$[\alpha]_D^{27}$ : -6.8 (*c* 0.18, MeOH).

IR (KBr)  $\nu_{\max}$ : 3479, 3359, 2960, 2964, 2870, 1734, 1697, 1645, 1466, 1454, 1381, 1261, 1061, 1024, 802 cm<sup>-1</sup>.

UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 207 (4.11) nm.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 2.

ESI(+)-MS *m/z*: 483.2 [M + Na]<sup>+</sup>.

HR-ESI(+)-MS *m/z*: 483.3436 [M + Na]<sup>+</sup> (calcd for C<sub>29</sub>H<sub>48</sub>O<sub>4</sub>Na, 483.3450).

### Euphyperin D (4)

White amorphous powder.

$[\alpha]_D^{25}$ : +74.6 (*c* 0.18, MeOH).

IR (KBr)  $\nu_{\max}$ : 3462, 2924, 2852, 1689, 1630, 1468, 1092 cm<sup>-1</sup>.

UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 255 (3.86) nm.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 2.

ESI(+)-MS *m/z*: 443.4 [M + H]<sup>+</sup>, 907.8 [2M + Na]<sup>+</sup>; EIMS *m/z* 442 [M]<sup>+</sup> (48), 424 (97), 406 (18), 391 (28), 302 (23), 272 (29), 177 (70), 123 (100).

HR-EIMS *m/z*: 442.3440 [M]<sup>+</sup> (calcd for C<sub>29</sub>H<sub>46</sub>O<sub>3</sub>, 442.3447).

**Bioassays:** The PTP1B inhibitory activity was measured according to the reported protocol [18]. XBP1 mRNA splicing inhibitory activity was also determined using a reported protocol [19].

**Supporting information:** IR, ESIMS/EIMS, HR-ESIMS/HR-EIMS, 1D and 2D NMR spectra of compounds **1–4** are provided.

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# A Fragmentation Study of Six C<sub>21</sub> Steroidal Aglycones by Electrospray Ionization Ion-Trap Time-of-Flight Tandem Mass Spectrometry

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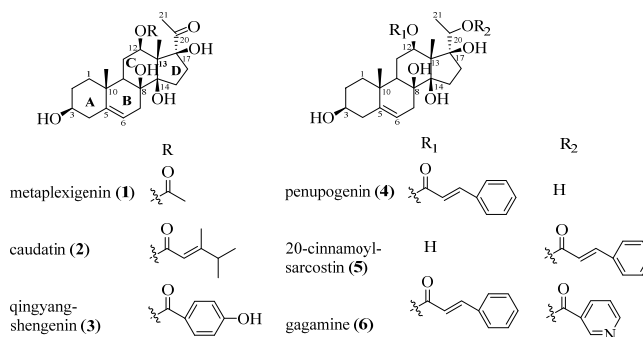
The fragmentation patterns of six C<sub>21</sub> steroidal aglycones, metaplexigenin (**1**), caudatin (**2**), qingyangshengenin (**3**), penupogenin (**4**), 20-cinnamoylsarcostin (**5**), and gagamine (**6**), were analyzed by high-resolution electrospray ionization ion-trap time-of-flight tandem mass spectrometry (HR-ESI-IT-TOF-MS<sup>n</sup>). The [M-H]<sup>−</sup> ions of steroids **1-3** that contain a carbonyl functional group at C-20 (Type I) and [M+H]<sup>+</sup> ions of steroids **5-6** that possess a hydroxyl group at C-20 (Type II) were readily observed in MS analyses. The fragmentation pathways and diagnostic fragment ions for these six steroidal aglycones were proposed on the basis of their MS<sup>n</sup> analyses. The common fragmentation pathways for type I steroidal aglycones include the neutral loss of the ester group at C-12 and the hydroxyl moieties on the steroid skeleton, as well as the cleavage of ring D. Their diagnostic fragment ions were identified as *m/z* 361 (**B**), 343 (**C**), 325 (**D**), 307 (**F**), 283 (**G**), 259 (**E**), and 243 (**H**). The fragmentation behavior of penupogenin (**4**) in type II was similar to those of type I, with *m/z* 363 (**B'**), 345 (**C'**), 327 (**D'**), 309 (**F'**), 283 (**G**), and 243 (**H**) as its diagnostic fragment ions. The ester group at C-20 was difficult to cleave in the MS<sup>n</sup> analyses of 20-cinnamoylsarcostin (**5**) and gagamine (**6**) so that the loss of this ester group was slower than that at C-12 and hydroxyl groups; the key ions at *m/z* 329 (**I**), 311 (**J**), 293 (**K**), and 275 (**L**) were characteristic for **5** and **6**. The base ion peaks were derived from the loss of the substituent group at either C-12 or C-17 for both type I and type II steroidal aglycones.

**Keywords:** ESI-IT-TOF-MS<sup>n</sup>, Fragmentation pathways, Diagnostic ions, C<sub>21</sub> Steroidal aglycones.

C<sub>21</sub> steroids, the major chemical components of the family Asclepiadaceae, have been proved to possess prolific bioactivities, such as antitumor [1], immunosuppressive [2], appetite-suppressant [3], and anti-hepatitis B virus [4,5]. Attention has been paid to the rapid detection and capture of C<sub>21</sub> steroids for bioactivity screening and new drug development. Nevertheless, the isolation and identification of C<sub>21</sub> steroids from natural materials are difficult due to their thermal instability and low abundance in natural materials. It is thus imperative to establish highly sensitive and accurate methods to analyze C<sub>21</sub> steroids.

Recently, electrospray ionization (ESI) techniques linked with multi-stage tandem mass spectrometry (MS<sup>n</sup>) have been extensively applied to analyze natural products including alkaloids [6], steroidal saponins [7], and triterpenoid saponins [8]. ESI-MS<sup>n</sup> techniques make it possible to determine the relationship between a precursor and its fragment ions, by which the fragmentation patterns can be sorted out and natural products in plants may be precisely analyzed [9,10].

Since most naturally-occurring C<sub>21</sub> steroids exist as glycosides, the MS<sup>n</sup> techniques have mainly been used to test the presence of C<sub>21</sub> steroidal glycosides in plants and to establish online methods to analyze the glycoside structures [11-16]. However, no systematic study of the ESI-MS<sup>n</sup> fragmentation of C<sub>21</sub> steroidal aglycones has been reported so far. Additionally, some limitations may arise from the low resolution of mass spectrometers utilized in previous molecular formula predictions [7, 13-16]. In this study, the fragmentation of six C<sub>21</sub> steroidal aglycones was explored using a LC/MS-IT-TOF mass spectrometer equipped with an electrospray ionization source and ion-trap and time-of-flight mass analyzers (ESI-IT-TOF). This mass spectrometer enables fast acquisition of multistage tandem spectra (MS<sup>1-10</sup>) with high accuracy and



**Figure 1:** Structures of compounds **1-6**

resolution. The six steroidal aglycones, metaplexigenin (**1**), caudatin (**2**), qingyangshengenin (**3**), penupogenin (**4**), 20-cinnamoylsarcostin (**5**), and gagamine (**6**) (as shown in Figure 1), are classified into type I that contain a carbonyl group at C-20 (steroids **1-3**) and type II that possess a hydroxyl group at C-20 (steroids **4-6**). A high resolution mass technique was first applied to investigate the fragmentation patterns of C<sub>21</sub> steroidal aglycones that may be used for the characterization of other structure-related steroids.

Prior to the MS<sup>n</sup> analyses, the full-scan mass spectra of steroids **1-6** in both positive and negative ion modes were acquired in automatic pattern. The [M-H]<sup>−</sup> ions for metaplexigenin (**1**), caudatin (**2**), qingyangshengenin (**3**), and penupogenin (**4**) were easily obtained. In contrast, only [M+H]<sup>+</sup> ions for 20-cinnamoylsarcostin (**5**) and gagamine (**6**) were observed. Therefore, the MS<sup>n</sup> investigations for steroids **1-4** in negative mode and for steroids **5-6** in positive mode were chosen, as shown in Tables 1-6. The mass spectra of steroids **1-6** are shown as Figures S1-S6 in the supporting information.

**Table 1:** Data for accurate masses and elemental composition of steroid **1** observed from tandem mass spectra in negative mode

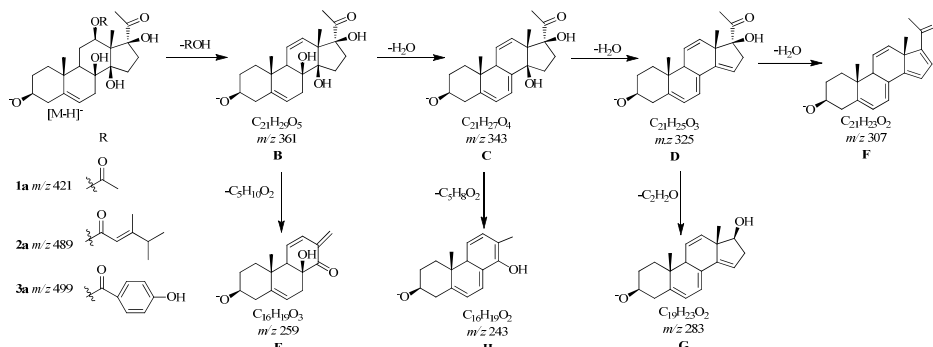
MS <sup>n</sup>	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Elemental composition	Measured ( <i>m/z</i> )	Calculated ( <i>m/z</i> )	Error (mDa)	Intensity (%)	Ion name	Assignment
(-)MS	422	421	C <sub>23</sub> H <sub>33</sub> O <sub>7</sub>	421.2214	421.2232	-1.8	100	<b>1a</b>	[M-H] <sup>-</sup>
MS <sup>2</sup>	421	361	C <sub>21</sub> H <sub>29</sub> O <sub>5</sub>	361.2008	361.2020	-1.2	100	<b>B</b>	<b>1a</b> -C <sub>2</sub> H <sub>4</sub> O <sub>2</sub>
		343	C <sub>21</sub> H <sub>27</sub> O <sub>4</sub>	343.1890	345.1915	-2.5	43	<b>C</b>	<b>B</b> -H <sub>2</sub> O
MS <sup>3</sup>	361	325	C <sub>21</sub> H <sub>25</sub> O <sub>3</sub>	325.1775	325.1809	-3.4	32	<b>D</b>	<b>C</b> -H <sub>2</sub> O
		343	C <sub>21</sub> H <sub>27</sub> O <sub>4</sub>	343.1891	345.1915	-2.4	100	<b>C</b>	<b>B</b> -H <sub>2</sub> O
		325	C <sub>21</sub> H <sub>25</sub> O <sub>3</sub>	325.1787	325.1809	-2.2	83	<b>D</b>	<b>C</b> -H <sub>2</sub> O
		259	C <sub>16</sub> H <sub>19</sub> O <sub>3</sub>	259.1323	259.1340	-1.7	21	<b>E</b>	<b>B</b> -C <sub>3</sub> H <sub>10</sub> O <sub>2</sub>
		343	C <sub>21</sub> H <sub>25</sub> O <sub>3</sub>	325.1787	325.1809	-2.2	100	<b>D</b>	<b>C</b> -H <sub>2</sub> O
	343	307	C <sub>21</sub> H <sub>23</sub> O <sub>2</sub>	307.1670	307.1704	-3.4	16	<b>F</b>	<b>D</b> -H <sub>2</sub> O
		283	C <sub>19</sub> H <sub>23</sub> O <sub>2</sub>	283.1676	283.1704	-2.8	23	<b>G</b>	<b>D</b> -C <sub>2</sub> H <sub>3</sub> O
		243	C <sub>16</sub> H <sub>19</sub> O <sub>2</sub>	243.1371	243.1391	-2.0	44	<b>H</b>	<b>C</b> -C <sub>3</sub> H <sub>5</sub> O <sub>2</sub>
		325	C <sub>21</sub> H <sub>23</sub> O <sub>2</sub>	307.1672	307.1704	-3.2	45	<b>F</b>	<b>D</b> -H <sub>2</sub> O
		283	C <sub>19</sub> H <sub>23</sub> O <sub>2</sub>	283.1686	283.1704	-1.8	100	<b>G</b>	<b>D</b> -C <sub>2</sub> H <sub>3</sub> O
MS <sup>4</sup>	307	291	C <sub>20</sub> H <sub>19</sub> O <sub>2</sub>	291.1373	291.1391	-1.8	100	<b>1k</b>	<b>F</b> -CH <sub>4</sub>
		265	C <sub>19</sub> H <sub>21</sub> O	265.1582	265.1598	-1.6	56	<b>1l</b>	<b>F</b> -C <sub>2</sub> H <sub>3</sub> O
	283	265	C <sub>19</sub> H <sub>21</sub> O	265.1574	265.1598	-2.4	100	<b>1l</b>	<b>G</b> -H <sub>2</sub> O

**Table 2:** Data for accurate masses and elemental composition of steroid **2** observed from tandem mass spectra in negative mode

MS <sup>n</sup>	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Elemental composition	Measured ( <i>m/z</i> )	Calculated ( <i>m/z</i> )	Error (mDa)	Intensity (%)	Ion name	Assignment
(-)MS	490	489	C <sub>28</sub> H <sub>41</sub> O <sub>7</sub>	489.2853	489.2858	-0.5	100	<b>2a</b>	[M-H] <sup>-</sup>
MS <sup>2</sup>	489	361	C <sub>21</sub> H <sub>29</sub> O <sub>5</sub>	361.1993	361.2020	-2.7	100	<b>B</b>	<b>2a</b> -C <sub>7</sub> H <sub>12</sub> O <sub>2</sub>
		343	C <sub>21</sub> H <sub>27</sub> O <sub>4</sub>	343.1878	345.1915	-3.7	32	<b>C</b>	<b>B</b> -H <sub>2</sub> O
MS <sup>3</sup>	361	325	C <sub>21</sub> H <sub>25</sub> O <sub>3</sub>	325.1771	325.1809	-3.8	23	<b>D</b>	<b>C</b> -H <sub>2</sub> O
		343	C <sub>21</sub> H <sub>27</sub> O <sub>4</sub>	343.1882	345.1915	-3.3	100	<b>C</b>	<b>B</b> -H <sub>2</sub> O
		325	C <sub>21</sub> H <sub>25</sub> O <sub>3</sub>	325.1776	325.1809	-3.3	93	<b>D</b>	<b>C</b> -H <sub>2</sub> O
		259	C <sub>16</sub> H <sub>19</sub> O <sub>3</sub>	259.1318	259.1340	-2.2	20	<b>E</b>	<b>B</b> -C <sub>3</sub> H <sub>10</sub> O <sub>2</sub>
		343	C <sub>21</sub> H <sub>25</sub> O <sub>3</sub>	325.1778	325.1809	-3.1	100	<b>D</b>	<b>C</b> -H <sub>2</sub> O
	343	307	C <sub>21</sub> H <sub>23</sub> O <sub>2</sub>	307.1664	307.1704	-4.0	12	<b>F</b>	<b>D</b> -H <sub>2</sub> O
		283	C <sub>19</sub> H <sub>23</sub> O <sub>2</sub>	283.1678	283.1704	-2.6	29	<b>G</b>	<b>D</b> -C <sub>2</sub> H <sub>3</sub> O
		243	C <sub>16</sub> H <sub>19</sub> O <sub>2</sub>	243.1360	243.1391	-3.1	33	<b>H</b>	<b>C</b> -C <sub>3</sub> H <sub>5</sub> O <sub>2</sub>
		325	C <sub>21</sub> H <sub>23</sub> O <sub>2</sub>	307.1664	307.1704	-4.0	66	<b>F</b>	<b>D</b> -H <sub>2</sub> O
		283	C <sub>19</sub> H <sub>23</sub> O <sub>2</sub>	283.1669	283.1704	-3.5	100	<b>G</b>	<b>D</b> -C <sub>2</sub> H <sub>3</sub> O
MS <sup>4</sup>	307	291	C <sub>20</sub> H <sub>19</sub> O <sub>2</sub>	291.1415	291.1391	+2.4	100	<b>2i</b>	<b>F</b> -CH <sub>4</sub>
		265	C <sub>19</sub> H <sub>21</sub> O	265.1580	265.1598	-1.8	56	<b>2j</b>	<b>F</b> -C <sub>2</sub> H <sub>3</sub> O
	283	265	C <sub>19</sub> H <sub>21</sub> O	265.1515	265.1598	-8.3	88	<b>2j</b>	<b>G</b> -H <sub>2</sub> O

**Table 3:** Data for accurate masses and elemental composition of steroid **3** observed from tandem mass spectra in negative mode

MS <sup>n</sup>	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Elemental composition	Measured ( <i>m/z</i> )	Calculated ( <i>m/z</i> )	Error (mDa)	Intensity (%)	Ion name	Assignment
(-)MS	500	499	C <sub>28</sub> H <sub>35</sub> O <sub>8</sub>	499.2344	499.2337	+0.7	100	<b>3a</b>	[M-H] <sup>-</sup>
MS <sup>2</sup>	499	361	C <sub>21</sub> H <sub>29</sub> O <sub>5</sub>	361.2005	361.2020	-1.5	26	<b>B</b>	<b>3a</b> -C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>
		343	C <sub>21</sub> H <sub>27</sub> O <sub>4</sub>	343.1885	345.1915	-3.0	85	<b>C</b>	<b>B</b> -H <sub>2</sub> O
MS <sup>3</sup>	361	325	C <sub>21</sub> H <sub>25</sub> O <sub>3</sub>	325.1781	325.1809	-2.8	100	<b>D</b>	<b>C</b> -H <sub>2</sub> O
		343	C <sub>21</sub> H <sub>27</sub> O <sub>4</sub>	343.1887	345.1915	-2.8	100	<b>C</b>	<b>B</b> -H <sub>2</sub> O
		325	C <sub>21</sub> H <sub>25</sub> O <sub>3</sub>	325.1777	325.1809	-3.2	52	<b>D</b>	<b>C</b> -H <sub>2</sub> O
		259	C <sub>16</sub> H <sub>19</sub> O <sub>3</sub>	259.1324	259.1340	-1.6	61	<b>E</b>	<b>B</b> -C <sub>3</sub> H <sub>10</sub> O <sub>2</sub>
		343	C <sub>21</sub> H <sub>25</sub> O <sub>3</sub>	325.1786	325.1809	-2.3	100	<b>D</b>	<b>C</b> -H <sub>2</sub> O
	343	307	C <sub>21</sub> H <sub>23</sub> O <sub>2</sub>	307.1675	307.1704	-2.9	14	<b>F</b>	<b>D</b> -H <sub>2</sub> O
		283	C <sub>19</sub> H <sub>23</sub> O <sub>2</sub>	283.1683	283.1704	-2.1	40	<b>G</b>	<b>D</b> -C <sub>2</sub> H <sub>3</sub> O
		243	C <sub>16</sub> H <sub>19</sub> O <sub>2</sub>	243.1371	243.1391	-2.0	45	<b>H</b>	<b>C</b> -C <sub>3</sub> H <sub>5</sub> O <sub>2</sub>
		325	C <sub>21</sub> H <sub>23</sub> O <sub>2</sub>	307.1675	307.1704	-2.9	100	<b>F</b>	<b>D</b> -H <sub>2</sub> O
		283	C <sub>19</sub> H <sub>23</sub> O <sub>2</sub>	283.1684	283.1704	-2.0	31	<b>G</b>	<b>D</b> -C <sub>2</sub> H <sub>3</sub> O
MS <sup>4</sup>	283	281	C <sub>19</sub> H <sub>21</sub> O <sub>2</sub>	281.1534	281.1547	-1.3	10	<b>3i</b>	<b>D</b> -C <sub>2</sub> H <sub>3</sub> O
		265	C <sub>19</sub> H <sub>21</sub> O	265.1558	265.1598	-4.0	50	<b>3j</b>	<b>G</b> -H <sub>2</sub> O
	281	265	C <sub>18</sub> H <sub>17</sub> O <sub>2</sub>	265.1233	265.1234	-0.1	100	<b>3j'</b>	<b>3i</b> -CH <sub>4</sub>
		259	C <sub>18</sub> H <sub>19</sub> O <sub>2</sub>	231.1363	231.1391	-2.8	100	<b>3k</b>	<b>E</b> -CO

**Figure 2:** Proposed common fragmentation pathways for steroids **1-3**.

**Common fragmentation pathways for type I steroids:** In the single-stage mass spectra, the [M-H]<sup>-</sup> ions for type I C<sub>21</sub> steroidal aglycones **1-3** were observed at *m/z* 421.2214 (**1a**), 489.2853 (**2a**), and 499.2344 (**3a**), corresponding to the molecular formulas of C<sub>23</sub>H<sub>33</sub>O<sub>7</sub>, C<sub>28</sub>H<sub>41</sub>O<sub>7</sub> and C<sub>28</sub>H<sub>35</sub>O<sub>8</sub>, respectively. These three

[M-H]<sup>-</sup> ions were selected as precursor ions to perform MS<sup>2</sup> experiments, from which the common ion at *m/z* 361 (**B**) was acquired as the base ion peak due to the loss of ROH (R= acetyl, tigloyl and *p*-hydroxybenzoyl) at C-12. Additionally, fragment ions **C** (*m/z* 343) and **D** (*m/z* 325) were also detected, which may be



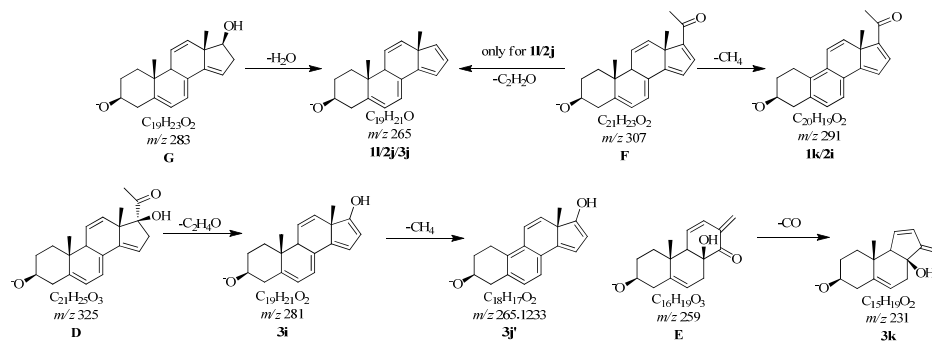


Figure 3: Other fragmentation pathways of compounds 1-3.

Table 4: Data for accurate masses and elemental composition of steroid 4 observed from tandem mass spectra in negative mode

MS <sup>n</sup>	Precursor ion ( $m/z$ )	Product ion ( $m/z$ )	Elemental composition	Measured ( $m/z$ )	Calculated ( $m/z$ )	Error (mDa)	Intensity (%)	Ion name	Assignment
(-)MS	512	511	$C_{30}H_{39}O_7$	511.2663	511.2701	-3.8	100	<b>4a</b>	[M-H]
MS <sup>2</sup>	511	363	$C_{21}H_{31}O_5$	363.2148	363.2177	-2.9	100	<b>B'</b>	<b>4a</b> - $C_9H_8O_2$
MS <sup>3</sup>	363	345	$C_{21}H_{29}O_4$	345.1960	345.2071	-11.1	17	<b>C'</b>	<b>B'</b> - $H_2O$
		327	$C_{21}H_{27}O_3$	327.1895	327.1966	-7.1	29	<b>D'</b>	<b>C'</b> - $H_2O$
		309	$C_{21}H_{25}O_2$	309.1812	309.1860	-4.8	29	<b>F'</b>	<b>D'</b> - $H_2O$
		299	$C_{19}H_{23}O_3$	299.1630	299.1653	-2.3	100	<b>4i</b>	<b>B'</b> - $C_2H_4O_2$
		283	$C_{19}H_{21}O_2$	283.1670	283.1704	-3.4	54	<b>G</b>	<b>D'</b> - $C_2H_4O$
		243	$C_{16}H_{19}O_2$	243.1316	243.1391	-7.5	17	<b>H</b>	<b>C'</b> - $C_5H_{10}O_2$
MS <sup>4</sup>	299	281	$C_{19}H_{21}O_2$	281.1519	281.1547	-2.8	100	<b>4j</b>	<b>4i</b> - $H_2O$

ascribed to the sequential elimination of 8-OH and 14-OH from **B**. To investigate further the fragmentation patterns of ions **B**, **C** and **D**, they were chosen as precursor ions in MS<sup>3</sup> analyses to generate ions **C** to **H**. Ions **B-H** were identified as the key fragment ions for steroids **1-3**, of which ion **E** at  $m/z$  259 was derived from ion **B** through ring D cleavage of the C<sub>21</sub> skeleton as  $C_5H_{10}O_2$  segment. Ion **F** at  $m/z$  307 was generated from ion **D** via the removal of one molecule of H<sub>2</sub>O from 17-OH and 16-H. Ion **G** also originated from ion **D** through the elimination of 17-OAc as a C<sub>2</sub>H<sub>2</sub>O segment and ion **H** could be generated by the cleavage of ring D as a C<sub>5</sub>H<sub>8</sub>O<sub>2</sub> segment in precursor ion **C** (Figure 2).

**Other fragmentation pathways for steroids 1-3:** The other fragmentation pathways of steroids **1-3** were mainly involved in methyl group loss on the C<sub>21</sub> steroidal skeleton. Ions at  $m/z$  291 (**1k/2i**) and  $m/z$  265 (**11/2j**) were both obtained in MS<sup>4</sup> experiments of precursor ions **F** and **G** for metaplexigenin (**1**) and caudatin (**2**). The 10-Me and 17-OAc in ion **F** were eliminated as CH<sub>4</sub> and C<sub>2</sub>H<sub>2</sub>O segments to produce **1k/2i** at  $m/z$  291 and **11/2j** at  $m/z$  265, respectively. The latter ion can also be generated from ion **G** by losing 17-OH. The ion **3j** at  $m/z$  265 for qingyangshengenin (**3**) was also generated from ion **G** in the MS<sup>4</sup> analysis. It was also found that precursor ion **D** can give rise to a peculiar ion at  $m/z$  281 (**3i**) corresponding to the departure of 17-OAc as a C<sub>2</sub>H<sub>4</sub>O segment in MS<sup>3</sup> investigation. Removal of the methyl group at C-10 in ion **3i** as a CH<sub>3</sub> segment yielded a fragment at  $m/z$  265.1233 (**3j'**), ascribed with the molecular formula C<sub>18</sub>H<sub>17</sub>O<sub>2</sub>, which was different from the ion **3j** ( $m/z$  265.1558, C<sub>19</sub>H<sub>21</sub>O). Precursor ion **E** lost one molecule of CO to afford ion **3k** at  $m/z$  231 in MS<sup>4</sup> experiment, and such a fragmentation pattern demonstrated the mechanism that ring D cleavage in ion **B** generated ion **E** by forming a carbonyl on ring C (Figure 3).

**Fragmentation pathways for steroid 4:** The fragmentation pathways for penupogenin (**4**) were similar to that of type **I** C<sub>21</sub> steroid aglycones. Its single-stage MS analysis led to the detection of a [M-H]<sup>-</sup> ion at  $m/z$  511.2663 (**4a**) as the base ion peak in negative mode with the chemical composition of C<sub>30</sub>H<sub>39</sub>O<sub>7</sub>. Ion **4a** lost the cinnamoyl group at C-12 as one molecule of cinnamic acid to afford ion **B'** at  $m/z$  363 in MS<sup>2</sup> analysis. The mass of fragment **B'** was 2 Da more than that of ion **B** owing to the presence of a

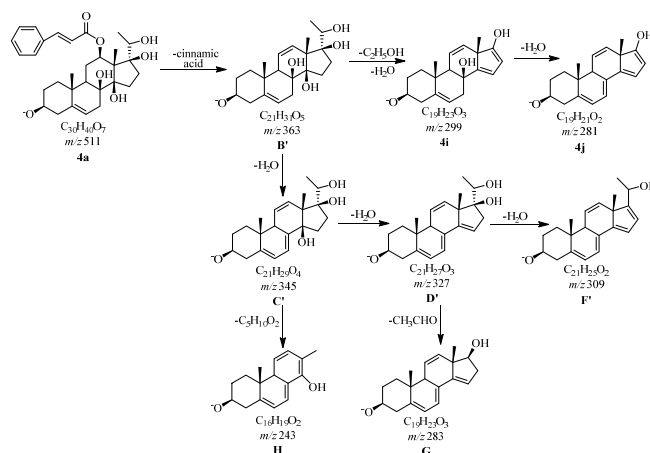


Figure 4: Proposed fragmentation pathways for compound 4.

hydroxyl group at C-20, and so the product ions of **B'** were ions **C'** at  $m/z$  345, **D'** at  $m/z$  327, and **F'** at  $m/z$  309 in MS<sup>3</sup> experiment, which could be interpreted by the same fragmentation routes as ions **C** ( $m/z$  343), **D** ( $m/z$  325) and **F** ( $m/z$  307). Furthermore, ions **G** at  $m/z$  283 and **H** at  $m/z$  243 were also found whose molecular formula and fragmentation pathways were similar to the above explanations, as described in Figure 2. In addition to ions **C'**, **D'**, **F'**, **G**, and **H**, ion **4i** at  $m/z$  299 was obtained as well due to the loss of the substituent group at C-17 as CH<sub>3</sub>CH<sub>2</sub>OH and of the hydroxyl group at C-14 as H<sub>2</sub>O. Ultimately, **4i** eliminated 8-OH to produce ion **4j** at  $m/z$  281 in the MS<sup>4</sup> experiment (Figure 4).

**Fragmentation pathways of steroids 5-6:** Fragmentation of 20-cinnamoylsarcostin (**5**) and gagamine (**6**) gave rise to [M+H]<sup>+</sup> ions **5a** at  $m/z$  513.2789 (C<sub>30</sub>H<sub>41</sub>O<sub>7</sub>) and **6a** at  $m/z$  618.3006 (C<sub>36</sub>H<sub>44</sub>NO<sub>8</sub>), in the first-stage mass spectrum. Due to the lack of an ester group at C-12, **5a** departed 8-OH to yield ion **5b** at  $m/z$  495 in MS<sup>2</sup> investigation. Additionally, ions **5c** at  $m/z$  477, **5d** at  $m/z$  459, **I** at  $m/z$  329, **J** at  $m/z$  311, and **K** at  $m/z$  293 were also detected. **5c** was generated from **5b** because of the loss of 7-OH, while **5d** was yielded from **5c** due to the loss of one molecule of H<sub>2</sub>O at C-14 and C-15. The loss of the cinnamoyl substituent at C-20 as cinnamic acid in **5c** and **5d** led to the generation of ions **I** and **J**, respectively.

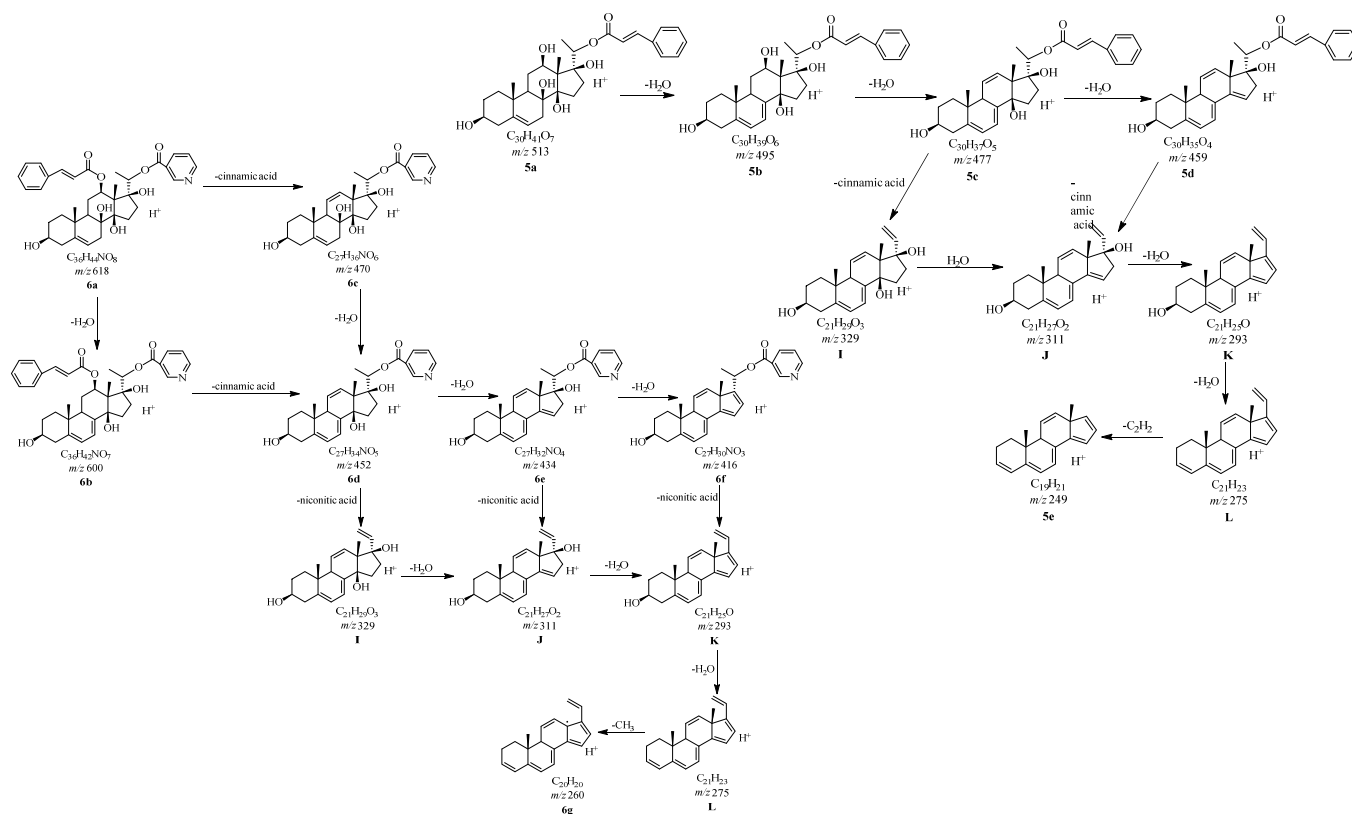


Figure 5: Proposed fragmentation pathways of ss 5-6

Table 5: Data for accurate masses and elemental composition of steroid **5** observed from tandem mass spectra in positive mode

MS <sup>n</sup>	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Elemental composition	Measured ( <i>m/z</i> )	Calculated ( <i>m/z</i> )	Error (mDa)	Intensity (%)	Ion name	Assignment
(+MS)	512	513	C <sub>30</sub> H <sub>41</sub> O <sub>7</sub>	513.2798	513.2847	-4.9	100	<b>5a</b>	[M+H] <sup>+</sup>
		495	C <sub>30</sub> H <sub>39</sub> O <sub>6</sub>	495.2675	495.2741	-6.6	43	<b>5b</b>	<b>5a</b> -H <sub>2</sub> O
		477	C <sub>30</sub> H <sub>37</sub> O <sub>5</sub>	477.2572	477.2636	-6.4	43	<b>5c</b>	<b>5b</b> -H <sub>2</sub> O
		459	C <sub>30</sub> H <sub>35</sub> O <sub>4</sub>	459.2476	459.2530	-5.4	17	<b>5d</b>	<b>5c</b> -H <sub>2</sub> O
		329	C <sub>21</sub> H <sub>29</sub> O <sub>3</sub>	329.2074	329.2111	-3.7	100	<b>I</b>	<b>5c</b> -cinnamic acid
		311	C <sub>21</sub> H <sub>27</sub> O <sub>2</sub>	311.1981	311.2006	-2.5	83	<b>J</b>	<b>I</b> -H <sub>2</sub> O or <b>5d</b> -cinnamic acid
		293	C <sub>21</sub> H <sub>25</sub> O	293.1867	293.1900	-3.3	26	<b>K</b>	<b>J</b> -H <sub>2</sub> O
MS <sup>3</sup>	477	459	C <sub>30</sub> H <sub>35</sub> O <sub>4</sub>	459.2454	459.2530	-7.6	28	<b>5d</b>	<b>5c</b> -H <sub>2</sub> O
		329	C <sub>21</sub> H <sub>29</sub> O <sub>3</sub>	329.2074	329.2111	-3.7	100	<b>I</b>	<b>5c</b> -CINN
		311	C <sub>21</sub> H <sub>27</sub> O <sub>2</sub>	311.1970	311.2006	-3.6	100	<b>J</b>	<b>I</b> -H <sub>2</sub> O or <b>5d</b> -cinnamic acid
		293	C <sub>21</sub> H <sub>25</sub> O	293.1872	293.1900	-2.8	45	<b>K</b>	<b>J</b> -H <sub>2</sub> O
		311	C <sub>21</sub> H <sub>27</sub> O <sub>2</sub>	311.1987	311.2006	-1.9	100	<b>J</b>	<b>5d</b> -cinnamic acid
		293	C <sub>21</sub> H <sub>25</sub> O	293.1885	293.1900	-1.5	80	<b>K</b>	<b>J</b> -H <sub>2</sub> O
		329	C <sub>21</sub> H <sub>29</sub> O <sub>3</sub>	329.2000	329.2006	-0.6	100	<b>J</b>	<b>I</b> -H <sub>2</sub> O
MS <sup>4</sup>	311	293	C <sub>21</sub> H <sub>25</sub> O	293.1864	293.1900	-3.6	54	<b>K</b>	<b>J</b> -H <sub>2</sub> O
		293	C <sub>21</sub> H <sub>25</sub> O	293.1855	293.1900	-4.5	100	<b>K</b>	<b>J</b> -H <sub>2</sub> O
		275	C <sub>21</sub> H <sub>23</sub>	275.1821	275.1794	+2.7	44	<b>L</b>	<b>K</b> -H <sub>2</sub> O
		249	C <sub>19</sub> H <sub>21</sub>	249.1547	249.1638	-9.1	43	<b>5e</b>	<b>L</b> -C <sub>2</sub> H <sub>2</sub>

The ion **J** might also be originated from ion **I** through the elimination of one molecule of H<sub>2</sub>O at C-14 and C-15. The ion **K** at *m/z* 293 could be interpreted by the hydroxyl group departure at C-17 in ion **J**. Similarly, ions **5d** and **I-K** were all observed in MS<sup>3</sup> analyses of **5c** (*m/z* 477), **5d** (*m/z* 459), and **J** (*m/z* 329). Finally, the MS<sup>4</sup> experiment of ion **J** (*m/z* 311) afforded ions **K** at *m/z* 293, **L** at *m/z* 275, and **5e** at *m/z* 249. Among them, fragment **L** arose from ion **K** because of the departure of 3-OH, and ion **5e** was generated by the elimination of the ethylene moiety at C-17 in ion **L** as a C<sub>2</sub>H<sub>2</sub> segment.

Two dissociation pathways were observed from ion **6a** (*m/z* 618) in MS<sup>2</sup> investigation, leading to the fragments at *m/z* 600 (**6b**) and *m/z* 470 (**6c**) due to the loss of H<sub>2</sub>O and cinnamic acid segments. Further elimination of 20-cinnamoyl and 8-OH in **6b** and **6c** yielded ion **6d**

at *m/z* 452. Elimination of 14-OH in **6d** led to the generation of ion **6e** at *m/z* 434, and the subsequent departure of 17-OH in **6e** produced ion **6f** at *m/z* 416 with a more stable conjugated structure. The above dissociation routes were confirmed by MS<sup>3</sup> experiments of precursor ions **6b**, **6c** and **6d**, in which ions **6d-6f** were all obtained. Apart from ions **6e** and **6f**, two non-nitrogen ions **I** at *m/z* 329 (C<sub>21</sub>H<sub>29</sub>O<sub>3</sub>) and **J** (C<sub>21</sub>H<sub>27</sub>O<sub>2</sub>) at *m/z* 311 were also found from precursor ion **6d**. In addition, the MS<sup>3</sup> analyses of ion **6f** provided ions **K** at *m/z* 293 and **L** at *m/z* 275. Ions **I** to **L** were the common product ions for 20-cinnamoylsarcostin (**5**) and gagamine (**6**), which could be regarded as the diagnostic ions for C<sub>21</sub> steroidal aglycones with the same skeleton, and 14-OH, 17-OH, and 3-OH were eliminated one by one from ions **I** to **L**, resulting in a more stable conjugated system. In MS<sup>4</sup> investigations, precursor ions **6e** and **K** gave rise to versatile fragments, **6f**, **J-L**, and **6g** at *m/z* 260,

**Table 6:** Data for accurate masses and elemental composition of steroid **6** observed from tandem mass spectra in positive mode

MS <sup>n</sup>	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Elemental composition	Measured ( <i>m/z</i> )	Calculated ( <i>m/z</i> )	Error (mDa)	Intensity (%)	Ion name	Assignment
(+)MS	617	618	C <sub>27</sub> H <sub>44</sub> NO <sub>3</sub>	618.3006	618.3061	-5.5	100	<b>6a</b>	[M+H] <sup>+</sup>
MS <sup>2</sup>	618	600	C <sub>27</sub> H <sub>42</sub> NO <sub>7</sub>	600.2879	600.2956	-7.7	8	<b>6b</b>	<b>6a</b> -H <sub>2</sub> O
		470	C <sub>27</sub> H <sub>36</sub> NO <sub>6</sub>	470.2472	470.2537	-6.5	16	<b>6c</b>	<b>6a</b> -cinnamic acid
		452	C <sub>27</sub> H <sub>34</sub> NO <sub>5</sub>	452.2359	452.2431	-7.2	74	<b>6d</b>	<b>6c</b> -H <sub>2</sub> O or <b>6b</b> -cinnamic acid
		434	C <sub>27</sub> H <sub>32</sub> NO <sub>4</sub>	434.2269	434.2326	-5.7	85	<b>6e</b>	<b>6d</b> -H <sub>2</sub> O
		416	C <sub>27</sub> H <sub>30</sub> NO <sub>3</sub>	416.2189	416.2220	-3.1	31	<b>6f</b>	<b>6e</b> -H <sub>2</sub> O
MS <sup>3</sup>	600	452	C <sub>27</sub> H <sub>34</sub> NO <sub>5</sub>	452.2359	452.2431	-7.2	24	<b>6d</b>	<b>6b</b> -cinnamic acid
		434	C <sub>27</sub> H <sub>32</sub> NO <sub>4</sub>	434.2287	434.2326	-3.9	24	<b>6e</b>	<b>6d</b> -H <sub>2</sub> O
		416	C <sub>27</sub> H <sub>30</sub> NO <sub>3</sub>	416.2121	416.2220	-9.9	16	<b>6f</b>	<b>6e</b> -H <sub>2</sub> O
	470	452	C <sub>27</sub> H <sub>34</sub> NO <sub>5</sub>	452.2387	452.2431	-4.4	100	<b>6d</b>	<b>6c</b> -H <sub>2</sub> O
		434	C <sub>27</sub> H <sub>32</sub> NO <sub>4</sub>	434.2295	434.2326	-3.1	67	<b>6e</b>	<b>6d</b> -H <sub>2</sub> O
		416	C <sub>27</sub> H <sub>30</sub> NO <sub>3</sub>	416.2165	416.2220	-5.5	13	<b>6f</b>	<b>6e</b> -H <sub>2</sub> O
	452	434	C <sub>27</sub> H <sub>32</sub> NO <sub>4</sub>	434.2274	434.2326	-5.2	65	<b>6e</b>	<b>6d</b> -H <sub>2</sub> O
		416	C <sub>27</sub> H <sub>30</sub> NO <sub>3</sub>	416.2181	416.2220	-3.9	20	<b>6f</b>	<b>6e</b> -H <sub>2</sub> O
		329	C <sub>21</sub> H <sub>29</sub> O <sub>3</sub>	329.2072	329.2111	-3.9	6	<b>I</b>	<b>6c</b> -nicotinic acid
		311	C <sub>21</sub> H <sub>27</sub> O <sub>2</sub>	311.1979	311.2009	-3.0	69	<b>J</b>	<b>I</b> -H <sub>2</sub> O or <b>6e</b> -nicotinic acid
		293	C <sub>21</sub> H <sub>25</sub> O	293.1867	293.1900	-3.3	100	<b>K</b>	<b>6f</b> -nicotinic acid
MS <sup>4</sup>	434	275	C <sub>21</sub> H <sub>23</sub>	275.1768	275.1794	-2.6	43	<b>L</b>	<b>K</b> -H <sub>2</sub> O
		416	C <sub>27</sub> H <sub>30</sub> NO <sub>3</sub>	416.2221	416.2220	+0.1	14	<b>6f</b>	<b>6e</b> -H <sub>2</sub> O
		311	C <sub>21</sub> H <sub>27</sub> O <sub>2</sub>	311.1982	311.2009	-2.7	36	<b>J</b>	<b>6e</b> -nicotinic acid
		293	C <sub>21</sub> H <sub>25</sub> O	293.1869	293.1900	-2.1	100	<b>K</b>	<b>J</b> -H <sub>2</sub> O or <b>6f</b> -nicotinic acid
	293	275	C <sub>21</sub> H <sub>23</sub>	275.1768	275.1794	-2.6	8	<b>L</b>	<b>K</b> -H <sub>2</sub> O
		260	C <sub>20</sub> H <sub>20</sub>	260.1538	260.1560	-2.2	100	<b>6g</b>	<b>L</b> -CH <sub>3</sub>

among which **6f** and **J** to **L** have been clarified in the MS<sup>3</sup> experiments and the ion **6g** could be explained by the loss of 13-Me as a CH<sub>3</sub> segment (Figure 5).

In conclusion, high resolution MS<sup>n</sup> fragmentation studies of C<sub>21</sub> steroidal aglycones showed characteristic fragmentation patterns different from those of C<sub>21</sub> steroidal glycosides. [M-H]<sup>-</sup> ions for metaplexigenin (**1**), caudatin (**2**), qingyangshengnin (**3**) and penupogenin (**4**), and [M+H]<sup>+</sup> ions for 20-cinnamoylsarcostin (**5**) and gagamine (**6**) were obtained in their single-stage mass spectra. The loss of ROH (R= acetyl, tigloyl, *p*-hydroxybenzoyl, cinnamoyl and nicotinoyl), H<sub>2</sub>O, C<sub>2</sub>H<sub>4</sub>O, C<sub>2</sub>H<sub>2</sub>O and CH<sub>4</sub> was the characteristic fragmentation from the precursor ions due to the presence of ester groups, hydroxyl, 1-*O*-ethyl and methyl groups. In particular, the most active 12-ester group was always first eliminated to give rise to the ion at [M-H-ROH]<sup>-</sup> as the base ion peak for compounds **1-4**. Ions at *m/z* 361 (**B**), 343 (**C**), 325 (**D**), 307 (**F**), 283 (**G**), 259 (**E**), and 243 (**H**) were the common product ions for steroids **1-3**. Steroid **4** displayed similar fragmentation pathways as steroids **1-3** to yield ions at *m/z* 363 (**B'**), 345 (**C'**), 327 (**D'**), 309 (**F'**), 283 (**G**), and 243 (**H**). All these ions could be recognized as the diagnostic ions for structural characterization. The substituent group at C-12 in compounds **5-6** was also the active group, leading to the departure of either a H<sub>2</sub>O or cinnamic acid segment, with the key fragments being *m/z* 329 (**I**), 311(**J**), 293 (**K**), and 275 (**L**). Besides, the substituent group loss at either C-12 or C-17 gave rise to high abundance ions, even as the base ion peak for all six steroids. These findings demonstrate the possible online analytical ability of ESI-IT-TOF tandem mass spectrometry for establishing an effective method to analyze C<sub>21</sub> steroidal alycones.

## Experimental

**General:** Acetonitrile, HPLC grade, was purchased from Merck Co. Ltd., Germany. Deionized water was purified using a MingCheTM-

D 24UV Merck Millipore system (Merck Millipore, Shanghai, China).

Steroids **1-6** were isolated in our laboratory from *Cynanchum auriculatum* Royle ex Wight., *Gymnema yunnanense* Tsiang, and *Marsdenia incisa* P. T. Li et Y. P. Li. Their structures were confirmed by the analysis of their spectroscopic data. Sample solutions with a final concentration of 0.25 mg·mL<sup>-1</sup> were prepared by dissolving each sample in 80% CH<sub>3</sub>CN in H<sub>2</sub>O. The samples were loaded onto the spectrometer via a syringe pump at the flow rate of 5 μL·min<sup>-1</sup>.

**Apparatus and analytical conditions:** MS<sup>n</sup> analyses were conducted on a LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan). Precise mass was corrected by calibration using the sodium trifluoroacetate clusters. The mass resolution was about 10 000 full width at half maximum (FWHM). The Shimadzu Composition Formula Predictor was used to speculate the molecular formula. MS experiments were achieved in automatic pattern, and MS2-5 experiments were performed in manual mode. The ESI-MS analytical conditions were as follows: drying gas pressure, 100.0 kPa; nebulizing gas (N<sub>2</sub>) flow, 0.5 L min<sup>-1</sup>; spray voltage, +4.50/-3.50 kV; detector voltage, 1.60 kV; equipment temperature, 40.0°C; heat block temperature, 200.0°C; curved desolvation line (CDL) temperature, 200.0°C; collision energy, 50%; collision gas (Ar), 50%; precursor ion selected width, *m/z* ±3.0 Da, and selected time, 20 ms; collision induced dissociation (CID) collision time, 30 ms; ion accumulation time, 10 ms; and *q* = 0.251; scan range, *m/z* 100-1000 for MS.

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## Three New Cytotoxic Withanolides from the Chinese Folk Medicine *Physalis angulata*

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Physagulides M-O, three new withanolides (**1-3**), were isolated from the aerial parts of *Physalis angulata* L. Their structures were elucidated through extensive spectroscopic techniques, including 1D and 2D NMR, and HRESIMS. The absolute configurations (22-*R*) of these new compounds were determined by CD analysis. Compounds **1** and **3** showed significant selective cytotoxic activities on the MG-63 cell line, with IC<sub>50</sub> values of 4.28 and 5.44  $\mu$ M, respectively.

**Keywords:** *Physalis angulata*, Withanolides, Cytotoxic activity.

Withanolides are a group of C<sub>28</sub> ergostane-type steroids with a  $\delta$ -lactone oxidized at C-22 and C-26, distributed mainly in plants belonging to the genera *Physalis*, *Withania*, *Datura*, *Hyoscyamus*, *Jaborosa*, *Nicandra* and *Tubocapsicum* of the family Solanaceae [1]. In recent years, withanolides have gained significant scientific interest due to their various structures and notable biological properties [2], including immunosuppression [3], quinine reductase induction [4], antiproliferative [5], anti-inflammation [6], and antitumor activities [7a, b]. *Physalis angulata* L., a widely distributed species throughout the east and southwest regions of China [8], is commonly used as a traditional Chinese medicine for antipyretic, anti-inflammatory and diuretic purposes [9]. Phytochemical research has indicated that withanolides, with diverse structures and significant antitumor activities, are the main constituents of this plant [10]. As a part of our research program on bioactive constituents from Chinese folk medicines, three new withanolides (**1-3**) were isolated from the aerial parts of the title plant. Their structures were elucidated through extensive spectroscopic techniques, including 1D and 2D NMR, and HRESIMS. The absolute configuration (22-*R*) of these new compounds was determined by CD analysis. Antiproliferative activities screen indicated that compounds **1** and **3** had selective significant cytotoxic activity on the MG-63 cell line with IC<sub>50</sub> values of 4.28 and 5.44  $\mu$ M, respectively [11]. Herein, we report the isolation, structure elucidation and cytotoxic activity of these new compounds.

Physagulide M (**1**) was obtained as a white amorphous solid, and its HRESIMS showed a [M+NH<sub>4</sub>]<sup>+</sup> peak at *m/z* 578.3326 (calcd 578.3324) corresponding to a molecular formula of C<sub>31</sub>H<sub>44</sub>O<sub>9</sub>. The strong IR absorptions at 3444 and 1707 cm<sup>-1</sup> indicated the presence of hydroxy groups and an  $\alpha$ ,  $\beta$ -unsaturated ketone [12], which was supported by an UV absorption at 227 nm [13]. The whole features of the <sup>1</sup>H and <sup>13</sup>C NMR (Table 1) data of **1**, especially the five methyl signals at  $\delta_H$  1.08, 1.30, 1.01, 1.86, and 1.93, and a set of carbon signals of an  $\alpha$ ,  $\beta$ -unsaturated ketone ( $\delta_C$  166.5, 122.2 and 149.1), indicated that compound **1** was a withanolide derivative, such as physagulin N [9]. Two characteristic olefinic protons of the  $\alpha$ ,  $\beta$ -unsaturated ketone in ring A seen in the <sup>1</sup>H NMR spectra of many normal withanolides [14] were absent in the spectrum of **1** (Table 1); instead of methylene protons at  $\delta_H$  3.08 (dd, *J* = 15.1, 5.3

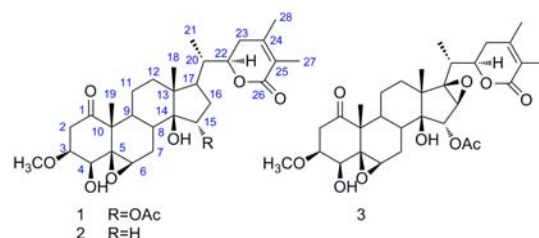


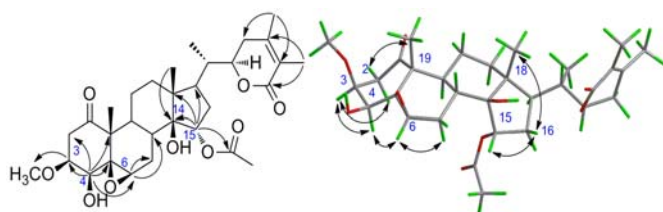
Figure 1: The structures of compounds **1-3**.

Hz, H-2 $\beta$ ), and  $\delta_H$  2.57 (dd, *J* = 15.2, 3.7 Hz, H-2 $\alpha$ ), one more oxymethine proton at  $\delta_H$  3.73 (ddd, *J* = 5.3, 3.7, 3.4 Hz, H-3), and an additional methoxyl signal ( $\delta_H$  3.32, s, 3H) were observed. Moreover, an HMBC correlation between a methoxyl signal at  $\delta_H$  3.32 (s, 3H) and C-3 ( $\delta_C$  77.3) was observed. The aforementioned evidence suggested that the typical  $\Delta^{2,3}$  of normal withanolides was absent and a methoxyl group was located at C-3. The signal at  $\delta_H$  3.51 (d, *J* = 3.4 Hz, H-4) and HMBC correlations between H-4 and C-2 ( $\delta_C$  39.1), C-3 ( $\delta_C$  77.3), C-5 ( $\delta_C$  64.3), and C-10 ( $\delta_C$  50.7) suggested that a hydroxyl group was located at C-4. Signals at  $\delta_H$  3.29 (s, H-6),  $\delta_C$  64.3 (C-5) and 61.8 (C-6) could be assigned for an epoxy group linked at C-5/6 [15], which was proved by the HMBC correlations from H-4 to C-5 and C-6, from H-6 to C-7 ( $\delta_C$  24.5) and C-8 ( $\delta_C$  35.1) (Figure 2). Two olefinic methyl signals at  $\delta_H$  1.86 (3H, s, Me-27) and  $\delta_H$  1.93 (3H, s, Me-28) and a double doublet at  $\delta_H$  4.32 (ddd, *J* = 13, 4.6, 3.5 Hz, H-22) indicated the presence of a typical  $\alpha$ ,  $\beta$ -unsaturated- $\delta$ -lactone in the side chain, which was confirmed by the HMBC correlations shown in Figure 2 [16]. HMBC correlations between H-15 ( $\delta_H$  4.99) and C-14 ( $\delta_C$  84.5), C-13 ( $\delta_C$  46.3), C-17 ( $\delta_C$  52.5) and the carbonyl group ( $\delta_C$  169.8) suggested that an acetoxyl group was located at C-15 ( $\delta_C$  80.6) (Figure 2). The remaining hydroxy group was placed at C-14 ( $\delta_C$  84.5) by the key HMBC correlations from Me-18 and H-15 to C-14 (Figure 2). Thus, the planar structure of **1** was elucidated as shown in Figure 1.

The relative stereochemistry of **1** was established by analysis of the ROESY spectrum [17]. Correlations of H-3 with H-4 and H-2 $\alpha$ , of H-4 with H-6, and of H-2 $\beta$  with Me-19 indicated the  $\alpha$ -orientations of H-3, H-4 and H-6. The  $\beta$ -orientation of H-15 was supported by the ROESY correlations between H-15, Me-18 and H-16 $\beta$  (Figure 2).

**Table 1:**  $^1\text{H}$  (500MHz) and  $^{13}\text{C}$  NMR (125 MHz) data for compounds **1–3** in  $\text{CDCl}_3$  ( $\delta$  in ppm,  $J$  in Hz).

Position	<b>1</b>		<b>2</b>		<b>3</b>	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$
1		209.9		210.2		210.2
2	3.08, dd (15.1, 5.3)	39.1	3.12, dd (14.6, 4.8)	38.9	3.12, dd (15.0, 4.7)	38.9
	2.57, dd (15.2, 3.7)		2.57, dd (14.6, 4.1)		2.57, dd (15.0, 3.8)	
3	3.73, ddd (5.3, 3.7, 3.4)	77.3	3.75, ddd (4.8, 4.1, 3.3)	77.7	3.74, ddd (4.7, 3.8, 3.5)	77.3
4	3.51, d (3.4)	75.0	3.50, d (3.3)	75.2	3.51, d (3.5)	75.0
5		64.3		64.6		64.1
6	3.29, br s	61.8	3.32, br s	62.7	3.27, s	62.1
7	2.51, m	25.9	2.48, m	27.1	2.56, m	25.6
	1.49, m		1.58, m		1.40, m	
8	1.60, m	36.3	1.58, m	35.3	1.58, m	34.4
9	1.82, m	38.5	1.50, m	39.0	1.90, m	37.3
10		50.7		51.0		50.4
11	1.93, m	21.4	1.50, m	21.7	1.59, m	20.6
	1.43, m		1.41, m		1.20, m	
12	1.81, m	41.2	1.44, m	41.5	1.60, m	31.6
	1.59, m		1.31, m		1.59, m	
13		46.3		46.9		46.6
14		84.5		84.8		81.4
15	4.99, d (4.5)	80.6	1.70, m	32.8	5.03, s	77.6
			1.54, m			
16	2.01, m	33.6	1.26, m	29.8	3.48, s	59.0
	1.67, m		1.25, m			
17	1.70, m	52.5	1.51, m	53.1		76.3
18	1.08, s	17.1	1.04, s	16.4	1.12, s	15.6
19	1.30, s	16.0	1.35, s	16.6	1.30, s	16.3
20	2.09, m	37.5	2.10, m	37.9	2.56, m	33.7
21	1.01, d (6.6)	16.1	0.99, d (6.6)	15.8	1.00, d (7.1)	13.4
22	4.32, ddd (13.0, 4.6, 3.5)	78.3	4.48, ddd (13.0, 4.3, 3.5)	78.5	4.50, dt (12.5, 4.0)	76.8
23	2.47, m	31.6	2.46, m	31.5	2.38, m	32.5
	1.96, m		2.03, m		2.13, m	
24		149.1		149.2		149.2
25		122.2		122.1		122.2
26		166.5		166.8		166.3
27	1.86, s	12.5	1.87, s	12.6	1.87, s	12.6
28	1.93, s	20.6	1.93, s	20.6	1.93, s	20.7
OAc	2.05, s	169.8			2.10, s	169.6
		21.8				21.0
OMe	3.32, s	56.8	3.35, s	56.8	3.32, s	56.7

**Figure 2:** The key HMBC and ROESY correlations of compound **1**.

The obvious positive Cotton effect at 249 nm in the ECD spectrum of **1** indicated that the absolute configuration of C-22 was assigned as *R* [18]. On the basis of all the above evidence, the structure of **1** was established as (20*S*, 22*R*)-15 $\alpha$ -acetoxy-5 $\beta$ , 6 $\beta$ -epoxy-4 $\beta$ , 14 $\beta$ -dyhydroxy-3 $\beta$ -methoxy-1-oxowitha-24-en-26, 22-olide, and named physagulide M.

The spectral characteristics of physagulide N (**2**) were very similar to those of **1**, with the major difference being the absence of signals corresponding to the acetoxy group in **1**, consistent with the 58.0061 mass units less than **1** in the HRESIMS. The upfield shifts of the C-15 from  $\delta_{\text{C}}$  80.6 in **1** to 32.8 suggested that there was no acetoxy group linked at C-15. The similar ROESY and ECD spectra indicated that the relative and absolute configuration of **2** were the same as those of **1**. Thus, compound **2** was established as (20*S*, 22*R*)-5 $\beta$ , 6 $\beta$ -epoxy-4 $\beta$ , 14 $\beta$ -dyhydroxy-3 $\beta$ -methoxy-1-oxowitha-24-en-26, 22-olide, and named as physagulide N.

Physagulide O (**3**) gave a molecular formula of  $\text{C}_{31}\text{H}_{42}\text{O}_{10}$  by HRESIMS ( $m/z$  592.3117  $[\text{M}+\text{NH}_4]^+$ , calcd. for 592.3116). Comparison of the NMR data of **3** and **1** indicated that they have

identical A-C rings and side chains. The key difference was the substitution pattern in ring D. A secondary epoxy group linked at C-16/17 was proposed by the NMR signals at  $\delta_{\text{H}}$  3.48 (br s, H-16),  $\delta_{\text{C}}$  59.0 (C-16) and  $\delta_{\text{C}}$  76.3 (s, C-17). This was consolidated by the HMBC spectral analysis, in which H-15 ( $\delta_{\text{H}}$  5.03) was correlated to C-14 ( $\delta_{\text{C}}$  81.4), C-13 ( $\delta_{\text{C}}$  46.6) and C-17 ( $\delta_{\text{C}}$  76.3), and H-16 ( $\delta_{\text{H}}$  3.48) to C-15 ( $\delta_{\text{C}}$  77.6). The orientation of the epoxy group was deduced to be  $\beta$  based on the ROESY cross-peaks for H-16 ( $\delta_{\text{H}}$  3.48) with Me-21 ( $\delta_{\text{H}}$  1.00). The absolute configuration of C-22 was established as *R* through the same ECD Cotton effects of **3** compared with **1** [18]. Thus, the structure of **3** was established as (20*S*, 22*R*)-15 $\alpha$ -acetoxy-5 $\beta$ , 6 $\beta$ :16 $\beta$ , 17 $\beta$ -diepoxy-4 $\beta$ , 14 $\beta$ -dyhydroxy-3 $\beta$ -methoxy-1-oxowitha-24-en-26, 22-olide, and named as physagulide O.

Cytotoxic activities of all compounds were examined against three human cancer cell lines (MG-63, HepG-2, and MDA-MB-231); doxorubicin was used as the positive control with  $\text{IC}_{50}$  values of 0.46, 3.72, and 3.70  $\mu\text{M}$ , respectively. Compounds **1** and **3** demonstrated significant selective cytotoxic activity on MG-63 with  $\text{IC}_{50}$  values of 4.28 and 5.44  $\mu\text{M}$ , respectively (Table 2). From the results of this cytotoxicity evaluation, these compounds may be valuable for cancer therapy.

**Table 2:** Cytotoxicity of compounds **1–3** against three human cancer cell lines.

Compound	$\text{IC}_{50}$ ( $\mu\text{M}$ )		
	MG-63	HepG-2	MDA-MB-231
<b>1</b>	4.28	>20	>20
<b>2</b>	>20	>20	>20
<b>3</b>	5.44	>20	>20
Doxorubicin <sup>a</sup>	0.46	3.72	3.70

<sup>a</sup>Doxorubicin was used as a positive control.

## Experimental

**General:** Optical rotations were measured with a JASCO P-1020 polarimeter. IR data were obtained on a Bruker Tensor 27 spectrometer, UV spectra on a Shimadzu UV-2501 PC spectrophotometer, and 1D and 2D NMR spectra, using CDCl<sub>3</sub> as solvent, on a Bruker Avance III NMR spectrometer at 500 MHz (<sup>1</sup>H) and 125 MHz (<sup>13</sup>C). HRESI mass spectra were collected with an Agilent 6520B UPLC-Q-TOF mass spectrometer. Circular dichroism(CD) spectra were recorded on a JASCO 810 spectropolarimeter. HPLC analysis was run on an Agilent 1200 instrument equipped with a multiple wavelength diode array detector (DAD). Preparative HPLC was performed on a Shimadzu instrument equipped with a Shim-pack RP-C<sub>18</sub> column (20×200 mm<sup>2</sup>, 10 μm), and a flow rate of 10.0 mL/min. Column chromatography (CC) was carried out using macroporous resin D-101 (pore size B 13-14 nm, 26–60 mesh, Qingdao Marine Chemical Co. Ltd., Qingdao, China), Silica gel (100-200 mesh and 200-300 mesh, Qingdao Marine Chemical Co. Ltd., Qingdao, China) and ODS RP-C<sub>18</sub> (40-63μm, Fuji, Japan).

**Plant material:** Whole plants of *Physalis angulata* were collected in August 2014, in Lin Yi, Shan Dong Province, China, and were identified by Professor Zhang Mian of the Research Department of Pharmacognosy, China Pharmaceutical University. A voucher specimen (No.PA-201407-LY) is deposited in the department of Natural Medicinal Chemistry, China Pharmaceutical University.

**Extraction and isolation:** The air-dried aerial parts of *P. angulata* (1 kg) were powdered and extracted with CH<sub>2</sub>Cl<sub>2</sub>-MeOH (1:1) at room temperature, 3 times. After removing the solvent under vacuum, the residue (70 g) was subjected to column chromatography (CC) on D-101 macroporous resin and eluted with a step gradient of EtOH-H<sub>2</sub>O (20:80, 40:60, 60:40, 80:20, 95:5, v/v) to yield 5 fractions: Fr. A-E. Fr. C (7g) was then chromatographed over silica gel with increasing polarities of CH<sub>2</sub>Cl<sub>2</sub>-MeOH (40:1, 20:1, 10:1, 0:100 v/v) to obtain 4 sub-fractions (Fr. C1-C4). Fr. C3 was applied to ODS MPLC eluted with isocratic MeOH-H<sub>2</sub>O (50:50 v/v) to afford 8 sub-fractions (Fr.C3A-H). Fr.C3C was chromatographed over ODS with acetonitrile-H<sub>2</sub>O (35:65, v/v) to give 2 sub-fractions (Fr.C3C 1-2). Fr.C3C1 was applied to preparative HPLC with MeOH-H<sub>2</sub>O (55:45 v/v) to yield **1** (6.2 mg) and **3** (14.7 mg). Fr.C3H was chromatographed over Sephadex LH-20 with MeOH to yield 2 sub-fractions (Fr.C3H 1-2). Fr.C3H1 was subjected to preparative HPLC with MeOH-H<sub>2</sub>O (60:40, v/v) to yield **2** (1.6 mg).

## Physagulide M (1)

White amorphous solid.

$[\alpha]_D^{20}$ : +10.5 (c 0.42, MeOH).

CD:  $\Delta\epsilon_{250}$  +4.92 (c 0.3, MeCN).

IR (KBr): 3444, 2925, 1707, 1383, 1256, 1095 cm<sup>-1</sup>.

UV/Vis  $\lambda_{max}$  (MeOH) nm (log  $\epsilon$ ): 227 (3.95), 206 (3.93).

<sup>1</sup>H and <sup>13</sup>C NMR data: see Table 1.

HRESIMS:  $m/z$  578.3326 [M+NH<sub>4</sub>]<sup>+</sup> (calcd for C<sub>31</sub>H<sub>48</sub>NO<sub>9</sub>, 578.3324).

## Physagulide N (2)

White amorphous solid.

$[\alpha]_D^{20}$ : -1.5 (c 0.18, MeOH).

CD:  $\Delta\epsilon_{250}$  +5.30 (c 0.3, MeCN).

IR (KBr): 3449, 2932, 1702, 1383, 1137 cm<sup>-1</sup>.

UV/Vis  $\lambda_{max}$  (MeOH) nm (log  $\epsilon$ ): 227 (4.21), 207 (4.20).

<sup>1</sup>H and <sup>13</sup>C NMR data: see Table 1.

HRESIMS:  $m/z$  520.3265 [M+NH<sub>4</sub>]<sup>+</sup> (calcd for C<sub>29</sub>H<sub>46</sub>NO<sub>7</sub>, 520.3269).

## Physagulide O (3)

White amorphous solid.

$[\alpha]_D^{20}$ : +21.6 (c 0.13, MeOH).

CD:  $\Delta\epsilon_{250}$  +4.84 (c 0.3, MeCN).

IR (KBr): 3489, 2929, 1707, 1381, 1225, 1097 cm<sup>-1</sup>.

UV/Vis  $\lambda_{max}$  (MeOH) nm (log  $\epsilon$ ): 227(4.58).

<sup>1</sup>H and <sup>13</sup>C NMR data: see Table 1.

HRESIMS:  $m/z$  592.3117 [M+NH<sub>4</sub>]<sup>+</sup> (calcd for C<sub>31</sub>H<sub>46</sub>NO<sub>10</sub>, 592.3116).

**Cytotoxicity assay:** The cytotoxicity bioassay of all new compounds against human cancer cells (MG-63, HepG-2, MDA-MB-231) was determined *in vitro* with the MTT assay, as described previously [19]. Doxorubicin was used as the positive control [20]. All experiments were carried out in triplicate.

**Supplementary data:** NMR spectra (<sup>1</sup>H and <sup>13</sup>C NMR, HSQC, HMBC and ROESY), HRESIMS and CD spectra for the new compounds (**1-3**).

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Diterpenoid Alkaloids from *Aconitum soongaricum* var. *pubescens*Lin Chen<sup>a,b,c</sup>, Lianhai Shan<sup>a</sup>, Jifa Zhang<sup>a</sup>, Wenliang Xu<sup>a</sup>, Mingyu Wu<sup>a</sup>, Shuai Huang<sup>a\*</sup> and Xianli Zhou<sup>a, b\*</sup><sup>a</sup>School of Life Science and Engineering, Southwest Jiaotong University, Chengdu, 610031 China<sup>b</sup>Key Laboratory of Advanced Technologies of Materials, Ministry of Education, School of Material Science and Engineering, Southwest Jiaotong University, Chengdu, 610031 China<sup>c</sup>School of Chemistry and Chemical Engineering, China West Normal University, Nanchong, 637002 China

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One new diterpenoid alkaloid, pubescensine (**1**), along with nine known diterpenoid alkaloids (**2**–**10**) were isolated from the roots of *Aconitum soongaricum* var. *pubescens*. Their structures were elucidated by spectroscopic analyses and comparison with previously reported data. All the compounds were evaluated for their antifeedant activities. The aconitine-type diterpenoid alkaloids (**1**–**6**) showed considerably potent antifeedant activity ( $EC_{50} < 1 \text{ mg/cm}^2$ ), while the activities of napelline-type diterpenoid alkaloids (comps. **7**, **9** and **10**) were not significant ( $EC_{50} > 50 \text{ mg/cm}^2$ ).

**Keywords:** *Aconitum soongaricum* var. *pubescens*, Diterpenoid alkaloids, Antifeedant activity.

Much attention has been paid to diterpenoid alkaloids not only for their complex structures, but also for their biological activities such as anti-inflammatory, analgesic, anti-arrhythmia, antifungal, and cytotoxic properties [1–3], as well as insecticidal and antifeedant activities [4–6]. Recently, in the course of our investigation of new bioactive diterpenoid alkaloids from plants of the genera *Aconitum* and *Delphinium*, we discovered that the crude alkaloid extract from *A. soongaricum* var. *pubescens* roots possesses significant feeding deterrent activity against *Pieris rapae* Linne. Chemical investigation of the active extract led to the isolation of ten diterpenoid alkaloids: pubescensine (**1**), 3-deoxyaconitine (**2**), aconitine (**3**), 15- $\alpha$ -hydroxyneoline (**4**), taurenine (**5**), bullatine B (**6**), songorine (**7**), 15-acetylsongorine (**8**), songoramine (**9**) and 12-*epi*-napelline (**10**) (Figure 1). Among these compounds, **1** is a new diterpenoid alkaloid. In this paper, we report the isolation, structure elucidation and antifeedant activities of these alkaloids.

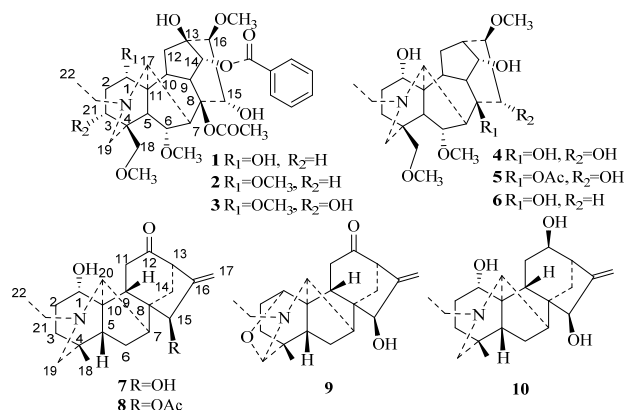


Figure 1: Structures of compounds **1**–**10**.

Pubescensine (**1**) was obtained as a white amorphous powder. Its molecular formula was determined to be  $C_{33}H_{45}NO_{10}$  from the  $[M+H]^+$  peak at  $m/z$  616.3123 (calcd. for  $C_{33}H_{46}NO_{10}$  616.3122) in the HR-ESI-MS. The IR spectrum indicated that **1** possesses hydroxyl ( $3485 \text{ cm}^{-1}$ ) and carbonyl ( $1723 \text{ cm}^{-1}$ ) groups. The  $^{13}\text{C}$ -NMR and DEPT spectra of **1** (Table 1) exhibited the presence of five methylenes ( $\delta_C$  29.5, 30.0, 36.3, 56.2, 79.8), ten methines

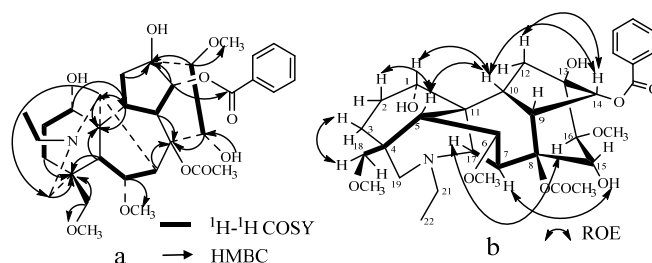


Figure 2: Key  $^1\text{H}$ - $^1\text{H}$  COSY, HMBC (a) and ROESY (b) correlations of **1**.

( $\delta_C$  39.6, 43.3, 43.6, 44.0, 62.9, 71.9, 78.7, 79.1, 83.7, 89.8), and four quaternary carbons ( $\delta_C$  38.1, 49.2, 74.0, 91.9). In addition, three methoxyl groups [ $\delta_H$  3.17(s), 3.31(s), 3.77(s)], an acetyl group [ $\delta_H$  1.42 (s)], a benzoyl group [ $\delta_H$  7.46 (t), 7.58 (t), 8.02 (d)] and a *N*-ethyl group [ $\delta_H$  1.15 (t), 2.44 (m), 2.82 (overlapped)] were present in the structure according to the  $^1\text{H}$  NMR spectrum. The above-mentioned data revealed that compound **1** is an aconitine-type diterpenoid alkaloid [7].

Comparison of the 1D-NMR data of **1** with those of the known alkaloid 3-deoxyaconitine (**2**) [8] indicated that there was a hydroxyl group at C(1) in **1** instead of the methoxyl group in **2**, which was also confirmed by the difference of 14 mass units between the two compounds. The hydroxyl group at C(1) was assigned an  $\alpha$ -orientation based on the signal of H-1 at  $\delta_H$  3.67 (broad singlet) and the resonance of C-1 at  $\delta_C$  71.9 in the NMR spectra [7], which was further supported by the cross-peaks between H-C(1) with H-C(10) and H-C(10) with H-C(14) in the ROESY spectrum. Their chemical shifts and multiplicity (Table 1) suggested that ring A possesses a boat conformation [7]. The complete planar structure of **1** was further verified by the analyses of the HMBC and  $^1\text{H}$ - $^1\text{H}$  COSY spectra (Figure 2). The configuration of **1** could be assigned as  $H_\beta$ -C(1),  $H_\beta$ -C(6),  $H_\beta$ -C(10),  $H_\beta$ -C(14),  $H_\beta$ -C(15) and  $H_\alpha$ -C(16) based on the observation of the related cross-peaks in its ROESY spectrum (Figure 2). Thus, the structure of pubescensine was assigned to be **1**. The structures of compounds **2**–**10** were identified by comparison of their spectral data with those described in the literature [8–16].

**Table 1:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for compound **1**.

Position	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult., $J = \text{Hz}$ )	Position	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult., $J = \text{Hz}$ )
1	71.9 d	3.67 br s	18	79.8 t	a 3.11 ABq (8.4)
2	30.0 t	$\alpha$ 1.62 m			b 3.55 ABq (8.4)
		$\beta$ 1.52 m	19	56.2 t	a 2.30 d (10.8)
3	29.5 t	$\alpha$ 1.26 m			b 2.61 d (10.8)
		$\beta$ 1.90 m	21	48.7 t	a 2.44 m
4	38.1 s	-			b 2.82 <sup>a</sup>
5	44.0 d	2.26 d (6.4)	22	13.0 q	1.15 t (7.2)
6	83.7 d	3.98 d (6.4)	6-OCH <sub>3</sub>	58.1 q	3.17 s
7	43.6 d	2.82 <sup>a</sup>	16-OCH <sub>3</sub>	61.5 q	3.77 s
8	91.9 s	-	18-OCH <sub>3</sub>	59.1 q	3.31 s
9	43.3 d	2.82 <sup>a</sup>	8-OAc	21.4 q	1.42 s
10	39.6 d	2.16 m			-
11	49.2 s	-	14-OBz	165.9 s	-
12	36.3 t	$\alpha$ 2.20 <sup>a</sup>	1'	129.7 s	-
		$\beta$ 2.27 <sup>a</sup>	2', 6'	129.6 d	8.02 d (7.2)
13	74.0 s	-	3', 5'	128.7 d	7.46 t (7.2)
14	79.1 d	4.89 d (4.8)	4'	133.3 d	7.58 t (7.2)
15	78.7 d	4.49 dd (2.7, 5.4)	13-OH	-	3.94 <sup>b</sup> s
16	89.8 d	3.41 d (5.4)	15-OH	-	4.45 <sup>b</sup> d (2.7)
17	62.9 d	2.91 s			

<sup>a</sup> Overlapped signals. <sup>b</sup> The signal disappeared after exchange with D<sub>2</sub>O

The antifeedant activities of the isolated compounds **1–10** were evaluated against *Pieris rapae* Linne (Table 2). The most antifeedant activity was found for compounds **3**, **1** and **2** ( $\text{EC}_{50} < 0.05 \text{ mg/cm}^2$ ), respectively, followed by **6**, **4** and **5** ( $\text{EC}_{50} < 1 \text{ mg/cm}^2$ ). The antifeedant activities of napelline-type diterpenoid alkaloids (**7**, **9** and **10**) were not significant ( $\text{EC}_{50} > 50 \text{ mg/cm}^2$ ). When the antifeedant activities of the compounds were compared for different classes, the napelline-type diterpenoid alkaloids were less active than the aconitine-type diterpenoid alkaloids.

**Table 2:** Antifeedant activities of the compounds isolated from *A. soongaricum* var. *pubescens* against *Pieris rapae* (n=3).

Comps.	$\text{EC}_{50}$ (mg/cm <sup>2</sup> ) (95% confidence limits)	Comps.	$\text{EC}_{50}$ (mg/cm <sup>2</sup> ) (95% confidence limits)
<b>1</b>	0.03 (0.01, 0.11)	<b>6</b>	0.41 (0.09, 2.10)
<b>2</b>	0.05 (0.01, 0.34)	<b>7</b>	>50
<b>3</b>	0.02 (0.01, 0.11)	<b>8</b>	nt
<b>4</b>	0.47 (0.33, 0.70)	<b>9</b>	>50
<b>5</b>	0.66 (0.32, 1.38)	<b>10</b>	>50

nt: not test (insufficient compound available)

## Experimental

**General:** Optical rotations, Perkin-Elmer 341 polarimeter; NMR, Bruker AV600; IR, Thermo Fisher Nicolet 6700; HR-ESI-MS, Waters ACQUITY UPLC/Xevo G2-S QTOF mass spectrometer.

**Plant material:** The roots of *A. soongaricum* Stapf var. *pubescens* were collected in Houxia, Xinjiang Uygur Autonomous Region of China, in August 2014, and were identified (voucher specimen: C. Ren & L. Wang 705) by Prof. Qing-Er Yang of the Institute of Botany, Chinese Academy of Sciences.

**Extraction and isolation:** Dried and powdered roots of *A. soongaricum* var. *pubescens* (5.3 kg) were extracted with 95% EtOH 3 times at rt, for 3 days each time. After removal of the solvent, the extract (2000 g) was suspended in water (3 L) and adjusted to pH 2 with hydrochloric acid solution, and then successively extracted with light petroleum (4×1 L) and ethyl acetate (4×1 L). Then, 28% aq. NH<sub>4</sub>OH soln. (2 L) was added to the aq. soln. to bring it to pH 10. The solutions were extracted with CH<sub>2</sub>Cl<sub>2</sub> (4×1 L). The CH<sub>2</sub>Cl<sub>2</sub> extracts were concentrated to produce the crude alkaloid extract (20 g). Column chromatography (CC) of the crude alkaloid extract over silica gel, using a CH<sub>2</sub>Cl<sub>2</sub>/MeOH

(60:1, v/v) mixture with increasing polarity afforded fractions A–E based on TLC analysis. Fraction A was separated by silica gel CC (light petroleum/Me<sub>2</sub>CO/Et<sub>2</sub>NH, 8:1:0.1, v/v/v) to obtain **2** (3-deoxyaconitine, 23 mg) and **9** (songoramine, 15 mg). CC (silica gel, light petroleum /Me<sub>2</sub>CO/Et<sub>2</sub>NH, 6:1:0.1, v/v/v) of fraction B afforded **7** (songorine, 1.5 g) and **8** (15-acetylsongorine, 2 mg). Fraction C was chromatographed on a silica gel column and eluted with light petroleum/Me<sub>2</sub>CO/Et<sub>2</sub>NH (3:1:0.1:0.1:0.1, v/v/v) to afford **1** (pubescensine, 14 mg), **5** (taurenine, 25 mg) and **3** (aconitine, 300 mg). Fr. D was subjected to CC on silica gel and eluted with light petroleum/CH<sub>2</sub>Cl<sub>2</sub> (1:1- 0:1, v/v) to give **6** (bullatine B, 460 mg). Fr. E was subjected to CC on silica gel and eluted with CH<sub>2</sub>Cl<sub>2</sub>/MeOH (10:1-1:1, v/v) to give **10** (12-epinapelline, 200 mg) and **4** (15- $\alpha$ -hydroxyneoline, 120 mg).

## Pubescensine

White amorphous powder.

$[\alpha]_{\text{D}}^{20}$ : -0.7 (*c* 0.3, CH<sub>2</sub>Cl<sub>2</sub>).

HR-ESI-MS  $m/z$   $[\text{M}+\text{H}]^+$ : 616.3123 (calcd. for C<sub>33</sub>H<sub>46</sub>NO<sub>10</sub> 616.3122).

$^1\text{H}$  (CDCl<sub>3</sub>, 600 MHz) and  $^{13}\text{C}$  NMR (CDCl<sub>3</sub>, 150 MHz): Table 1.

IR (KBr)  $\nu_{\text{max}}$ : 3485, 2927, 2823, 2584, 1723, 1602, 1452, 1382, 1315, 1279, 1240, 1180, 1108, 1027, 984, 960, 919, 897, 710 cm<sup>-1</sup>.

**Antifeedant bioassays:** A *Pieris rapae* colony was reared on cabbage foliage and maintained at  $24 \pm 1^\circ\text{C}$ , > 70% relative humidity with a photoperiod of 16:8 h (L: D) in a growth chamber.

The antifeedant properties of the test compounds were evaluated using the choice leaf-disc method described by González-Coloma *et al.* [17, 18]. Choice experiments were conducted with newly-emerged third-instar larvae of *P. rapae*. Fresh cabbage leaves were cut into leaf discs (2 cm diameter) and then treated on the upper surface with 15  $\mu\text{L}$  of either the test substance emulsions or deionized water containing acetone and Tween-20 (10: 0.012, v/v) for control. After air drying for 1 h, 2 treated leaves and 2 control leaves were arranged alternatively on 2% agar beds (2-3 mm) in 15 cm diameter Petri dishes. Four healthy and starved 3 h instars were placed in each dish and allowed to feed in a growth chamber (environmental conditions as described above). Three replicates were prepared for each treatment. Feeding was terminated after consumption of 50–70% of the control disks, and then the area of leaves consumed was examined with a LI-3000 portable area meter (American Lincoln Co. Ltd). Percent feeding reduction (%FR) was determined for each arena by the equation:

$$\%FR = (CK - T) / CK \times 100$$

Where *CK* and *T* are control leaf disc areas eaten and treated leaf disc areas eaten, respectively.

Compounds with a FR > 50% were tested in a dose-response experiment to calculate their relative potency ( $\text{EC}_{50}$  values, the effective dose for 50% feeding reduction), which was determined from linear regression analysis (%FR on log dose).

**Supplementary data:** NMR, IR and HR-ESI-MS for compound **1**.

**Acknowledgments** - This work was financially supported by the National Natural Science Foundation of China (31171695), the Science and Technology Support Programs of Sichuan Province (2013SZ0083), the Research Foundation for Educational Commission of Sichuan Province (15TD0048 and 15ZB0140) and the Fundamental Research Funds for Central Universities (2682014RC15).

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Two New C<sub>18</sub>-Diterpenoid Alkaloids from *Delphinium anthriscifolium*Lianhai Shan<sup>a,b</sup>, Jifa Zhang<sup>a</sup>, Lin Chen<sup>c</sup>, Jiaxi Wang<sup>a</sup>, Shuai Huang<sup>a\*</sup> and Xianli Zhou<sup>a,b\*</sup><sup>a</sup>School of Life Science and Engineering, Southwest Jiaotong University, Chengdu, 610031 China<sup>b</sup>Key Laboratory of Advanced Technologies of Materials, Ministry of Education, School of Material Science and Engineering, Southwest Jiaotong University, Chengdu, 610031 China<sup>c</sup>School of Chemistry and Chemical Engineering, China West Normal University, Nanchong, 637002 China

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Two new C<sub>18</sub>-diterpenoid alkaloids, anthriscifoltine A (**1**) and anthriscifoltine B (**2**), along with three known diterpenoid alkaloids, deoxydelcorine (**3**), anthriscifolcine A (**4**) and anthriscifolcine G (**5**), were isolated from the whole herbs of *Delphinium anthriscifolium* var. *majus*. Their structures were elucidated by spectroscopic methods, including 1D, 2D NMR, and HR-ESI-MS.

**Keywords:** *Delphinium anthriscifolium* var. *majus*, Diterpenoid alkaloids, Anthriscifoltine, NMR.

*Delphinium anthriscifolium* var. *majus* is an herbaceous plant belonging to the family Ranunculaceae. It is widely distributed in Guizhou, Sichuan, Hubei and Shanxi provinces in China, but currently is also cultivated in other regions. It contains many diterpenoid alkaloids having biological activities such as anti-inflammatory, analgesic, anti-arrhythmia, antifungal, and cytotoxic properties [1,2]. As a part of our efforts to study the chemical composition of *D. anthriscifolium*, we have now isolated and identified two C<sub>18</sub>-diterpenoid alkaloids, anthriscifoltine A (**1**) and anthriscifoltine B (**2**), along with three known compounds, deoxydelcorine (**3**), anthriscifolcine A (**4**) and anthriscifolcine G (**5**). In this paper, we report the extraction, isolation, and structure elucidation of these alkaloids.

The molecular formula of anthriscifoltine A (**1**), was determined as C<sub>30</sub>H<sub>45</sub>NO<sub>9</sub> from the HR-ESI-MS ion at *m/z* 564.3185 [M+H]<sup>+</sup> (calcd. for C<sub>30</sub>H<sub>46</sub>NO<sub>9</sub>, 564.3173). The IR spectrum indicated that **1** possesses hydroxyl (3462 cm<sup>-1</sup>) and carbonyl (1736 cm<sup>-1</sup>) groups. The <sup>1</sup>H NMR and <sup>13</sup>C NMR data (Table 1) of **1** indicated the presence of the signals of a 2-methylbutanoyloxy group (MbO) at [δ<sub>H</sub> 2.38 (1H, m), 1.47 (2H, m), 0.91 (3H, t, *J* = 7.2 Hz), 1.14 (3H, d, *J* = 6.6 Hz) and δ<sub>C</sub> 177.0 (s), 41.2 (d), 26.6 (t), 11.6 (q), 16.5 (q)] [3], an *N*-ethyl group [δ<sub>H</sub> 1.07 (3H, t, *J* = 7.2 Hz), 2.75 (2H, m), δ<sub>C</sub> 14.0 (q) and 50.8 (t)], two methoxyl groups [δ<sub>H</sub> 3.27 (3H, s) and 3.28 (3H, s), δ<sub>C</sub> 55.8 (q) and 55.9 (q)], an acetyl group [δ<sub>H</sub> 2.04 (3H, s), δ<sub>C</sub> 21.8 (q) and 170.5 (s)] and a methylenedioxy group [4.89 (1H, br s) and 4.94 (1H, br s), δ<sub>C</sub> 94.0 (t)]. The remaining 18 carbons were assigned based on 1D- and 2D-NMR data. The presence of only one non-oxygenated quaternary carbon signal (δ<sub>C</sub> 55.5 s) indicates that compound **1** is a C<sub>18</sub>-diterpenoid alkaloid [4a,b]. The locations of the acetoxyl group at C-6 and the 2-methylbutanoyloxy group at C-14 were determined by the correlations in the HMBC experiment. Besides the two ester groups, the two methoxyl groups were attributed to C-1 and C-16, respectively, and the methylenedioxy group was assigned to be at C-7 and C-8 as revealed by the long-range HMBC correlations. The existence of seven oxygenated carbons deduced from its <sup>13</sup>C NMR spectrum suggests that **1** has one hydroxyl group, in addition to two methoxy groups, two ester groups, and a methylenedioxy group. The location of the hydroxyl group at C-10 was further confirmed by the HMBC correlations.

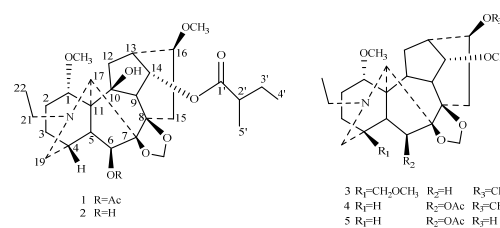
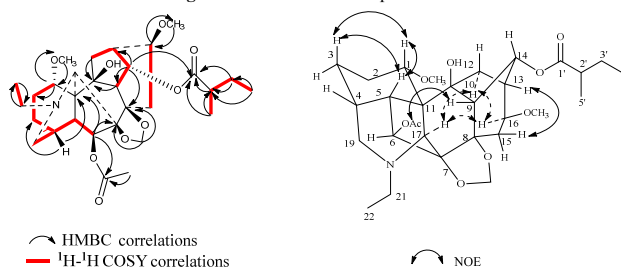


Figure 1: Structures of compounds 1–5.

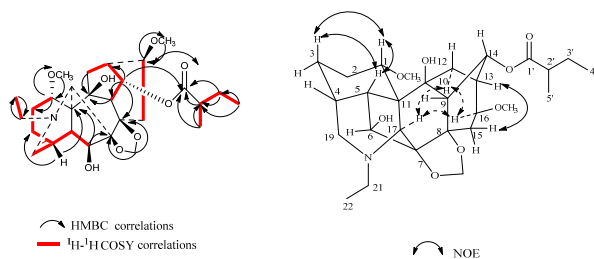
Figure 2: Key <sup>1</sup>H-<sup>1</sup>H COSY, HMBC and NOESY correlations of **1**.

The relative configuration of anthriscifoltine A was deduced from the vicinal coupling constants (Table 1) and a NOESY experiment. In the NOESY spectrum of **1**, the cross-peak between H-17 and H-16 proved that 16-OCH<sub>3</sub> has a β-position. The acetoxyl group at C-6 was determined to have a β-orientation as well based on the multiplicity of H-6 (singlet) in the <sup>1</sup>H NMR spectrum [5a]. The coupling constants of H-1 at δ<sub>H</sub> 3.55 (1H, t, *J* = 8.4 Hz) and H-14 at δ<sub>H</sub> 5.28 (1H, t, *J* = 4.8 Hz) confirmed the β-position of axial H-1 [5b] and the α-position of H-14, respectively [5c]. Therefore, the structure of anthriscifoltine A (**1**) was determined as shown in Figure 1. The full assignment of anthriscifoltine A (**1**) was based on the 1D- and 2D NMR spectral data (Table 1, Figure 2).

The molecular formula of anthriscifoltine B (**2**) was determined to be C<sub>28</sub>H<sub>43</sub>NO<sub>8</sub> from the [M+H]<sup>+</sup> peak at *m/z* 522.3084 (calcd. for C<sub>28</sub>H<sub>44</sub>NO<sub>8</sub> 522.3067) in the HR-ESI-MS. The IR absorption spectrum indicated that **2** possesses hydroxyl (3445 cm<sup>-1</sup>) and carbonyl (1731 cm<sup>-1</sup>) groups. The <sup>13</sup>C NMR data of anthriscifoltine B (**2**) were very similar to those of **1** except for lacking a signal for an acetyl group. The proton signal of H-6 at δ<sub>H</sub> 5.27 in compound **1** was shifted upfield to δ<sub>H</sub> 4.27 in compound **2** suggesting that 6-OAc

**Table 1:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for compounds **1** and **2**.

Position	<b>1</b>		<b>2</b>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult., J (Hz))	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (mult., J (Hz))
1	77.4 d	3.55 t (8.4)	77.5 d	3.62 t (9.0)
2	26.4 t	2.06 $\alpha$ m, 2.12 $\beta$ m	26.5 t	2.06 $\alpha$ m, 2.12 $\beta$ m
3	28.9 t	1.43 $\beta$ m, 1.80 $\alpha$ m	29.8 t	1.40 $\beta$ m, 1.73 $\alpha$ m
4	34.1 d	2.09 m	34.7 d	2.08 m
5	44.8 d	1.87 m	46.1 d	1.81 m
6	81.3 d	5.27 s	82.4 d	4.27 s
7	91.9 s	-	92.8 s	-
8	81.7 s	-	82.6 s	-
9	50.4 d	3.49 d (5.4)	51.0 d	3.55 d (5.4)
10	83.4 s	-	83.0 s	-
11	55.5 s	-	55.8 s	-
12	35.5 t	1.71 $\alpha$ m, 2.56 $\beta$ m	34.9 t	1.74 $\alpha$ m, 2.57 $\beta$ m
13	37.5 d	2.80 m	36.6 d	2.74 m
14	74.3 d	5.28 t (4.8)	74.3 d	5.28 t (4.8)
15	39.1 t	1.84 $\beta$ dd (7.8, 16.2)	38.5 t	1.83 $\beta$ dd (7.8, 16.2)
		3.13 $\alpha$ d (16.2)		3.01 $\alpha$ d (16.2)
16	81.7 d	3.23 d (8.4)	81.3 d	3.21 d (8.4)
17	64.8 d	3.08 d (1.8)	64.4 d	3.03 d (1.8)
19	50.7 t	2.83 m	51.0 t	2.75 m
21	50.6 t	2.75 m	50.8 t	2.68 m
22	14.0 q	1.07 t (7.2)	14.0 q	1.06 t (7.2)
1-OCH <sub>3</sub>	55.8 q	3.27 s	55.8 q	3.25 s
16-OCH <sub>3</sub>	55.9 q	3.28 s	55.9 q	3.26 s
O-CH <sub>2</sub> -O	94.0 t	4.89 br s	93.5 t	5.05 br s
		4.94 br s		5.13 br s
6-OAc	170.5 s	-	-	-
	21.8 q	2.04 s	-	-
14-MbO 1'	177.0 s	-	176.8 s	-
2'	41.2 d	2.38 m	41.2 d	2.35 m
3'	26.6 t	1.47 m	26.5 t	1.47 m
4'	11.6 q	0.91 t (7.2)	11.7 q	0.90 t (7.2)
5'	16.5 q	1.14 d (6.6)	16.4 q	1.14 d (7.2)

**Figure 3:** Key  $^1\text{H}$ - $^1\text{H}$  COSY, HMBC and NOESY correlations of **2**.

in **1** was substituted by a hydroxyl group, which was confirmed by the difference of 42 mass units between those two compounds. Thus, the structure of anthriscifoltine B was determined as compound **2**. The full assignment of anthriscifoltine B (**2**) was based on the 1D- and 2D-NMR spectral data (Table 1, Figure 3). The structures of compounds **3–5** were identified by comparison of their spectral data with those described in the literature [5a,b,d].

## Experimental

**General:** Optical rotations, Perkin-Elmer 341 polarimeter; NMR, Bruker AV600; IR, Thermo Fisher Nicolet 6700; HR-ESI-MS, Waters ACQUITY UPLC/Xevo G2-S QTOF mass spectrometer.

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**Plant material:** The whole herbs of *D. anthriscifolium* var. *majus* were collected in Longshanwa, Zhuxi county, Hubei province of China, in April 2015, and were identified (voucher specimen: L H. Shan & J X. Wang 801) by Prof. Qing-Er Yang of the Institute of Botany, Chinese Academy of Sciences.

**Extraction and isolation:** Dried and powdered whole herbs of *D. anthriscifolium* (21.5 kg) were extracted with 95% EtOH, 4 times at room temperature, for a week each time. After removal of the solvent, the extract (2000 g) was suspended in water (3 L) and adjusted to pH 2 with HCl, and then successively extracted with light petroleum (4 $\times$ 1 L) and ethyl acetate (4 $\times$ 1 L). The pH value of the aqueous layer was adjusted to 10 with aqueous ammonium hydroxide solution and the subsequent mixture was extracted with  $\text{CH}_2\text{Cl}_2$  (4 $\times$ 1 L). The  $\text{CH}_2\text{Cl}_2$  extracts were concentrated to produce the crude alkaloid extract (28.5 g). Column chromatography of the crude alkaloid extract over silica gel, using a  $\text{CH}_2\text{Cl}_2$ :MeOH (80:1, v/v) mixture with increasing polarity afforded fractions A–G based on TLC analysis. Fraction A was separated by silica gel CC (light petroleum/Me<sub>2</sub>CO/Et<sub>2</sub>N 50: 1: 0.1, v/v/v) to obtain compounds **1** (25 mg), **2** (30 mg), **3** (8 mg), **4** (16 mg) and **5** (20 mg).

### Anthriscifoltine A (**1**)

White amorphous powder.

$[\alpha]_{\text{D}}^{20}$ : -15.3 (c 0.56,  $\text{CHCl}_3$ ).

IR (KBr)  $\nu_{\text{max}}$ : 3462, 2958, 2924, 2873, 2854, 2821, 1736, 1670, 1461, 1368, 1242, 1199, 1156, 1130, 1091, 1057, 962  $\text{cm}^{-1}$ .

$^1\text{H}$  ( $\text{CDCl}_3$ , 600 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 150 MHz): Table 1.

HR-ESI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  564.3185 (calcd. for  $\text{C}_{30}\text{H}_{46}\text{NO}_9$  564.3173).

### Anthriscifoltine B (**2**)

White amorphous powder.

$[\alpha]_{\text{D}}^{20}$ : -4.9 (c 0.56,  $\text{CHCl}_3$ ).

IR (KBr)  $\nu_{\text{max}}$ : 3445, 2956, 2924, 2854, 2823, 1731, 1668, 1463, 1379, 1239, 1199, 1156, 1128, 1091, 1058, 964  $\text{cm}^{-1}$ .

$^1\text{H}$  ( $\text{CDCl}_3$ , 600 MHz) and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 150 MHz): Table 1.

HR-ESI-MS  $m/z$ :  $[\text{M}+\text{H}]^+$  522.3084 (calcd. for  $\text{C}_{28}\text{H}_{44}\text{NO}_8$  522.3067).

**Supplementary data:** NMR, IR and HR-ESI-MS for compounds **1** and **2**.

**Acknowledgments** - This work was financially supported by the National Natural Science Foundation of China (31171695), the Science and Technology Support Programs of Sichuan Province (2013SZ0083), Innovation Team Fund of Sichuan Province Education Department (15TD0048), Applied Basic Research of Sichuan Province (2014JY0125) and the Fundamental Research Funds for Central Universities (2682013CX033, 2682014RC15).

Majusine D: A New C<sub>19</sub>-diterpenoid Alkaloid from *Delphinium majus*

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A new C<sub>19</sub>-diterpenoid alkaloid, designated as majusine D (1), has been isolated from *Delphinium majus* W. T. Wang. The structure was elucidated by detailed NMR-spectroscopic studies.

**Keywords:** *Delphinium majus*, C<sub>19</sub>-diterpenoid alkaloid, Majusine D.

Diterpenoid alkaloids are believed to be the major bioactive components of the genus *Delphinium* [1–4], a large genus within the Ranunculaceae family. *D. majus* W. T. Wang is distributed mainly in southwest Sichuan and northwest Yunnan of mainland China, especially around the Jinsha River basin [5]. In our previous papers, three new C<sub>19</sub>-diterpenoid alkaloids and six new C<sub>20</sub>-diterpenoid alkaloid have been reported [6]. Continuing investigations seeking new bioactive compounds of *D. majus* have now led to the isolation of one other new C<sub>19</sub>-diterpenoid alkaloid, majusine D (1). This paper deals with the separation and structural elucidation of this new alkaloid.

Compound 1 was obtained as an amorphous powder. Its positive-ion HRESI-MS showed a quasi-molecular ion peak at  $m/z$  466.2812, corresponding to the molecular formula, C<sub>25</sub>H<sub>39</sub>NO<sub>7</sub>. Its NMR spectra exhibited the presence of an *N*-ethyl group [ $\delta_{\text{H}}$  1.03 (3H, t,  $J$  7.6 Hz);  $\delta_{\text{C}}$  13.7 q, 50.3 t], four methoxyl groups [ $\delta_{\text{H}}$  3.33, 3.35, 3.36, 3.42 (each 3H, s);  $\delta_{\text{C}}$  56.3 q, 59.3 q, 59.2 q, 51.3 q], two unsaturated methines [ $\delta_{\text{H}}$  5.39, 5.66 (each 1H);  $\delta_{\text{C}}$  131.5d, 125.0 d]. Its <sup>13</sup>C NMR spectrum displayed seven oxygenated carbon signals ( $\delta_{\text{C}}$  72.2d, 74.7 d, 78.5d, 81.0 s, 82.2 d, 90.5 d, 91.2s), suggesting that 1 possessed three hydroxyl groups in addition to four methoxyl groups. All of the available evidence revealed that 1 was a lycottonine-type C<sub>19</sub>-diterpenoid alkaloid [2]. A triplet signal at  $\delta_{\text{H}}$  3.95 ( $J$  = 5.6 Hz) was attributed to H-14 $\beta$ , implying the presence of an oxygen substituted group at the C-14 position. Comparison of the NMR data of 1 (Table 1) with those of deltatsine (2) [7] revealed that they were similar (Figure 1). The major difference between them was that methylenes at C-2 and C-3 in deltatsine (2) were replaced by two unsaturated methines in 1. Correlations between OCH<sub>3</sub>-6 and C-6, OCH<sub>3</sub>-8 and C-8, OCH<sub>3</sub>-16 and C-16, OCH<sub>3</sub>-18 and C-18 in the HMBC spectrum (Figure 2) suggested that the methoxyl groups could be assigned to C-6, and C-8, and C-16, and C-18, respectively. The occurrence of a  $\Delta^{(2,3)}$  double bond was corroborated by the correlations from H-2 to C-4 and from H-19 to C-3 in the HMBC spectrum (Figure 2). The secondary hydroxyl group was located at C-1 on the basis of the correlation with H-2 in the COSY spectrum, while the  $\alpha$ -orientation of OH-1 was confirmed in the NOESY spectrum by the coupling between H-1 $\beta$  (3.62 d) and H-10 $\beta$  (2.27 m). The molecular framework of majusine D is elucidated as Figure 1. Moreover, the <sup>13</sup>C NMR spectrum showed that the compound contain an ester carbonyl ( $\delta_{\text{C}}$  172.4s), three oxygenated carbon signals ( $\delta_{\text{C}}$  74.1 d, 71.4 d, 64.5 t),

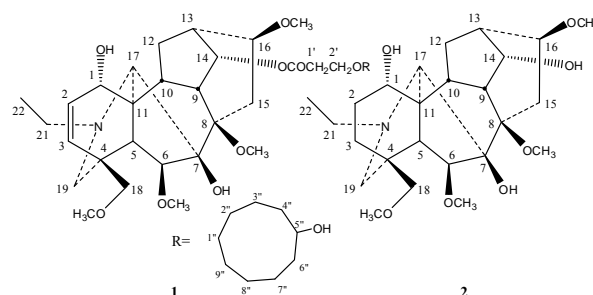
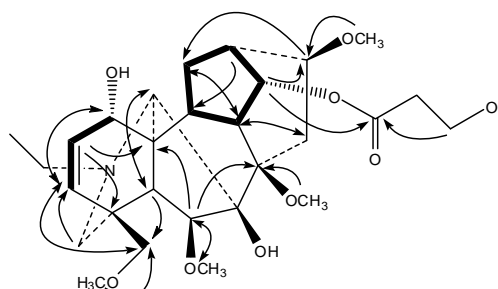
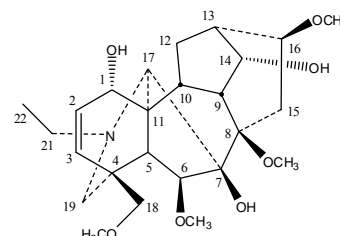


Figure 1: The structures of compounds 1 and 2.

Figure 2: Key <sup>1</sup>H - <sup>1</sup>H COSY (—) and HMBC (---) correlations of 1.Figure 3: The hydrolysis product of the C<sub>14</sub>-ester group from 1.

eight secondary carbons ( $\delta_{\text{C}}$  33.9, 32.4, 29.1, 28.3, 27.0, 26.8, 25.5, 20.2). In the HMBC spectrum, there are connections of H-14 $\beta$ , H-1' ( $\delta_{\text{H}}$  2.43 2H), and H-2' ( $\delta_{\text{H}}$  4.20, 2H, t,  $J$  = 5.2 Hz) to ester carbonyl ( $\delta_{\text{C}}$  172.4). In the IR spectrum, a peak at 1726 cm<sup>-1</sup> displayed that the compound contained a series of four connected secondary carbons. Therefore, the structure of majusine D may be deduced as 1 (Figure 1). Compound 1 is a new C<sub>19</sub>-diterpenoid alkaloid, and the hydrolysis product of the C<sub>14</sub>-ester group (Figure 3) from compound 1 is also a new alkaloid.

**Table 1:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopic data for compounds **1** and **2**.

Position	1		2
	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{C}}$
1	3.62 d (4.4)	72.2d	72.2 d
2	5.66 dd (9.2, 4.4)	125.0 d	27.0 t
3	5.39 d (9.2)	131.5 d	29.2 t
4	-	37.0 s	37.1 s
5	1.65 br s	48.7 d	48.8 d
6	3.79 br s	90.5 d	90.6 d
7	-	91.2 s	91.2 s
8	-	81.0 s	81.1 s
9	2.01 m	45.0 d	45.1 d
10	2.27 m	39.7 d	39.8 d
11	-	48.4 s	48.5 s
12	1.61 m, 1.41 m	28.5 t	28.4 t
13	2.26 m	39.7 d	39.8 d
14	3.95 t (5.6)	74.7 d	74.6 d
15	2.58 m, 1.78 m	31.2 t	30.9 t
16	3.38 (hidden)	82.2 d	82.2 d
17	2.82 br s	66.4 d	66.4 d
18	3.11, 3.27 ABq, (8.8)	78.5 t	78.6 t
19	2.37, 2.39ABq, (7.6)	57.2 t	57.2 t
21	2.77 m, 2.91 m	50.3 t	50.2 t
22	1.03 t (7.6)	13.7 q	13.7 q
6-OCH <sub>3</sub>	3.36 s	59.2 q	59.2 q
8-OCH <sub>3</sub>	3.42 s	51.3 q	51.3 q
16-OCH <sub>3</sub>	3.33 s	56.3 q	56.2 q
18-OCH <sub>3</sub>	3.35 s	59.3 q	59.1 q
CO	-	174.2 s	
1'	2.43 (hidden)	33.9 t	
2'	4.20 t (5.2)	64.5 t	

(400 MHz for  $^1\text{H}$  NMR, 50 MHz for  $^{13}\text{C}$  NMR,  $\text{CDCl}_3$ ,  $\delta$  in ppm,  $J$  in Hz)

## Experimental

**General:** Optical rotation was measured on a Autopol Automatic Polarimeter. IR spectra were obtained on a Perkin-Elmer FT-IR Spectrum Two.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were taken on a Varian Unity INOVA 400/45 NMR spectrometer in  $\text{CDCl}_3$  with TMS as the internal standard. The ESIMS and HRESIMS were recorded on either a VG Auto Spec 3000 or a Finnigan-MAT 90 instrument. Silica gel H (Qingdao Sea Chemical Factory, Qingdao, People's Republic of China) was used for column chromatography. Zones on TLC (silica gel G) were detected using modified Dragendorff's reagent.

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**Plant material:** *Delphinium majus* was collected in Yanbian County, Sichuan Province, People's Republic of China, in June 2012. The plant was authenticated by associate professor Qi Zhao of the Key Laboratory of Medicinal and Edible Plant Resources, Development of Sichuan Education Department, Chengdu University, where a voucher specimen (201201) has been deposited.

**Extraction and isolation:** Air-dried and powdered whole herbs of *Delphinium majus* (2.0 kg) were percolated with 0.1 M HCl (7 L). The obtained acid aqueous solution was basified with 10% aqueous  $\text{NH}_4\text{OH}$  to pH 9–10 and then extracted with ethyl acetate ( $4 \text{ L} \times 3$ ). Removal of the solvent under reduced pressure afforded the total crude alkaloids (8.2 g) as a yellowish amorphous powder, which was chromatographed over a silica gel column, eluting with cyclohexane-acetone (8:1→1:1) gradient system, to give fractions A (105 mg), B (2.3 g), C (1.8 g), and D (2.9 g). Fraction D was separated over a silica gel H column, eluting with  $\text{CHCl}_3$ - $\text{CH}_3\text{OH}$  (40:1 → 10:1), to yield majusine D (**1**) (11 mg).

## Majusine D (1)

White amorphous powder.

$[\alpha]_{\text{D}}^{20}$ : +22.6 ( $c$  1.0,  $\text{CHCl}_3$ ).

IR (KBr): 3478, 2939, 1636, 1233, 726  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR: Table 1.

$^{13}\text{C}$  NMR: Table 1.

ESI-MS  $m/z$  (%): 466 (100), 678 $[\text{M}+\text{H}]^+$  (1).

HR-ESI-MS  $m/z$ : found 466.2812 (100), calcd. for  $\text{C}_{25}\text{H}_{39}\text{NO}_7$ , 466.2804; 678.4166 (1.2) $[\text{M}+\text{H}]^+$ , calcd. for  $\text{C}_{37}\text{H}_{60}\text{NO}_{10}$ , 678.4139.

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Epoxide Opening of a 7,17-*Seco*-7,8-Epoxy- $C_{19}$ -Diterpenoid AlkaloidHong Ji<sup>a</sup>, Feng-Peng Wang<sup>b\*</sup> and Qiao-Hong Chen<sup>c\*</sup><sup>a</sup>Pharmaceutical Research Center, School of Pharmaceutical Science, Guangzhou Medical University, Guangzhou, Guangdong 511436, China<sup>b</sup>Department of Chemistry of Medicinal Natural Products, West China College of Pharmacy, Sichuan University, Chengdu, Sichuan 610041, China<sup>c</sup>Department of Chemistry, California State University, Fresno, 2555 E. San Ramon Ave. M/S SB70, Fresno, California USA 93740

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A new and effective approach toward epoxide opening of a 7,17-*seco*-7,8-epoxy- $C_{19}$ -diterpenoid alkaloid is herein described. The starting epoxide was prepared from naturally occurring yunnacotinine via a nine-step transformation. Treatment of this epoxide with trifluoroacetic anhydride in dioxane at 110°C followed by reduction with sodium boron hydride generated two epoxide opening compounds **7** and **8**. Each of their structures is characteristic of a  $\Delta^{8,15}$  bridgehead double bond and a 7 $\beta$ -oxygen-substituted group.

**Keywords:**  $C_{19}$ -diterpenoid alkaloid, Epoxide-opening.

Numerous  $C_{19}$ -diterpenoid alkaloids have been isolated from a variety of *Aconitum* and *Delphinium* (Ranunculaceae). Certain species have long been used as traditional Chinese medicines for the treatment of various diseases [1-2]. It has been demonstrated that  $C_{19}$ -diterpenoid alkaloids exhibited a plethora of biological activities [3-4]. We started to develop conversational synthesis of taxoids starting from  $C_{19}$ -diterpenoid alkaloids since early 1990s. During the course, several novel approaches to the taxane ABC ring system and numerous intriguing reactions of  $C_{19}$ -diterpenoid alkaloids have been reported [5-13]. We have found that modifications of ring B, including cleavage of C7–C17, enlargement of ring B, functionalization of C-7 and C-8 for assembly of the  $\Delta^{8,15}$  bridgehead double bond, and introduction of functional groups with specific configurations, were part of the critical steps to our successful conversions from aconitine-type  $C_{19}$ -diterpenoid alkaloids [5-7] to the core structure of taxoids. During the B-ring modifications of yunnacotinine [11], an efficient and convenient approach to the 7,8-epoxide opening was found. We herein describe this epoxide-opening reaction, together with the structure characterization of the novel  $C_{19}$ -diterpenoid alkaloid derivatives resulted from this reaction.

Naturally occurring yunnacotinine (**1**) was readily converted to its derivative **2** through a six-step procedure in 80% overall yield according to the procedure as previously described by us [7]. In hope to facilitate the functionalization of C-7 and C-8, the C7–C17 bond in alkaloid **2** was cleaved by reacting with thionyl chloride in benzene followed by reducing with NaBH<sub>4</sub> to furnish compound **3** (Figure 1) [7]. The oxygenated groups at C-7 and C-8 was desired for the construction of the  $\Delta^{8,15}$  bridgehead double bond in taxoid ABC ring system. Several attempts to introduce oxygenated groups at C-7 and C-8 were unfortunately failed. For example, addition of the  $\Delta^{7,8}$  double bond in **3** through oxymercuration reaction did not occurred. The attempted addition with HOBr or Br<sub>2</sub> was also not successful probably due to the participation of the nitrogen atom. Dihydroxylation of 7,17-*seco*-diterpenoid alkaloid **3** produced expected 7,8-oxygenated alkaloid **4**. As compared with **3**, the NMR spectra of **4** showed the absence of the  $\Delta^{7,8}$  double bond as well as an acetyl group, and the presence of two additional oxygenated carbons ( $\delta_H$  3.75 d and  $\delta_C$  71.3 d for a tertiary carbon;  $\delta_C$  76.0 s for a quaternary carbon), indicating the structure of compound **4** as described in Figure 1. However, we had to give up this synthetic method due to the extremely low yield of **4**.

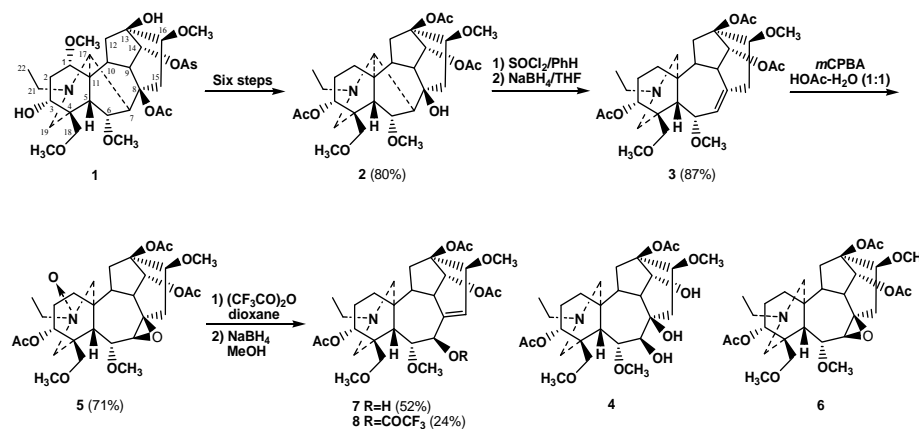
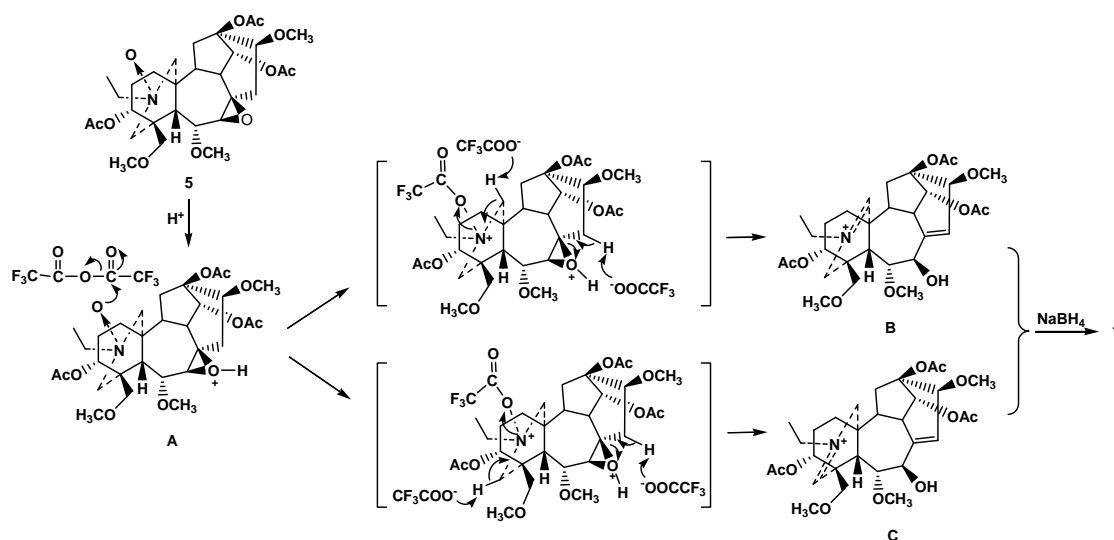


Figure 1

Scheme 1: A Plausible Mechanism from **5** to Compounds **7** and **8****Table 1:** NMR spectral data of compound **7** in CDCl<sub>3</sub> (400 MHz for <sup>1</sup>H, 100 MHz for <sup>13</sup>C).

No.	$\delta_C$	$\delta_H$ mult. ( <i>J</i> in Hz)	HMBC (H→C)
1	27.7 t	1.59 m	C-2, C-3, C-5, C-11
2	34.9 t	1.77 m	C-2, C-3, C-5, C-11
3	72.5 d	1.82 m	C-1, C-3, C-4, C-11
4	42.5 s	5.02 dd (10.8, 6.8)	C-2, C-4, C-18, C-19
5	39.4 d	1.71 brs	C-3, C-4, C-6, C-11, C-18
6	81.0 d	3.60 d (5.2)	C-4, C-5, C-7, C-8, C-11, 6-OCH <sub>3</sub>
7	74.1 d	3.97 d (5.2)	C-6, C-8, C-9
8	137.1 s	—	—
9	47.5 d	2.62 m	C-7, C-8, C-11, C-12, C-15
10	38.5 d	2.47 m	C-8, C-9, C-11, C-12, C-14
11	37.4 s	—	—
12	45.5 t	1.23 m	C-10, C-14
13	85.1 s	1.94 m	C-9, C-11, C-13, C-14
14	74.8 d	5.47 d (3.6)	C-8, C-10, C-13
15	122.3 d	5.78 d (5.2)	C-8, C-9, C-13, C-16
16	80.9 d	4.45 d (5.2)	C-8, C-12, C-14, 16-OCH <sub>3</sub>
17	52.2 t	2.12 m	C-5, C-10, C-11
18	73.2 t	2.38 m	C-5, C-11
19	57.3 t	3.37 ABq (9.2)	C-3, C-4, C-5, C-19, 18-OCH <sub>3</sub>
20	—	3.52 ABq (9.2)	C-3, C-4, C-5, C-19, 18-OCH <sub>3</sub>
21	50.9 t	2.32 m	C-3, C-5, C-18
22	12.1 q	2.50 m	C-3, C-4, C-5, C-18
23	50.9 t	2.42 q (6.8)	C-22
24	12.1 q	0.98 t (6.8)	C-21
25	56.1 q	3.26 s	C-6
26	58.8 q	3.46 s	C-16
27	59.2 q	3.33 s	C-18
28	171.0 s	—	—
29	21.1 q	2.03 s	3-OCO
30	170.9 s	—	—
31	21.9 q	2.07 s	13-OCO
32	170.3 s	—	—
33	21.2 q	2.06 s	14-OCO

In order to avoid the possible oxidation of nitrogen and subsequently to improve the yield, acidic conditions were selected for the epoxidation of alkaloid **3**. Exposure of compound **3** to 30% H<sub>2</sub>O<sub>2</sub> in formic acid for 2 h generated epoxides **5** (59%) and **6** (10%). The molecular formulas of compounds **5** and **6** (C<sub>30</sub>H<sub>45</sub>NO<sub>11</sub> and C<sub>30</sub>H<sub>45</sub>NO<sub>10</sub>) were determined by their HR-MS data. In the NMR spectra of **6**, the signals for the  $\Delta^{7,8}$  double bond disappeared while the characteristic signals at  $\delta_H$  3.75 (1H, d, *J* = 8.8 Hz), and  $\delta_C$  65.6 d and 60.4 s were observed. Compound **5** possesses the same unsaturated degree as **6**, but with 16 more mass units as compared with **6** as determined by their mass spectra, indicating that epoxide **5** is the *N*-oxidation derivative of **6**. The NMR spectra of **5** exhibited characteristic signals at  $\delta_H$  1.61 (3H, t,

*J* = 7.0 Hz) and  $\delta_C$  8.7 q for an *N*-ethyl group [14]. The comparison of the <sup>13</sup>C-NMR spectra of **5** and **6** showed different  $\delta$  values at C-1, C-2, and C-3, especially at C-17, C-19, C-21, and C-22, due to the *N*-oxidation effect [14]. This led to the determination of the structure for epoxide **5**. Treatment of **3** with an excess *m*CPBA in HOAc-H<sub>2</sub>O (1:1) under refluxing conditions also afforded compound **5** in 71% yield. Considering that the nitrogen oxidation was inevitable and that attempt to reduce **5** into **6** failed, epoxide **5** was directly used as a substrate for the subsequent epoxide ring-opening reaction. We attempted to treat **5** with acid activated silica gel or various kinds of acids (98% formic acid, 20% H<sub>2</sub>SO<sub>4</sub> and trifluoroacetic acid *etc.*) respectively, but no reactions occurred in such cases. An acetic anhydride instead of acids was used in the reaction, resulting in an unidentified product. We then tried to use trifluoroacetic anhydride which yielded two inseparable, highly polar products. The <sup>1</sup>H NMR spectrum of the crude product suggested that they are the corresponding iminium salts containing the  $\Delta^{8,15}$  double bond. Without further purification, the crude product was directly reduced with NaBH<sub>4</sub> in MeOH, leading to two expected 7-oxygenated-7,17-*seco*-diterpenoid alkaloids **7** and **8**. The molecular formulas for these two desired products are C<sub>30</sub>H<sub>45</sub>NO<sub>10</sub> and C<sub>32</sub>H<sub>44</sub>F<sub>3</sub>NO<sub>11</sub>, respectively, as determined by their HR-MS data. The NMR spectra of **7** displayed the absence of the signals for the epoxide moiety but the presence of characteristic signals at  $\delta_H$  5.78 (1H, d, *J* = 5.2 Hz), and  $\delta_C$  137.1 s and 122.3 d for a trisubstituted double bond. This double bond was attributed to C-8 and C-15 due to the multi-bond <sup>1</sup>H-<sup>13</sup>C correlations of H-15 with C-8, C-9, C-13 and C-16 in its HMBC spectrum. In the NMR spectra of **7**, in addition to the distinctive upfield shifts of C-17, C-19, and C-21, the chemical shifts for *N*-ethyl group at  $\delta_H$  0.98 (3H, t, *J* = 6.8 Hz) and  $\delta_C$  12.1 q were also greatly changed as compared with those of **5**, suggesting that the *N*-oxide in **5** has been reduced back to tertiary amine. The structure of **7** was confirmed by its 2D NMR data, as shown in Table 1. The NMR spectra of **8** are very similar to those of **7**, except for the existence of signals for an additional trifluoroacetyl group. The structure of **8** was thus determined as illustrated in Figure 1. The plausible mechanism for the formation of 7-oxygenated compounds **7** and **8** was proposed. As shown in Scheme 1, in the presence of trifluoroacetic anhydride and a trace amount of H<sub>2</sub>O in dioxane, protonation of epoxide oxygen in **5**, followed by ring opening and elimination resulted in the formation of the  $\Delta^{8,15}$  double bond. Additionally, esterification of *N*-oxide in **5** with trifluoroacetic anhydride and subsequent

Polonovski-like process can produce a pair of regioisomeric immonium salts **B** and **C**. Reduction of immonium salts may yield compound **7** while reduction of the immonium salts followed by acylation may generate compound **8**.

**Table 2:** <sup>13</sup>C NMR spectral data of compounds **4-6** and **8** (50 MHz, CDCl<sub>3</sub>).

No.	4	5	6	8
1	27.9 t	23.2 t	26.4 t	26.8 t
2	32.5 t	36.7 t	38.0 t	34.3 t
3	74.4 d	72.5 d	74.5 d	71.9 d
4	42.7 s	41.6 s	42.5 s	41.0 s
5	44.2 d	45.0 d	45.3 d	37.7 d
6	81.8 d	79.9 d	79.6 d	77.7 d
7	71.3 d	65.6 d	65.6 d	76.1 d
8	76.0 s	60.4 s	60.5 s	137.6 s
9	42.6 d	54.2 d	53.8 d	47.9 d
10	39.1 d	38.8 d	39.3 d	42.2 d
11	37.4 s	38.5 s	37.4 s	37.4 s
12	34.5 t	37.7 t	37.4 t	43.8 t
13	84.3 s	84.2 s	83.8 s	85.0 s
14	80.9 d	78.9 d	79.2 d	74.5 d
15	46.4 t	46.1 t	46.7 t	121.9 d
16	81.3 d	81.2 d	80.9 d	79.3 d
17	58.4 t	69.6 t	54.7 t	51.6 t
18	73.5 t	72.4 t	72.0 t	70.0 t
19	52.3 t	65.2 t	48.1 t	57.7 t
21	50.8 t	62.4 t	51.5 t	50.5 t
22	12.1 q	8.7 q	13.3 q	12.2 q
6-OCH <sub>3</sub>	56.3 q	56.2 q	55.8 q	56.1 q
16-OCH <sub>3</sub>	58.1 q	57.3 q	57.6 q	58.6 q
18-OCH <sub>3</sub>	59.2 q	59.1 q	58.9 q	59.1 q
3-OAc	170.8 s	170.7 s	170.6 s	170.7 s
	21.2 q	21.2 q	21.1 q	21.1 q
13-OAc	170.2 s	170.5 s	170.4 s	170.9 s
	21.7 q	21.6 q	21.4 q	21.5 q
14-OAc	–	170.1 s	170.1 s	170.3 s
	–	21.4 q	21.2 q	21.3 q
7-OCOCH <sub>3</sub>	–	–	–	159.3 s
	–	–	–	114.7 s

In conclusion, a convenient and effective epoxide-opening method of 7,17-*seco*-7,8-epoxy-C<sub>19</sub>-diterpenoid alkaloid was found. The products **7** and **8** achieved from this study serve as critical intermediates for the assembly of a taxoid ring system bearing a Δ<sup>8,15</sup> bridgehead double bond and an oxygenated group at 7β position. Higher reactivity of trifluoroacetic anhydride and the rigid structure of the C<sub>19</sub>-diterpenoid alkaloid derivatives may be the main reasons for the formation of products **7** and **8** with the Δ<sup>8,15</sup> bridgehead double bond and with the hydroxyl group at the 7β position.

## Experimental

**General:** Melting points were determined on a Kofler block (uncorrected); IR spectra were recorded on a Nicolet FT-IR 200 SXV spectrometer; Optical rotations were measured in a 1.0-dm cell by a PE-314 polarimeter at 20 ± 1 °C; <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were acquired on a Varian INOVA 400/54 or a Bruker AC-E 200 spectrometer in CDCl<sub>3</sub> with TMS as internal standard; MS spectra were obtained on Finnigan LCQ and Micromass Auto Spec Ultima-Tof spectrometer; Silica gel GF<sub>254</sub> and H (10–40 μm, Qingdao Sea Chemical Factory, China) were used for TLC, Chromatotron, and CC.

**Compound 4:** A solution of KMnO<sub>4</sub> (131 mg, 0.83 mmol) in H<sub>2</sub>O (13 mL) was added dropwise to a solution of compound **3** (235 mg, 0.41 mmol) and NaCO<sub>3</sub> (470 mg 5.66 mmol) in dioxane-H<sub>2</sub>O (1:1, 20 mL). The mixture was stirred at room temperature for 20 min. The precipitate was removed by vacuum filtration and washed with dioxane. The filtrate and washings were combined, and the solvent was removed under reduced pressure. This residue was suspended in H<sub>2</sub>O (20 mL) and extracted with ethyl acetate (20 mL×3), the combined organic extracts were dried with anhydrous Na<sub>2</sub>SO<sub>4</sub>, and the solvent was removed under reduced pressure. Chromatography

of the residue (200 mg) on silica gel (6 g) using cyclohexane–acetone (4:1) as eluent afforded compound **4** (white amorphous powder, 30 mg, 13%).

MP: 166–168°C.

[α]<sub>D</sub><sup>20</sup>: -32.5 (c 1.20, CHCl<sub>3</sub>).

IR (KBr): 3462, 1742, 1451, 1253 cm<sup>-1</sup>.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 0.99 (3H, t, *J* = 7.2 Hz, NCH<sub>2</sub>CH<sub>3</sub>), 2.04, 2.10 (each 3H, s, 2×OAc), 3.26, 3.37, 3.38 (each 3H, s, 3×OMe), 3.75 (1H, d, *J* = 5.2 Hz, H-7α), 3.89 (1H, d, *J* = 5.2 Hz, H-14β), 4.31 (1H, t, *J* = 4.8 Hz, H-16α), 4.35 (1H, d, *J* = 6.0 Hz, H-6β), 4.99 (1H, dd, *J*<sub>1</sub> = 11.2 Hz, *J*<sub>2</sub> = 7.2 Hz, H-3β), 5.56 (1H, d, *J* = 4.8 Hz, OH-7).

<sup>13</sup>C NMR: Table 2.

ESI-MS: *m/z* (%) = 556 [M + H<sup>+</sup>] (100).

HRMS-ESI: *m/z* [M + H<sup>+</sup>] calcd for C<sub>28</sub>H<sub>46</sub>NO<sub>10</sub>: 556.2998; found: 556.2989.

**Compound 5 and 6:** A solution of compound **3** (135 mg, 0.24 mmol) in HCOOH (3 mL) was cooled in an ice-water bath, and 30% H<sub>2</sub>O<sub>2</sub> (2 mL) was added dropwise. After stirring for 10 min, the mixture was allowed to warm to room temperature and stirred for 2 h. After pouring into ice water (5 mL), the solution was basified with conc. NH<sub>4</sub>OH to pH 10. The aqueous layer was extracted with CHCl<sub>3</sub> (8 mL×3). The combined extracts were dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated. The residue (153 mg) was subjected to column chromatography (silica gel H, 3 g), eluted with CHCl<sub>3</sub>–MeOH (18:1) to afford **5** (white amorphous powder, 84 mg, 59%) and **6** (white amorphous powder, 14 mg, 10%).

### Compound 5

MP: 116–118°C.

[α]<sub>D</sub><sup>20</sup>: +18.6 (c 2.30, CHCl<sub>3</sub>).

IR (KBr): 1738, 1261, 892, 811 cm<sup>-1</sup>.

<sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): 1.61 (3H, t, *J* = 7.0 Hz, NCH<sub>2</sub>CH<sub>3</sub>), 2.08, 2.11, 2.13 (each 3H, s, 3×OAc), 3.25, 3.35, 3.44 (each 3H, s, 3×OMe), 3.91 (1H, t, *J* = 8.0 Hz, H-16α), 4.49 (1H, d, *J* = 6.2 Hz, H-6β), 4.67 (1H, brs, H-7), 4.84 (1H, d, *J* = 5.0 Hz, H-14β), 5.18 (1H, dd, *J*<sub>1</sub> = 10.8 Hz, *J*<sub>2</sub> = 7.2 Hz, H-3β).

<sup>13</sup>C NMR: Table 2.

ESI-MS: *m/z* (%) = 596 [M + H<sup>+</sup>] (100).

HRMS-ESI: *m/z* [M + H<sup>+</sup>] calcd for C<sub>30</sub>H<sub>46</sub>NO<sub>11</sub>: 596.3075; found: 596.3087.

### Compound 6

MP: 132–134°C.

[α]<sub>D</sub><sup>20</sup>: -27.3 (c 0.68, CHCl<sub>3</sub>).

IR (KBr): 1738, 1245, 1193, 936, 797 cm<sup>-1</sup>.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 1.10 (3H, t, *J* = 7.2 Hz, NCH<sub>2</sub>CH<sub>3</sub>), 2.04, 2.06, 2.09 (each 3H, s, 3×OAc), 3.21, 3.25, 3.32 (each 3H, s, 3×OMe), 3.75 (1H, d, *J* = 8.8 Hz, H-7), 3.82 (1H, t, *J* = 8.0 Hz, H-16α), 4.01 (1H, d, *J* = 6.4 Hz, H-6β), 4.89 (1H, d, *J* = 5.2 Hz, H-14β), 4.93 (1H, dd, *J*<sub>1</sub> = 12.4 Hz, *J*<sub>2</sub> = 5.6 Hz, H-3β).

<sup>13</sup>C NMR: Table 2.

ESI-MS: *m/z* (%) = 580 [M + H<sup>+</sup>] (100).

HRMS-ESI: *m/z* [M + H<sup>+</sup>] calcd for C<sub>30</sub>H<sub>46</sub>NO<sub>10</sub>: 580.2995; found: 580.2982.

**Compound 5:** To a solution of compound **3** (135 g, 0.24 mmol) in HOAc–H<sub>2</sub>O (1:1, 5 mL) was added *m*CPBA (165 mg, 0.96 mmol). The mixture was refluxed for 2 h and cooled prior to being poured into ice water (5 mL). The solution was basified with conc. NH<sub>4</sub>OH solution to pH 10 and extracted with CHCl<sub>3</sub> (8 mL×3). The extracts were dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated. Column chromatography

(silica gel H, 3 g) of the residue (148 mg) using  $\text{CHCl}_3$ -MeOH (18:1) as eluent afforded **5** as a white amorphous powder (102 mg, 71%).

**Compound 7 and 8:** To a solution of compound **5** (142 mg, 0.24 mmol) in dioxane (10 mL) was added trifluoroacetic anhydride (1 mL). The mixture was refluxed for 3 h and then concentrated under reduced pressure. The residue was diluted with  $\text{H}_2\text{O}$  (10 mL), basified with conc.  $\text{NH}_4\text{OH}$  solution to pH 10, and extracted with  $\text{CHCl}_3$  (8 mL $\times$ 3). The extracts were dried ( $\text{Na}_2\text{SO}_4$ ) and concentrated to give a crude. The crude was dissolved in MeOH (5 mL), to which was added  $\text{NaBH}_4$  (36 mg, 0.96 mmol). The solution was stirred at room temperature for 4 h. After removal of the solvent, the residue was diluted with water (5 mL). The subsequent mixture was extracted with ethyl acetate (4 mL $\times$ 3), the extracts were dried ( $\text{Na}_2\text{SO}_4$ ), and the organic solvent was removed *in vacuo*. Chromatography of the residue (138 mg) on silica gel (4 g) using cyclohexane-acetone (6:1) as eluent afforded **7** (a white amorphous powder, 71 mg, 52%) and **8** (a white amorphous powder, 39 mg, 24%).

#### Compound 7

MP: 123-124°C.

$[\alpha]_{\text{D}}^{20}$ : +23.3 (*c* 0.82,  $\text{CHCl}_3$ ).

IR (KBr): 3468, 1740, 1452, 1366, 1092  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (400 MHz) and  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ): Table 1.

ESI-MS:  $m/z$  (%) = 580 [ $\text{M} + \text{H}^+$ ] (100).

HRMS-ESI:  $m/z$  [ $\text{M} + \text{H}^+$ ] calcd for  $\text{C}_{30}\text{H}_{46}\text{NO}_{10}$ : 580.2995; found: 580.2981.

#### Compound 8

MP: 137-139°C.

$[\alpha]_{\text{D}}^{20}$ : +10.5 (*c* 0.24,  $\text{CHCl}_3$ ).

IR (KBr): 1788, 1525, 1374, 1078  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ): 0.98 (3H, t,  $J$  = 6.8 Hz,  $\text{NCH}_2\text{CH}_3$ ), 2.02, 2.04, 2.08 (each 3H, s, 3 $\times$ OAc), 3.10, 3.41, 3.47 (each 3H, s, 3 $\times$ OMe), 3.85 (1H, d,  $J$  = 5.2 Hz, H-6 $\beta$ ), 4.04 (1H, d,  $J$  = 4.8 Hz, H-16 $\alpha$ ), 4.94 (1H, dd,  $J_1$  = 11.2 Hz,  $J_2$  = 7.2 Hz, H-3 $\beta$ ), 5.47 (1H, d,  $J$  = 3.6 Hz, H-14 $\beta$ ), 5.57 (1H, d,  $J$  = 4.8 Hz, H-7), 5.96 (1H, d,  $J$  = 5.2 Hz, H-15).

$^{13}\text{C}$  NMR: Table 2.

ESI-MS:  $m/z$  (%) = 698 [ $\text{M} + \text{Na}^+$ ] (100).

HRMS-ESI:  $m/z$  [ $\text{M} + \text{Na}^+$ ] calcd for  $\text{C}_{32}\text{H}_{44}\text{F}_3\text{NO}_{11}\text{Na}$ : 698.2259; found: 698.2272.

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## Further Studies on Structure-Cardiac Activity Relationships of Diterpenoid Alkaloids

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The cardiac effect of thirty-eight diterpenoid alkaloids was evaluated on the isolated bullfrog heart model. Among them, twelve compounds exhibited appreciable cardiac activity, with compounds **3** and **35** being more active than the reference drug lanatoside. The structure-cardiac activity relationships of the diterpenoid alkaloids were summarized based on our present and previous studies [2]: i) 1 $\alpha$ -OMe or 1 $\alpha$ -OH, 8-OH, 14-OH, and NH (or NMe) are key structural features important for the cardiac effect of the aconitine-type C<sub>19</sub>-diterpenoid alkaloids without any esters. C<sub>18</sub>-diterpenoid alkaloids, lycotonine-type C<sub>19</sub>-diterpenoid alkaloids, and the veatchine- and denudatine-type C<sub>20</sub>-diterpenoid alkaloids did not show any cardiac activity; ii) the presence of 3 $\alpha$ -OH is beneficial to the cardiac activity; iii) the effect on the cardiac action of 6 $\alpha$ -OMe, 13-OH, 15 $\alpha$ -OH, and 16-demethoxy or a double bond between C-15 and C-16 depends on the substituent pattern on the nitrogen atom.

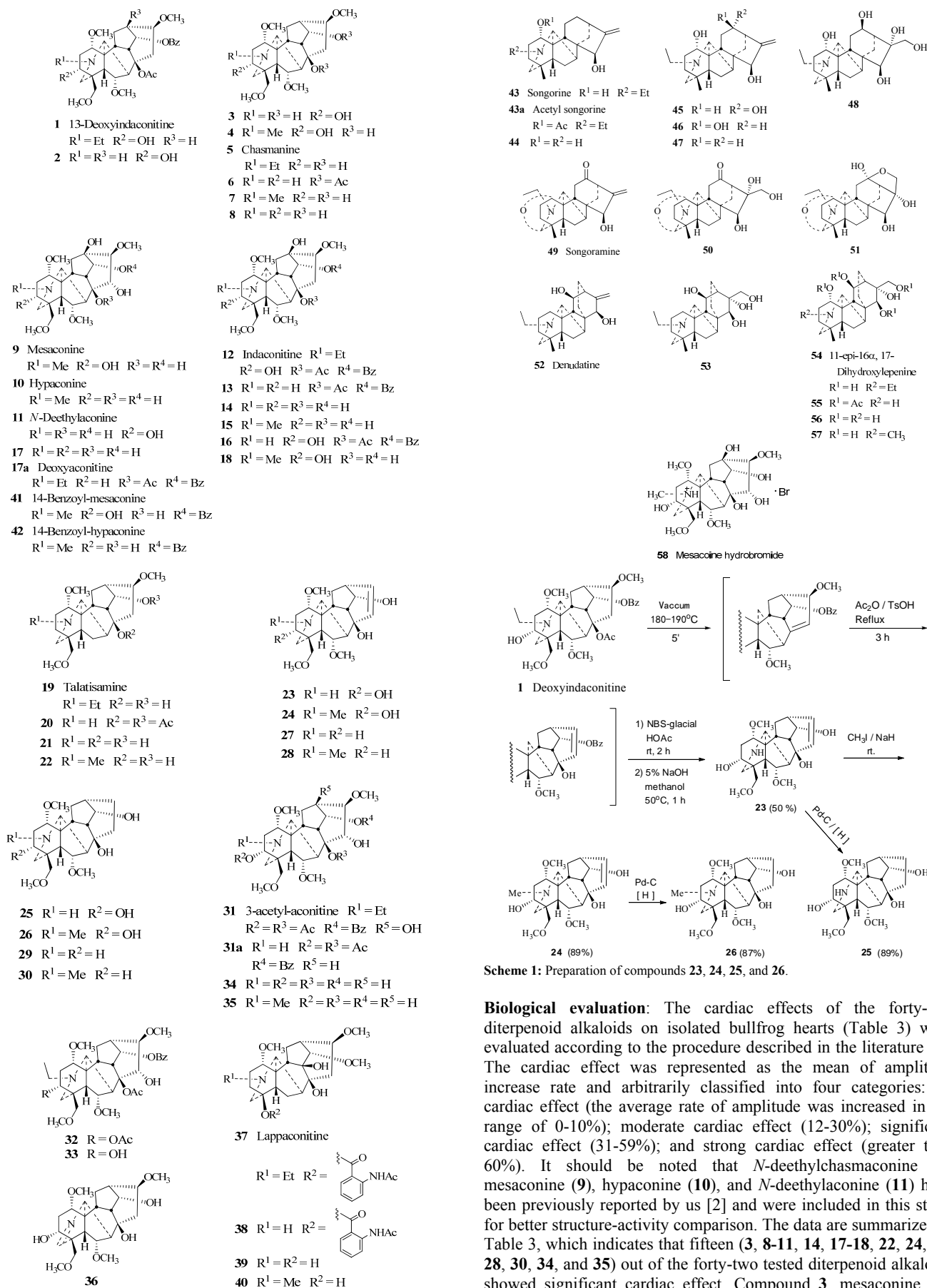
**Keywords:** Diterpenoid alkaloid, Cardioactive evaluation, Structure-activity relationships.

“Fu<sup>zi</sup>” (*Aconitum carmichaeli*) is a profound folk medicine throughout China, Japan, and Korea, as well as other regions in Southeast Asia. In search of diterpenoid alkaloids with considerable cardiac effect, we have previously isolated C<sub>19</sub>-diterpenoid alkaloids as its chemical components with cardiac activity [1] and initiated the associated structure-activity relationships [2]. For further in-depth structure-activity studies and search for possible candidates for clinical development, thirty-eight C<sub>18</sub>, C<sub>19</sub>, and C<sub>20</sub>-diterpenoid alkaloids were designed and synthesized using either mesaconine and hypanine or lappaconitine, songorine, acetylsongorine, denudatine, and 11-epi-16 $\alpha$ ,17-dihydroxylepenine as the starting materials. Their cardiac effect was assessed on the isolated bullfrog heart model. The preparation, biological evaluation, and systematic structure-activity relationships of these alkaloids are presented herein.

The known and naturally occurring diterpenoid alkaloids, 13-deoxyindaconitine, chasmanine, 14-benzoylmesaconine, 14-benzoylmesaconine, indaconitine, deoxyaconitine, talatisamine, 3-acetylaconitine, lappaconitine, songorine, acetylsongorine, songoramine, denudatine, 11-epi-16 $\alpha$ ,17-dihydroxylepenine, and mesaconine hydrobromide, were extracted from either *Aconitum* or *Delphinium* spp. by one of our laboratories.

All *N*-deethylation of diterpenoid alkaloids in this paper was achieved according to the method reported by us [3, 4]; all *N*-methylation was accomplished by reacting with methyl iodide in the presence of sodium hydride. Compound **3** was prepared by *N*-deethylation of 13-deoxyindaconitine (**1**) followed by hydrolysis. Compound **4** was prepared by methylation of **2** followed by hydrolysis. Compound **6** was made by acylation of chasmanine (**5**), followed by *N*-deethylation. Compounds **7** and **8** were obtained by methylation of **6** followed by hydrolysis. Compound **13** was prepared by treatment of indaconitine (**12**) with thionyl chloride, followed by hydrogenation (H<sub>2</sub>/Pd). Compounds **14** and **15** were

made from **13** using a similar procedure as that illustrated for the preparation of compounds **3** and **4**, respectively. Similarly, compounds **17** and **18** were prepared from **17a** and **12**, respectively. Compounds **21** and **22** were produced from talatisamine (**19**) via a three-step procedure including acetylation, *N*-deethylation, and hydrogenation. Compounds **23**, **24**, **25**, and **26** were prepared from 13-deoxyindaconitine (**1**) through a reaction sequence as shown in Scheme 1, including pyrolysis, isopyro rearrangement, *N*-deethylation, *N*-methylation or Pd/C-catalyzed hydrogenation. Similarly, compounds **27**, **28**, **29**, and **30** were obtained from chasmanine (**5**) through diacetylchasmanine (**6**). Compound **34** was prepared from 3-acetylaconitine (**31**) by a three-step reaction sequence including Barton's oxygenation [5], *N*-deethylation, and hydrolysis. Methylation of **31a** (prepared from **31**) with CH<sub>3</sub>I/NaH, followed by hydrolysis, gave compound **35**. Compound **36** was prepared from 13-deoxyindaconitine (**1**) by *N*-deethylation and hydrolysis. Compound **38** was produced by *N*-deethylation of lappaconitine (**37**). Compounds **39** and **40** were prepared from compound **38** via hydrolysis, and methylation and hydrolysis, respectively. Compound **44** was prepared from songorine (**43**) according to the procedure described in the literature [3, 4]. Reduction of songorine (**43**) with NaBH<sub>4</sub> resulted in compounds **45** and **46**, while reduction of songorine (**43**) using the Huang-Minlon method with modification led to compound **47**. Oxidation of compound **46**, songoramine (**49**), and denudatine (**52**) with H<sub>2</sub>O<sub>2</sub>-MeOH produced compounds **48**, **50** and **51**, as well as **53**, respectively. Treatment of **55** [prepared from 11-epi-16 $\alpha$ ,17-dihydroxylepenine (**54**)] with 5% NaOH solution in methanol followed by CH<sub>3</sub>I-NaH gave compounds **56** and **57**. Their structures were established by comparison of their <sup>1</sup>H (<sup>13</sup>C) NMR data with those reported in the literature [6], as well as the high resolution mass data. The <sup>13</sup>C NMR spectral data for all compounds (Tables 1, 2) were assigned based on comparing them with those of analogues.

Scheme 1: Preparation of compounds **23**, **24**, **25**, and **26**.

**Biological evaluation:** The cardiac effects of the forty-two diterpenoid alkaloids on isolated bullfrog hearts (Table 3) were evaluated according to the procedure described in the literature [7]. The cardiac effect was represented as the mean of amplitude increase rate and arbitrarily classified into four categories: no cardiac effect (the average rate of amplitude was increased in the range of 0-10%); moderate cardiac effect (12-30%); significant cardiac effect (31-59%); and strong cardiac effect (greater than 60%). It should be noted that *N*-deethylchasmaconine (**8**) mesaconine (**9**), hypaconine (**10**), and *N*-deethylaconine (**11**) have been previously reported by us [2] and were included in this study for better structure-activity comparison. The data are summarized in Table 3, which indicates that fifteen (**3**, **8-11**, **14**, **17-18**, **22**, **24**, **25**, **28**, **30**, **34**, and **35**) out of the forty-two tested diterpenoid alkaloids showed significant cardiac effect. Compound **3**, mesaconine (**9**),

hyaconine (**10**), and compound **35** exhibit better cardiac effect than the reference drug lanatoside C.

A broad range (potent-to-inactive) of cardiac effects has been observed for the forty-two diterpenoid alkaloids with the following structure-activity relationships:

- 1) C<sub>18</sub>-diterpenoid alkaloids (e.g. **39**, **40**) or the aconitine-type alkaloids containing a monoester [e.g. 14-benzoylmesaconine (**41**) and 14-benzoylhyaconine (**42**)], the veatchine-type C<sub>20</sub>-diterpenoid alkaloids (e.g. **43-48**, **50**, and **51**), and the denudatine-type C<sub>20</sub>-diterpenoid alkaloids (e.g. **53**, **56** and **57**) did not exhibit any apparent cardiac effect. Additionally, no considerable effect was observed for the aconitine-type alkaloids that contain an imine moiety (e.g. **36**).
- 2) Consistent with the results that we previously reported [2], the aminoalcohol group of aconitine-type C<sub>19</sub>-diterpenoid alkaloids bearing both hydroxyl groups at C-8 and C-14 are most essential structure features for the cardiac activity. The substituent pattern on the nitrogen atom (NH, NMe, or NEt), the hydroxyl group at C-3, C-15, and C-13, and the methoxyl group at C-6 can appreciably contribute to or abolish the activity. These can be specified as below: i) the alkaloids with a 3□-OH (e.g. **3**, **4** and **18**) are more potent than those without it (e.g. **8**, **7**, and **15**), suggesting the presence of a 3□-OH is beneficial to the cardiac effect, which is consistent with the conclusions in our previous paper [2]; ii) in addition to alkaloids **15** and **28**, the alkaloids that possess a methyl group on the nitrogen atom [e.g. mesaconine (**9**), **22**, **28**, and **35**] are more active than the corresponding compounds containing a hydrogen instead [e.g. N-deethylamine (**11**), **21**, **27**, and **34**]. The similar effect of the NMe on the cardiac activity can also be concluded by comparing the activity of **24/23**, **22/21**, and **30/29**. However, alkaloids that contain a NH moiety but no 13, 15□-OH (e.g. **3**, **8**, and **29**) have greater activity than those with a NMe moiety (e.g. **4**, **7**, and **30**); iii) except for compound **7**, an alkaloid possessing a 16□-OMe (e.g. **4**) is more active than the corresponding one without it (e.g. **26**) and the one with a double bond between C-15 and C-16 (e.g. **24**); iv) the alkaloids **7** and **8** that contain a 13-OH have significantly improved activity compared with their

corresponding alkaloids **15** and **14** that lack the 13-OH, suggesting that this group is also a determining factor for the cardiac action. V) the effect of 6□-OMe on the cardiac activity depends on the substituent pattern on the nitrogen atom. Both NMe-containing compounds **7** and **22** did not show any cardiac activity, no matter with either the presence or absence of a 6□-OMe. However, introduction of 6□-OMe into the NH-containing compound **21** led to compound **8** with enhanced cardiac effect.

In conclusion, the structure-cardiac activity relationships of diterpenoid alkaloids are summarized as below based on our previous and current studies: i) 1□-OMe or 1□-OH, 8-OH, 14-OH, and NH (or NMe) are critical structural features necessary for the cardiac effect of the aconitine-type C<sub>19</sub>-diterpenoid alkaloids without any esters. C<sub>18</sub>-diterpenoid alkaloids, lycotonine-type C<sub>19</sub>-diterpenoid alkaloids, and the veatchine- and denudatine-type C<sub>20</sub>-diterpenoid alkaloids did not possess any significant cardiac activity; ii) the existence of 3□-OH can significantly improve the cardiac activity; iii) the effects on the cardiac activity of 6□-OMe, 13-OH, 15□-OH, and 16-demethoxy or a double bond between C-15 and C-16 heavily depend upon the substituent pattern on the nitrogen atom; iv) interestingly, mesaconine hydrobromide (**58**) without showing any *in vitro* cardiac activity exhibited significant and quick *in vivo* efficacy that is comparable with the free base mesaconine (**9**) [8].

## Experimental

**General methods:** Melting points were determined on a Koffler block (uncorrected). IR spectra were obtained on a Nicolet FT-IR 200 SXV spectrophotometer. <sup>1</sup>H and <sup>13</sup>C NMR spectra were taken on a Varian Unity INOVA 400/54 NMR spectrometer in CDCl<sub>3</sub> with TMS as the internal standard. The ESIMS and HRESIMS were recorded on either a VG Auto Spec 3000 or a Finnigan-MAT 90 instrument. Silica gel H (Qingdao Marine Chemical Factory, Qingdao, China) was used for column chromatography (CC). Zones on TLC (silica gel G) plates were detected with modified Dragendorff's reagent.

**Table 1:** <sup>13</sup>C NMR spectral data of compounds **2-4**, **6**, **7**, **13-15**, **17**, **18**, and **20-25** (100 MHz, CDCl<sub>3</sub>).

No.	2 <sup>a</sup>	3	4	6	7	13 <sup>b</sup>	14	15	17	18	20 <sup>c</sup>	21	22	23	24	25
1	83.2	75.2	83.1	83.7	82.1	83.1	83.0	85.7	83.4	84.1	83.1	84.6	86.2	81.8	82.7	82.3
2	37.5	34.7	33.9	28.9	34.7	35.1	29.3	34.8	28.4	35.3	28.0	30.5	32.5	35.3	34.0	34.3
3	71.4	71.5	72.3	23.3	25.8	23.4	23.6	25.8	22.8	72.3	24.9	26.1	25.8	71.6	71.8	71.5
4	43.6	43.6	43.5	38.9	39.4	39.0	39.1	39.4	38.6	43.4	37.8	38.3	38.2	43.8	43.5	43.7
5	47.5	47.9	48.4	43.8	48.5	44.0	49.2	50.4	51.5	50.1	44.3	38.5	37.5	54.9	47.9	57.0
6	81.3	82.1	81.9	82.4	82.1	82.5	82.6	82.1	82.2	82.8	24.6	27.0	24.6	81.3	81.5	81.3
7	55.5	56.3	51.2	53.4	51.4	43.6	57.3	51.1	47.4	50.8	47.4	52.7	45.1	47.5	47.5	48.2
8	85.5	81.7	71.9	85.8	72.4	85.6	74.3	72.8	76.1	72.3	85.9	73.2	72.7	73.8	73.8	75.1
9	42.6	47.7	48.0	43.8	49.2	43.6	44.8	49.1	42.7	47.8	42.4	46.3	46.8	46.6	47.1	47.2
10	39.5	38.5	37.8	39.0	37.8	40.2	41.1	42.3	41.0	41.6	38.8	44.0	44.7	38.5	38.5	34.8
11	49.7	49.4	50.1	50.4	50.2	50.3	48.9	50.0	48.8	49.9	37.8	48.2	48.6	49.2	49.8	50.1
12	27.5	27.2	27.8	28.5	28.0	29.0	35.9	35.6	36.7	33.7	28.5	24.9	27.4	31.4	32.6	30.5
13	44.6	45.2	45.1	43.4	45.4	74.5	76.3	76.7	78.2	76.5	41.1	45.6	45.6	45.6	45.2	45.3
14	75.5	72.6	75.3	75.4	75.4	78.9	78.9	79.4	78.4	79.2	75.3	75.4	75.4	74.0	74.1	74.7
17	56.6	58.4	63.2	57.0	63.5	57.7	59.1	63.4	61.8	63.1	56.4	55.8	63.7	57.6	64.4	59.0
18	77.5	77.7	77.1	79.8	80.4	79.9	80.1	80.4	79.8	77.0	78.8	79.1	79.2	77.8	77.1	78.1
19	40.8	40.7	49.7	*	56.3	*	50.0	56.1	50.1	49.5	49.0	47.2	*	66.9	49.8	48.9
NCH <sub>3</sub>	-	-	42.1	-	42.3	-	-	42.1	-	42.0	-	-	42.5	-	42.2	-
1-OMe	55.1	55.7	56.3	55.2	56.3	55.3	55.3	56.5	55.3	56.3	55.6	56.3	56.4	55.7	56.3	55.6
6-OMe	56.3	56.3	57.2	56.4	56.4	57.5	57.4	57.2	56.2	57.0	-	-	-	57.5	57.4	57.3
16-OMe	57.5	57.2	56.3	57.9	57.3	58.8	57.6	57.6	58.0	57.5	56.9	57.0	56.5	-	-	-
18-OMe	59.1	59.1	59.1	59.0	59.1	59.0	58.0	59.1	59.0	59.0	59.3	59.3	59.4	59.2	59.1	59.2
15	34.6	38.9	38.8	37.5	38.8	39.7	42.6	39.6	80.7	39.5	37.5	39.0	38.5	129.6	129.6	30.1
16	82.3	81.7	81.8	82.2	81.8	83.0	82.4	84.3	91.0	81.8	82.4	82.0	82.1	131.4	131.5	23.8

a: OAc: 169.5 s, 21.5 q; OBz: 166.1 s (COO), 130.1 s (1"), 129.5 d (2", 6"), 128.4 d (3", 5"), 133.0 d (4"); b: OAc: 169.5 s, 21.3 q; OBz: 166.0 s, (COO), 129.8 s (1"), 129.5 d (2", 6"), 128.4 d (3", 5"), 139.1 d (4"); c: OAc×2: 170.8 s, 169.3 s, 22.2 q, 21.2 q).

**Table 2:**  $^{13}\text{C}$  NMR spectral data of compounds **27-30**, **34-36**, and **38-40** (100 MHz,  $\text{CDCl}_3$ )

NO	26	27	28	29	30	34	35	36	38 <sup>a</sup>	39	40
1	82.7	83.1	85.4	82.6	85.8	82.2	83.1	82.7	82.4	82.5	82.7
2	34.0	24.1	26.2	25.6	25.8	30.4	31.0	33.3	29.5	34.1	36.8
3	71.8	29.8	34.8	31.8	30.6	70.6	70.5	72.3	26.2	25.7	26.8
4	43.5	39.3	39.4	38.8	39.5	44.8	44.7	49.5	77.3	70.4	70.8
5	47.9	53.9	48.5	57.4	52.4	53.3	50.2	57.0	36.7	52.1	49.6
6	81.5	82.0	81.7	82.4	82.4	85.2	84.7	81.8	23.6	23.4	26.1
7	47.5	44.8	48.1	44.7	49.8	45.6	45.7	45.7	52.3	36.4	35.8
8	73.7	74.4	73.9	76.3	75.1	79.4	79.3	71.2	83.5	75.5	75.4
9	47.1	46.8	47.8	47.4	47.9	49.4	49.0	47.7	76.0	77.9	78.5
10	38.5	38.3	38.7	34.6	34.9	42.0	41.7	37.3	49.1	49.4	46.4
11	49.5	49.9	49.8	50.2	50.7	50.8	51.4	51.4	52.7	52.7	50.7
12	32.6	31.5	32.9	32.3	34.9	33.9	34.0	26.9	24.4	25.6	23.4
13	45.2	44.8	45.6	44.8	45.4	46.5	46.5	44.1	44.2	47.5	49.5
14	74.1	74.1	74.2	75.4	74.8	76.3	76.1	75.2	90.0	89.9	89.9
15	129.6	128.3	129.7	29.0	30.4	79.9	79.9	37.1	44.0	43.6	44.4
16	131.5	132.8	131.6	23.3	22.9	91.9	92.2	81.5	82.2	82.5	84.8
17	64.4	58.7	64.4	58.3	63.8	57.5	65.3	62.0	57.1	57.7	62.9
18	77.1	80.3	80.5	80.0	80.6	77.3	76.1	78.8	-	-	-
19	49.8	49.3	56.2	49.0	56.2	41.6	42.5	166.3	50.8	52.0	60.1
NCH <sub>3</sub>	42.2	-	42.4	-	42.4	-	49.6	-	-	-	42.2
1-OMe	56.3	55.4	56.4	55.3	56.5	55.5	55.9	55.8	55.8	55.6	55.9
6-OMe	57.4	57.6	57.5	57.7	57.3	57.3	57.1	57.0	-	-	-
14-OMe	-	-	-	-	-	-	-	-	56.0	56.3	57.7
16-OMe	-	-	-	-	-	59.3	59.2	56.5	57.8	55.8	56.7
18-OMe	59.1	59.1	59.1	59.1	59.2	58.3	58.3	59.3	-	-	-

a: NHAc: 171.3 s (NCO), 25.4 (25.2) q ( $\text{CH}_3\text{CON}$ ); 169.0 (167.2) s (COO), 141.5 (140) s (2''), 134.3 (133.1) d (4''), 130.9 (127.3) d (6''), 122.4 (122.2) d (5''), 121.3 (120.1) (3''), 115.5 s (1'').

**Table 3:**  $^{13}\text{C}$  NMR spectral data of compounds **44-48**, **50**, **51**, **53**, **56** and **57** (100 MHz,  $\text{CDCl}_3$ )

NO	44	45	46	47	48 <sup>a</sup>	50	51 <sup>a</sup>	53 <sup>a</sup>	56 <sup>b</sup>	57 <sup>a</sup>
1	67.3	69.8	69.6	69.6	69.2	67.8	67.8	26.3	68.4	69.1
2	29.6	29.8	30.6	20.4	27.9	29.8	28.9	19.7	31.1	29.8
3	30.6	31.1	31.4	31.5	28.9	24.1	23.2	39.3	38.0	36.7
4	33.5	33.6	33.5	34.6	35.9	37.7	36.9	33.4	34.4	34.4
5	46.4	48.1	47.9	49.6	48.9	48.7	49.0	50.6	51.3	49.7
6	21.8	23.3	19.1	22.3	22.7	23.9	22.9	21.8	24.2	23.0
7	45.6	43.3	44.0	44.1	45.8	45.7	45.5	41.9	45.8	44.5
8	48.2	50.5	50.7	47.6	49.8	50.7	49.5	41.9	44.5	42.7
9	36.0	37.0	36.2	35.3	39.1	30.4	31.6	52.3	52.8	51.8
10	51.0	52.4	52.9	48.9	53.3	51.5	50.0	44.6	51.9	52.1
11	36.0	32.3	23.4	37.2	31.2	29.6	28.4	71.0	71.4	69.8
12	210.3	67.3	31.9	210.2	79.7	213.0	107.7	38.8	46.7	44.9
13	53.0	43.7	40.6	53.1	47.3	53.8	53.4	20.0	21.2	20.0
14	36.8	35.5	35.3	40.1	37.2	37.4	32.3	25.2	28.1	27.3
15	77.5	76.8	77.6	77.0	82.3	75.4	81.1	75.3	85.2	83.8
16	148.4	153.8	164.8	150.2	88.4	78.8	86.9	77.5	80.2	79.3
17	111.3	111.9	106.1	114.4	71.4	68.1	68.6	65.3	68.8	67.8
18	24.4	26.3	26.6	26.2	25.6	18.8	17.7	25.4	25.7	25.1
19	46.1	58.2	58.6	57.4	57.9	92.8	92.5	56.7	47.7	58.1
20	57.7	66.3	66.3	69.3	66.2	65.6	65.6	71.2	61.3	67.5
21	-	50.9	50.7	51.1	54.6	48.2	47.7	50.3	-	42.5
22	-	13.2	13.4	13.5	13.7	14.1	13.0	12.1	-	-

a:  $\text{CDCl}_3 + \text{CD}_3\text{OD}$ , b:  $\text{CD}_3\text{OD}$ .

**Table 4:** Cardiac effects of 42 diterpenoid alkaloids and lanatoside on isolated bullfrog hearts

Compound	Rate of amplitude increase (%)	Result <sup>a</sup>	Compound	Rate of amplitude increase (%)	Result <sup>a</sup>
control <sup>b</sup>	0	0	30	17.0	(+)
3	85.0	(+++)	34	31.5	(++)
4	31.0	(++)	35	176.7	(++++)
7	-57.0	(-)	36	-42.0	(-)
8	23.9	(+)	38	-50.0	(-)
9	82.0 [2]	(+++)	39	-27.0	(-)
10	118.0 [2]	(+++)	40	-53.0	(-)
11	28.0 [2]	(+)	41	-27.0	(-)
14	45.0	(++)	42	-18.0	(-)
15	0	(-)	43	-55	(-)
17	36.0	(++)	44	-29	(-)
18	23.6	(+)	45	-34	(-)
18a	0 [2]	(-)	46	(-)	(-)
21	0	(-)	47	-56	(-)
22	19.0	(+)	48	(-)	(-)
23	8.0	(-)	50	-5	(-)
24	25.0	(+)	51	-30	(-)
25	20.0	(+)	53	-40	(-)
26	-48.0	(-)	56	-26	(-)
27	8.0	(-)	57	-43%	(-)
28	31.0	(++)	58	0	(-)
29	-29.0	(-)	Lanatoside C	73.3	(+++)

a: The results were judged based on the average rate of amplitude increase: (-): 0-10%, (+): 12-30%, (++): 31-59%, (+++): 60-120%; b: distilled water; c: the cardioactive duration of alkaloids can reach 30'; the concentration of the tested compounds: 0.01 mol/mL; 0.0002 mol/mL for lanatoside C.



**Preparation of compound 2:** To a solution of 13-deoxyindoconitine (**1**, 680 mg, 1.10 mmol) in glacial acetic acid (20 mL) was added NBS (600 mg, 3.4 mmol), and the mixture was kept stirred at room temperature for 3 h prior to being basified to pH 10 with conc. ammonium hydroxide. The mixture was extracted with chloroform; the combined extracts were dried and concentrated. The residue was purified by CC on silica gel, eluting with light petroleum: acetone (3:1, saturated with conc. ammonium hydroxide) to give compound **2** (476 mg, 74%).

#### Compound 2

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 8.05 (2H, d, *J* = 7.2 Hz, H-2", 6"), 7.55 (1H, t, *J* = 7.6 Hz, H-4"), 7.49 (2H, t, *J* = 7.6 Hz, H-3", 5"), 5.07 (1H, t, *J* = 4.8 Hz, H-14β), 4.13 (1H, d, *J* = 6.8 Hz, H-6), 3.82 (1H, dd, *J* = 10.0, 5.6 Hz, H-3), 3.85, 3.32, 3.29, 3.19 (each 3H, s, OMe × 4), 1.33 (3H, s, OAc-8).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

ESIMS: *m/z* 586 [M+H]<sup>+</sup>.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>32</sub>H<sub>44</sub>NO<sub>9</sub>: 586.3016, found: 586.3016

**Preparation of compound 3:** Compound **2** (320 mg, 0.55 mmol) was dissolved in a solution of sodium hydroxide in methanol (5%, 10 mL), and the reaction solution was stirred at 60°C for 1 h. After concentration *in vacuo*, the residue was subjected to CC on silica gel eluting with CHCl<sub>3</sub>: MeOH: diethylamine (97:7:1) to yield compound **3** (242 mg, 77%).

#### Compound 3

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 3.17 (1H, d, *J* = 6.4 Hz, H-1), 1.97, 2.51 (each 1H, m, H<sub>2</sub>-2), 3.71 (1H, t, *J* = 5.6 Hz, H-3), 1.95 (1H, t, *J* = 6.8 Hz, H-5), 4.25 (1H, d, *J* = 6.8 Hz, H-6), 3.17 (1H, hidden, H-7), 2.13 (1H, t, *J* = 5.2 Hz, H-9), 1.64 (1H, m, H-10), 1.65, 1.97 (each 1H, m, H<sub>2</sub>-12), 2.29 (1H, m, H-13), 4.10 (1H, t, *J* = 4.8 Hz, H-14β), 1.78, 2.39 (each 1H, m, H<sub>2</sub>-15), 3.38 (1H, hidden, H-16), 1.88 (1H, s, H-17), 3.69, 3.81 (each 1H, ABq, *J* = 9.2 Hz, H<sub>2</sub>-18), 2.93, 3.38 (each 1H, ABq, *J* = 13.2 Hz, H<sub>2</sub>-19), 3.31 (3H, s, OCH<sub>3</sub>-1), 3.32 (3H, s, OCH<sub>3</sub>-6), 3.24 (3H, s, OCH<sub>3</sub>-16), 3.29 (3H, s, OCH<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>23</sub>H<sub>38</sub>NO<sub>7</sub>: 440.2648, found: 440.2650.

**Preparation of compound 4:** To a solution of compound **2** (150 mg, 0.26 mmol) in anhydrous THF (5 mL) was added sodium hydride (80%, 7 mg, 0.23 mmol), and the mixture was stirred at room temperature before the addition of methyl iodide (18 μL). The reaction was allowed to proceed with stirring for 2 h. Removal of the solvent afforded a residue, to which was added a solution of sodium hydroxide in methanol (5%, 6 mL). A general work-up afforded a crude product, which was purified by CC (silica gel, CHCl<sub>3</sub>-MeOH, 9:1) to give compound **4** (100 mg, 85%).

#### Compound 4

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.57 (1H, brs, OH), 3.02 (1H, dd, *J* = 10.0, 6.8 Hz, H-1), 4.23 (1H, d, *J* = 6.8 Hz, H-6), 4.07 (1H, t, *J* = 4.8 Hz, H-14β), 3.66 (1H, dd, *J* = 11.6, 6.0 Hz, H-3β), 3.33, 3.24 (each 3H, s, OMe × 2), 3.31 (6H, s, OCH<sub>3</sub> × 2), 2.30 (3H, s, NCH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HR-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>24</sub>H<sub>40</sub>NO<sub>7</sub>: 454.2805; found: 454.2800.

**Preparation of compound 6:** To a solution of chasmanine (**5**, 400 mg, 0.88 mmol) in acetic anhydride (6 mL) was added TsOH (400 mg), and the reaction was allowed to proceed with stirring at room

temperature for 16 h. A general work-up provided a white solid, to which was added glacial acetic acid (20 mL) and NBS (710 mg). The subsequent reaction mixture was stirred at room temperature for 3 h before being concentrated. The white residue was subjected to CC on silica gel eluting with light petroleum: acetone:diethylamine (80:20:1) to furnish compound **6** (250 mg, 65%).

#### Compound 6

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.81 (1H, t, *J* = 4.8 Hz, H-14β), 4.06 (1H, d, *J* = 5.6 Hz, H-6), 3.01, 3.58 (each 1H, ABq, *J* = 8.4 Hz, H<sub>2</sub>-18), 3.32, 3.31, 3.26, 3.22 (each 3H, s, OMe × 4), 2.04, 1.97 (each 3H, s, OAc × 2).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HR-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>24</sub>H<sub>40</sub>NO<sub>6</sub>: 438.2856; found: 438.2859.

**Preparation of compound 7:** To a solution of compound **6** (114 mg, 0.26 mmol) in anhydrous THF (20 mL) was added sodium hydride (80%, 6 mg, 0.2 mmol), and the mixture was stirred at room temperature for 15 min before the addition of methyl iodide (14 μL). The reaction was allowed to proceed for 2 h at room temperature. A general work-up procedure gave a residue, to which was added a solution of sodium hydroxide in methanol (5%, 3 mL). The subsequent reaction mixture was stirred at 60°C for 1 h and the solvent was removed *in vacuo*. The white foam was purified by CC eluting with CHCl<sub>3</sub>: MeOH: diethylamine (97:3:1) to yield compound **7** (83 mg, 74%).

#### Compound 7

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.60 (1H, brs, OH), 4.20 (1H, d, *J* = 6.8 Hz, H-6), 4.13 (1H, t, *J* = 4.5 Hz, H-14β), 3.60, 3.70 (each 1H, ABq, *J* = 8.0 Hz, H<sub>2</sub>-18), 3.40 (1H, d, *J* = 9.2 Hz, H-16), 3.34, 3.31, 3.30, 3.25 (each 3H, s, OMe × 4), 2.31 (3H, s, NCH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>24</sub>H<sub>40</sub>NO<sub>6</sub>: 428.2856; found: 438.2859.

**Preparation of compound 8:** To a solution of chasmanine (**5**, 600 mg, 1.32 mmol) in acetic anhydride (60 mL) was added TsOH (40 mg) and the reaction was allowed to proceed for 16 h. After a general work-up, the white residue was dissolved in glacial acetic acid (20 mL). To this solution was added NBS (713 mg, 4.00 mmol), and the subsequent reaction solution was stirred at room temperature for an additional 3 h. After the second standard work-up, the crude product was purified by CC (silica gel, light petroleum: acetone, 4:1 with 1% diethylamine) to give compound **8** (440 mg, 65%).

#### Compound 8

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 3.23, 3.31, 3.33, 3.35 (each 3H, s, OMe × 4), 3.65 (1H, ABq, *J* = 8.4 Hz, H-18), 4.18 (1H, t, *J* = 5.2 Hz, H-14β), 4.23 (1H, t, *J* = 6.8 Hz, H-6).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): 81.7 (C-1), 23.2 (C-2), 28.6 (C-3), 38.7 (C-4), 44.7 (C-5), 82.3 (C-6), 40.1 (C-7), 73.9 (C-8), 47.6 (C-9), 44.4 (C-10), 48.9 (C-11), 28.9 (C-12), 57.1 (C-13), 75.3 (C-14), 41.4 (C-15), 82.4 (C-16), 57.4 (C-17), 79.8 (C-18), 49.9 (C-19), 55.2 (C-1'), 56.1 (C-6'), 57.6 (C-16'), 59.0 (C-18').

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>23</sub>H<sub>38</sub>NO<sub>6</sub>: 424.2699; found: 424.2705.

**Preparation of compound 13:** To a solution of indoconitine (**12**, 4 g, 6.4 mmol) in THF (12 mL) was added thionyl chloride (6 mL), and the reaction mixture was stirred at 40°C for 2 h. Removal of volatile solvent gave a white solid, which was dissolved in ethanol

(95%, 10 mL) and glacial acetic acid (2 mL). To the mixture was added 10% Pd-C and the reaction was allowed to proceed in the presence of hydrogen. A general work-up gave white foam, which was dissolved in glacial acetic acid (9 mL). To the subsequent mixture was added NBS (260 mg), and the reaction mixture was stirred at room temperature for 3 h. After a general work-up, the crude residue was purified by CC eluting with cyclohexane: acetone: diethylamine (75:25:1) to yield compound **13** (2.73 g, 71%).

#### Compound 13

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 8.05 (1H, d, *J* = 7.2 Hz, H-2", 6"), 7.56 (1H, t, *J* = 7.6 Hz, H-4"), 7.43 (2H, t, *J* = 7.6 Hz, H-3", 5"), 4.93 (1H, d, *J* = 5.2 Hz, H-14β), 4.03 (1H, brs, NH), 4.00 (1H, d, *J* = 6.8 Hz, H-6), 3.55, 3.28, 3.24, 3.18 (each 3H, s, OMe × 4), 1.31 (3H, s, OAc-8).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>32</sub>H<sub>44</sub>NO<sub>9</sub>: 586.3016; found: 586.3021

**Preparation of compound 14:** Compound **13** (95 mg, 0.16 mmol) was dissolved in a solution of sodium hydroxide in methanol (1%, 3 mL). The reaction mixture was stirred at 60°C for 1 h before removal of solvent. The crude residue was purified by CC on silica gel eluting with CHCl<sub>3</sub>: MeOH: diethylamine (95:5:1) to provide compound **14** (51 mg, 73%).

#### Compound 14

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.13 (1H, d, *J* = 6.4 Hz, H-6), 3.99 (1H, d, *J* = 4.8 Hz, H-14β), 3.81 (1H, brs, OH), 3.64 (1H, ABq, *J* = 8.4 Hz, H-18), 3.42, 3.34, 3.31, 3.21 (each 3H, s, OCH<sub>3</sub> × 4).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>23</sub>H<sub>38</sub>NO<sub>7</sub>: 440.2648; found: 440.2649.

**Preparation of compound 15:** To a solution of compound **13** (95 mg, 0.16 mmol) in anhydrous THF (5 mL) was added sodium hydride (80%, 5 mg), and the mixture was stirred for 15 min before the addition of methyl iodide (11 μL). The reaction was allowed to proceed for an additional 2 h. A standard work-up provided a white solid, to which was added a solution of sodium hydroxide in methanol (5%, 5 mL). The subsequent reaction mixture was stirred at 60°C for 1 h prior to another standard work-up. The crude residue was purified by CC eluting with CHCl<sub>3</sub>: MeOH: diethylamine (97:3:1) to give compound **15** (24 mg, 71%).

#### Compound 15

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.11 (1H, d, *J* = 7.2 Hz, H-6), 4.02 (1H, brs, OH), 3.97 (1H, t, *J* = 4.8 Hz, H-14β), 3.69, 3.33 (each 1H, ABq, *J* = 8.4 Hz, H<sub>2</sub>-18), 3.42, 3.30, 3.30, 3.25 (each 3H, s, OCH<sub>3</sub> × 4), 2.99 (1H, dd, *J* = 10.8, 6.8 Hz, H-1), 2.31 (3H, s, NCH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>24</sub>H<sub>40</sub>NO<sub>7</sub>: 454.2805; found: 454.2806.

**Preparation of compound 17:** To a solution of dexoyaconitine (**17a**, 315 mg, 0.50 mmol) in glacial acetic acid (10 mL) was added NBS (267 mg, 1.5 mmol), and the reaction mixture was kept stirred at room temperature for 3 h. A standard work-up procedure gave a crude residue, which was subjected to CC eluting with light petroleum: acetone: diethylamine (66:33:1) to furnish *N*-deethyl-deoxyaconitine (24 mg, 80%). This compound was dissolved in a solution of sodium hydroxide in methanol (5%, 10 mL) and refluxed for 30 sec. After removing the solvent, the residue was dissolved in CHCl<sub>3</sub>: MeOH (9:3, 10 mL) and filtered. The filtrate

was concentrated and subjected to CC eluting with CHCl<sub>3</sub>: MeOH (95:5, saturated with conc. NH<sub>4</sub>OH) to yield compound **17** (115 mg, 76%).

#### Compound 17

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.61 (1H, d, *J* = 6.8 Hz, H-15), 4.20 (1H, brs, OH), 3.85 (1H, d, *J* = 6.4 Hz, H-14β), 4.14 (1H, d, *J* = 6.4 Hz, H-6), 3.70, 3.35, 3.32, 3.19 (each 3H, s, OCH<sub>3</sub> × 4), 3.07, 3.60 (each 1H, ABq, *J* = 8.4 Hz, H<sub>2</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>23</sub>H<sub>38</sub>NO<sub>8</sub>: 456.2597; found: 456.2599

**Preparation of compound 18:** To a solution of indaconitine (**12**, 440 mg, 0.7 mmol) in glacial acetic acid (12 mL) was added NBS (375 mg, 2.1 mmol), and the reaction was allowed to proceed at room temperature for 4 h prior to being basified with conc. ammonium hydroxide to pH 10. The subsequent mixture was extracted with CHCl<sub>3</sub> (20 mL × 3); the combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was purified by CC eluting with light petroleum: acetone: diethylamine (75:25:1) to yield *N*-deethylindaconitine (329 mg, 78%). To the solution of this compound (300 mg, 0.5 mmol) in THF (10 mL) was added sodium hydride (80%, 15 mg, 0.5 mmol) followed by methyl iodide (35 μL, 0.6 mmol) under argon. The subsequent reaction was allowed to proceed for 2 h before the solvent was removed. To the residue was added a solution of sodium hydroxide in methanol (15 mL), and the reaction was allowed to proceed with refluxing for an additional 1 h. The reaction mixture was first acidified with conc. HCl to pH 2 then basified with conc. ammonium hydroxide to pH 10. The mixture was extracted with chloroform, and the combined extracts were dried over anhydrous sodium sulfate and concentrated. The crude residue was purified by CC eluting with CHCl<sub>3</sub>: MeOH (95:5, saturated with conc. ammonium hydroxide) to give compound **18** (185 mg, 78%).

#### Compound 18

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.15 (1H, d, *J* = 6.8 Hz, H-6), 3.66 (1H, dd, *J* = 11.2, 6.8 Hz, H-3β), 3.41, 3.32, 3.31, 3.25 (each 3H, s, OCH<sub>3</sub> × 4), 2.32 (3H, s, NCH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>24</sub>H<sub>40</sub>NO<sub>8</sub>: 470.2754; found: 470.2750.

**Preparation of compound 20:** To a solution of talasamine (**19**, 1.1 g, 2.61 mmol) in acetic anhydride (6 mL) was added TsOH (600 mg), and the reaction was allowed to proceed at room temperature for 16 h. After a standard work-up, the white residue was dissolved again in glacial acetic acid (40 mL). To this solution was added 7 equivalents of NBS, and the reaction was continued with stirring at room temperature for 4 h. After the second standard work-up, the white foam was subjected to CC eluting with cyclohexane: acetone: diethylamine to furnish an imine (230 mg). Hydrogenolysis of this imine catalyzed by Pt<sub>2</sub>O at 60°C for 24 h yielded a white residue, which was purified by CC eluting with CHCl<sub>3</sub>: MeOH: diethylamine (90:10:1) to furnish compound **20** (250 mg, 20%).

#### Compound 20

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.80 (1H, t, *J* = 4.8 Hz, H-14β), 3.31, 3.28, 3.27 (each 3H, s, OCH<sub>3</sub> × 3), 2.03, 1.94 (each 3H, s, OAc).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>26</sub>H<sub>40</sub>NO<sub>7</sub>: 478.2805; found: 478.2804.

**Preparation of compound 21:** Compound **20** (100 mg, 0.20 mmol) was dissolved in a solution of sodium hydroxide in methanol (5%, 10 mL), and the reaction mixture was stirred at 60°C for 1 h. After a general work-up procedure, the white residue was purified by CC eluting with CHCl<sub>3</sub>: MeOH: diethylamine (90:10:1) to generate compound **21** (24 mg, 30%).

#### Compound 21

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.56 (1H, brs, OH), 4.18 (1H, d, *J* = 4.5 Hz, H-14β), 3.34, 3.29, 3.27 (each 3H, s, OMe × 3), 3.95, 3.09 (each 1H, ABq, *J* = 8.8 Hz, H<sub>2</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>36</sub>NO<sub>5</sub>: 394.2593; found: 394.2591.

**Preparation of compound 22:** To a solution of compound **20** (105 mg, 0.22 mmol) in THF (5 mL) was added sodium hydride (80%, 7 mg, 0.22 mmol), and the mixture was stirred for 15 min before the addition of methyl iodide (15 μL). The reaction was allowed to proceed with stirring at room temperature for 2 h. A general work-up gave a white solid, to which was added a solution of sodium hydroxide in methanol (5%, 3 mL). The subsequent reaction mixture was stirred at 60°C for an additional 1 h and then concentrated under reduced pressure. The crude residue was purified by CC on silica gel eluting with CHCl<sub>3</sub>: MeOH (97:3) to yield compound **22** (59 mg, 65%).

#### Compound 22

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.81 (1H, brs, disappeared with D<sub>2</sub>O, OH), 4.13 (1H, t, *J* = 4.8 Hz, H-14β), 3.34, 3.29, 3.27 (each 3H, s, OMe × 3), 2.26 (3H, s, NCH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>33</sub>H<sub>38</sub>NO<sub>5</sub>: 408.2750; found: 408.2755.

**Preparation of compounds 23, 24, 25 and 26:** Pyrolysis of 13-deoxyindaconitine (**1**, 1.2 g, 1.95 mmol) at 180-190°C under vacuum for 5 min followed by CC (light petroleum: acetone: diethylamine, 80:16:1) provided a white powder (650 mg, 62%). This was dissolved in acetic anhydride (25 mL), to which was added TsOH (200 mg). The subsequent solution was refluxed for 3 h prior to being cooled to 0°C. The mixture was basified with conc. NH<sub>4</sub>OH to pH > 10, and extracted with chloroform 3 times. The combined extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The crude residue was subjected to CC eluting with light petroleum: acetone (5:1) to give a rearranged product (white amorphous powder, 590 mg, 80%). To a solution of this product in glacial acetic acid (10 mL) was added NBS (450 mg, 3 eq), and the reaction mixture was stirred at room temperature for 2 h. The mixture was cooled to 0°C and basified with conc ammonium hydride to pH > 10, which was extracted with chloroform 3 times. The combined extracts were dried over anhydrous sodium sulfate and concentrated. The crude residue (520 mg) was dissolved in a solution of sodium hydroxide in methanol (5%) and stirred at 50°C for 1 h. After removal of solvent, the residue was partitioned between water and ethyl acetate. The organic layers were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by CC eluting with light petroleum: acetone: diethylamine (60:30:1) to yield compound **23** (white amorphous powder, 400 mg, 50%). To a solution of compound **23** (200 mg, 0.24 mmol) in THF was added sodium hydride (8.0 mg) and methyl iodide (20 μL), and the reaction was allowed to proceed at room temperature for 2 h. After removal of solvent, the residue was purified by CC eluting with chloroform: methanol (50:1) to give compound **24** (white amorphous

powder, 180 mg, 89%). To a solution of compound **23** (100 mg, 0.23 mmol) in 95% ethanol (3 mL) was added 2 drops of glacial acetic acid and Pt<sub>2</sub>O (10 mg). The reaction was allowed to proceed in the presence of hydrogen under 30 MPa for 20 h. The mixture was basified with conc. ammonium hydroxide to pH > 10. A standard work-up procedure followed by CC [chloroform: methanol (50:1)] yielded compound **25** (white amorphous powder, 90 mg, 89%). According to a similar procedure, hydrogenation of **24** (100 mg, 0.23 mmol), followed by CC (silica gel, CHCl<sub>3</sub>: MeOH = 100:1) gave compound **26** (87 mg, 87 %).

#### Compound 23

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 5.89 (1H, d, *J* = 9.6 Hz, H-16), 5.72 (1H, d, *J* = 9.6 Hz, H-15), 4.34 (1H, d, *J* = 6.8 Hz, H-6), 4.01 (1H, t, *J* = 4.8 Hz, H-14β), 3.36, 3.31, 3.24 (each 3H, s, OCH<sub>3</sub> × 3).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>34</sub>NO<sub>6</sub>: 408.2386; found: 408.2381.

#### Compound 24

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 5.87 (1H, d, *J* = 9.6 Hz, H-16), 5.63 (1H, d, *J* = 9.6 Hz, H-15), 4.30 (1H, d, *J* = 6.8 Hz, H-6), 4.03 (1H, t, *J* = 4.8 Hz, H-14β), 3.34, 3.32, 3.23 (each 3H, s, OMe × 3), 2.26 (3H, s, NCH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>23</sub>H<sub>36</sub>NO<sub>6</sub>: 422.2543; found: 422.2540.

#### Compound 25

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.27 (1H, d, *J* = 6.4 Hz, H-6), 4.02 (1H, t, *J* = 4.8 Hz, H-14β), 3.34, 3.31, 3.29 (each 3H, s, OCH<sub>3</sub> × 3).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 1.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>36</sub>NO<sub>6</sub>: 410.2543; found: 410.2560.

#### Compound 26

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.23 (1H, d, *J* = 6.8 Hz, H-6), 4.03 (1H, t, *J* = 4.8 Hz, H-14β), 3.32, 3.31, 3.27 (each 3H, s, OCH<sub>3</sub> × 3), 3.01 (1H, dd, *J* = 9.2, 6.0 Hz, H-1), 2.36 (3H, s, NCH<sub>3</sub>). 2.90, 2.59 (each 1H, ABq, *J* = 11.2 Hz, H<sub>2</sub>-19).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 2.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>23</sub>H<sub>38</sub>NO<sub>6</sub>: 424.2699; found: 424.2700.

**Preparation of compounds 27, 28, 29 and 30:** These 4 compounds were prepared from chasmaning (**5**, 2.0 g, 4.45 mmol) using the same procedure described for the preparation of compounds **23-26**.

#### Compound 27

White amorphous powder, 300 mg, 17% yield.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 5.84 (1H, m, H-16), 5.76 (1H, d, *J* = 9.6 Hz, H-15), 4.30 (1H, d, *J* = 6.8 Hz, H-6), 3.97 (1H, t, *J* = 5.2 Hz, H-14β), 3.33, 3.29, 3.16 (each 3H, s, OCH<sub>3</sub> × 3).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 2.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>34</sub>NO<sub>5</sub>: 392.2437; found: 392.2435.

#### Compound 28

White amorphous powder, 140 mg, 93% yield.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 5.89 (1H, m, H-16), 5.64 (1H, d, *J* = 9.6 Hz, H-15), 4.25 (1H, d, *J* = 6.8 Hz, H-6), 4.04 (1H, t, *J* = 4.4 Hz, H-14β), 3.33, 3.31, 3.23 (each 3H, s, OCH<sub>3</sub> × 3), 3.01 (1H, dd, *J* = 10.0, 6.8 Hz, H-1), 2.26 (3H, s, NCH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 2.

HRMS-ESI:  $m/z$   $[M+H]^+$  calcd for  $C_{23}H_{36}NO_5$ : 406.2593; found: 406.2591.

#### Compound 29

White amorphous powder, 65 mg, 91% yield.

$^1H$  NMR (400 MHz,  $CDCl_3$ ): 4.22 (1H, d,  $J = 6.4$  Hz, H-6), 4.06 (1H, t,  $J = 4.8$  Hz, H-14 $\beta$ ), 3.35, 3.31, 3.22 (each 3H, s,  $OCH_3 \times 3$ ).

$^{13}C$  NMR (100 MHz,  $CDCl_3$ ): Table 2.

HRMS-ESI:  $m/z$   $[M+H]^+$  calcd for  $C_{22}H_{36}NO_5$ : 394.2593; found: 394.2599.

#### Compound 30

White amorphous powder, 63 mg, 91% yield.

$^1H$  NMR (400 MHz,  $CDCl_3$ ): 4.18 (1H, d,  $J = 6.8$  Hz, H-6), 4.04 (1H, t,  $J = 4.8$  Hz, H-14 $\beta$ ), 3.31, 3.30, 3.28 (each 3H, s,  $OCH_3 \times 3$ ), 3.01 (1H, dd,  $J = 10.4, 6.4$  Hz, H-1), 2.72, 2.41 (each 1H, ABq,  $J = 10.4$  Hz, H<sub>2</sub>-19), 2.31 (3H, s, H<sub>3</sub>-21).

$^{13}C$  NMR (100 MHz,  $CDCl_3$ ): Table 2.

HRMS-ESI:  $m/z$   $[M+H]^+$  calcd for  $C_{23}H_{38}NO_7$ : 408.2648; found: 408.2642.

**Preparation of compound 31a:** To a solution of 3-acetylaconitine (**31**, 600 mg, 0.87 mmol) in THF (72 mL) was added imidazole (8 mg), NaH (696 mg),  $CS_2$  (7.2 mL) and  $CH_3I$  (5.4 mL, 0.08 mmol), and the reaction was allowed to proceed at room temperature for 3 h. A general work-up yielded a residue (400 mg), to which was added benzene (20 mL) and  $n-Bu_3SnH$  (0.24 mg), and the reaction mixture was refluxed for 1 h. After removal of solvent, a general work-up gave a residue (300 mg). To a solution of this residue (180 mg) in glacial acetic acid (5.5 mL) was added NBS (144 mg, 0.81 mmol), and the reaction mixture was stirred at room temperature for 1.5 h. After removal of the solvent, the residue was diluted with water (15 mL), basified with ammonium hydroxide to pH > 10, and extracted with  $CHCl_3$  (10 mL  $\times$  3). The combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was purified by CC eluting with light petroleum: acetone: diethylamine (75:15:1) to produce compound **31a** (150 mg, 26 %).

#### Compound 31a

$^1H$  NMR (400 MHz,  $CDCl_3$ ): 8.02 (2H, d,  $J = 7.6$  Hz), 7.56 (1H, t,  $J = 7.2$  Hz), 7.44 (2H, t,  $J = 7.6$  Hz), 5.20 (1H, t, H-3), 5.06 (1H, t,  $J = 4.4$  Hz, H-14 $\beta$ ), 4.38 (1H, d,  $J = 5.2$  Hz, H-15), 4.18 (1H, d,  $J = 6.4$  Hz, H-6), 3.73 (1H, d,  $J = 8.8$  Hz, H-18), 3.53, 3.28, 3.26, 3.21 (each 3H, s,  $OCH_3 \times 4$ ), 2.06 (3H, s,  $OAc$ -3), 1.42 (3H, s,  $OAc$ -8).

HRMS-ESI:  $m/z$   $[M+H]^+$  calcd for  $C_{34}H_{46}NO_{11}$ : 644.3071; found: 644.3123.

**Preparation of compound 34:** Compound **31a** (70 mg, 0.11 mmol) was dissolved in a solution of sodium hydroxide (5%, 2 mL), and the reaction was allowed to proceed at room temperature overnight. A general work-up gave a residue, which was subjected to CC on silica gel eluting with  $CHCl_3$ : MeOH (10:1, saturated with conc.  $NH_4OH$ ) to yield compound **34** (40 mg, 81%).

#### Compound 34

$^1H$  NMR (400 MHz,  $CDCl_3$ ): 8.02 (2H, d,  $J = 7.2$  Hz), 7.56 (1H, t,  $J = 7.2$  Hz), 7.44 (2H, t,  $J = 7.6$  Hz), 5.04 (1H, t,  $J = 4.4$  Hz, H-14 $\beta$ ), 4.91 (1H, dd,  $J = 12.0, 6.0$  Hz, H-3), 4.40 (1H, d,  $J = 2.4$  Hz, OH-15), 4.34 (1H, dd,  $J = 6.0, 2.4$  Hz, H-15), 4.13 (1H, d,  $J = 6.4$  Hz, H-6), 3.82 (1H, d,  $J = 8.8$  Hz, H-18), 3.53, 3.29, 3.22, 3.20 (each 3H, s,  $OCH_3 \times 4$ ), 2.34 (3H, s,  $NCH_3$ ), 2.06 (3H, s,  $OAc$ -3), 1.44 (3H, s,  $OAc$ -8);

$^{13}C$  NMR (100 MHz,  $CDCl_3$ ): Table 2.

HRMS-ESI:  $m/z$   $[M+H]^+$  calcd for  $C_{23}H_{38}NO_7$ : 440.2648; found: 440.2650.

**Preparation of compound 35:** To a solution of compound **31a** (110 mg, 0.17 mmol) in THF (5.5 mL) was added sodium hydride (4.1 mg, 0.17 mmol) and methyl iodide (10.7  $\mu$ L, 0.17 mmol), and the reaction mixture was stirred at room temperature for 2 h. A general work-up gave a crude residue, to which was added a solution of sodium hydroxide in methanol (5%, 2 mL). The subsequent reaction mixture was stirred overnight and concentrated under reduced pressure. The crude residue was subjected to CC on silica gel eluting with  $CHCl_3$ : MeOH (20:1) to yield compound **35** (34 mg, 84.1%).

#### Compound 35

$^1H$  NMR (400 MHz,  $CDCl_3$ ): 3.82 (1H, br.s, H-3), 4.27 (1H, d,  $J = 6.4$  Hz, H-6), 4.04 (1H, d,  $J = 4.8$  Hz, H-14 $\beta$ ), 3.45, 3.77 (each 1H, ABq,  $J = 8.4$  Hz, H<sub>2</sub>-18), 3.32, 3.31, 3.37, 3.39 (each 3H, s,  $OMe \times 4$ ).

$^{13}C$  NMR (100 MHz,  $CDCl_3$ ): Table 2.

HRMS-ESI:  $m/z$   $[M+H]^+$  calcd for  $C_{24}H_{40}NO_8$ : 470.2754; found: 470.2750.

**Preparation of compound 36:** To a solution of 13-deoxyindaconitine (**1**, 200 mg, 0.28 mmol) in glacial acetic acid (10 mL) was added NBS (310 mg, 1.22 mmol), and the reaction was allowed to proceed at 130°C for 10 h. A general work-up provided a residue, to which was added a solution of sodium hydroxide in methanol (5%, 10 mL). The subsequent reaction mixture was stirred at 50°C for 1 h before removing the methanol. The crude residue was purified by CC eluting with  $CHCl_3$ : MeOH (40:1) to give compound **36** (115 mg, 70%).

#### Compound 36

$^1H$  NMR (400 MHz,  $CDCl_3$ ): 7.47 (1H, s, H-19), 3.67 (1H, dd,  $J = 10.4, 6.4$  Hz, H-1), 3.38, 3.36, 3.25, 3.20 (each 3H, s,  $OCH_3 \times 4$ ).

$^{13}C$  NMR (100 MHz,  $CDCl_3$ ): Table 2.

HRMS-ESI:  $m/z$   $[M+H]^+$  calcd for  $C_{23}H_{36}NO_7$ : 438.2492; found: 438.2496.

**Preparation of compound 38:** To a solution of lappaconitine (**37**, 2.336 g, 4 mmol) in glacial acetic acid (70 mL) was added NBS (2.136 g, 12 mmol), and the reaction was allowed to proceed at room temperature for 2 h. The reaction was quenched by pouring the reaction mixture into ice water, which was basified with conc ammonium hydroxide to pH 10 and extracted with chloroform (20 mL  $\times$  3). The combined extracts were dried over anhydrous sodium sulfate and concentrated. The residue (2.3 g) was purified by CC on silica gel eluting with light petroleum: acetone: diethylamine (75:25:1) to give *N*-deethylappaconitine (**38**, 1.87 g, 84%).

#### Compound 38

$^1H$  NMR (400 MHz,  $CDCl_3$ ): 8.63 (8.56) (1H, d,  $J = 8.4$  Hz, H-6"), 7.90 (1H, d,  $J = 8.0$  Hz, H-3"), 7.50 (7.44) (1H, t,  $J = 8.0$  Hz, H-4"), 7.01 (6.99) (1H, t,  $J = 8.0$  Hz, H-5"), 6.51 (6.66) (each 1H, brs, NH), 3.37 (3H, s,  $OCH_3$ ), 3.27, 3.26 (each 3H, s,  $OCH_3 \times 2$ ), 2.91 (3H, s,  $NCOCH_3$ ).

$^{13}C$  NMR (100 MHz,  $CDCl_3$ ): Table 2.

HRMS-ESI:  $m/z$   $[M+H]^+$  calcd for  $C_{30}H_{41}N_2O_8$ : 557.2863; found: 557.2959

**Preparation of compound 39:** Compound **38** (300 mg, 0.54 mmol) was dissolved in a solution of sodium hydroxide in methanol (10%), and the mixture was stirred at 60°C for 1 h and then basified with conc.  $NH_4OH$  to pH 11. After removing the solvents, the residue was purified by CC eluting with  $CHCl_3$ : MeOH (95:5 - 9:1, saturated with conc. ammonium hydroxide) to furnish compound **39** (168 mg, 79%).

**Compound 39**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 3.43 (1H, ABq, *J* = 8.4 Hz, H-18), 3.41, 3.30, 3.28 (each 3H, s, OCH<sub>3</sub> × 3).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 2.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>21</sub>H<sub>34</sub>NO<sub>6</sub>: 396.2386; found: 396.2388.

**Preparation of compound 40:** To a solution of compound **38** (420 mg, 0.755 mmol) in THF (10 mL) was added sodium hydride (18 mg, 0.755 mmol) under argon, and the mixture was stirred for 15 min before the addition of methyl iodide (50 µl, 0.801 mmol). The reaction was allowed to proceed with stirring at room temperature for 2 h, and the solvent was removed under reduced pressure. To the residue was added a solution of sodium hydroxide in methanol (5%, 15 mL), and the subsequent mixture was stirred at 60°C for 1 h prior to being basified with conc. ammonium hydroxide to pH 11. The solvent was removed *in vacuo*, and the residue was subjected to CC on silica gel eluting with chloroform: methanol (95:5, saturated with conc. ammonium hydroxide) to yield compound **40** (263 mg, 85%).

**Compound 40**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 3.40, 3.30, 3.29 (each 3H, s, OCH<sub>3</sub> × 3), 2.29 (3H, s, NCH<sub>3</sub>).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 2.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>36</sub>NO<sub>6</sub>: 410.2543; found: 410.2541.

**Preparation of compound 44:** To a solution of acetylsongorine (**43a**, 600 mg, 1.38 mmol) in isopropanol (15 mL) was added NBS (740 mg, 4.17 mmol), and the reaction mixture was stirred at 30°C for 2 h. After being basified with conc. ammonium hydroxide to pH > 10, the mixture was extracted with chloroform: methanol (10:1, 30 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated. The crude residue was subjected to CC eluting with CHCl<sub>3</sub>: MeOH (20:1) to provide a white amorphous powder (280 mg). This was dissolved in a solution of sodium hydroxide in methanol (5%, 10 mL) and the consequent mixture was stirred at 50°C for 2 h. After removal of solvent, the residue was subjected to CC on silica gel eluting with CHCl<sub>3</sub>: MeOH (20:1) to yield compound **44** (150 mg, 25%).

**Compound 44**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 5.19, 5.14 (each 1H, s, H<sub>2</sub>-17), 4.28 (1H, s, H-15), 3.57 (1H, s, H-20), 0.67 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 3.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>20</sub>H<sub>28</sub>NO<sub>3</sub>: 330.2069; found: 330.2072.

**Preparation of compounds 45 and 46:** To a solution of songorine (**43**, 420 mg, 1.18 mmol) in methanol (30 mL) was added NaBH<sub>4</sub> (100 mg, 2.63 mmol), and the reaction mixture was stirred at room temperature for 35 min. The mixture was basified with conc. ammonium hydroxide to pH 10 and extracted with chloroform (20 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The crude residue was purified by CC on silica gel eluting with CHCl<sub>3</sub>: MeOH (30:1) to yield compounds **45** (160 mg, 40%) and **46** (250 mg, 60%).

**Compound 45**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 5.16, 5.14 (each 1H, s, H<sub>2</sub>-17), 4.17 (1H, s, H-15), 3.93 (1H, dd, *J* = 7.6, 6.4 Hz, H-12), 3.52 (1H, dd, *J* = 9.6, 6.8 Hz, H-1), 3.36 (1H, s, H-20), 1.06 (3H, t, *J* = 7.2 Hz, H<sub>3</sub>-22), 0.77 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 3.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>34</sub>NO<sub>3</sub>: 360.2539; found: 360.2538.

**Compound 46**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 5.33, 5.12 (each 1H, s, H<sub>2</sub>-17), 4.21 (1H, s, H-15), 4.18 (1H, dd, *J* = 9.2, 5.6 Hz, H-12), 3.87 (1H, dd, *J* = 8.8, 6.4 Hz, H-1), 3.47 (1H, s, H-20), 1.14 (3H, t, *J* = 7.2 Hz, H<sub>3</sub>-22), 0.80 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 3.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>34</sub>NO<sub>3</sub>: 360.2539; found: 360.2540.

**Preparation of compound 47:** To a solution of songorine (**43**, 50 mg, 0.14 mmol) in 2 mL TEG was added NH<sub>2</sub>NH<sub>2</sub> · H<sub>2</sub>O (0.4 mL, 7 mmol) and KOH (40 mg, 0.7 mmol), and the reaction was allowed to proceed under reflux at 160°C for 1 h. After removal of water and hydrazine, the reaction was allowed to proceed at 180°C for an additional 1 h. After cooling to room temperature, the reaction mixture was diluted with water and extracted with ethyl acetate (20 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was purified by CC eluting with light petroleum: ethyl acetate: diethylamine (75:25:1) to furnish compound **47** (40 mg, 82%).

**Compound 47**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 5.01 (2H, s, H<sub>2</sub>-17), 4.19 (1H, d, *J* = 8.4 Hz, H-15), 3.97 (1H, t, *J* = 6.0 Hz, H-1), 3.34 (1H, s, H-20), 1.06 (3H, t, *J* = 7.2 Hz, H<sub>3</sub>-22), 0.78 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 3.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>34</sub>NO<sub>2</sub>: 344.2590; found: 344.2585.

**Preparation of compound 48:** To a solution of compound **46** (90 mg, 0.25 mmol) in formic acid (85%, 10 mL) was added hydrogen peroxide (30%, 0.4–5 mL), and the reaction was allowed to proceed at room temperature for 5 h. The reaction mixture was basified with conc. ammonium hydroxide to pH > 10 and extracted with chloroform: methanol (5:1, 10 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was purified by CC eluting with CHCl<sub>3</sub>: MeOH (20:1, saturated with conc. NH<sub>4</sub>OH) to give compound **48** (30 mg, 35%).

**Compound 48**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.41 (1H, d, *J* = 13.2 Hz, H-12), 3.97, 3.36 (each 1H, ABq, *J* = 8.4 Hz, H<sub>2</sub>-17), 3.73 (1H, s, H-15), 0.74 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 3.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>36</sub>NO<sub>5</sub>: 394.2593; found: 374.2585.

**Preparation of compounds 50 and 51:** To a solution of songoramine (**49**, 100 mg, 0.28 mmol) in formic acid (85%, 10 mL) was added hydrogen peroxide (30%, 0.5 mL), and the reaction was allowed to proceed at room temperature for 20 h prior to being basified with 25% ammonium hydroxide to pH > 10. The subsequent mixture was extracted with ethyl acetate (20 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo*. CC of the crude residue on silica gel eluting with CHCl<sub>3</sub>: MeOH (30:1) furnished compounds **50** (60 mg, 85%) and **51** (35 mg, 32%).

**Compound 50**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.10, 3.70 (each 1H, ABq, *J* = 8.0 Hz, H<sub>2</sub>-17), 4.03 (1H, d, *J* = 5.2 Hz, H-1), 3.83 (1H, s, H-19), 3.70 (1H, s, H-15), 1.03 (3H, t, *J* = 7.2 Hz, H<sub>3</sub>-22), 0.82 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 3.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>32</sub>NO<sub>5</sub>: 390.2280; found: 390.2279.

**Compound 51**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 3.97 (1H, d, *J* = 5.2 Hz, H-1), 3.70 (1H, s, H-15), 3.55, 3.50 (each 1H, ABq, *J* = 7.6 Hz, H<sub>2</sub>-17), 1.02 (3H, t, *J* = 7.2 Hz, H<sub>3</sub>-22), 0.84 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 3.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>32</sub>NO<sub>5</sub>: 390.2280; found: 390.2279.

**Preparation of compound 53:** To a solution of denudatine (**52**, 20 mg, 0.06 mmol) in methanol (1 mL) was added hydrogen peroxide (0.1 mL), and the reaction was allowed to proceed at 60°C for 20 h prior to being quenched with 25% ammonium hydroxide to pH > 10. The mixture was extracted with chloroform: methanol (5:1, 10 mL × 3). The combined extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was purified by CC on silica gel, using CHCl<sub>3</sub>: MeOH (50:1) as eluent, to provide compound **53** (13 mg, 60%).

**Compound 53**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 3.82 (1H, d, *J* = 9.2 Hz, H-11), 3.38, 3.43 (each 1H, ABq, *J* = 12.0 Hz, H<sub>2</sub>-17), 3.26 (1H, s, H-15), 1.06 (3H, t, *J* = 7.2 Hz, H<sub>3</sub>-22), 0.73 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 3.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>22</sub>H<sub>36</sub>NO<sub>4</sub>: 378.2644; found: 378.2648.

**Preparation of compound 55:** To a solution of compound **54** (70 mg, 0.12 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (2 mL) was added *m*CPBA (70 mg, 0.39 mmol), and the reaction mixture was kept stirred at room temperature for 30 min prior to being concentrated. To the residue was added Fe<sub>2</sub>SO<sub>4</sub>·7H<sub>2</sub>O (130 mg, 0.46 mmol) and the subsequent mixture was stirred at room temperature for 3 h. After general workup, the crude product was purified by CC over silica gel eluting with CHCl<sub>3</sub>: MeOH (100:1) to furnish compound **55** (23 mg, 35%).

**Compound 55**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 5.16 (1H, s, H-15), 5.12 (1H, m, H-1), 5.00 (1H, m, H-11), 3.91 (1H, m, H-20), 2.05, 2.05, 1.98, 1.99 (each 3H, s, OAc × 4), 1.03 (3H, t, *J* = 7.2 Hz, H<sub>3</sub>-22), 0.72 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>): Table 3.

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HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>28</sub>H<sub>40</sub>NO<sub>9</sub>: 534.2703; found: 534.2740.

**Preparation of compound 56:** Compound **55** (30 mg, 0.06 mmol) was dissolved in a solution of sodium hydroxide in methanol (5%, 10 mL), and the subsequent reaction solution was stirred at 60°C for 2 h. The reaction mixture was concentrated *in vacuo*, and the residue was purified by CC over silica gel, eluting with CHCl<sub>3</sub>: MeOH (20:1, saturated with 25% ammonium hydroxide) to furnish compound **56** (7 mg, 35%).

**Compound 56**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.55 (1H, d, *J* = 9.2 Hz, H-11), 4.21 (1H, dd, *J* = 10.4, 6.8 Hz, H-1), 4.16 (1H, s, H-15), 3.80, 3.77 (each 1H, ABq, *J* = 12.0 Hz, H<sub>2</sub>-17), 3.86 (1H, s, H-20), 0.83 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>+CD<sub>3</sub>OD): Table 3.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>20</sub>H<sub>32</sub>NO<sub>5</sub>: 366.2280; found: 366.2276.

**Preparation of compound 57:** To a solution of compound **55** (58 mg, 0.10 mmol) in THF (2 mL) was added sodium hydride (3.0 mg, 0.13 mmol), followed by methyl iodide (7 μL, 0.10 mmol). The reaction mixture was stirred at room temperature for 3 h prior to removing THF *in vacuo*. To the residue was added a solution of sodium hydroxide (5%) in methanol (10 mL), and the subsequent reaction mixture was stirred at 60°C for 2 h. After removing methanol, the residue was subjected to CC, eluting with CHCl<sub>3</sub>: MeOH (50:1, v/v, saturated with 25% ammonium hydroxide) to yield compound **57** (35 mg, 92 %).

**Compound 57**

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): 4.38 (1H, d, *J* = 9.2 Hz, H-11), 4.06 (1H, dd, *J* = 10.4, 6.8 Hz, H-1), 2.25 (3H, s, H<sub>3</sub>-21), 0.65 (3H, s, H<sub>3</sub>-18).

<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD): Table 3.

HRMS-ESI: *m/z* [M+H]<sup>+</sup> calcd for C<sub>21</sub>H<sub>32</sub>NO<sub>5</sub>: 378.2280; found: 378.2271.

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# Monoterpenoid Indole Alkaloids from *Catharanthus roseus* Cultivated in Yunnan

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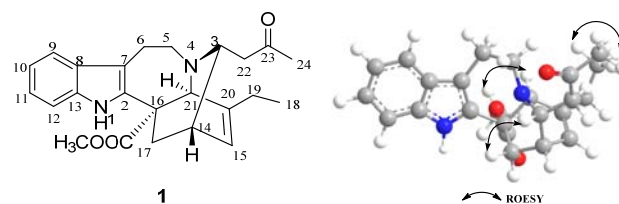
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A new monoterpenoid indole alkaloid, 15,20-dehydro-3 $\alpha$ -(2-oxopropyl) coronaridine (**1**), along with sixteen analogues (**2**–**17**) were isolated from the leaves of *Catharanthus roseus* cultivated in Yunnan. The new alkaloid was elucidated on the basis of extensive spectroscopic analysis, and the known alkaloids were identified by comparison with the reported spectroscopic data. Among them, alkaloid **16** was isolated from *Catharanthus* for the first time.

**Keywords:** *Catharanthus roseus*, Apocynaceae, Monoterpenoid indole alkaloids, 15,20-Dehydro-3 $\alpha$ -(2-oxopropyl) coronaridine.

*Catharanthus roseus* (L.) G. Don. (Apocynaceae), a perennial evergreen herb with medicinal and ornamental values, is widely distributed in continental Africa, America, Asia, Australia, Europe, and in some islands of the Pacific Ocean [1]. *C. roseus* has been studied widely in view of its remarkable anticancer properties, especially the bisindole alkaloids, vinblastine and vincristine [1]. In previous investigations, we found that plant secondary metabolites would be influenced significantly by the ecological environments [2]. *C. roseus* has also been cultivated widely in China as an outside ornamental, which inspired us to search for structurally diverse indole alkaloids from the same plant in different ecological environments. Phytochemical investigation of the alkaloids of *C. roseus* cultivated in Xishuangbanna, a tropical rainforest zone, afforded a new alkaloid 15,20-dehydro-3 $\alpha$ -(2-oxopropyl) coronaridine (**1**), along with sixteen analogues (**2**–**17**), coronaridine (**2**) [3], tetrahydroalstonine (**3**) [4], ajmalicine (**4**) [4], ajmalicinine (**5**) [5], serpentine (**6**) [6], 19-*epi*-alstonine (**7**) [7], yohimbine (**8**) [8], 19,20-dehydro-16-*epi*-yohimbine (**9**) [9], 16*R*-sitsirikine (**10**) [10], isositsirikine (**11**) [10], 16*R*-dihydrositsirikine (**12**) [11], pericyclivine (**13**) [12], akuammidine (**14**) [12], 17-acetyllochnerine (**15**) [13], 21-carbonylnormavacurine (**16**) [14], and 19*S*-*epi*-misiline (**17**) [15]. Among them, alkaloid (**16**) was isolated from this plant for the first time.

Alkaloid **1** was isolated as a white amorphous powder. Its molecular formula was deduced as C<sub>24</sub>H<sub>28</sub>N<sub>2</sub>O<sub>3</sub> based on <sup>13</sup>C NMR and HRESIMS data. The <sup>1</sup>H NMR spectrum of **1** showed characteristic signals of an unsubstituted indole alkaloid moiety at  $\delta_H$  7.47 (1H, d, 7.8 Hz, H-9), 7.08 (1H, td, 7.2 Hz, H-10), 7.14 (1H, td, 7.2 Hz, H-11), and 7.23 (1H, d, 7.8 Hz, H-12). Additionally, the <sup>1</sup>H spectrum displayed signals for an ethyl side chain at  $\delta_H$  2.25/2.10 (2H, m, H-19) and 1.07 (3H, t, Me-18), a bridgehead proton adjacent to a nitrogen at  $\delta_H$  4.15 (1H, br s, H-21), a methoxy group at  $\delta_H$  3.71 (3H, s), a -COCH<sub>3</sub> group ( $\delta_H$  2.13, 3H, s), and an olefinic proton at  $\delta_H$  5.77 (1H, d, 6.0 Hz, H-15). The <sup>1</sup>H-<sup>1</sup>H COSY spectrum suggested two main spin systems:  $\delta_H$  2.76/1.85 (dd/dd, H<sub>2</sub>-17)/ $\delta_H$  2.66 (m, H-14) [ $\delta_H$  3.36 (m, H-3/N)]/ $\delta_H$  2.48/2.33 (dd/dd, H<sub>2</sub>-22)/H-15 and H<sub>2</sub>-19/Me-18. The HMBC correlations from H-3 to  $\delta_C$  51.5 (t, C-5) and  $\delta_C$  62.1 (d, C-21) revealed that C-3, C-21, and C-5 originated from carbons attached to N-4. Likewise, the HMBC cross-peaks of H-21/C-2,  $\delta_C$  54.8 (s, C-16),  $\delta_C$  38.7 (t, C-17) and



**Figure 1:** Structure and ROESY correlations of compound **1**.

**Table 1:** <sup>1</sup>H and <sup>13</sup>C NMR data for **1** (CDCl<sub>3</sub>, 600 and 150 MHz, respectively).

position	$\delta_H$ , mult. (J in Hz)	$\delta_C$ , type	HMBC
1	7.62, br s		7, 8
2		136.2, s	
3	3.36, m	55.9, d	5, 21
5	3.44, m; 3.32, m	51.5, t	7, 21
6	3.26, m; 2.90, m	21.5, t	
7		110.7, s	
8		128.9, s	
9	7.47, br d (7.8)	118.2, d	
10	7.08, td (7.2)	119.5, d	
11	7.14, td (7.2)	121.9, d	
12	7.23, br d (7.8)	110.4, d	
13		134.9, s	
14	2.66, m	34.7, d	16, 20
15	5.77, d (6.0)	121.7, d	3, 21
16		54.8, s	
17	2.76, dd (13.2, 3.6)	38.7, t	
	1.85, dd (13.2, 2.4)		
18	1.07, t (7.2)	11.2, q	
19	2.25, m; 2.10, m	26.3, t	15
20		150.1, s	
21	4.15, br s	62.1, d	
22	2.48, dd (16.2, 4.8)	49.3, t	14
	2.33, dd (16.2, 7.8)		
23		209.1, s	
24	2.13, s	31.1, q	
-COOCH <sub>3</sub>		174.0, s	
-COOCH <sub>3</sub>	3.71, s	52.3, q	

H-17/C-2, C-16, C-21,  $\delta_C$  174.0 (s, -COOCH<sub>3</sub>) implied that CH-21, CH<sub>2</sub>-17 and the methoxycarbonyl group were connected via C-16, H-14/ $\delta_C$  49.3 (t, C-22), C-16,  $\delta_C$  150.1 (s, C-20) and H-15/ $\delta_C$  26.3 (t, C-19),  $\delta_C$  55.9 (d, C-3); C-21 showed a double bond located at C-15/C-20 and a 3 $\alpha$ -(2-oxopropyl). These observations suggested that **1** possessed an ibogan-type monoterpenoid indole alkaloid carbon skeleton, similar to that of 3-(2-oxopropyl) coronaridine [3]. The differences between them were that a methylene and a methine in 3-(2-oxopropyl) coronaridine [3] were replaced by a double bond ( $\delta_C$  150.1, s, C-20; 127.1, d, C-15) in **1**. In its ROESY spectrum, the



NOE correlations between H-21/ $\delta_{\text{H}}$  3.71 ( $-\text{COOCH}_3$ ), placed H-21 and  $\text{COOCH}_3$  on the same face. Biogenetically, they were assigned with an  $\alpha$ -orientation, and H-14 with a  $\beta$ -orientation of coronaridine derivatives isolated from Apocynaceae [16]. Moreover, NOE correlations between H-14 and H-3 suggested that H-14 and H-3 were on the same face, whereas the correlations between Me-18 and Me-24 positioned them on the opposite side (Figure 1). The specific rotation of **1**  $[\alpha]_{\text{D}}^{21} -11$  ( $c$  0.13,  $\text{CHCl}_3$ ) was also similar to that observed for ervatamine G  $[\alpha]_{\text{D}}^{20} -23$  ( $c$  0.3,  $\text{CHCl}_3$ ) [16]. Based on the above evidence, the structure of **1** was elucidated as shown.

## Experimental

**General experimental procedures:** Optical rotation, JASCO P-1020 digital polarimeter; UV, Shimadzu UV-2401 PC spectrophotometer; IR, Bruker Tensor-27 infrared spectrophotometer; 1D and 2D NMR, Bruker AM-400 and DRX-600 spectrometers; ESIMS, Bruker HTC/Esquire spectrometer; HREIMS, Waters AutoSpec Premier P776 spectrometer; Semipreparative HPLC was performed on an Agilent 1260 HPLC with a ZORBAX SB-C18 ( $9.4 \times 250$  mm). TLC were visualized by Dragendorff's reagent and 10%  $\text{H}_2\text{SO}_4$  in ethanol.

**Plant material:** Air-dried stems and branches of *C. roseus* were collected in November 2012 from Xishuangbanna, Yunnan province, P. R. China. The plant was identified by Mr Hai-Chuan Tai. A voucher specimen (No. Tai2012110503) has been deposited in KIB, CAS.

**Extraction and isolation:** The air-dried and powdered stems and branches of *C. roseus* (9.0 kg) were extracted with MeOH under reflux conditions, and the solvent was evaporated *in vacuo*. The residue was dissolved in 0.37% HCl (pH 2-3) and the solution was subsequently basified using 10% ammonia to pH 9-10. The basic solution was partitioned with EtOAc, affording a two-phase mixture. The EtOAc fraction (190 g) was subjected to CC over silica gel (Fr. A-F). Fr. A (6.5 g) was subjected to silica gel CC

(light petroleum/acetone, 9:1-0:1, v/v) and recrystallization to yield **3** (700 mg), and **4** (26.5 mg). Fr. B (9.8 g) was eluted *via* silica gel CC under isocratic conditions (light petroleum/acetone, 7:1, v/v) to afford **1** (1.8 mg), **2** (5.6 mg), **8** (1.5 mg), **10** (4.7 mg), and **17** (10.0 mg). Compounds **14** (6.3 mg), **5** (10.3 mg), **9** (4.5 mg), and **11** (3.0 mg) were obtained from Fr. C (11.2 g) by silica gel CC with isocratic elution using light petroleum/acetone, 5:1. Fr. D (35.5 g) was subjected to silica gel CC ( $\text{CHCl}_3/\text{MeOH}$ , 20:1-0:1) and further resolved by semi-HPLC to yield compounds **15** (2.4 mg), **12** (1.7 mg), **13** (159.6 mg), and **16** (30.7 mg). Fr. E (95.3 g) was chromatographed on a RP-18 column with  $\text{MeOH}/\text{H}_2\text{O}$  (20:1-1:0, v/v) gradient system to give fractions E1-E8. Fractions E3 (6.3 g) and E5 (9.6 g) were separated on a Sephadex LH-20 column eluting with  $\text{CHCl}_3/\text{MeOH}$  (1:1, v/v) and further by PTLC to yield **6** (10.8 mg), and **7** (4.8 mg).

## 15, 20-Dehydro-3 $\alpha$ -(2-oxopropyl) coronaridine (1)

White amorphous powder.

$[\alpha]_{\text{D}}^{21} -11$  ( $c$  0.13,  $\text{CHCl}_3$ ).

Rf: 0.5 (light petroleum-ethyl acetate, 10:1).

IR (KBr): 3425, 2962, 2925, 1714, 1632, 1461, 1383, 1262, 1095, 1028, 804  $\text{cm}^{-1}$ .

UV/Vis  $\lambda_{\text{max}}$  (MeOH) nm (log  $\epsilon$ ): 240 (3.70), 284 (3.59).

$^1\text{H}$  and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): Table 1.

ESIMS:  $m/z$  (%) = 393  $[\text{M} + \text{H}]^+$  (100).

HRESIMS: found  $m/z$  393.2181 (Calcd. for 393.2178).

**Supplementary data:** NMR and mass spectra of the new compound **1**, and  $^{13}\text{C}$  NMR data of compounds **2–17**.

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Two New Oxindole Alkaloid Glycosides from the Leaves of *Nauclea officinalis*Long Fan<sup>a,b,c</sup>, Xiao-Jun Huang<sup>a,b</sup>, Chun-Lin Fan<sup>a</sup>, Guo-Qiang Li<sup>a</sup>, Zhen-Long Wu<sup>a,b</sup>, Shuo-Guo Li<sup>a,b</sup>, Zhen-Dan He<sup>c</sup>, Ying Wang<sup>a,b,\*</sup> and Wen-Cai Ye<sup>a,b,\*</sup><sup>a</sup>Institute of Traditional Chinese Medicine & Natural Products, College of Pharmacy, Jinan University, Guangzhou 510632, P. R. China<sup>b</sup>JNU-HKUST Joint Laboratory for Neuroscience & Innovative Drug Research, Jinan University, Guangzhou 510632, P. R. China<sup>c</sup>Department of Pharmacy, School of Medicine, Shenzhen University, Shenzhen 518060, P. R. China

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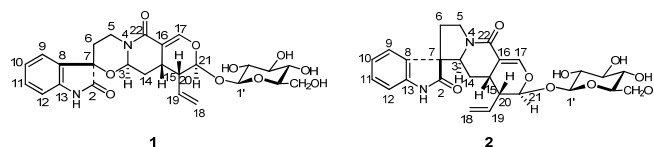
Received: July 18<sup>th</sup>, 2015; Accepted: August 6<sup>th</sup>, 2015

Two new oxindole alkaloid glycosides, nauclealomide A and (3*S*,7*R*)-javaniside, were isolated from the leaves of *Nauclea officinalis*. Their structures and absolute configurations were elucidated by means of NMR, HRESIMS, X-ray diffraction, acid hydrolysis and quantum chemical CD calculation. Nauclealomide A is a novel monoterpenoid oxindole alkaloid possessing a rare tetrahydro-2*H*-1,3-oxazine ring.

**Keywords:** *Nauclea officinalis*, Rubiaceae, Oxindole alkaloid, Tetrahydro-2*H*-1,3-oxazine ring.

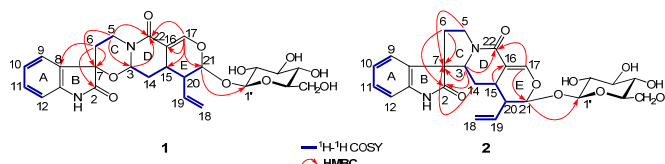
The genus *Nauclea* (Rubiaceae) comprises thirty-five species which are mainly distributed in *Africa* and tropical regions of Asia and Australia [1]. The plants of this genus are rich in indole alkaloids, which exhibit antimalarial, antiproliferative and renin-inhibitory activities [2-5]. To date, phytochemical investigations have led to the isolation of more than 70 indole alkaloids from the genus. The bark and twigs of *Nauclea officinalis* Pierre ex Pitard is used as a traditional medicine in China for treatment of colds, swelling of the throat, pink eye and other ailments [1]. During the course of an ongoing search for new alkaloids from medicinal plants in China [6-12], the EtOH extract of the leaves of *N. officinalis* was further investigated, which led to the isolation of two new oxindole alkaloid glycosides, nauclealomide A (**1**) and (3*S*,7*R*)-javaniside (**2**) (Figure 1). The structures of indole alkaloids **1** and **2** were elucidated by NMR, HRESIMS, X-ray diffraction, acid hydrolysis, as well as CD analysis. Herein, the isolation and structural elucidation of the new indole alkaloids are described.

Nauclealomide A (**1**) showed a molecular formula of C<sub>26</sub>H<sub>30</sub>N<sub>2</sub>O<sub>10</sub> based on the HRESIMS (*m/z* 553.1791 [M + Na]<sup>+</sup>; calculated for C<sub>26</sub>H<sub>30</sub>N<sub>2</sub>O<sub>10</sub>Na: *m/z* 553.1793). The UV spectrum of **1** showed absorption maxima at 211 and 242 nm. The IR spectrum exhibited absorptions at 3394 and 1648 cm<sup>-1</sup>, which suggested the presence of amino and α,β-unsaturated carbonyl functional groups. The <sup>1</sup>H and <sup>13</sup>C NMR spectra of **1** displayed signals due to two carbonyl groups (δ<sub>C</sub> 180.1 and 166.1), a trisubstituted double bond [δ<sub>H</sub> 7.44 (1H, d, *J* = 2.4 Hz); δ<sub>C</sub> 149.7 and 109.0], a terminal vinyl group [δ<sub>H</sub> 5.56 (1H, dt, *J* = 17.0, 10.1 Hz), 5.29 (1H, dd, *J* = 17.0, 1.8 Hz) and 5.24 (1H, dd, *J* = 10.1, 1.8 Hz); δ<sub>C</sub> 134.1 and 120.7] and an *ortho*-substituted benzene ring [δ<sub>H</sub> 7.25 (1H, br d, *J* = 7.5 Hz), 7.25 (1H, br t, *J* = 7.5 Hz), 7.02 (1H, br t, *J* = 7.5 Hz) and 6.84 (1H, br d, *J* = 7.5 Hz); δ<sub>C</sub> 142.3, 131.4, 131.1, 125.4, 123.9 and 111.0]. In addition, the NMR spectra of **1** displayed signals due to a sugar unit [δ<sub>H</sub> 4.69 (1H, d, *J* = 7.9 Hz), 3.87 (1H, dd, *J* = 11.8, 1.7 Hz), 3.66 (1H, dd, *J* = 11.8, 5.3 Hz), and 3.37-3.20 (4H, overlapped); δ<sub>C</sub> 100.4, 78.3, 78.2, 74.7, 71.5 and 62.6]. Acid hydrolysis of **1** afforded D-glucose, which was identified by HPLC analysis using authentic samples as references.

Figure 1: Structures of **1** and **2**.

In addition, the β-configuration for D-glucose was further determined based on the large <sup>3</sup>*J*<sub>H1,H2</sub> coupling constant (*J* = 7.9 Hz) of the anomeric proton. Taken together, these spectral data suggested that **1** was a monoterpenoid oxindole alkaloid with a β-D-glucose unit [13-15].

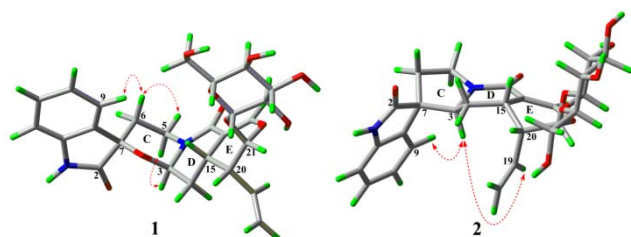
With the aid of <sup>1</sup>H-<sup>1</sup>H COSY, HSQC, HMBC, and ROESY experiments, all of the <sup>1</sup>H and <sup>13</sup>C NMR signals of **1** were assigned as shown in Table 1. The <sup>1</sup>H-<sup>1</sup>H COSY spectrum of **1** revealed the presence of three spin coupling systems (C-9 to C-12, C-5 to C-6, and C-3 to C-21/C-18, Figure 2). In the HMBC spectrum, the correlations between H-17 and C-15/C-16/C-21/C-22, and between H-3 and C-22 revealed the presence of ring E, which was fused to ring D. In addition, the HMBC correlation between H-21 and C-1' indicated that the glucose was located at C-21. Moreover, HMBC correlations between Ha-5 and C-3/C-7/C-22, as well as between Ha-6 and C-2/C-7/C-8 were also observed (Figure 2).

Figure 2: <sup>1</sup>H-<sup>1</sup>H COSY and selected HMBC correlations of **1** and **2**.

Comparison of the NMR data of **1** with those of javaniside [13,14] indicated that they were very similar, except for the obvious down-field shift of H-3 (from δ<sub>H</sub> 4.09 to 6.28), C-3 (from δ<sub>C</sub> 65.5 to 80.0) and C-7 (from δ<sub>C</sub> 58.0 to 77.6). Furthermore, according to the

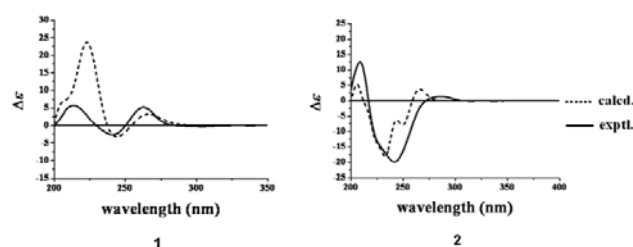
**Table 1:** NMR spectroscopic data for **1** and **2** in CD<sub>3</sub>OD (400 MHz for <sup>1</sup>H, 100 MHz for <sup>13</sup>C, δ in ppm, *J* in Hz).

Position	<b>1</b>		<b>2</b>	
	δ <sub>C</sub>	δ <sub>H</sub>	δ <sub>C</sub>	δ <sub>H</sub>
2	180.1	-	181.5	-
3	80.0	6.28 t (2.8)	63.8	3.95 m
5	39.3	a 4.60 ddd (13.0, 4.9, 1.8) b 3.75 dd (13.0, 3.0)	44.9	a 3.93 m b 3.83 m
6	32.4	a 2.11 td (14.1, 5.4) b 1.77 m	34.3	a 2.36 dt (13.0, 9.8) b 2.21 ddd (13.0, 7.5, 1.6)
7	77.6	-	57.7	-
8	131.4	-	129.8	-
9	125.4	7.25 br d (7.5)	124.0	7.25 br d (7.5)
10	123.9	7.02 br t (7.5)	123.7	7.05 td (7.5, 1.0)
11	131.1	7.25 br t (7.5)	130.0	7.24 td (7.5, 1.0)
12	111.0	6.84 br d (7.5)	111.0	6.91 dt (7.5, 1.0)
13	142.3	-	143.4	-
14	29.7	1.76 m	24.7	a 1.77 dt (13.7, 9.4) b 1.51 dt (13.7, 4.5)
15	23.4	3.27 m	24.9	3.18 m
16	109.0	-	109.9	-
17	149.7	7.44 d (2.4)	147.5	7.14 d (2.6)
18	120.7	a 5.29 dd (17.0, 1.8) b 5.24 dd (10.1, 1.8)	120.6	a 5.11 dd (10.1, 1.9) b 5.06 dd (17.0, 1.9)
19	134.1	5.56 dt (17.0, 10.1)	133.8	5.59 dt (17.0, 10.1)
20	44.3	2.61 ddd (10.1, 5.8, 1.7)	45.7	2.41 ddd (10.1, 5.3, 1.6)
21	98.1	5.46 d (1.7)	97.4	5.37 d (1.6)
22	166.1	-	167.7	-
1'	100.4	4.69 d (7.9)	99.9	4.65 d (7.9)
2'	74.7	3.20 m	74.6	3.15 dd (9.0, 7.9)
3'	78.2	3.37 m	77.8	3.35 t (9.0)
4'	71.5	3.30 m	71.5	3.25 t (9.0)
5'	78.3	3.31 m	78.3	3.28 dd (5.7, 2.0)
6'	62.6	a 3.87 dd (11.8, 1.7) b 3.66 dd (11.8, 5.3)	62.6	a 3.84 dd (12.0, 2.0) b 3.62 dd (12.0, 5.7)

**Figure 3:** Selected ROESY correlations of **1** and **2**.

molecular formula and the degree of unsaturation, the remaining oxygen atom could be assigned as a bridge between C-3 and C-7 to form a tetrahydro-2*H*-1,3-oxazine ring (Figure 2).

The relative configuration of **1** was deduced from coupling constants and ROESY data. The coupling constants of <sup>3</sup>*J*<sub>20,21</sub> (*J* = 1.7 Hz) and <sup>3</sup>*J*<sub>20,15</sub> (*J* = 5.8 Hz) suggested the presence of β/β/α orientation for H-15, H-20 and H-21 [13-15], while the coupling constant of <sup>3</sup>*J*<sub>3,14</sub> (*J* = 2.8 Hz) indicated the presence of α configuration of H-3. In the ROESY spectrum, the correlations between H-3 and Hb-5, as well as between Ha-6 and H-9/Ha-5 indicated the presence of β/α/β configuration of Ha-5, Hb-5 and Ha-6 (Figure 3). The absolute configuration of C-15 in these indole alkaloids was determined as *S* biosynthetically based on secologanin [16]. Thus, the absolute configuration of **1** was elucidated as 3*R*,7*R*,15*S*,20*R*,21*S*, which was consistent with the results obtained by the following quantum chemical CD calculation experiment (Figure 4).

**Figure 4:** Calculated and experimental CD spectra of **1** and **2**.

The molecular formula of (3*S*,7*R*)-javaniside (**2**) was established as C<sub>26</sub>H<sub>30</sub>N<sub>2</sub>O<sub>9</sub> (*m/z* 537.1823 [M + Na]<sup>+</sup>; calculated for C<sub>26</sub>H<sub>30</sub>N<sub>2</sub>O<sub>9</sub>Na: *m/z* 537.1844) by HRESIMS. Acid hydrolysis of **2** also afforded D-glucose. Similar to **1**, the NMR spectra of **2** revealed the presence of two carbonyl groups (δ<sub>C</sub> 181.5 and 167.7), a trisubstituted double bond [δ<sub>H</sub> 7.14 (1H, d, *J* = 2.6 Hz); δ<sub>C</sub> 147.5 and 109.9], a terminal vinyl group [δ<sub>H</sub> 5.59 (1H, dt, *J* = 17.0, 10.1 Hz), 5.11 (1H, dd, *J* = 10.1, 1.9 Hz) and 5.06 (1H, dd, *J* = 17.0, 1.9 Hz); δ<sub>C</sub> 133.8 and 120.6], and an *ortho*-substituted benzene ring, which suggested that **2** was also a monoterpene indole alkaloid with a glucose moiety [13-15].

With the aid of 1D and 2D NMR experiments, all of the <sup>1</sup>H and <sup>13</sup>C NMR signals of **2** were assigned (Table 1). The <sup>1</sup>H-<sup>1</sup>H COSY spectrum of **2** revealed the presence of three spin coupling systems in bold (Figure 2). In the HMBC spectrum, the correlations between H-17 and C-21/C-22, between H-15 and C-16, as well as between H-3 and C-22 revealed the presence of ring E, which was fused to ring D. Moreover, the HMBC correlations between H<sub>2</sub>-6/H-5b/H-3/H<sub>2</sub>-14 and C-7, and between H-3/H<sub>2</sub>-6 and C-2 were observed, which indicated that ring C was fused to ring D and then linked to ring B via the spiro-carbon (C-7). In addition, the HMBC correlation between H-21 and C-1' indicated that the glucose was located at C-21.

The relative configuration of **2** could be established by a ROESY experiment. In the ROESY spectrum, the correlation between H-3 and H-19 indicated the α orientation of H-3. In addition, the ROESY correlation between H-9 and H-3 suggested that the carbonyl group at C-2 was above the C/D/E plane (Figure 3). Considering the biogenetic pathway of these indole alkaloids, the absolute configuration of C-15 was determined as *S* [16]. Thus, the absolute configuration of **2** was elucidated as 3*S*,7*R*,15*S*,20*R*,21*S*, which was consistent with the results obtained by the single crystal X-ray diffraction experiment (Figure 5) and the quantum chemical CD calculation experiment (Figure 4).

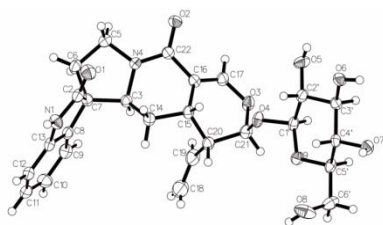


Figure 5: Perspective view of the crystal structure of **2**.

## Experimental

**General experimental procedures:** Optical rotations were measured on a JASCO P-1020 polarimeter, UV spectra on a JASCO V-550 UV/VIS spectrophotometer with a 1 cm length cell, and IR spectra on a JASCO FT/IR-480 plus FT-IR spectrometer.  $^1\text{H}$ ,  $^{13}\text{C}$ , and 2D NMR spectra were recorded on a Bruker AV-400 spectrometer and HRESIMS on an Agilent 6210 ESI/TOF mass spectrometer. CD spectra were obtained using a JASCO J-810 circular dichroism spectrometer. For column chromatography, silica gel (300–400 mesh; Qingdao Marine Chemical Group Corporation) and Sephadex LH-20 (Pharmacia) were used. TLC analyses were carried out using precoated silica gel GF<sub>254</sub> plates (Yantai Chemical Industry Research Institute). Analytical high-performance liquid chromatography (HPLC) was performed on an Agilent chromatograph equipped with a G1311C pump and a G1315D diode-array detector (DAD) with a Cosmosil 5C<sub>18</sub>-MS-II column (4.6 × 250 mm, 5 μm). Preparative HPLC was carried out on an Agilent instrument equipped with a G1310B pump and a G1365D detector with a Cosmosil 5C<sub>18</sub>-MS-II Waters column (10 × 250 mm, 5 μm). X-ray crystallographic analysis was carried out on an Agilent Gemini S Ultra diffractometer with Cu Kα radiation ( $\lambda = 1.5418 \text{ \AA}$ ).

**Plant material:** The leaves of *N. officinalis* were collected in Sanya city, Hainan province of P. R. China, in July of 2008, and authenticated by Professor Wei-ping Chen (Hainan Branch Institute of Medicinal Plants, Chinese Academy of Medical Science). A voucher specimen (No. 20090223) was deposited in the Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, P. R. China.

**Extraction and isolation:** Air-dried and powdered leaves of *N. officinalis* (4.8 kg) were extracted with 95% EtOH (50 L × 3, 3 days each) at room temperature. The EtOH solution was evaporated under reduced pressure to afford a residue (250.0 g), which was suspended in water and partitioned successively with light petroleum and EtOAc. The basic solution was then partitioned with *n*-BuOH to afford *n*-BuOH and emulsion fractions. The emulsion fraction (5.0 g) was subjected to silica gel CC, eluting with CHCl<sub>3</sub> - MeOH (100:0→80:20) to give 9 fractions (Fr. 1–9). Fr. 7 (669.0 mg) were further purified on a Sephadex LH-20 column using MeOH as eluent, then nauclealomide A (**1**, 10.0 mg) and (3*S*,7*R*)-javaniside (**2**, 15.0 mg) were finally obtained by preparative HPLC using MeOH-H<sub>2</sub>O (43:57, 3 mL/min) as the mobile phase.

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## Nauclealomide A (**1**)

yellowish amorphous solid.

$[\alpha]_D^{27}$ : -55 (*c* 0.17, MeOH).

IR (KBr):  $\nu_{\text{max}}$  3394, 1707, 1648, 1482, 1072  $\text{cm}^{-1}$ .

UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 211 (4.32), 242 (4.10) nm.

$^1\text{H}$  and  $^{13}\text{C}$  NMR data (CD<sub>3</sub>OD, 400 and 100 MHz): Table 1.

HRESIMS *m/z*: 553.1791 [*M* + Na]<sup>+</sup> (calcd for C<sub>26</sub>H<sub>30</sub>N<sub>2</sub>O<sub>10</sub>Na: 553.1793).

## (3*S*,7*R*)-Javaniside (**2**)

colourless plates.

$[\alpha]_D^{27}$ : -212 (*c* 0.25, MeOH).

IR (KBr):  $\nu_{\text{max}}$  3394, 1703, 1652, 1587, 1470, 1072, 1015  $\text{cm}^{-1}$ .

UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 211 (4.50), 244 (4.33) nm.

$^1\text{H}$  and  $^{13}\text{C}$  NMR data (CD<sub>3</sub>OD, 400 and 100 MHz): Table 1.

HRESIMS *m/z*: 537.1823 [*M* + Na]<sup>+</sup> (calcd for C<sub>26</sub>H<sub>30</sub>N<sub>2</sub>O<sub>9</sub>Na: 537.1844).

**Crystal data of (3*S*,7*R*)-javaniside (**2**):** The X-ray data of indole alkaloid **2** were collected using a Sapphire CCD with a graphite monochromated Cu Kα radiation,  $\lambda = 1.54184 \text{ \AA}$  at 173.00(10) K. The structure was solved by direct methods using the SHELXS-97 program, and refined by the SHELXL-97 program and full-matrix least-squares calculations [17]. C<sub>26</sub>H<sub>32</sub>N<sub>2</sub>O<sub>10</sub>, *M<sub>r</sub>* = 532.54, Orthorhombic, *a* = 10.99153 (12) Å, *b* = 13.26367 (14) Å, *c* = 17.28232 (16) Å,  $\alpha = \beta = \gamma = 90.00^\circ$ , *V* = 2519.56 (4) Å<sup>3</sup>, space group *P*2(1)2(1)2(1), *Z* = 4,  $\mu$  (Cu Kα) = 0.912 mm<sup>-1</sup>, 20419 reflections collected, 4036 independent reflections (*R<sub>int</sub>* = 0.0270). The final *R<sub>1</sub>* value was 0.0297 [*I* > 2σ(*I*)]. The final *wR*(*F*<sup>2</sup>) value was 0.0769 [*I* > 2σ(*I*)]. The final *R<sub>1</sub>* value was 0.0310 (all data). The final *wR*(*F*<sup>2</sup>) value was 0.0781 (all data). The goodness of fit on *F*<sup>2</sup> was 1.069. Flack parameter = -0.09 (15). Crystallographic data of (3*S*,7*R*)-javaniside have been deposited at the Cambridge Crystallographic Data Centre (deposition number: CCDC 937396).

**Acid hydrolysis:** Indole alkaloids **1** and **2** (2 mg each) in 2 N HCl (5 mL) were heated at 80°C for 2 h, after which the residue was dissolved in pyridine (1 mL) and stirred with L-cysteine methyl ester hydrochloride (2 mg) at 60°C for 1 h. *O*-tolyl isothiocyanate (5 μL) was then added to the mixture and heated at 60°C for an additional 1 h. The reaction mixture was subsequently analyzed by HPLC and detected at 250 nm. Analytical HPLC was performed on a Cosmosil 5C<sub>18</sub>-MS-II column (4.6 × 250 mm, 5 μm) at 20°C using CH<sub>3</sub>CN-0.05% CH<sub>3</sub>COOH in H<sub>2</sub>O (25:75, 1.0 mL/min) as the mobile phase. Peaks were detected with a G1315D DAD. D-Glucose (*t<sub>R</sub>* 16.4 min) was identified as the sugar moiety of indole alkaloids **1** and **2** by comparison with authentic samples of D-glucose (*t<sub>R</sub>* 16.4 min) and L-glucose (*t<sub>R</sub>* 14.9 min) [18].

**Supplementary data:** HRESIMS, UV, IR, 1D and 2D NMR spectra of indole alkaloids **1** and **2**, and quantum chemical CD calculations of **1** and **2**.

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*Lycopodium* Alkaloids from *Diphasiastrum complanatum*

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One new lycopodine-type *Lycopodium* alkaloid, dehydroisofawcettiine *N*-oxide (**1**) and eleven known analogues (**2**–**12**) were isolated from the whole plant of *Diphasiastrum complanatum*. The new structure was established on the basis of spectroscopic methods, including 2D NMR techniques. The absolute configurations of **2** and its new *N*-oxide derivative (**1**) were deduced by chemical transformation combined with Cotton effects in their electronic circular dichroism (ECD) spectra.

**Keywords:** *Lycopodium* alkaloids, *Diphasiastrum complanatum*, Absolute configuration, Chemical transformation, ECD.

*Diphasiastrum complanatum* (L.) Holub (syn.: *Lycopodium complanatum* L.) belonging to the family Lycopodiaceae and commonly known as “Guo-Jiang-Long” in Chinese, is a club moss mainly distributed in temperate and subtropical areas. This plant has been used as a folk medicine for the treatment of arthritic pain, quadriplegia, contusion, and blood stasis [1]. Previous investigations of this plant led to the isolation of a number of *Lycopodium* alkaloids [2] with interesting bioactivities, such as enhancement of mRNA expression for nerve growth factor (NGF) [2d,2i], cytotoxicity against murine leukemia L1210 cells [2i], and antimicrobial activity [2i]. During the continuing program of discovery of novel bioactive alkaloids from club mosses [3], the chemical constituents of *D. complanatum* were reinvestigated. Herein described are the isolation and structural determination of one new (**1**) and eleven known (**2**–**12**) *Lycopodium* alkaloids (Figure 1) from the title plant.

The air-dried and pulverized whole plant of *D. complanatum* was extracted with 90% MeOH at room temperature and then worked up as usual [3,4] to give the crude alkaloid-containing extract. From this, 12 *Lycopodium* alkaloids were isolated and characterized (**1**–**12**, Figure 1). Comparing their spectroscopic data and physicochemical properties observed and reported, the known alkaloids were identified as dehydroisofawcettiine (**2**) [5], lycopodine (**3**) [6], lycopodine *N*-oxide (**4**) [7], L.20 (= 6*α*-hydroxylycopodine, **5**) [8], clavolonine (**6**) [9,10], flabelliformine (**7**) [10], 6-*epi*-8*β*-acetoxylycoclavine (**8**) [11], acetyl fawcettiine (**9**) [6], lycoclavine (**10**) [12], 12-deoxyhuperzine O (**11**) [13], and lycodine (**12**) [14]. Among them, alkaloids **2**, **4**, **6**, **8** and **9** are reported here for the first time from the title plant.

Compound **1** showed an  $[M+H]^+$  ion peak at  $m/z$  322.2016 (calcd 322.2013) in its positive mode HRESIMS, corresponding to the molecular formula  $C_{18}H_{27}NO_4$ . The IR absorption bands at 1707 and 1733  $cm^{-1}$  implied the presence of ester and ketone carbonyl groups. The  $^1H$  and  $^{13}C$  NMR data of **1** (Table 1), with the aid of an HSQC NMR experiment, showed the presence of one methyl doublet at  $\delta_H$  0.94 (3H, d,  $J$  = 6.2 Hz, Me-16;  $\delta_C$  19.0), eight  $sp^3$  methylenes, five  $sp^3$  methines (including one oxymethine at  $\delta_H$  4.62 (1H, dd,  $J$  = 11.0, 4.1 Hz, H-8),  $\delta_C$  79.2), one  $sp^3$  ( $\delta$  74.4) quaternary carbon, and one carbonyl group ( $\delta_C$  207.9) for the skeleton, along with signals assignable to an acetyl group [ $\delta_H$  2.06

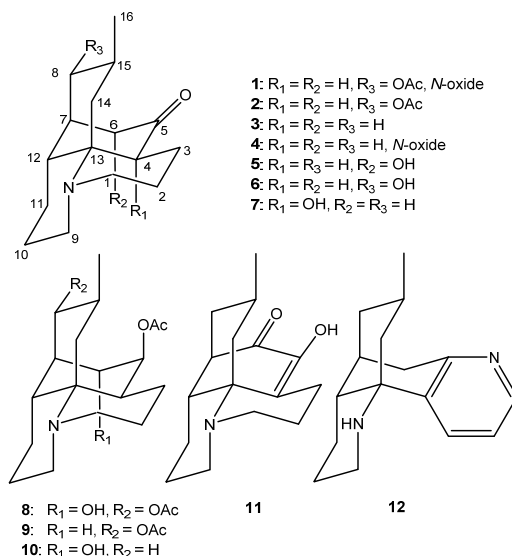


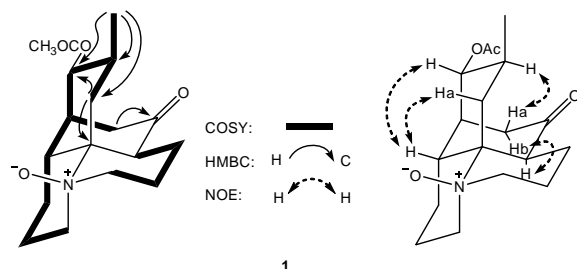
Figure 1: Chemical structures of compounds **1**–**12**.

(3H, s);  $\delta_C$  21.3 and 170.4]. The above spectroscopic data showed high similarity to those of a known lycopodine-type alkaloid, dehydroisofawcettiine (**2**) [5]; however, the complete  $^1H$  and  $^{13}C$  NMR data of **2** have never been reported until the present study. Differing from **2**, the molecular formula of **1** contains one more oxygen atom. Meanwhile, the  $^{13}C$  NMR shifts of C-1 ( $\delta_C$  64.4), C-9 ( $\delta_C$  59.8) and C-13 ( $\delta_C$  74.2) neighboring the N-atom in **1** were all shifted to lower field compared with the corresponding carbons [ $\delta_C$  47.0 (C-1), 46.9 (C-9) and 59.3 (C-13)] of compound **2** (see Experimental section). Thus, compound **1** was assumed to be the *N*-oxide derivative of **2**, which was confirmed by further detailed 2D NMR (COSY and HMBC) spectroscopic analyses of **1** (Figure 2). The relative configuration of **1** was then found to be the same as that of dehydroisofawcettiine (**2**) from the magnitudes of  $J_{H-8, H-15}$  (11.2 Hz) and  $J_{H-14a, H-15}$  (13.0 Hz), and diagnostic NOE correlations of H-8/H-12 ( $\delta$  2.85, br d,  $J$  = 13.8 Hz), H-12/H<sub>a</sub>-14 ( $\delta$  2.63, dd,  $J$  = 14.4, 14.4 Hz), H-15 ( $\delta$  1.56, m)/H<sub>a</sub>-6 ( $\delta$  2.57, br d,  $J$  = 16.4 Hz), and H<sub>b</sub>-6 ( $\delta$  2.35, dd,  $J$  = 16.4, 6.3 Hz)/H-4 ( $\delta$  3.01, dd,  $J$  = 12.2, 3.7 Hz) (Figure 2).



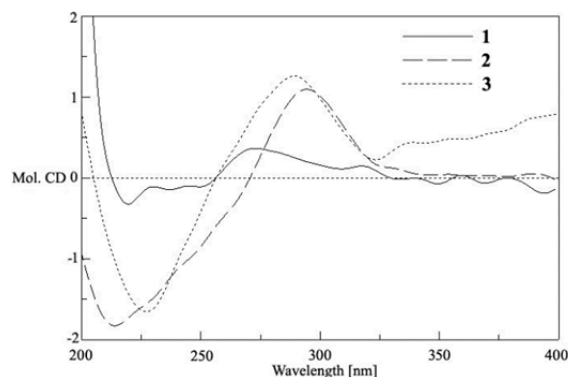
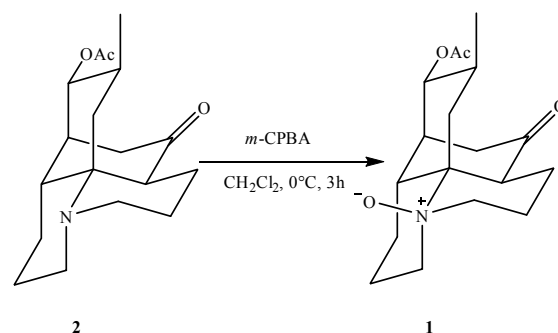
**Table 1:**  $^1\text{H}$  (400 MHz) and  $^{13}\text{C}$  (100 MHz) NMR data <sup>a, b</sup> for alkaloid **1**.

No.	$\delta_{\text{H}}$ , mult ( $J$ in Hz)	$\delta_{\text{C}}$
1a	3.60, ddd (13.5, 13.5, 4.4)	64.4 CH <sub>2</sub>
1b	3.19, br d (13.5)	
2a	1.92, m	21.3 CH <sub>2</sub>
2b	1.85, m	
3a	2.22, br d (14.6)	17.5 CH <sub>2</sub>
3b	1.70, m, m	
4	3.01, dd (12.2, 3.7)	48.8 CH
5		207.9 C
6a	2.57, br d (16.4)	36.2 CH <sub>2</sub>
6b	2.35, dd (16.4, 6.3)	
7	2.44, m	39.1 CH
8	4.62, dd (11.0, 4.1)	79.1 CH
9a	3.96, ddd (12.6, 12.6, 3.0)	59.8 CH <sub>2</sub>
9b	3.08, br d (12.6)	
10a	2.78, m	20.1 CH <sub>2</sub>
10b	1.78, m	
11a	1.91, m	23.3 CH <sub>2</sub>
11b	1.68, m	
12	2.85, br d (13.8)	36.4 CH
13		74.2 C
14a	2.63, dd (14.4, 14.4)	33.1 CH <sub>2</sub>
14b	2.10, dd (14.4, 5.0)	
15	1.56, m	30.4 CH
16	0.94, d (6.2)	19.0 CH <sub>3</sub>
8-OAc	2.06, s	21.3 CH <sub>3</sub> 170.4 C

<sup>a</sup>Assignments were made by a combination of 1D and 2D NMR experiments;<sup>b</sup>Recorded in CDCl<sub>3</sub>.**Figure 2:** COSY, key HMBC and NOE correlations of compound **1**.

As previously described by Ayer and Altenkirk [15], the lycopodine-like alkaloids with a carbonyl group at C-5 and in which the nitrogen lone pair is equatorial to ring A consistently display two Cotton effects above 200 nm, a positive around 288 nm due to  $n \rightarrow \pi$  transition and a negative centered at 223 nm associated with  $\sigma$ -coupled p interaction. In good agreement with this assumption, the electronic circular dichroism (ECD) spectrum of dehydroisofawcettiine (**2**) exhibited a positive Cotton effect at 295 nm ( $\Delta\epsilon +1.09$ ) and a negative one at 214 nm ( $\Delta\epsilon -1.85$ ), which matched well with that of lycopodine (**3**) [15], as depicted in Figure 3. Thus, the absolute configuration of **2** could be unequivocally determined as 4*S*,7*S*,8*R*,12*R*,13*R*,15*S*. In the case of alkaloid **1**, due to the disappearance of the nitrogen lone pair after oxidation, it is hard to judge the related Cotton effects since its ECD curve almost flattened (Figure 3). Nevertheless, the absolute configuration of **1** was established to be the same as that of **2** by chemical transformation. In a supplementary oxidation experiment, compound **2** was treated with one equivalent of *m*-CPBA to produce its *N*-oxide derivative (Scheme 1), which was identical to **1** by means of HPLC, optical rotation, and spectroscopic analyses. Accordingly, the structure of **1** was defined as (4*S*,7*S*,8*R*,12*R*,13*R*,15*S*)-dehydroisofawcettiine *N*-oxide. Indeed, naturally occurring *N*-oxide derivatives of *Lycopodium* alkaloids have often been encountered [2h,7,16].

Similar to casuarinines A–J, lycodine-type alkaloids from *Lycopodium casuarinoides* [3], all the isolates were evaluated for their neuroprotective and anti-acetylcholinesterase (AChE) effects, but none of them were active. They also did not show any significant cytotoxicity against human A-549 and NCI-H460 cancer cell lines.

**Figure 3:** ECD spectra of compounds **1–3**.**Scheme 1:** Chemical transformation from **2** to **1**.

## Experimental

**General experimental procedures:** Optical rotations were measured on an Autopol IV automatic polarimeter, IR spectra on an Avatar 360 ESP FTIR spectrometer, ECD spectra on a JASCO-810 spectropolarimeter, and NMR spectra on a Bruker Avance III 400 MHz spectrometer. Chemical shifts are expressed in  $\delta$  (ppm), and referenced to the residual solvent signals. ESIMS were measured on an Agilent 1100 series mass spectrometer, and HRESIMS on an AB SCIEX Triple TOF 5600+ spectrometer. Semi-preparative HPLC was performed on a Waters e2695 system coupled with a Waters 2998 Photodiode Array Detector and an ODS column (5  $\mu\text{m}$ , 250  $\times$  10 mm, SunFire). Column chromatography (CC) was performed using silica gel (200–300 mesh, Kang-Bi-Nuo Silysia Chemical Ltd., Yantai, China), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Silica gel-precoated plates (GF254, 0.25 mm, Kang-Bi-Nuo Silysia Chemical Ltd., Yantai, China) were used for TLC. Compounds were visualized using UV light (254 and/or 365 nm) and by spraying with Dragendorff's reagent.

**Plant materials:** The whole plant of *D. complanatum* was collected in October 2013 from Bijie in Guizhou Province of China. A voucher specimen (No. 20131007) was deposited at the Herbarium of the Department of Natural Products Chemistry, School of Pharmacy at Fudan University. The plant was identified by Prof. Qiang Luo (Guizhou University of Engineering Science, Bijie, Guizhou Province of China).

**Extraction and isolation:** The air-dried and pulverized whole plant of *D. complanatum* (1.6 kg) was extracted with 90% MeOH (5  $\times$  8 L) at room temperature, and the MeOH extract (170 g) was partitioned between EtOAc and 3% tartaric acid. The water-soluble portion, adjusted to pH 9 with sat. Na<sub>2</sub>CO<sub>3</sub>, was partitioned with CHCl<sub>3</sub>. The CHCl<sub>3</sub>-soluble portion (3.9 g) was loaded on a silica gel column, eluted with a gradient of CH<sub>2</sub>Cl<sub>2</sub>/MeOH (1:0–0:1) to

afford fractions 1–8. Fraction 2 (40 mg) was chromatographed on Sephadex LH-20 ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$ , 2:1) to afford **2** (1.9 mg) and **11** (6.1 mg). Fraction 3 (90 mg) was subjected to silica gel CC ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$ , 50:1) and then purified by semi-preparative HPLC [ $\text{MeOH}-\text{H}_2\text{O}$  (containing 0.05%  $\text{Et}_2\text{NH}$ , v/v) 60:40, v/v; flow rate, 3.0 mL/min] to furnish **1** (1.7 mg,  $t_R = 10.4$  min) and **4** (5.6 mg,  $t_R = 12.0$  min). Compounds **3** (16.0 mg,  $t_R = 13.1$  min) and **12** (2.7 mg,  $t_R = 10.7$  min) were isolated from fraction 4 (80 mg) by semi-preparative HPLC [ $\text{MeOH}-\text{H}_2\text{O}$  (containing 0.05%  $\text{Et}_2\text{NH}$ , v/v) 50:50, v/v; flow rate, 3.0 mL/min]. Fraction 5 (200 mg) was subjected to gel permeation chromatography (GPC) on Sephadex LH-20 ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$ , 2:1) to afford **9** (29.5 mg). Compound **5** (7.2 mg,  $t_R = 14.4$  min) was obtained from fraction 6 (210 mg) by semi-preparative HPLC [ $\text{MeCN}-\text{H}_2\text{O}$  (containing 0.05%  $\text{Et}_2\text{NH}$ , v/v) 30:70, v/v; flow rate, 3.0 mL/min]. Fraction 7 (50 mg) was chromatographed on Sephadex LH-20 ( $\text{MeOH}$ ) to furnish **6** (9.4 mg). Fraction 8 (300 mg) was separated by Sephadex LH-20 ( $\text{MeOH}$ ) and further purified by semi-preparative HPLC [ $\text{MeCN}-\text{H}_2\text{O}$  (containing 0.05%  $\text{Et}_2\text{NH}$ , v/v) 45:55, v/v; flow rate, 3.0 mL/min] to afford **7** (6.1 mg,  $t_R = 16.0$  min), **8** (2.1 mg,  $t_R = 10.8$  min), and **10** (1.7 mg,  $t_R = 20.4$  min).

#### Dehydroisofawcettiine *N*-oxide (**1**)

$[\alpha]_D^{25}$ : +30.0 ( $c$  0.1,  $\text{MeOH}$ ).

IR (film): 3417, 2923, 1733, 1707, 1627, 1377, 1244, 1048  $\text{cm}^{-1}$ .

$^1\text{H}$  and  $^{13}\text{C}$  NMR: Table 1.

HRESIMS:  $m/z$   $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{18}\text{H}_{27}\text{NO}_4$ : 322.2013; found: 322.2016.

*m*-CPBA oxidation of dehydroisofawcettiine (**2**): Similar to the reported literature [7], compound **2** (4.0 mg, 0.01312 mmol) was dissolved in  $\text{CH}_2\text{Cl}_2$  (2 mL) to which *m*-CPBA (2.9 mg, 0.0142 mmol) was added. The reaction mixture was left at  $0^\circ\text{C}$  for 3 h and then evaporated to give a residue, which was applied to semi-preparative HPLC [ $\text{MeOH}-\text{H}_2\text{O}$  (containing 0.05% diethylamine, v/v) 60:40, v/v; flow rate, 3.0 mL/min], giving 3.4 mg (yield 80%)

of the *N*-oxide product of **2**. All the spectroscopic data ( $^1\text{H}$  NMR, ESIMS and  $[\alpha]_D^{25}$ ) were identical with those of natural **1**.

#### Dehydroisofawcettiine (**2**)

$[\alpha]_D^{25}$ : +34.0 ( $c$  0.1,  $\text{MeOH}$ ).

ECD ( $c$   $6.56 \times 10^{-4}$  M,  $\text{MeOH}$ )  $\lambda_{\text{max}}$  ( $\Delta\epsilon$ ): 214 (−1.85), 295 (+1.09) nm.

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  4.59 (1H, dd,  $J = 11.0, 4.2$  Hz, H-8), 3.31 (1H, ddd,  $J = 14.2, 14.2, 3.7$  Hz, H-1a), 3.14 (1H, ddd,  $J = 12.4, 12.4, 2.7$  Hz, H-9a), 2.93 (1H, dd,  $J = 11.8, 3.1$  Hz, H-4), 2.67 (1H, dd,  $J = 13.9, 4.9$  Hz, H-14a), 2.63 (1H, br d,  $J = 12.4$  Hz, H-9b), 2.55 (1H, dd,  $J = 14.2, 4.9$  Hz, H-1b), 2.49 (1H, dd,  $J = 17.9, 3.6$  Hz, H-6a), 2.33 (1H, d,  $J = 17.9$  Hz, H-6b), 2.32 (1H, m, H-3a), 2.10 (1H, m, H-7), 2.07 (3H, s,  $\text{CH}_3\text{CO}$ ), 1.88 (1H, m, H-2a), 1.84 (1H, m, H-10a), 1.77 (1H, m, H-12), 1.72 (1H, m, H-10b), 1.65 (1H, m, H-11a), 1.63 (1H, m, H-13b), 1.58 (1H, m, H-11b), 1.53 (1H, m, H-15), 1.39 (1H, br d,  $J = 13.8$  Hz, H-2b), 1.09 (1H, dd,  $J = 13.9, 13.9$  Hz, H-14b), 0.89 (3H, d,  $J = 6.2$  Hz, Me-16);

$^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  212.6 (C-5, C), 170.8 ( $\text{CH}_3\text{CO}$ , C), 80.4 (C-8, CH), 59.3 (C-13, C), 47.0 (C-9,  $\text{CH}_2$ ), 46.9 (C-1,  $\text{CH}_2$ ), 43.3 (C-12, CH), 42.9 (C-4, CH), 41.6 (C-14,  $\text{CH}_2$ ), 39.6 (C-7, CH), 37.0 (C-6,  $\text{CH}_2$ ), 30.1 (C-15, CH), 25.9 (C-10,  $\text{CH}_2$ ), 24.7 (C-11,  $\text{CH}_2$ ), 21.1 ( $\text{CH}_3\text{CO}$ ,  $\text{CH}_3$ ), 19.2 (C-3,  $\text{CH}_2$ ), 18.9 (C-16,  $\text{CH}_3$ ), 18.5 (C-2,  $\text{CH}_2$ )

ESIMS:  $m/z$  306  $[\text{M}+\text{H}]^+$ .

**Supplementary data:** NMR spectra of compounds **1** and **2** are available in electronic form on the publisher's website.

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# Effects of Adding Vindoline and MeJA on Production of Vincristine and Vinblastine, and Transcription of their Biosynthetic Genes in the Cultured CMCs of *Catharanthus roseus*

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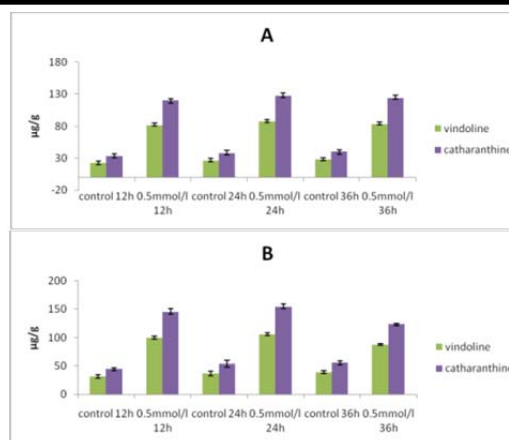
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Vincristine and vinblastine were found by Liquid Chromatography-Mass Spectrometry (LC-MS) in *Catharanthus roseus* cambial meristem cells (CMCs) jointly treated with 0.25 mM vindoline and methyl jasmonate (MeJA), suggesting that *C. roseus* CMCs contain a complete set of the enzymes which are in response to convert vindoline into vincristine and vinblastine. Based on the facts that the transcript levels of vindoline-biosynthetic genes (*STR*, *SGD* and *D4H*) were up-regulated instead of being down-regulated by adding itself to the culture, and that the transcriptional factor *ORCA3* was up-regulated simultaneously, we further confirmed that the transcription of *STR*, *SGD*, *D4H* was manipulated by *ORCA3*.

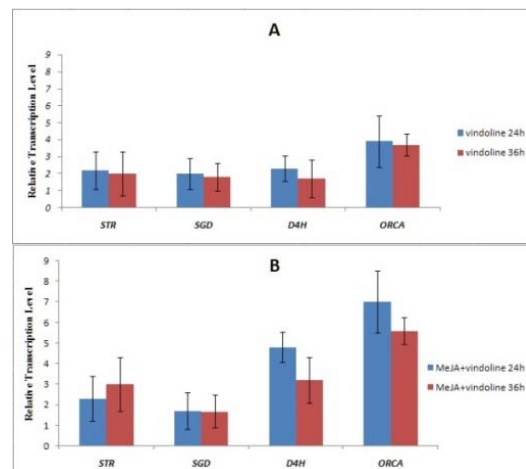
**Keywords:** *Catharanthus roseus*, CMCs, TIAs, Quantitative RT-PCR, Precursor feeding, Gene expression profiling.

The terpene indole alkaloids (TIAs) from *Catharanthus roseus* are important secondary metabolites with diverse structures and biological activities [1,2]. In the complex biosynthetic process of TIAs, strictosidine aglycone is considered as a key intermediate from which at least two biosynthetic pathways are derived. From these two pathways, vindoline and cathenamine are synthesized, respectively. The coupling of vindoline and cathenamine finally leads to the production of bisindole alkaloids vincristine and vinblastine, and cathenamine is also hypothesized as the precursor of ajmalicine and serpentine. Previous results have proved that vindoline can be detected from CMCs of *C. roseus*, but not from either suspension cells or hairy roots due to deficiency of the corresponding enzymes in the latter two cells / tissues [3,4]. Unfortunately, vincristine and vinblastine are not found in the CMCs cultures which was possibly due to the low concentration of vindoline. Since the enzymes necessary for converting vindoline into vincristine and vinblastine have not been characterized yet, the levels of their transcription or expression could not be directly analyzed by biological methods, such as quantitative reverse transcription (RT)-PCR and Western blot. To clarify whether CMCs of *C. roseus* contain a complete set of response system of enzymes for converting vindoline into vincristine and vinblastine, vindoline was fed to the culture of CMCs of *C. roseus* in either the presence or absence of methyl jasmonate (MeJA). Also, the effects of excess vindoline on the transcription of the genes involved in its biosynthesis were investigated.

In the presence or absence of 250 mM MeJA, CMCs of *C. roseus* were treated with 0.5 mM vindoline for 12 h, 24 h and 36 h, respectively. Vinblastine and vincristine were observed by LC-MS. As time went on, the change in accumulation of both vindoline and catharanthine was not significantly observed, suggesting that the production and consumption of the two compounds were in a state of equilibrium (Figure 1). Interestingly, the treatment of vindoline enhanced the accumulation of catharanthine (Figure 1A). In general, additional feeding of one product can inhibit the transcription and translation of genes located at its up-stream steps due to a negative



**Figure 1:** The accumulation of vindoline and catharanthine in *C. roseus* CMCs (A) treated with vindoline or (B) jointly treated with both vindoline and MeJA for 12, 24 and 36 h.



**Figure 2:** In comparison with untreated *C. roseus* CMCs, the relative transcription levels of *STR*, *SGD*, *D4H* and *ORCA3* in *C. roseus* CMCs (A) treated with either vindoline or (B) jointly treated with both vindoline and MeJA for 24 and 36 h, respectively.

feedback mechanism, which proposed that the transcription levels of the genes involved in the synthesis of vindoline were reduced due to the addition of vindoline. We monitored the transcription levels of *STR*, *SGD* and *D4H* in both vindoline-treated and vindoline-untreated CMCs by quantitative real time reverse transcription PCR (qRT-PCR) [5,6]. Unexpectedly, the transcription levels of *STR*, *SGD* and *D4H* were up-regulated by treated with vindoline. Eleven-day old CMCs were treated with either ethanol or 0.5 mM vindoline in ethanol, respectively. After 24 or 36 h, the CMCs were harvested and the transcript levels of *STR*, *SGD* and *D4H* were analyzed. For 24-h cells treated with vindoline enhanced the transcript levels of *STR*, *SGD* and *D4H* by 2.2, 2.0 and 2.3 folds of those of the ethanol-treated CMCs; for 36-h cells treated with vindoline led to 2.1-, 1.9- and 1.9-fold increases, respectively (Figure 2).

It has been reported that the transcriptional regulator ORCA3 is capable of manipulating the transcription of *STR*, *SGD* and *D4H* [7]. We monitored the change in *ORCA3* transcript level. The result indicated that in the CMCs with 24-h and 36-h treatment with vindoline, the transcription levels of *ORCA3* increased by 3.9 and 3.8 folds of those of the control cells, respectively (Figure 2A). Therefore, we supposed that vindoline may firstly enhance *ORCA3* transcription which not only overcomes the negative feedback effect caused by feeding vindoline, but also improves the transcription of *STR*, *SGD* and *D4H*. Transcription of genes responsible for biosynthesis of catharanthine might be up-regulated by ORCA3 as well, which could be the reason that the treatment with vindoline enhanced the accumulation of catharanthine (Figure 1A). Regretfully, no enzyme involved in conversion of strictosidine aglycone to catharanthine has been characterized, so this assumption could not be verified.

MeJA can be used as an elicitor to enhance production of TIAs and transcription of their biosynthetic genes [3]. By jointly treating with 250 mM MeJA and 0.5 mM vindoline, enhancement of the accumulation of vindoline was not observed (Figure 1) and the transcript levels of *STR*, *SGD* and *D4H* increased by magnitudes comparable with those of treating solely with vindoline, while the transcript level of *ORCA3* was induced by 7 folds of that of the control cells (Figure 2). These results indicated that MeJA, in combination with vindoline, was not capable of enhancing the accumulation of vindoline and the transcript levels of its biosynthetic genes. However, in the presence of both vindoline and

MeJA, the further increase of the transcript level of *ORCA3*, together with the fact that vincristine and vinblastine were observed only in the CMCs treated with both MeJA and vindoline, indicated that ORCA3 also improved the transcription of genes locating at downstream of vindoline, although these genes have not been characterized as yet. In conclusion, with treatment by both vindoline and MeJA, CMCs of *C. roseus* produced vinblastine and vincristine, suggesting that CMCs of *C. roseus* contain a complete set of the enzymes involved in conversion of vindoline to vinblastine and vincristine. However, CMCs cannot produce enough vindoline to yield vinblastine and vincristine in detectable amounts, and so feeding with vindoline is necessary. The transcriptions of the genes responsible for synthesizing vindoline, such as *STR*, *SGD* and *D4H*, are up-regulated by ORCA3, which may play a key role in manipulating the transcription of the genes involved in transforming vindoline into vinblastine and vincristine, as well.

## Experimental

**Plant material:** CMCs of *C. roseus* were prepared in our research group [8]. Initially, these CMCs were cultured in solid MS medium with 1-naphthalene acetic acid (NAA, 2.0 mg/L), sucrose (10.0 g/L) and gelrite (4.0 g/L); the pH was regulated to 5.8. After that, cultures were sub-cultured every 12 days in 250 mL Erlenmeyer flasks in liquid MS medium. The cells were cultured at 25°C with a 12/12 h light / dark cycle, agitated at 100 rpm.

**Extraction procedure:** Alkaloids were extracted from medium and cells, respectively, following the reported method [9].

**Analysis of TIAs:** HPLC and LC-MS experiments were performed as previously report [3].

**Monitor gene expression:** The quantitative real time reverse transcription PCR (qRT-PCR) experiments were conducted as previously report [3].

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Structures and Chemotaxonomic Significance of Stemona Alkaloids from *Stemona japonica*Min Yi<sup>a,†</sup>, Xue Xia<sup>a,†</sup>, Hoi-Yan Wu<sup>b,c</sup>, Hai-Yan Tian<sup>a</sup>, Chao Huang<sup>d</sup>, Paul Pui-Hay But<sup>b</sup>, Pang-Chui Shaw<sup>b,c\*</sup> and Ren-Wang Jiang<sup>a</sup><sup>a</sup>College of Pharmacy, Jinan University, Guangzhou, 510632, P.R. China<sup>b</sup>School of Life Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, P.R. China<sup>c</sup>Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, P.R. China<sup>d</sup>Sinopharm Shenzhen Ltd., Shenzhen, P.R. China<sup>†</sup> These authors contributed equally to this work.

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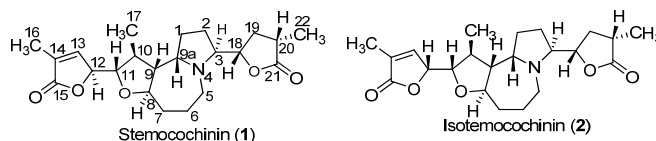
A pair of new alkaloid stereo-isomers, stemocochinin (**1**) and isostemocochinin (**2**), was obtained from the roots of *Stemona japonica* Miq., along with seven known alkaloids, stemonamine (**3**), isostemonamine (**4**), maistemonine (**5**), isomaistemonine (**6**), croomine (**7**), stemonine (**8**), and protostemonine (**9**). The complete structure and stereochemistry of the pair of isomers were established by extensive analysis of the spectral data. Furthermore, our results indicated that *S. japonica* is chemically closer to *S. sessilifolia* than *S. tuberosa*, which are consistent with our previous DNA study on *Stemona* species.

**Keywords:** *Stemona japonica*, Stemona alkaloid, Chemotaxonomic significance, DNA phylogenetics.

Plants of the genus *Stemona*, belonging to the medicinally important family Stemonaceae, are a rich source of a class of structurally unique pyrrolo[1,2- $\alpha$ ]azepine alkaloids known as Stemona alkaloids [1]. Three species in this genus, i.e. *S. tuberosa*, *S. japonica* and *S. sessilifolia*, are collectively recorded as Radix Stemonae ('Bai-Bu' in Chinese) in the Chinese Pharmacopoeia [2]. This herb is often used as an antitussive drug to treat respiratory disorders, such as cough and tuberculosis, and is also used as an anthelmintic agent for domestic animals [3]. The prominent clinical and pharmacological properties of these plants have prompted many phytochemical studies, and over one hundred Stemona alkaloids have been isolated from these herbs [4].

Our group has been engaged in this class of intriguing alkaloids for many years [5-8]. A series of alkaloids were identified from *S. tuberosa*, and some of them were found to show antitussive activities [9,10]. Interestingly, significant chemical diversity was observed for *S. tuberosa* collected from different places of production [5]. During our further systematic investigation of alkaloids from the genus *Stemona*, the chemical constituents of *S. japonica* were studied. A pair of stereoisomers, stemocochinin (**1**, Figure 1) and isostemocochinin (**2**), was isolated together with seven known alkaloids (**3-9**). Their chemical structures and stereochemistry were established by spectroscopic analysis. The chemotaxonomic significance of these alkaloids is discussed based on the distribution of these alkaloids in the genus and a DNA phylogenetic study.

A 95% ethanol extract of the herb was acidified with dilute hydrochloric acid. The acid soluble fraction was adjusted to pH 9 with ammonia solution, and then extracted with CH<sub>2</sub>Cl<sub>2</sub>. Compounds **1-9** were obtained from the CH<sub>2</sub>Cl<sub>2</sub> fraction by silica gel column chromatography.

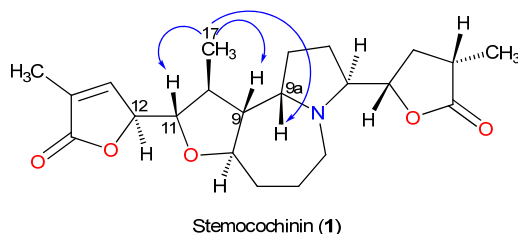
**Figure 1:** Chemical structures stemocochinin (**1**) and isostemocochinin (**2**).

Compound **1**, an amorphous powder, had the molecular formula C<sub>22</sub>H<sub>31</sub>NO<sub>5</sub>, based on the quasimolecular ion [M+H]<sup>+</sup> at *m/z* 389 in its EI-MS. The base peak at *m/z* 290 [M-C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>], a characteristic cleavage fragment of Stemona alkaloids, indicated the presence of an  $\alpha$ -methyl- $\gamma$ -lactone ring annexed to C-3. Another prominent peak at *m/z* 292 [M-C<sub>5</sub>H<sub>5</sub>O<sub>2</sub>] can be attributed to the loss of the unsaturated  $\alpha$ -methyl- $\gamma$ -lactone ring from the molecular ion.

The <sup>1</sup>H NMR spectrum (Table 1) shows the presence of two primary methyl groups at  $\delta_H$  1.06, d, *J* = 6.6 Hz, H-17) and  $\delta_H$  1.25 (3H, d, *J* = 7.1 Hz, H-22), an olefinic methyl at  $\delta_H$  1.92 (3H, d, *J* = 2.4 Hz, H-16), four oxymethines at  $\delta_H$  3.89 (1H, m, H-8), 3.72 (1H, m, H-11), 4.83 (1H, dd, *J* = 6.4, 1.8 Hz, H-12) and 4.17 (1H, m, H-18), a *sp*<sup>2</sup> methine at  $\delta_H$  7.14 (1H, dq, *J* = 1.9, 1.5 Hz, H-13), a methine and two geminal protons attached to carbon atoms bearing a nitrogen function at  $\delta_H$  3.24 (1H, m, H-3),  $\delta_H$  3.45 (1H, dd, *J* = 15.6, 5.5 Hz, H-5 $\beta$ ) and  $\delta_H$  2.89 (1H, dd, *J* = 15.6, 11.7 Hz, H-5 $\alpha$ ). The <sup>13</sup>C NMR and DEPT spectra of **1** show 22 carbon atoms: two lactonic carbonyl atoms ( $\delta_C$  179.5 and 174.1), two olefinic carbon atoms conjugated with carbonyl group ( $\delta_C$  146.9 and 130.7), four carbon atoms bearing oxygen ( $\delta_C$  80.1, 85.1, 83.2 and 83.1), six methylene groups ( $\delta_C$  26.8, 26.9, 47.3, 20.2, 35.2 and 34.3), three methyl groups ( $\delta_C$  10.7, 16.7 and 14.9) and four general methine carbons ( $\delta_C$  55.4, 58.9, 40.7 and 34.9). These spectroscopic data are reminiscent of the pentacyclic Stemoamide-type alkaloids bearing two lactone rings [11].

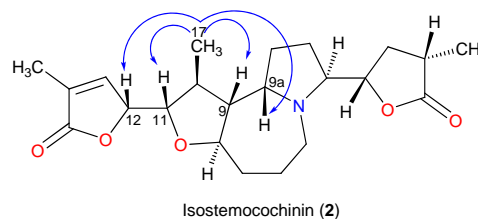
**Table 1:** The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of compounds **1** and **2** ( $J$  in Hz).

Compound 1			Compound 2		
	$^1\text{H}$	$^{13}\text{C}$		$^1\text{H}$	$^{13}\text{C}$
1	1.75, m 1.58, m	26.8, t		1.81, m 1.56, m	26.8, t
2	1.89, m 1.41, m	26.9, t		1.90, m 1.37, m	26.9, t
3	3.24, m	64.5, d		3.21, m	64.4, d
5	3.45, dd (15.6, 5.5) 2.89, dd (11.7, 15.6)	47.3, t		3.44, dd (15.6, 5.2) 2.88, dd (11.4, 15.6)	47.4, t
6	1.55, m 1.33, m	20.2, t		1.54, m 1.33, m	20.2, t
7	2.11, m 1.28, m	35.2, t		2.05, m 1.26, m	35.4, t
8	3.89, ddd (5.6, 5.6, 1)	80.1, d		3.79, m	80.5, d
9	1.99, ddd (4.7, 10.2)	55.4, d		1.98, dd (5.2, 10.6)	55.1, d
9a	3.65, m	58.9, d		3.64, dd (5.0, 10.3)	59.1, d
10	1.82, m	40.7, d		2.19, m	39.6, d
11	3.52, m	85.1, d		3.77, m	83.4, d
12	4.83, m (6.4, 1.8)	83.2, d		4.89, m (2.2, 1.8)	80.6, d
13	7.14, dq (1.9, 1.5)	146.9, d		7.00, dq (1.8, 1.5)	146.1, d
14		130.7, s			131.0, s
15		174.1, s			174.2, s
16	1.92, dd (1.5, 1.8)	10.7, q		1.94, dd (1.8, 1.8)	10.8, q
17	1.06, d (6.6)	16.7, q		1.08, d (6.6)	15.9, q
18	4.17, m	83.1, d		4.15, m	83.5, d
19	2.37, m 1.53, m	34.3, t		2.36, m 1.52, m	34.4, t
20	2.62, m	34.9, d		2.69, m	35.0, d
21		179.5, s			179.4, s
22	1.25, d (6.9)	14.9, q		1.25, d (6.9)	14.9, q

**Figure 2:** Key ROESY correlations of **1**.

The full assignments and connectivities were determined by  $^1\text{H}$  -  $^1\text{H}$  COSY, HMQC and HMBC spectra. The  $^1\text{H}$  -  $^1\text{H}$  COSY spectrum established spin systems involving H-5, H-6, H-7, H-8, H-9, H-10, H-11, H-12, H-13 and H<sub>3</sub>-16, and H-1, H-2, H-3, H-19, H-18, H-19, H-20 and H<sub>3</sub>-22. The HMQC spectrum revealed a signal at  $\delta_{\text{H}}$  4.83 (H-12) attached to a carbon at  $\delta_{\text{C}}$  83.2 (C-12), and the HMBC spectrum showed H-12 correlated to C-11, C-13, C-14 and C-15, suggesting that the  $\gamma$ -lactone was formed by ring closure involving the oxygen atoms bridged to C-12 and C-15. Furthermore, the HMBC correlation H-12 $\rightarrow$ C-11 suggested the location of this lactone at C-11. The HMBC correlation of the characteristic olefinic signal at  $\delta$  7.14 (H-13) to C-12, C-14, C-15 and C-16 suggested that the double bond is within the lactone ring and conjugated with the carbonyl group at C-15. Similarly, the HMQC spectrum revealed that the signal at  $\delta_{\text{H}}$  4.17 (H-18) was correlated with a carbon at  $\delta_{\text{C}}$  80.4 (C-18), and the HMBC spectrum showed that H-18 correlated with C-3, C-19, C-20 and C-21, suggesting that another  $\gamma$ -lactone attached to C-3 was formed by ring closure involving the oxygen atoms bridged to C-18 and C-21.

The stereochemistry of compound **1** was determined from the ROESY spectrum in which the protons of Me-17 had correlations with H-9a, H-11 and H-9, but not with H-12, indicating that Me-17, H-9a, H-11 and H-9 are on the same side and are  $\beta$ -orientated, whereas H-12 should be  $\alpha$ -orientated (Figure 2). Accordingly, compound **1** was determined to be stemocochinin. This alkaloid has been reported before [11], but the full assignment of NMR data and detailed stereochemistry were not determined.

**Figure 3:** The key ROESY correlations of **2**.

Compound **2** had the same quasimolecular ion as that of **1** at  $m/z$  389. It also showed the same prominent peaks at  $m/z$  290 and 292 as alkaloid **1** in its ESI-MS. The  $^1\text{H}$ -,  $^{13}\text{C}$ - and DEPT-NMR spectra of **2** were similar to those of **1** except that the chemical shifts of H-10 and H-11 are slightly different (Table 1). Comparison of the ROESY spectra of alkaloids **1** and **2** revealed a significant difference. Clear correlation was observed between methyl-17 and H-12 in the ROESY spectrum (Figure 3) of alkaloid **2**, suggesting that H-12 should be  $\beta$ -orientated. Thus, the only difference between alkaloids **1** and **2** was the relative configuration of C-12, (*S* in **1** and *R* in **2**).

Accordingly, compound **2** was determined to be an isomer of stemocochinin (**1**) and was accorded the trivial name isostemocochinin (**2**).

The known alkaloids were identified as stemonamine (**3**) [13, 14], isostemonamine (**4**) [13], maistemonine (**5**) [13-16], isomaistemonine (**6**) [14, 17, 18], croomine (**7**) [12, 19, 20], stemonine (**8**) [21, 22] and protostemonine (**9**) [15, 21, 22] by comparing their MS and NMR spectral data with those reported in literature (Figure 1).

Six of the seven known alkaloids have been identified from *S. sessilifolia*, while only compound **7** has been isolated from *S. tuberosa* (Table 2), indicating that *S. japonica* is chemically closer to *S. sessilifolia* than to *S. tuberosa*. Current phytochemical results are consistent with our previous DNA study on *Stemona* species [23], which showed that the 5S rRNA sequence of *S. tuberosa* shares a similarity of 76% and 78.5% with those of *S. japonica* and *S. sessilifolia*, respectively. In contrast, the similarity between *S. japonica* and *S. sessilifolia* is 84.5%. Thus, both phytochemical and DNA phylogenetic data confirm a closer relationship between *S. japonica* and *S. sessilifolia* than *S. tuberosa*.

**Table 2:** Distribution of compounds **1-9** in the three *Stemona* species recorded in Chinese Pharmacopeia.

Compound	Name	Source	Reference
<b>1</b>	Stemocochinin	<i>S. japonica</i>	12
<b>2</b>	Isostemocochinin	<i>S. japonica</i>	Not found
<b>3</b>	Stemonamine	<i>S. japonica</i> <i>S. sessilifolia</i>	13 14
<b>4</b>	Isostemonamine	<i>S. japonica</i>	13
<b>5</b>	Maistemonine	<i>S. japonica</i> <i>S. sessilifolia</i>	13,15 16,14
<b>6</b>	Isomaistemonine	<i>S. sessilifolia</i> <i>S. japonica</i>	14,17 18
<b>7</b>	Croomine	<i>C. japonica</i> <i>S. tuberosa</i>	19,12 20
<b>8</b>	Stemonine	<i>S. japonica</i>	21,22
<b>9</b>	Protostemonine	<i>S. japonica</i> <i>S. sessilifolia</i>	15,21,22 14,17

## Experimental

**General procedure:** TLC was performed on pre-coated silica gel GF254 plates (Qingdao Marine Chemical Factory, Qingdao, China). The NMR spectra were obtained on a Bruker 300 spectrometer with chemical shifts reported in ppm using TMS as an internal standard.

ESIMS were recorded on a Finnigan MAT TSQ 7000 instrument. Column chromatography (CC) was performed on silica gel (200–400 mesh, Qingdao Marine Chemical Plant, Qingdao, People's Republic of China). All solvents used in CC and high-performance liquid chromatography (HPLC) were of analytical grade (Shanghai Chemical Plant, Shanghai, People's Republic of China).

**Plant material:** The roots of *S. japonica* were collected in Guangxi Province, China in September 2004. The herbs (voucher specimen No. SJ-1) were identified at the Institute of Traditional Chinese Medicine and Natural Products, Jinan University.

**Isolation of compounds 1-9:** One kg of dried roots of *S. japonica* were ground and percolated with 95% EtOH at room temperature for 3 days, and then filtered and concentrated under reduced pressure. The residue was acidified with 500 mL of 4% HCl solution, and then extracted with Et<sub>2</sub>O (400 mL x 3). The Et<sub>2</sub>O was evaporated to give a crude non-alkaloid extract (6 g). The pH of the water layer was adjusted to 9–10 with 35% NH<sub>4</sub>OH, and extracted with diethyl ether to give the total alkaloids (4.5 g). Part of these (4.0 g) was chromatographed on a silica gel column and eluted with CH<sub>2</sub>Cl<sub>2</sub>: MeOH: NH<sub>4</sub>OH (94: 6: 0.04). The eluates were monitored by TLC and grouped into 6 fractions (Fr. 1 - Fr. 6). Fr. 1 was re-chromatographed on a silica gel column using *n*-hexane: EtOAc (6:4) as eluent to yield alkaloid **3** (10 mg). Fr. 2 was chromatographed on a silica gel column eluted with *n*-hexane:

EtOAc (5: 5) to give alkaloids **4** (12 mg), **5** (20 mg) and **6** (20 mg). Fr. 4 was chromatographed on a silica gel column eluted with *n*-hexane: EtOAc: acetone (2: 2: 0.5) to give alkaloids **7** (20 mg), **8** (26 mg) and **9** (93 mg). Fr. 6 was separated by silica gel CC eluting with *n*-hexane: EtOAc: acetone (2: 2: 1) to afford alkaloids **1** (20 mg) and **2** (40 mg).

#### Stemochocochinin (1)

Amorphous powder.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

FAB-MS: *m/z*: 390 [M+H]<sup>+</sup>.

EI-MS: *m/z*: 389 [M]<sup>+</sup> (3), 292 [M-C<sub>5</sub>H<sub>5</sub>O<sub>2</sub>]<sup>+</sup> (100), 290 [M-C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>]<sup>+</sup> (75), 278, 164, 134.

#### Isostemochocochinin (2)

Amorphous powder.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

FAB-MS: *m/z*: 390 [M+H]<sup>+</sup>.

EI-MS: *m/z*: 389 [M]<sup>+</sup> (2), 292 [M-C<sub>5</sub>H<sub>5</sub>O<sub>2</sub>]<sup>+</sup> (75), 290 [M-C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>]<sup>+</sup> (100), 278, 164, 136.

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Chemical Constituents of *Euonymus glabra*Jie Ren<sup>a</sup>, Yang-Guo Xie<sup>a</sup>, Xing Wang<sup>a</sup>, Shi-Kai Yan<sup>a</sup>, Hui-Zi Jin<sup>a,\*</sup> and Wei-Dong Zhang<sup>a,b,\*</sup><sup>a</sup>School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, China<sup>b</sup>Department of Phytochemistry, Second Military Medical University, Shanghai 200433, China

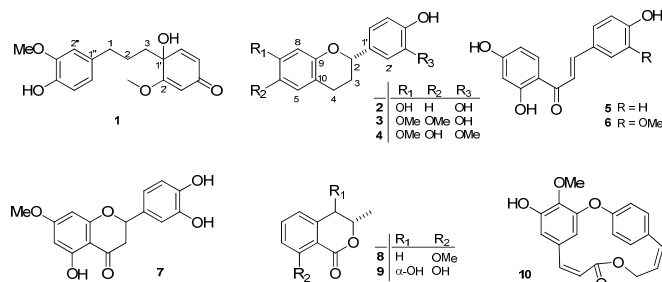
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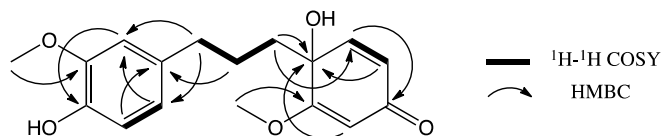
One new phenolic compound (**1**) and one new flavan (**2**), together with eight known compounds (**3–10**) were isolated from the stems and twigs of *Euonymus glabra* Roxb. Their structures were elucidated mainly on the basis of 1D and 2D spectroscopic methods and circular dichroism analysis. In addition, compounds **1–10** were tested for their inhibitory effects against LPS-induced NO production in RAW264.7 macrophages. Compounds **1–5** and **7** exhibited moderate inhibitory activities with IC<sub>50</sub> values ranged from 5.1 to 11.9  $\mu$ M.

**Keywords:** *Euonymus glabra*, Euonyphenylpropane A, Flavan, NO production.

*Euonymus glabra* Roxb. (Celastraceae) is a deciduous shrub, which is mainly distributed in Xishuangbanna Dai Autonomous Prefecture in Yunnan province at an elevation of 900–1800 meters. It has been recorded that the twigs and leaves of this species are poisonous [1]. Previous investigations showed that *Euonymus* species exhibited a high diversity of both secondary metabolites (including sesquiterpenes, alkaloids, terpenoids, flavonoids) and biological activities (such as anti-tumor, anti-diabetes, and insecticidal effects [2]. In the continuing search for further bioactive natural products from this genus, phytochemical investigations of *E. glabra* were carried out and led to the isolation of one new phenolic compound euonyphenylpropane A (**1**), one new flavan (2*R*)-3',4',7-trihydroxyflavan (**2**), along with eight known compounds, griffinoid C (**3**) [5], griffinoid B (**4**) [5], isoliquiritigenin (**5**) [6], 2',4,4'-trihydroxy-3-methoxy-chalcone (**6**) [7], 7-*O*-methyl-eriodictyol (**7**) [8], (*R*)-(-)-mellein methyl ether (**8**) [9], *trans*-4-hydroxymellein (**9**) [10], and combretastatin D-3 (**10**) [11]. Here, we report the isolation and structural elucidation of these compounds, as well as their inhibitory activities against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 macrophages.

Figure 1: Structures of compounds **1–10**.

Compound **1** was obtained as a light yellow solid. Its molecular formula was determined to be C<sub>17</sub>H<sub>20</sub>O<sub>5</sub> by HRESIMS analysis (*m/z* 305.1392 [M+H]<sup>+</sup>), indicating eight degrees of unsaturation. The IR absorptions suggested the presence of hydroxyl groups (3448 cm<sup>-1</sup>) and a conjugated carbonyl group (1445 cm<sup>-1</sup>). The <sup>1</sup>H and <sup>13</sup>C NMR spectral data showed great similarities to those of broussonone A [3], except for an additional methoxyl group in compound **1** (Table 1). HMBC correlation between  $\delta_{\text{H}}$  3.81 and C-3'' ( $\delta_{\text{C}}$  148.8)

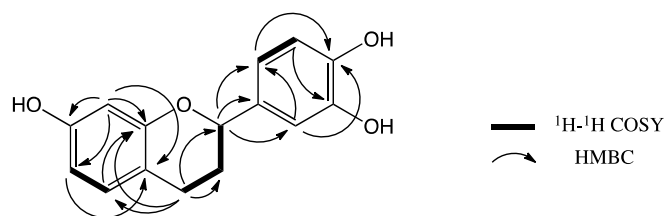
Figure 2: Key <sup>1</sup>H–<sup>1</sup>H COSY and HMBC correlations of compound **1**.Table 1: <sup>13</sup>C and <sup>1</sup>H NMR data [ $\delta_{\text{H}}$  (J in Hz)] for compound **1**.

No.	$\delta_{\text{C}}$	$\delta_{\text{H}}$
1	36.1	2.5 m
2	26.7	1.26 m; 1.40 m
3	38.9	1.71 m; 1.95 m
1'	72.2	
2'	178.5	
3'	102.1	5.53 d (1.7)
4'	190.3	
5'	128.1	6.08 dd (10.0, 1.7)
6'	150.1	6.63 d (10.0)
1''	134.6	
2''	113.1	6.68 d (1.9)
3''	148.8	
4''	145.6	
5''	116.1	6.66 d (8.0)
6''	121.8	6.54 dd (8.0, 1.9)
2'-OMe	56.7	3.76 s
3''-OMe	56.4	3.81 s

<sup>13</sup>C and <sup>1</sup>H NMR data were measured in CD<sub>3</sub>OD at 100 and 400 MHz, respectively.

was observed, which indicated that the additional methoxyl group was attached at C-3'' (Figure 2). Thus, the chemical structure of **1** was confirmed and this compound was named as euonyphenylpropane A.

Compound **2** was obtained as a light yellow solid. Its molecular formula C<sub>15</sub>H<sub>14</sub>O<sub>4</sub> was determined from the positive HR-ESIMS ion at *m/z* 259.0951 [M+H]<sup>+</sup>, indicating nine degrees of unsaturation. The IR spectrum exhibited absorption bands ascribed to hydroxy groups (3445 cm<sup>-1</sup>) and benzene rings (1644 cm<sup>-1</sup>). The <sup>1</sup>H NMR data of **2** exhibited two sets of ABX-substituted aromatic protons [ring A:  $\delta_{\text{H}}$  6.25 (1H, d, *J* = 2.4 Hz), 6.31 (1H, dd, *J* = 8.1, 2.4 Hz), 6.85 (1H, d, *J* = 8.1 Hz) and ring B:  $\delta_{\text{H}}$  6.72 (1H, dd, *J* = 8.0, 1.8 Hz), 6.75 (1H, d, *J* = 8.0 Hz), 6.85 (1H, d, *J* = 1.8 Hz)]. In addition, signals of an oxymethine [ $\delta_{\text{H}}$  4.80 (1H, dd, *J* = 10.0, 2.5 Hz),  $\delta_{\text{C}}$  79.0] and two methylenes [ $\delta_{\text{H}}$  2.65, 2.80 (2H, m),  $\delta_{\text{C}}$  25.4;  $\delta_{\text{H}}$  1.90, 2.10 (2H, m),  $\delta_{\text{C}}$  31.3] were observed, which suggested compound **2** as a flavan.

Figure 3: Key  $^1\text{H}$ - $^1\text{H}$  COSY and HMBC correlations of compound 2.Table 2:  $^{13}\text{C}$  and  $^1\text{H}$  NMR data [ $\delta_{\text{H}}$  (J in Hz)] for compound 2.

No.	$\delta_{\text{C}}$	$\delta_{\text{H}}$
2	79.0	4.80 dd (10.0, 2.5)
3	31.3	1.90 m; 2.10 m
4	25.4	2.65 m; 2.80 m
5	130.9	6.85 d (8.1)
6	109.0	6.31 dd (8.1, 2.4)
7	157.5	
8	104.0	6.25 d (2.4)
9	157.1	
10	114.3	
1'	135.0	
2'	114.4	6.85 d (1.8)
3'	146.3	
4'	146.0	
5'	116.1	6.72 dd (8.0, 1.8)
6'	118.7	6.75 d (8.0)

 $^{13}\text{C}$  and  $^1\text{H}$  NMR data were measured in  $\text{CD}_3\text{OD}$  at 100 and 400 MHz, respectively.

The corresponding carbon signals were assigned by HMQC experiment (Table 2). Analysis of the  $^1\text{H}$ - $^1\text{H}$  COSY spectrum revealed the spin system of H-2/H<sub>2</sub>-3/H<sub>2</sub>-4 as shown in Figure 3. The chemical structure of compound 2 was further confirmed by the HMBC spectrum (Figure 3). In the CD spectrum, a positive Cotton effect observed at 280 nm indicated a 2R configuration, in comparison with published data [4] and 2 was defined as (2R)-3',4',7-trihydroxyflavan.

Compounds 1–10 were tested for their inhibitory effects against LPS-induced NO production in RAW264.7 macrophages with aminoguanidine as positive control. As shown in Table 2, compounds 1–5 and 7 exhibited moderate inhibitory activities with  $\text{IC}_{50}$  values ranged from 5.1 to 11.9  $\mu\text{M}$ .

Table 3: Inhibitory effects of compounds 1–10 isolated from *E. glabra* against LPS-induced NO production in RAW264.7 macrophages.

Compounds	$\text{IC}_{50}^a$ ( $\mu\text{M}$ )
1	10.3
2	8.1
3	11.9
4	5.1
5	11.9
6	20.1
7	5.4
8	21.8
9	19.3
10	19.1
aminoguanidine <sup>a</sup>	21.7

<sup>a</sup> Positive control

## Experimental

**General procedures:** TLC analysis utilized HSGF<sub>254</sub> silica gel plates (10–40  $\mu\text{m}$ , Yantai, China). Column chromatography (CC) was performed using silica gel (100–200, 200–300 mesh, Yantai, China), silica gel H (10–40  $\mu\text{m}$ , Qingdao, China) and Sephadex LH-20 (Pharmacia Co. Ltd.). Preparative HPLC (Shimadzu LC-6AD) was performed on a preparative column (Shimadzu PRC-ODS EV0233). 1D and 2D NMR spectra were recorded on Bruker Avance-400 spectrometers in either  $\text{CD}_3\text{OD}$  or  $\text{DMSO}-d_6$  with TMS as internal standard. ESIMS were recorded on an Agilent LC/MSD Trap XCT spectrometer (Waters, USA), and HR-ESIMS on a Q-Tof micro YA019 mass spectrometer (Waters, USA). Optical rotations were recorded on a JASCO P-2000 polarimeter. IR spectra were

measured on a Bruker FTIR Vector 22 spectrometer with KBr pellets. CD spectra were determined on a JASCO J-815 spectrometer.

**Plant material:** The stems and twigs of *E. glabra* Roxb. were collected in Xishuangbanna Dai Autonomous Prefecture, Yunnan province, PR China, in August 2010, and were authenticated by Prof. Haiou Yang, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 201008SDWM) was deposited at the School of Pharmacy, Shanghai Jiao Tong University.

**Extraction and isolation:** Dried stems and twigs of *E. glabra* (4.5 kg) were powdered and extracted with 95% EtOH 3 times at room temperature and then evaporated under reduced pressure to give crude extract (350.0 g). This was further partitioned with light petroleum (PE), EtOAc and *n*-BuOH to obtain 3 fractions. The EtOAc fraction (90.7 g) was subjected to silica gel CC, and eluted with a step gradient of  $\text{CH}_2\text{Cl}_2$ -MeOH (100:0–0:100) to yield 7 fractions (EA-A–EA-G). EA-A (3.9 g) was chromatographed on macroporous resin MCI (MeOH- $\text{H}_2\text{O}$ , 4:1) and a silica gel column eluting with a step gradient of  $\text{CH}_2\text{Cl}_2$ -MeOH (100:0–20:1), followed by a Sephadex LH-20 column (MeOH) to give 10 sub-fractions (EA-A1–EA-A10). EA-A5 was purified by preparative HPLC (MeOH- $\text{H}_2\text{O}$ , 55:45) to yield compound 10 (5.0 mg). EA-A8 and EA-A9 were purified by preparative HPLC (MeOH- $\text{H}_2\text{O}$ , 65:35) to obtain compounds 8 (5.0 mg) and 9 (3.5 mg), respectively. EA-B (7.0 g) was subjected to macroporous resin MCI (MeOH- $\text{H}_2\text{O}$ , 4:1) and a silica gel column eluting with a step gradient of  $\text{CH}_2\text{Cl}_2$ -MeOH (100:1–5:1) followed by Sephadex LH-20 column (MeOH) to give 9 sub-fractions (EA-B1–EA-B9). EA-B6 was further purified by silica gel CC eluting with  $\text{CH}_2\text{Cl}_2$ -MeOH (100:1) to afford compounds 5 (5.3 mg) and 6 (3.2 mg). EA-B7 was purified by preparative HPLC (MeOH- $\text{H}_2\text{O}$ , 60:40) to yield compound 3 (11.8 mg). EA-B8 was subjected to preparative HPLC (MeOH- $\text{H}_2\text{O}$ , 60:40) to obtain compound 7 (1.6 mg). EA-C (7.1 g) was chromatographed on macroporous resin MCI (MeOH- $\text{H}_2\text{O}$ , 4:1) and a silica gel column eluting with a step gradient of  $\text{CH}_2\text{Cl}_2$ -MeOH (50:1–0:100) followed by a Sephadex LH-20 column (MeOH) to give 7 sub-fractions (EA-C1–EA-C7). EA-C7 was purified by preparative HPLC (MeOH- $\text{H}_2\text{O}$ , 60:40) to yield compounds 1 (6.1 mg), 2 (5.2 mg) and 4 (7.8 mg).

### Euonyphenylpropane A (1)

Light yellow solid.

$[\alpha]_{\text{D}}^{20}$ : –3.7 (*c* 0.10, MeOH).

IR (KBr): 3448, 1620, 1505, 1645, 1455, 1383, 1189, 1121, 1075, 1032  $\text{cm}^{-1}$ .

$^1\text{H}$  and  $^{13}\text{C}$  NMR: Table 1.

HR-ESIMS (positive)  $m/z$ : 305.1392  $[\text{M}+\text{H}]^+$  (calcd for  $\text{C}_{17}\text{H}_{21}\text{O}_5$ , 305.1384).

### (2R)-3',4',7-Trihydroxyflavan (2)

Light yellow solid.

$[\alpha]_{\text{D}}^{20}$ : +8.1 (*c* 0.10, MeOH).

CD (1.0  $\times 10^{-4}$  M, MeOH):  $\lambda$  ( $\Delta \epsilon$ ) 280 (2.1).

IR (KBr): 3445, 1644, 1383, 1189, 1153, 1119, 1077, 1022, 610  $\text{cm}^{-1}$ .

$^1\text{H}$  and  $^{13}\text{C}$  NMR: Table 2.

HR-ESIMS (positive)  $m/z$ : 259.0951  $[\text{M}+\text{H}]^+$  (calcd for  $\text{C}_{15}\text{H}_{15}\text{O}_4$ , 259.0965).

**Determination of nitric oxide (NO) production:** The assay was performed using the MTT method, as previously described [12–14].



Briefly, RAW264.7 cells grown on a 100 mm culture dish were harvested and seeded in 96-well plates ( $2 \times 10^5$  cells/ well) for NO production. The plates were pretreated with various concentrations of samples for 30 min and incubated with LPS (1  $\mu\text{g/mL}$ ) for 24 h. The amount of NO was determined by the nitrite concentration in the cultured RAW264.7 macrophage supernatants with the Griess reagent. The cell viability was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich] reduction [15].

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Isoprenylated Flavonoids with PTP1B Inhibition from *Ficus tikoua*Lu-Qin Wu<sup>a</sup>, Chun Lei<sup>a</sup>, Li-Xin Gao<sup>b</sup>, Hai-Bing Liao<sup>a</sup>, Jing-Ya Li<sup>b</sup>, Jia Li<sup>b</sup> and Ai-Jun Hou<sup>a,\*</sup><sup>a</sup>Department of Pharmacognosy, School of Pharmacy, Fudan University, 826 Zhang Heng Road, Shanghai 201203, PR China<sup>b</sup>National Center for Drug Screening, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 189 Guo Shou Jing Road, Shanghai 201203, PR China

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Two new isoprenylated flavanones, ficutikousins A and B (**1** and **2**), together with seven known compounds (**3**–**9**) were isolated from the whole plant of *Ficus tikoua* (Moraceae). The structures of the new compounds were elucidated on the basis of spectroscopic methods. Compounds **1**–**7** exhibited moderate inhibitory activities against PTP1B *in vitro*.

**Keywords:** *Ficus tikoua*, Moraceae, Isoprenylated flavonoids, Protein tyrosine phosphatase 1B.

*Ficus tikoua* Bur. belongs to the family Moraceae and is a creeping vine plant mainly distributed in south China, India, Vietnam and Laos [1]. This plant is used as traditional Chinese medicine and ethnomedicine in more than ten nationalities in China for the treatment of edema, jaundice, amenorrhea, and bruise [2,3]. Previous phytochemical studies on this plant resulted in the isolation of some flavonoids and phenolic glycosides, some of which showed antifungal and cytotoxic activities [4]. In the course of discovering structurally and biologically interesting compounds from the family Moraceae [5], we investigated the chemical constituents of *F. tikoua*.

Fractionation of an ethanol extract of this plant afforded eight flavonoids and one coumarin, including two new isoprenylated flavanones, ficutikousins A (**1**) and B (**2**), along with seven known compounds, derrone (**3**) [6a], alpinumisoflavone (**4**) [6b], (*S*)-5,7,3',4'-tetrahydroxy-2'-(3-methylbut-2-enyl)flavanone (**5**) [6c], (*S*)-paratocarpin K (**6**) [6d], 3'-(3-methylbut-2-enyl)biochanin A (**7**) [6e], genistein (**8**) [6f], and bergapten (**9**) [6g]. The known compounds **3**–**7** were isolated from this plant for the first time. Protein tyrosine phosphatase 1B (PTP1B) plays an important role in regulating the sensitivity of insulin signaling and fat metabolism and is considered to be a significant target in treating type 2 diabetes and obesity [7]. Recently, a series of isoprenylated flavonoids with PTP1B-inhibiting activities were isolated from the family Moraceae by our group [5a, d]. As a continuing research on the discovery of effective PTP1B inhibitors, compounds isolated from *F. tikoua* were evaluated *in vitro* for the inhibition on PTP1B enzyme activity. Compounds **1**–**7** showed moderate inhibitory effects. We herein report the structural elucidation and biological evaluation of these compounds.

Ficutikousin A (**1**), an optically active compound ( $[\alpha]_D^{25} = -8.7$ ), was obtained as white amorphous powder. Its molecular formula was determined as C<sub>20</sub>H<sub>18</sub>O<sub>6</sub> by HRESIMS ( $m/z$  353.1033 [M – H]<sup>–</sup>; calcd. 353.1031). The IR spectrum of **1** indicated the presence of hydroxyl (3421 cm<sup>–1</sup>), carbonyl (1653 cm<sup>–1</sup>), and benzene ring (1539 and 1467 cm<sup>–1</sup>) moieties. The UV spectrum showed absorption maxima at λ<sub>max</sub> 212 (sh), 231, 282 and 331 (sh) nm, which were similar to those of flavanones [8]. The <sup>1</sup>H NMR

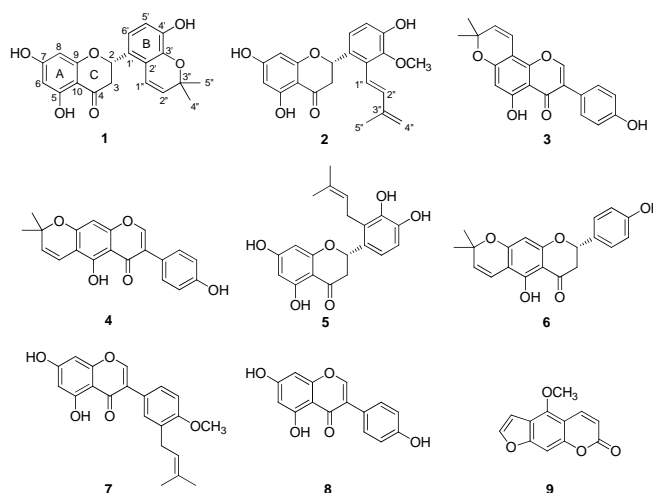
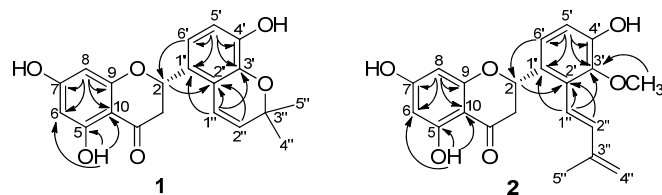


Figure 1: Structures of compounds **1**–**9**.

spectrum (Table 1) revealed signals of an ABX spin system at δ<sub>H</sub> 5.73 (1H, dd, *J* = 13.2, 2.8 Hz, H-2), 3.21 (1H, dd, *J* = 17.2, 13.2 Hz, H-3), and 2.70 (1H, dd, *J* = 17.2, 2.8 Hz, H-3), characteristic of ring C on a flavanone skeleton. It also showed resonances for a hydrogen-bonded hydroxyl group at δ<sub>H</sub> 12.20 (1H, s), two *ortho*-coupled aromatic protons at δ<sub>H</sub> 6.99 (1H, d, *J* = 8.4 Hz, H-6') and 6.79 (1H, d, *J* = 8.4 Hz, H-5'), two *meta*-coupled aromatic protons at δ<sub>H</sub> 5.96 (2H, br s), and a 2,2-dimethylpyran ring at δ<sub>H</sub> 6.81 (1H, d, *J* = 10.0 Hz, H-1''), 5.85 (1H, d, *J* = 10.0 Hz, H-2''), and 1.42, 1.43 (each 3H, s, H<sub>3</sub>-4'', 5''). The <sup>13</sup>C NMR spectrum (Table 1) exhibited 20 carbon signals attributable to one carbonyl group, eight quaternary sp<sup>2</sup>, one quaternary sp<sup>3</sup>, six methine sp<sup>2</sup>, one methine sp<sup>3</sup>, one methylene sp<sup>3</sup>, and two methyl carbon atoms. These spectroscopic data suggested that **1** is a monoprenylated flavanone. The two *ortho*-coupled protons at δ<sub>H</sub> 6.99 and 6.79 were assigned to H-6' and H-5', respectively, as established by the HMBC correlations from H-6' (δ<sub>H</sub> 6.99) to C-2 and from H-5' (δ<sub>H</sub> 6.79) to C-1', C-3', C-4', and C-6' (Figure 2). The HMBC correlations from H-1'' (δ<sub>H</sub> 6.81) to C-1', C-2', and C-3' and from H-2'' (δ<sub>H</sub> 5.85) to C-2' indicated that the 2,2-dimethylpyran ring was fused at C-2' and

**Table 1:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR Data (400 and 100 MHz, resp.) of compounds **1** and **2** in acetone- $d_6$  ( $\delta$  in ppm, J in Hz).

Position	<b>1</b>		<b>2</b>	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$
2	5.73, dd (13.2, 2.8)	77.3	5.65, dd (13.6, 2.8)	77.7
3	2.70, dd (17.2, 2.8)	43.5	2.68, dd (17.2, 2.8)	43.3
	3.21, dd (17.2, 13.2)		3.31, dd (17.2, 13.6)	
4		197.8		198.0
5		165.9		165.9
6	5.96, br s	97.4	5.96, d (2.0)	97.5
7		167.9		167.8
8	5.96, br s	96.4	5.99, d (2.0)	96.3
9		164.9		164.9
10		103.8		103.8
1'		126.3		129.3
2'		121.3		133.1
3'		141.5		146.9
4'		147.6		151.9
5'	6.79, d (8.4)	116.4	6.93, d (8.8)	123.2
6'	6.99, d (8.4)	120.0	7.32, d (8.8)	124.6
1''	6.81, d (10.0)	120.5	6.73, d (16.4)	116.5
2''	5.85, d (10.0)	133.3	6.81, d (16.4)	139.6
3''		77.0		143.7
4''	1.42, s <sup>a</sup>	28.0	5.06, br s	118.8
5''	1.43, s <sup>a</sup>	28.0	1.94, s	18.9
OH-5	12.20, s		12.17, s	
OMe-3'			3.71, s	60.9

<sup>a</sup> Assignments are exchangeable.**Figure 2:** Key HMBC (H→C) correlations of compounds **1** and **2**.

C-3'. The substitution of ring A was further supported by the HMBC correlations shown in Figure 2. The stereochemistry at C-2 was assigned as *S*-configuration by the ECD data with Cotton effects at 288 (–) and 330 (+) nm [8]. Thus, the structure of **1** was determined and named ficustikousin A.

Ficustikousin B (**2**), obtained as white amorphous powder, was assigned the molecular formula  $\text{C}_{21}\text{H}_{20}\text{O}_6$  by HRESIMS ( $m/z$  367.1202 [ $\text{M} - \text{H}$ ]<sup>–</sup>; calcd.  $m/z$  367.1187). The NMR spectroscopic data of **2** (Table 1) suggested a flavanone skeleton with the same rings A and C as those of **1**. It differed from **1** in the substituents on the ring B, as deduced from the NMR data of a 3-methylbutadienyl group [ $\delta_{\text{H}}$  6.73 (1H, d,  $J = 16.4$  Hz, H-1''), 6.81 (1H, d,  $J = 16.4$  Hz, H-2''), 5.06 (2H, br s, H<sub>2</sub>-4''), and 1.94 (3H, s, H<sub>3</sub>-5'')];  $\delta_{\text{C}}$  116.5 (C-1''), 139.6 (C-2''), 143.7 (C-3''), 118.8 (C-4''), and 18.9 (C-5'')] and a methoxyl group [ $\delta_{\text{H}}$  3.71 (3H, s);  $\delta_{\text{C}}$  60.9 (OMe-3')]. In the HMBC spectrum (Figure 2), the correlations from H-1'' to C-1', C-2' and C-3' and from H-2'' to C-2' indicated that the 3-methylbutadienyl group was attached to C-2'. The methoxyl group was located at C-3' according to the HMBC correlation from the methoxyl protons to C-3'. The (*E*)-configuration of the C(1'')=C(2'') bond was indicated by the coupling constant of H-1'' and H-2'' ( $J = 16.4$  Hz). Compound **2** showed no Cotton effect in the ECD spectrum. The stereochemistry at C-2 was proposed to be *S*-configuration on the basis of the negative specific optical rotation ( $[\alpha]_{\text{D}}^{25} = -5.0$ ) and the *trans* diaxial coupling constant of H-2 and H-3 ( $J = 13.6$  Hz) [9]. Thus, the structure of **2** was assigned and named ficustikousin B.

The structures of compounds **3–9** were identified by comparison of their spectral data with those described in the literature.

**Table 2:** Inhibitory Effects of Compounds **1–7** against PTP1B.

Compound	$\text{IC}_{50} \pm \text{SD}$ ( $\mu\text{M}$ )
<b>1</b>	$40.37 \pm 4.66$
<b>2</b>	$16.33 \pm 1.47$
<b>3</b>	$24.64 \pm 0.57$
<b>4</b>	$11.16 \pm 1.88$
<b>5</b>	$19.27 \pm 3.26$
<b>6</b>	$25.12 \pm 2.28$
<b>7</b>	$18.55 \pm 0.74$
Oleanolic acid	$2.61 \pm 0.59$

The isolated compounds (**1–9**) were examined *in vitro* for the inhibition on PTP1B. Oleanolic acid is used as positive control in this test. The isoprenylated flavonoids (**1–7**) showed moderate PTP1B inhibitory activities ( $10 \mu\text{M} < \text{IC}_{50} < 50 \mu\text{M}$ ) (Table 2).

In summary, two new isoprenylated flavanones and seven known compounds including five isoprenylated flavonoids were isolated from the whole plant of *F. tikoua*. Although isoprenylated flavonoids from *F. tikoua* had been reported previously [4a, b, c], the flavanones with 2,2-dimethylpyran moiety (**1** and **6**) and methylbutadienyl group (**2**) were found for the first time from this plant. Furthermore, isoprenylated flavonoids (**1–7**) were found to inhibit PTP1B, while flavonoid and coumarin without isoprenoid group (**8** and **9**) were inactive.

## Experimental

**General:** Optical rotations were measured on Autopol IV. UV spectra were taken on a Hitachi U-2900 spectrophotometer. IR spectra were measured on a Nicolet Avatar 360 spectrometer with KBr pellets. ECD spectra were manipulated on a Jasco J-810 automatic digital polarimeter. NMR spectra were recorded on a Bruker DRX-400 and Varian Mercury Plus 400 instruments. ESIMS and HRESIMS were performed on an Agilent 1100 LC/MSD and on a Bruker Daltonics Apex 7.0 TESLA FTMS mass spectrometer, respectively. Column chromatography (CC) was performed on silica gel (200–300 mesh; Yantai Institute of Chemical Technology, Yantai, PR China), Sephadex LH-20 gel (GE Healthcare Bio-Science AB, Uppsala, Sweden), and Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan). TLC analysis was run on precoated silica gel GF254 plates (10–40  $\mu\text{m}$ , Yantai Institute of Chemical Technology, Yantai, PR China), and spots were visualized by spraying with 10%  $\text{H}_2\text{SO}_4$  in EtOH, followed by heating. Semi-preparative HPLC was run on an Agilent 1200 and a Promosil C<sub>18</sub> column (10  $\times$  150 mm, 5  $\mu\text{m}$ , Agela Technologies, China), using a UV detector set at 210 nm.

**Plant material:** The whole plant of *F. tikoua* was collected from Nanchong, Sichuan province, PR China, in August 2012. The plant material was identified by Dr. Yun Kang, Fudan University, and a voucher specimen (TCM 12-08-25 Hou) has been deposited in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Fudan University.

**Extraction and isolation:** The milled and air-dried plant material (4.7 kg) was percolated with 95% EtOH (20 L  $\times$  5 times) at room temperature. The filtrate was concentrated under reduced pressure to yield a residue (400 g), which was suspended in  $\text{H}_2\text{O}$  and extracted with EtOAc (2 L  $\times$  3 times). The EtOAc extract (96 g) was subjected to column chromatography (CC) on Diaion HP-20 eluted with 85% EtOH and then was applied to CC over silica gel eluted with a gradient system of  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (100:1, 20:1, 9:1, 5:1, 2:1, v/v) to provide fractions 1–5. Fraction 1 (11 g) was purified by silica gel CC (petroleum ether (PE)/  $\text{Me}_2\text{CO}$  30:1, v/v) to give **9** (3.1 mg). Fraction 2 (8 g) was subjected successively to Sephadex LH-20 CC (MeOH), silica gel CC (PE/ $\text{Me}_2\text{CO}$  50:1, v/v),

and semi-preparative HPLC (MeOH/H<sub>2</sub>O 7:3, v/v, flow rate 1 mL/min) to give **6** (7 mg, *t<sub>R</sub>* 70 min) and **7** (6 mg, *t<sub>R</sub>* 50 min). Purification of fraction 3 (3.3 g) by Sephadex LH-20 CC (MeOH) and semi-preparative HPLC (MeOH/H<sub>2</sub>O 6:4, v/v, flow rate 1 mL/min) yielded **3** (20 mg, *t<sub>R</sub>* 56 min) and **8** (4 mg, *t<sub>R</sub>* 10 min). Fraction 4 (8.4 g) was subjected to Sephadex LH-20 CC (MeOH) to give fractions 4.1–4.5. Fraction 4.1 was isolated by semi-preparative HPLC (MeOH/H<sub>2</sub>O 7:3, v/v, flow rate 1 mL/min) to provide **4** (3.9 mg, *t<sub>R</sub>* 27 min) and **5** (8 mg, *t<sub>R</sub>* 29 min). Fraction 4.3 was separated by semi-preparative HPLC (MeOH/H<sub>2</sub>O 6:4, v/v, flow rate 1 mL/min) to give **2** (3.5 mg, *t<sub>R</sub>* 67 min). Fraction 4.4 was purified by semi-preparative HPLC (MeOH/H<sub>2</sub>O 5.5:4.5, v/v, flow rate 1 mL/min) to provide **1** (4 mg, *t<sub>R</sub>* 46 min).

#### Ficustikousin A (1)

White amorphous powder.

[ $\alpha$ ]<sub>D</sub><sup>25</sup>: −8.7 (c 0.2, MeOH).

IR (KBr)  $\nu_{\max}$ : 3421, 1653, 1539, 1467, 1327, 1287, 1157, 1087 cm<sup>−1</sup>.

UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 212 (sh 3.35), 231 (3.51), 282 (3.42), 331 (sh 2.95) nm.

ECD (MeOH)  $\lambda_{\max}$  ( $\Delta\epsilon$ ): 260 (+1.27), 288 (−1.39), 330 (+0.46) nm.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

HRESIMS: *m/z* 353.1033 [M − H]<sup>−</sup>; calcd for C<sub>20</sub>H<sub>17</sub>O<sub>6</sub>: 353.1031.

#### Ficustikousin B (2)

White amorphous powder.

[ $\alpha$ ]<sub>D</sub><sup>25</sup>: −5.0 (c 0.2, MeOH).

IR (KBr)  $\nu_{\max}$ : 3421, 1653, 1579, 1467, 1411, 1331, 1301, 1255, 1163, 1087 cm<sup>−1</sup>.

UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 212 (sh 3.80), 228 (3.82), 282 (3.67), 332 (sh 2.87) nm.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

HRESIMS: *m/z* 367.1202 [M − H]<sup>−</sup>; calcd for C<sub>21</sub>H<sub>19</sub>O<sub>6</sub>: 367.1187.

**Assay of PTP1B activity:** The procedure was the same as that reported previously [5d, 10]. The result of PTP1B inhibition was expressed as IC<sub>50</sub>, which was calculated with Prism 4 software (Graphpad, San Diego, CA).

**Supplementary data:** ESIMS, HRESIMS, NMR (<sup>1</sup>H and <sup>13</sup>C NMR, HMBC) and ECD spectra for compounds **1** and **2** are available.

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Phenolic Derivatives from *Hypericum japonicum*Guoyong Luo<sup>a,b</sup>, Min Zhou<sup>a</sup>, Qi Ye<sup>a</sup>, Jun Mi<sup>c</sup>, Dongmei Fang<sup>a</sup>, Guolin Zhang<sup>a,\*</sup> and Yinggang Luo<sup>a,\*</sup><sup>a</sup>Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, PR China<sup>b</sup>University of the Chinese Academy of Sciences, Beijing 100049, PR China<sup>c</sup>Chengdu Nanshan Pharmaceutical Co., Ltd., Chengdu 610041, PR China

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Three new acylphloroglucinol glycosides, hypericumols A - C, together with fifteen known phenolic derivatives, were isolated from the total phenolic extract of *Hypericum japonicum*. Hypericumols A, B, and C were characterized as 4,6-dimethyl-2-methylpropanoylphloroglucinol-1-*O*- $\beta$ -D-glucopyranoside (**1**), 4-methyl-2-methylpropanoylphloroglucinol-1-*O*- $\beta$ -D-glucopyranoside (**2**), and (2'*S*)-4,6-dimethyl-2-methylbutyrylphloroglucinol-1-*O*- $\beta$ -D-glucopyranoside (**3**), respectively, on the basis of spectroscopic data interpretation and chemical degradation reaction.

**Keywords:** *Hypericum japonicum*, Hypericumol, Acylphloroglucinol, Flavonoid, Phenol.

*Hypericum japonicum* Thunb., di er cao or tian ji huang in Chinese, is an annual / perennial herbal plant [1]. It is widely distributed in Asia, Oceania, and North America [1]. The whole plant material has been used in traditional Chinese medicine for heat-relieving, detoxification, hemostasis, and detumescence [1]. Phytochemical and pharmacological investigations showed that the chemical components of *H. japonicum* have diverse biological activities such as antioxidant [2], antiviral [3], antitumor [4], antimicrobial [5], and hepatoprotection [2a, 6]. Flavonoids and chromones are the primary chemical components of *H. japonicum*, whereas phloroglucinols and xanthenes are two characteristic metabolites present in *H. japonicum* [7]. Recent studies demonstrated that the total phenolic extract of *H. japonicum* showed potential application to cure chronic kidney disease (CKD), which may be attributed to its effects on interfering with renal fibrosis, and enhancing cellular and humoral immunity [8]. As part of our continuing interests in the chemistry of traditional Chinese medicines [9], here we report the isolation and structure characterization of three new acylphloroglucinol glycosides (**1** - **3**), together with fifteen known compounds, including an acylphloroglucinol (**4**), nine flavonoids (**5** - **12**, **16**), three benzoic acid derivatives (**13** - **15**), a pyrone (**17**), and a chromone glycoside (**18**), from the phenolic extract of *H. japonicum* (Figure 1).

Compound **1** was isolated as a yellowish gum. Its molecular formula  $C_{18}H_{26}O_9$  was calculated from the  $[M+Na]^+$  ion at  $m/z$  409.1480 in its HRESIMS (positive ion mode). The hydroxyl and conjugated carbonyl groups were deduced from the IR bands at 3410 and 1615  $cm^{-1}$ , respectively. Besides the  $^1H$  NMR signals for a hexose moiety, two singlet methyls at  $\delta_H = 2.03$  (3H, s) and 2.20 (3H, s), two doublet methyls at  $\delta_H = 0.99$  (3H, d,  $J = 6.9$  Hz) and 1.16 (3H, d,  $J = 6.9$  Hz), and a heptet methine group at  $\delta_H = 4.05$  (1H, h,  $J = 6.9$  Hz) were recognized from the  $^1H$  NMR spectrum (Table 1). In addition to the  $^{13}C$  NMR signals for the groups mentioned above, seven downfield quaternary carbon signals ( $\delta_C > 109$ ) were observed in the  $^{13}C$  NMR spectrum (Table 1), which indicated the presence of a phenyl and a conjugated keto carbonyl group. Compound **1** might be an acylphloroglucinol derivative in view of its characteristic UV bands at 290 and 329 nm for acylphloroglucinol [10]. A 2-methylpropionyl group was established from the two doublet methyls, the

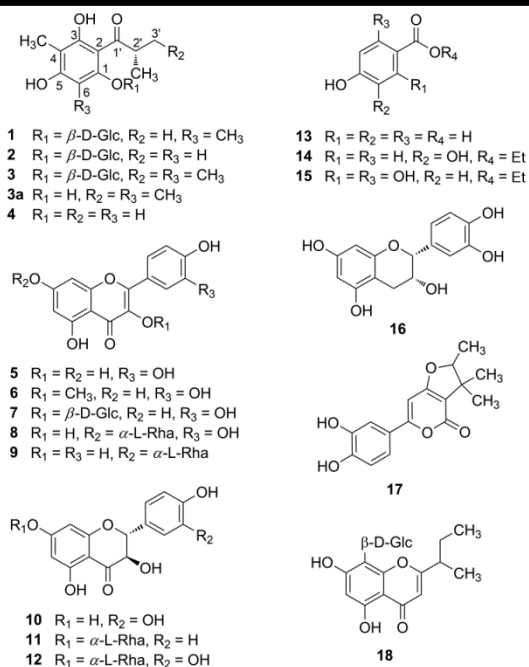


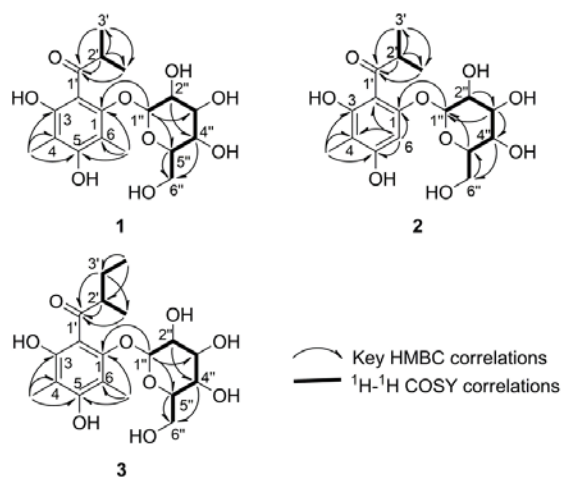
Figure 1. Chemical structures of phenolic derivatives from *Hypericum japonicum*.

heptet methine, and the keto carbonyl group on the basis of  $^1H$ - $^1H$  COSY correlations of H-3' / H-2' / H-2'- $CH_3$  and the key HMBC correlations of H-2' / C-1', C-3', C-2'- $CH_3$ ; H-3' / C-1', C-2'- $CH_3$ ; and H-2'- $CH_3$  / C-1' shown in Figure 2. A 2,4-dimethyl-1,3,5-trihydroxyphenyl moiety was established from the key HMBC correlations of H-4- $CH_3$  / C-3, C-4, C-5 and H-6- $CH_3$  / C-1, C-5, C-6 (Figure 2). Acid hydrolysis of compound **1** afforded an acylphloroglucinol and D-glucose with  $\beta$ -orientation in view of the  $^1H$  NMR signal of the anomeric proton at  $\delta_H = 4.38$  (1H, d,  $J = 7.7$  Hz). The 1-OH was glucosylated in view of the key HMBC correlation of H-1'' / C-1 (Figure 2). Thus compound **1**, named as hypericumol A, was characterized as 4,6-dimethyl-2-methylpropanoylphloroglucinol-1-*O*- $\beta$ -D-glucopyranoside (Figure 1).

**Table 1:** NMR spectroscopic data of compounds **1–3** in CD<sub>3</sub>OD.

<b>1</b>		<b>2</b>		<b>3</b>	
	$\delta_{\text{H}}^a$		$\delta_{\text{H}}^a$		$\delta_{\text{H}}^a$
1	153.72, C	1	159.29, C	1	153.83, C
2	112.71, C	2	105.57, C	2	112.45, C
3	158.52, C	3	165.38, C	3	159.08, C
4	109.30, C	4	106.30, C	4	109.20, C
5	160.41, C	5	163.31, C	5	160.50, C
6	111.13, C	6	94.50, CH	6	111.05, C
4-CH <sub>3</sub>	2.03, s	8.35, CH <sub>3</sub>	1.95, s	7.48, CH <sub>3</sub>	2.05, s
6-CH <sub>3</sub>	2.20, s	9.43, CH <sub>3</sub>			2.21, s
1'	214.98, C				214.29, C
2'	4.05, h (6.9)	40.65, CH	4.05, h (6.7)	40.32, CH	3.92, m
3'	0.99 d (6.9) <sup>b</sup>	21.16, CH <sub>3</sub> <sup>c</sup>	1.14, d (6.7) <sup>c</sup>	19.55, CH <sub>3</sub> <sup>c</sup>	1.90, m
2''-CH <sub>3</sub>	1.16 d (6.9) <sup>b</sup>	17.87, CH <sub>3</sub> <sup>c</sup>	1.15, d (6.7) <sup>c</sup>	20.42, CH <sub>3</sub> <sup>c</sup>	1.02, d (7.1)
3''-CH <sub>3</sub>					0.97, t (7.5)
1''	4.38 d (7.7)	105.64, CH	5.00, d (7.5)	101.60, CH	4.48, d (7.7)
2''	3.48 dd (9.2, 7.7)	75.56, CH	3.51, dd (9.1, 7.5)	74.76, CH	3.52, dd (9.2, 7.7)
3''	3.40 t (9.2)	77.67, CH	3.46, m	78.79, CH	3.43, t (9.2)
4''	3.27 t (9.2)	72.04, CH	3.38, m	71.23, CH	3.27, t (9.2)
5''	3.10 ddd (9.2, 6.2, 2.7)	77.81, CH	3.44, m	78.37, CH	3.14, ddd (9.2, 6.4, 2.8)
6''	3.73 dd (11.9, 2.7)	63.15, CH <sub>2</sub>	3.94, dd (12.0, 2.1)	62.56, CH <sub>2</sub>	3.76, dd (11.9, 2.8)
	3.56 dd (11.9, 6.2)		3.71, dd (12.0, 5.7)		3.57, dd (11.9, 6.4)

<sup>a</sup> Recorded at 400 MHz and reported in ppm. The coupling constants are reported in Hz; s, singlet; d, doublet; t, triplet; h, heptet; m, multiplet; <sup>b</sup> Recorded at 100 MHz; <sup>c</sup> Data in the same column are exchangeable.

**Figure 2.** <sup>1</sup>H-<sup>1</sup>H COSY and HMBC correlations of compounds **1–3**.

Compound **2** showed similar IR and UV bands to that of compound **1**, which suggested that **2** might be an acylphloroglucinol derivative. The molecular formula of **2** was determined to be C<sub>17</sub>H<sub>24</sub>O<sub>9</sub> from the [M+Na]<sup>+</sup> ion at *m/z* 395.1305 from the HRESIMS (positive ion mode). Comparison of the NMR data of compound **2** with that of compound **1** (Table 1) showed that a singlet methyl group of **1** was replaced by an aromatic proton at  $\delta_{\text{H}}$  = 6.23 (1H, s) to form **2**, which was consistent with the molecular formula of **2**. As shown in Figure 2, the spectroscopic data interpretation confirmed that the 6-methyl of **1** was replaced by the proton mentioned above to form **2**. Thus compound **2**, named as hypericumol B, was characterized as 4-methyl-2-methylpropanoylphloroglucinol-1-*O*- $\beta$ -D-glucopyranoside (Figure 1).

Compound **3** had similar spectroscopic characteristics to that of compounds **1** and **2**. The molecular formula of **3**, C<sub>19</sub>H<sub>28</sub>O<sub>9</sub>, was determined from the [M+Na]<sup>+</sup> ion at *m/z* 423.1614 in its HRESIMS (positive ion mode). This indicated an additional CH<sub>2</sub> group present in the structure of **3**, relative to that of **1**. Comparison of the NMR signals of **3** with those of **1** showed that a doublet methyl of **1** was replaced by a triplet methyl at  $\delta_{\text{H}}$  = 0.97 (3H, t, *J* = 7.5 Hz) in **3**. A CH<sub>2</sub> group at  $\delta_{\text{H}}$  = 1.90 (1H, m) and 1.41 (1H, m) /  $\delta_{\text{C}}$  = 25.94 was deduced from the NMR spectroscopic data (Table 1) and it was confirmed to be adjacent to the triplet methyl group by the <sup>1</sup>H-<sup>1</sup>H COSY and HMBC correlations (Figure 2). A 2-methylbutyryl group was established from a doublet methyl, a triplet methyl, a methine, a methylene, and a keto carbonyl group on the basis of detailed spectroscopic data interpretation (Figure 2). Acid hydrolysis of **3**

led to the identification of D-glucose and 4,6-dimethylmultifidol (**3a**) [11]. The absolute configuration of C-2' in **3a** was determined to be *S* by its optical rotation ( $[\alpha]_{\text{D}}^{20}$  +2.4), according to the empirical rule established by Pei *et al.* to determine the absolute configuration of 2-methylbutyrylphloroglucinols [12]. Thus, the structure of compound **3** was elucidated as (2'*S*)-4,6-dimethyl-2-methylbutyrylphloroglucinol-1-*O*- $\beta$ -D-glucopyranoside. Compound **3** was a product of the acid hydrolysis of 4,6-dimethyl-1-*O*-[ $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranosyl]multifidol [13]. However, no spectroscopic data were reported and assigned to the product [13]. Here we report the compound as a naturally occurring product from the plant with detail spectroscopic data and named it as hypericumol C (**3**) (Figure 1).

The known compounds isolated from the phenolic extract were identified as 2-(2-methylpropionyl)-4-methylphloroglucinol (**4**) [14], quercetin (**5**) [15], 3-*O*-methylquercetin (**6**) [16], isoquercitrin (**7**) [17], quercetin-7-*O*- $\alpha$ -L-rhamnopyranoside (**8**) [18], kaempferol-7-*O*- $\alpha$ -L-rhamnopyranoside (**9**) [19], dihydroquercetin (**10**) [2c], dihydrokaempferol-7-*O*- $\alpha$ -L-rhamnopyranoside (**11**) [20], (2*R*,3*R*)-taxifolin-7-*O*- $\alpha$ -L-rhamnopyranoside (**12**) [21], *p*-hydroxybenzoic acid (**13**) [22], ethyl 3,4-dihydroxybenzoate (**14**) [23], ethyl 2,4,6-trihydroxybenzoate (**15**) [24], epicatechin (**16**) [25], saropyrone (**17**) [26], and 5,7-dihydroxy-2-(1-methylpropyl)chromone-8- $\beta$ -D-glucopyranoside (**18**) [27] on the basis of spectroscopic analyses.

In summary, three new acylphloroglucinol glycosides and fifteen known phenolic natural products were isolated and identified from the phenolic extract of *H. japonicum*. Among them, quercetin (**5**), isoquercitrin (**7**), and dihydroquercetin (**10**) are the major chemical components of the extract. Quercetin (**5**) has been reported to recover renal amyloidosis and collagen deposition [28] and exhibit anti-fibrotic and kidney protection activities related to renal dysfunction [29], indicating its therapeutic potential in treating CKD. Meanwhile, oxidative stress induced by free radicals play a vital role in the progression of kidney diseases such as renal fibrosis and chronic renal failure [30], which suggested that antioxidant therapy using phenolic derivatives might be a practical strategy for CKD. The chemical investigations presented here support, in part, the potent application of the phenolic extract of *H. japonicum* in the treatment of CKD.

## Experimental

**General:** Optical rotations were recorded on a Perkin-Elmer 341 automatic polarimeter. UV-Vis spectra were collected on a Perkin-Elmer Lambda 35 UV-Vis spectrometer with  $\lambda_{\text{max}}$  given in nanometers. IR spectra were recorded on a Perkin-Elmer Spectrum One spectrometer with  $\nu_{\text{max}}$  given in cm<sup>-1</sup>. NMR spectra were recorded on a Bruker Ascend 400 spectrometer with chemical shifts  $\delta$  in ppm. HRESIMS were measured on a Bruker Bio TOF IIIQ (quadrupole time of flight) mass spectrometer and ESIMS on a Waters Xevo TQ (tandem quadrupole) mass spectrometer. Column chromatography (CC) was performed on silica gel (Qindao Haiyang Chemical Co., Ltd., P. R. China (QHCC)), silica gel 60 (Merck, German), ODS gel (YMC Co., Ltd, Japan), or Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden). Thin layer chromatography (TLC) was conducted on plates precoated with 10 - 40  $\mu\text{m}$  of silica gel GF254 from QHCC. Preparative HPLC separation was carried out on a LC3000 liquid chromatograph (Beijing Chuangxin Tongheng Science and Technology Co., Ltd) with a YMC C<sub>18</sub> column (20  $\times$  250 mm; 10  $\mu\text{m}$ ), and semi-preparative HPLC on a Perkin-Elmer 200 liquid chromatograph with a Welch C<sub>18</sub> column (10  $\times$  250 mm; 5  $\mu\text{m}$ ), and detected at 208 nm. All solvents were



commercially purchased and distilled under normal atmospheric pressure prior to use.

**Extraction and isolation:** The crude phenolic extract was provided by Chengdu Nanshan Pharmaceutical Co., Ltd. The crude extract was prepared, according to the following procedure. The whole plant material of *H. japonicum* was air-dried and extracted with 65% aqueous EtOH with refluxing. The filtrates were concentrated to remove EtOH under reduced pressure. The aqueous residue was subjected to CC on HPD100 macroporous resin eluted with H<sub>2</sub>O, 30% and 80% EtOH, respectively. The eluents of 80% EtOH were collected and concentrated to afford the crude phenolic extract. This (350 g) was separated by CC over silica gel (160-200 mesh) eluted with light petroleum-acetone (2:1, 1:1, 0:1; v/v) to afford 15 sub-fractions S1 - S15. Fraction S3 (3.9 g) was subjected to preparative HPLC eluted with CH<sub>3</sub>OH-H<sub>2</sub>O (60:40, v/v; 15 mL/min) to give compound **14** (*t<sub>R</sub>* = 7.6 min; 23 mg) and three sub-fractions S3B - S3D. Fraction S3B was purified with semi-preparative HPLC with CH<sub>3</sub>CN-H<sub>2</sub>O (31:69, v/v; 5 mL/min) as eluents to afford compound **15** (*t<sub>R</sub>* = 30 min; 6 mg). Fraction S3D was separated by semi-preparative HPLC eluted with CH<sub>3</sub>CN-H<sub>2</sub>O (31:69, v/v; 5 mL/min) to afford compound **4** (*t<sub>R</sub>* = 32 min; 70 mg). Fraction S4 was separated by preparative HPLC eluted with CH<sub>3</sub>OH-H<sub>2</sub>O (54:46, v/v; 20 mL/min) to give compounds **6** (*t<sub>R</sub>* = 30 min; 60 mg) and **17** (*t<sub>R</sub>* = 32 min; 6 mg), and four sub-fractions S4A - S4D. Fraction S4A was separated by semi-preparative HPLC eluted with CH<sub>3</sub>OH-H<sub>2</sub>O (38:62, v/v; 4 mL/min) to afford a sub-fraction S4A1 (*t<sub>R</sub>* = 8 min) that was then subjected to CC on silica gel 60 (15 - 40 μm) eluted with CHCl<sub>3</sub>-MeOH (10:1, v/v) to afford compound **13** (2 mg). Compound **5** (9.4 g) was precipitated from the light petroleum-acetone (2:1) solution of fraction S6. Compound **10** (3.8 g) was precipitated from the light petroleum-acetone (2:1) solution of fraction S7. The remaining sub-fraction of S7 was separated by CC over silica gel (160-200 mesh) with CHCl<sub>3</sub>-MeOH (15:1, v/v) as solvent to give 4 sub-fractions S7A - S7D. Fraction S7B was subjected to preparative HPLC eluted with CH<sub>3</sub>OH-H<sub>2</sub>O (50:50, v/v; 15 mL/min) to afford compound **3** (*t<sub>R</sub>* = 29 min; 63 mg) and 3 sub-fractions S7B1 - S7B3. Fraction S7B3 was separated by semi-preparative HPLC eluted with CH<sub>3</sub>OH-H<sub>2</sub>O (50:50, v/v; 3 mL/min) to afford subfraction S7B3C (*t<sub>R</sub>* = 20 min) that was subjected to semi-preparative HPLC eluted with CH<sub>3</sub>CN-H<sub>2</sub>O (32:68, v/v; 3 mL/min) to give compounds **1** (*t<sub>R</sub>* = 11 min; 463 mg) and **2** (*t<sub>R</sub>* = 26 min; 6 mg). Compound **8** (88 mg) was crystallized from the methanol solution of fraction S9. Fraction S10 was separated over a self-packed ODS column using CH<sub>3</sub>OH-H<sub>2</sub>O (40:60, v/v) as solvent to give 4 sub-fractions S10A - S10D. Fraction S10C was subjected to semi-preparative HPLC with CH<sub>3</sub>CN-H<sub>2</sub>O (30:70, v/v; 4 mL/min) to afford compound **9** (*t<sub>R</sub>* = 24 min; 26 mg) and a sub-fraction (*t<sub>R</sub>* = 4 min) that was separated by semi-preparative HPLC with CH<sub>3</sub>OH-H<sub>2</sub>O (31:69, v/v; 3 mL/min) to afford compounds **11** (*t<sub>R</sub>* = 40 min; 11 mg) and **18** (*t<sub>R</sub>* = 44 min; 8 mg). Compound **7** (3.3

g) was precipitated from the methanol solution of fraction S15. The remaining sub-fraction of S15 was further subjected to CC over ODS eluted with CH<sub>3</sub>OH-H<sub>2</sub>O (28:72, v/v) to give a sub-fraction that was separated by semi-preparative HPLC with CH<sub>3</sub>CN-H<sub>2</sub>O (11:89, v/v; 5 mL/min) to afford compounds **16** (*t<sub>R</sub>* = 16 min; 9 mg) and **12** (*t<sub>R</sub>* = 24 min; 80 mg).

**Hypericumol A [4,6-dimethyl-2-methylpropanoylphloroglucinol-1-O-β-D-glucopyranoside] (1)**

Yellowish gum.

[α]<sub>D</sub><sup>20</sup>: +79 (c 0.23, MeOH).

UV (MeOH) λ<sub>max</sub> (log ε): 290 (4.10), 329 (3.68) nm.

IR (KBr) ν<sub>max</sub>: 3410, 2969, 2931, 2875, 1615, 1382, 1180, 1150, 1073, 1039 cm<sup>-1</sup>.

<sup>1</sup>H NMR and <sup>13</sup>C NMR data: Table 1.

ESIMS (positive ion mode) *m/z*: 409.22 [M+Na]<sup>+</sup>, 425.12 [M+K]<sup>+</sup>.

ESIMS (negative ion mode) *m/z*: 385.31 [M-H]<sup>-</sup>, 223.19 [M-H-Glc]<sup>-</sup>.

HRESIMS (positive ion mode) *m/z*: 409.1480 (calcd for C<sub>18</sub>H<sub>26</sub>NaO<sub>9</sub>, 409.1469, error -2.8 ppm).

**Hypericumol B [4-methyl-2-methyl propanoylphloroglucinol-1-O-β-D-glucopyranoside] (2)**

Yellowish gum.

[α]<sub>D</sub><sup>20</sup>: -93 (c 0.32, MeOH).

UV (MeOH) λ<sub>max</sub> (log ε): 289 (4.00), 329 (3.49) nm.

IR (KBr) ν<sub>max</sub>: 3430, 2974, 2933, 2880, 1622, 1384, 1242, 1100, 1080, 1042 cm<sup>-1</sup>.

<sup>1</sup>H NMR and <sup>13</sup>C NMR data: Table 1.

ESIMS (positive ion mode) *m/z*: 395.19 [M+Na]<sup>+</sup>, 411.11 [M+K]<sup>+</sup>.

ESIMS (negative ion mode) *m/z*: 371.27 [M-H]<sup>-</sup>, 209.19 [M-H-Glc]<sup>-</sup>.

HRESIMS (positive ion mode) *m/z*: 395.1305 (calcd for C<sub>17</sub>H<sub>24</sub>NaO<sub>9</sub>, 395.1313, error -1.9 ppm).

**Hypericumol C [(2'S)-4,6-dimethyl-2-methylbutyrylphloroglucinol-1-O-β-D-glucopyranoside] (3)**

Yellowish gum.

[α]<sub>D</sub><sup>20</sup>: +67 (c 0.22, MeOH).

UV (MeOH) λ<sub>max</sub> (log ε): 290 (3.87), 329 (3.60) nm.

IR (KBr) ν<sub>max</sub>: 3418, 2965, 2931, 2876, 1615, 1378, 1177, 1148, 1074, 1039 cm<sup>-1</sup>.

<sup>1</sup>H NMR and <sup>13</sup>C NMR data: Table 1.

ESIMS (positive ion mode) *m/z*: 423.16 [M+Na]<sup>+</sup>, 439.09 [M+K]<sup>+</sup>.

ESIMS (negative ion mode) *m/z*: 399.25 [M-H]<sup>-</sup>, 237.18 [M-H-Glc]<sup>-</sup>.

HRESIMS (positive ion mode) *m/z*: 423.1614 (calcd for C<sub>19</sub>H<sub>28</sub>NaO<sub>9</sub>, 423.1626, error -2.6 ppm).

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## Synthesis and Anti-Proliferative Effects of Quercetin Derivatives

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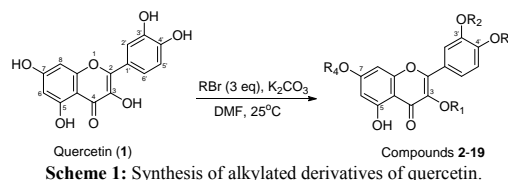
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Prostate cancer is the most common diagnosed invasive cancer in American men and is the second leading cause of cancer-related deaths. Although there are several therapies successful in treating early, localized stage prostate cancer, current treatment of advanced metastatic castration-resistant prostate cancer remains ineffective due to inevitable progression of resistance to first-line treatment with docetaxel. The natural product quercetin (3,3',4',5,7-pentahydroxyflavone), a flavonoid compound ubiquitous in dietary plants, possesses evidenced potential in treating advanced metastatic castration-resistant prostate cancer. However, its poor bioavailability and moderate potency hinder its advancement into clinical therapy. In order to engineer quercetin derivatives with improved potency and pharmacokinetic profiles for the treatment of advanced metastatic prostate cancer, we started this study with creating a small library of alkylated derivatives of quercetin for *in vitro* evaluation. The biological data and chemical reactivity of quercetin and its derivatives reported in literature directed us to design 3,4',7-*O*-trialkylquercetins as our first batch of targets. Consequently, nine 3,4',7-*O*-trialkylquercetins, together with four 3,7-*O*-dialkylquercetins, four 3,3',4',7-tetraalkylquercetins, and one 3,3',4'-*O*-trialkylquercetin, were prepared by one step *O*-alkylation of commercially available quercetin mediated by potassium carbonate. Their structures were determined by 1D and 2D NMR data, and HRMS. Their anti-proliferative activities towards both androgen-refractory and androgen-sensitive prostate cancer cells were evaluated using WST-1 cell proliferation assay. The acquired structure-activity relationships indicate that 3,7-*O*-dialkylquercetins rather than 3,4',7-*O*-trialkylquercetins were much more potent than quercetin towards prostate cancer cells.

**Keywords:** Quercetin, Dietary natural product, Prostate cancer, Derivatives, Cell proliferation.

Prostate cancer, the most common diagnosed invasive cancer in American men, is the second leading cause of cancer-related deaths. Even though most patients can recover from localized androgen-sensitive prostate cancer after an appropriate therapy, the prostate cancer will unfortunately relapse in around 2-3 years after recovery from the early, androgen-sensitive stage [1]. At this point, prostate cancer is no longer responding to hormone therapy and/or has metastasized to different areas of the body. This was referred to as Hormone-Refractory Prostate Cancer or Androgen-Independent Prostate Cancer, and recently was designated as Castration-Resistant Prostate Cancer (CRPC). The US FDA did approve numerous drugs for the treatment of both androgen-dependent and castration-resistant prostate cancer. Sadly, there are no FDA-approved drugs that can actually cure advanced metastatic CRPC. The current first-line chemotherapy for CRPC, docetaxel, can barely increase survival by an average of 2.4 years [2]. The patients' inevitable progression of resistance to docetaxel makes it become an even less effective chemotherapeutic for advanced metastatic CRPC. Very recently, FDA approved cabazitaxel as a second-line treatment for the patients refractory to the first-line docetaxel chemotherapy due to the fact that carbazitaxel is the only available chemotherapeutic that can improve survival (even though by only 2.4 months) for docetaxel-resistant patients [3]. There is thus an urgent need to search for effective chemotherapeutics for clinical treatment of advanced metastatic castration-resistant prostate cancer.

Quercetin, 3,3',4',5,7-pentahydroxyflavone (**1** in Scheme 1), is a flavonoid compound ubiquitous in plant food sources [4]. Quercetin was capable of inhibiting cell growth in the mouse prostate cancer cell line TRAMP-C2 with an IC<sub>50</sub> of 20 μM [5-6]. Numerous studies demonstrated that quercetin was also capable of suppressing

**Table 1:** Alkyl groups for compounds 2-19.

Comps	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
2	Methyl	H	H	Methyl
3	Methyl	H	Methyl	Methyl
4	Methyl	Methyl	Methyl	Methyl
5	Ethyl	H	H	Ethyl
6	Ethyl	H	Ethyl	Ethyl
7	Ethyl	Ethyl	Ethyl	Ethyl
8	Ethyl	Ethyl	Ethyl	H
9	Propyl	H	H	Propyl
10	Propyl	H	Propyl	Propyl
11	Butyl	H	H	Butyl
12	Butyl	H	Butyl	Butyl
13	Butyl	Butyl	Butyl	Butyl
14	Pentyl	H	Pentyl	Pentyl
15	Hexyl	H	Hexyl	Hexyl
16	Isopropyl	H	Isopropyl	Isopropyl
17	Isopentyl	H	Isopentyl	Isopentyl
18	Benzyl	H	Benzyl	Benzyl
19	Benzyl	Benzyl	Benzyl	Benzyl

the growth of both androgen-sensitive (LNCaP) and androgen-refractory human prostate cancer cell lines (PC-3 and DU145) and that quercetin has no apparent toxicity against normal prostate epithelial cells [7-8]. Maggolini and co-workers first demonstrated that quercetin functioned as agonists for the mutant androgen receptor (AR) T877A so that it could inhibit the proliferation of LNCaP cells at low concentration. However, high concentration of quercetin may independently cause significant cytotoxicity without involving hormone receptor expression [9].

Wang *et al.* reported that quercetin exhibited potential *in vivo* anti-prostate tumor efficacy in an androgen-sensitive LAPC-4 xenograft prostate tumor mouse model using severe combined immunodeficiency (SCID) mice. It inhibited tumor growth by 3% when 0.2% was administered as a supplement in diet and by 15% when 0.4% was taken [10]. Quercetin can also significantly reduce PC-3 tumor volume and weight in 6-week old BALB/cA nude mice. The average weight of prostate tumors decreased from 0.242 g for the control group to 0.099 g for the quercetin treated group, implying the *in vivo* anti-prostate tumor potential of quercetin [11]. The above-described cell-based and animal-based studies have evidenced that quercetin could be a good candidate for prostate cancer treatment. However, its poor bioavailability and moderate potency hinder its advancement into clinical therapy. In order to engineer quercetin derivatives with improved potency and a better pharmacokinetic profile for the treatment of advanced metastatic prostate cancer, we started this project by creating a small panel of alkylated derivatives of quercetin for *in vitro* evaluation. The structure-activity relationships will be used to direct our further structure modulation.

The structure-cytotoxicity relationships of methylquercetins summarized by Beutler *et al.* [12] showed that 3,4',7-trimethylquercetin possessed better cytotoxicity than quercetin and other methylquercetins. Intriguingly, Rao *et al.* [13] summarized that methylation of the five phenolic hydroxyl groups in quercetin occurred gradually with the following sequential positions order: 4'

> 7 > 3 > 3' > 5. The superposition of cytotoxic potency and chemical reactivity of quercetin derivatives directed us to synthesize a group of new 3,4',7-*O*-trialkylquercetins as our first batch of targets. Different alkyl groups were introduced to investigate the effect of their length and steric hindrance on the cell proliferation. The targets were proposed to be achieved by one-step alkylation of quercetin.

As shown in Scheme 1, we planned to synthesize our expected 3, 4',7-*O*-trialkylquercetins by treating one equivalent of quercetin with three equivalents of alkyl halide using DMF as solvent and potassium carbonate as base. Reaction of quercetin with the appropriate alkyl halide did provide us with the expected 3,4',7-*O*-trialkylquercetins as the major products, but in moderate yields (less than 25%). Assignments of three alkyl groups were based on the key HMBC correlations between CH<sub>2</sub> protons of ethyl groups ( $\delta$  4.10, 4.10, and 4.22) and C-3, C-7, and C-4' in derivative **6** as summarized in Figure 1. Four 3,7-*O*-dialkylquercetins, four 3,3',4',7-*O*-tetraalkylquercetins, and one 3,3',4'-*O*-trialkylquercetin were also collected for structure-activity relationship studies. Similarly, the locations of the alkyl groups in 3,7-*O*-dibutylquercetin (**11**) and in 3,3',4'-*O*-triethylquercetin (**8**) were also confirmed by the existence of key correlations in their HMBC spectra (Figure 1). As shown in Table 2, all signals in the <sup>1</sup>H- and <sup>13</sup>C-NMR spectra for compounds **6**, **8**, and **11** have been fully assigned based on the extensive interpretation of their COSY, HMQC, and HMBC data.

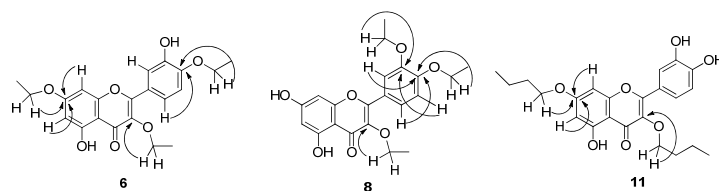


Figure 1: Key HMBC correlations in compounds **6**, **8**, and **11**.

Table 2: NMR data for derivatives **6**, **8**, and **11** (<sup>1</sup>H NMR: 300 MHz; <sup>13</sup>C NMR: 75 MHz).

Position	<b>6</b> (CDCl <sub>3</sub> )		<b>8</b> (acetone-d <sub>6</sub> )		<b>11</b> (acetone-d <sub>6</sub> )	
	$\delta_C$ , type	$\delta_H$ , <i>J</i> in Hz	$\delta_C$ , type	$\delta_H$ , ( <i>J</i> in Hz)	$\delta_C$ , type	$\delta_H$ , ( <i>J</i> in Hz)
2	156.5, C		155.7, C		156.3, C	
3	138.7, C		137.5, C		137.7, C	
4	179.6, C		178.7, C		178.8, C	
4a	106.6, C		104.9, C		105.6, C	
5	162.6, C		162.3, C		162.0, C	
6	98.8, CH	6.33, d (1.8)	98.6, CH	6.26, s	97.9, CH	6.30, d (2.1)
7	165.4, C		164.3, C		165.1, C	
8	93.1, CH	6.42, d (1.8)	93.7, CH	6.51, s	92.2, CH	6.62, d (2.1)
8a	157.4, C		157.8, C		156.8, C	
1'	124.3, C		123.0, C		122.3, C	
2'	115.1, CH	7.72, br.s	113.8, CH	7.79, d (1.8)	115.7, CH	7.74, d (2.1)
3'	146.1, C		148.4, C		144.9, C	
4'	148.6, C		151.4, C		148.1, C	
5'	111.6, CH	6.94, d (9.3)	112.6, CH	7.12, d (8.4)	115.2, CH	7.00, d (8.4)
6'	122.2, CH	7.73, dd (9.3, 1.8)	122.2, CH	7.74, dd (8.4, 1.8)	121.4, CH	7.61, dd (8.4, 2.1)
3-O-CH <sub>2</sub>	64.8, CH <sub>2</sub>	4.10, q (6.9)	67.9, CH <sub>2</sub>	4.14, overlapped	-	-
CH <sub>3</sub>	15.2, CH <sub>3</sub>	1.45, t (6.9)	14.3, CH <sub>3</sub>	1.32, t (6.9)	-	-
7-O-CH <sub>2</sub>	65.3, CH <sub>2</sub>	4.10, q (6.9)	-	-	-	-
CH <sub>3</sub>	15.4, CH <sub>3</sub>	1.51, t (6.9)	-	-	-	-
7-O-CH <sub>2</sub>	-	-	-	-	68.3, CH <sub>2</sub>	4.14, t (6.6)
CH <sub>2</sub>	-	-	-	-	30.9, CH <sub>2</sub>	1.85-1.67, m
CH <sub>2</sub>	-	-	-	-	18.9, CH <sub>2</sub>	1.56-1.42, m
CH <sub>3</sub>	-	-	-	-	13.15, CH <sub>3</sub>	0.92, t (7.2)
3'-O-CH <sub>2</sub>	-	-	64.1, CH <sub>2</sub>	4.17, overlapped	-	-
CH <sub>3</sub>	-	-	14.2, CH <sub>3</sub>	1.43, t (6.9)	-	-
4'-O-CH <sub>2</sub>	69.2, CH <sub>2</sub>	4.22, q (6.9)	64.4, CH <sub>2</sub>	4.18, overlapped	-	-
CH <sub>3</sub>	16.2, CH <sub>3</sub>	1.36, t (6.9)	14.3, CH <sub>3</sub>	1.43, t (6.9)	-	-
3-O-CH <sub>2</sub>	-	-	-	-	71.9, CH <sub>2</sub>	4.05, t (6.6)
CH <sub>2</sub>	-	-	-	-	31.9, CH <sub>2</sub>	1.85-1.67, m
CH <sub>2</sub>	-	-	-	-	18.9, CH <sub>2</sub>	1.56-1.42, m
CH <sub>3</sub>	-	-	-	-	13.19, CH <sub>3</sub>	0.99, t (7.2)
3'-OH	-	5.81, s	-	-	-	8.50, brs
5-OH	OH	12.68, s	-	12.81, s	-	12.80, s
4'-OH	-	-	-	-	-	8.50, brs

Among the eighteen quercetin derivatives that we prepared, five of them (**8**, **14-17**) are new compounds. Derivatives **2-7**, **9-13**, and **18-19** are known, but there are no reports available on their anti-proliferative activity against prostate cancer cell lines, even though their *in vitro* effects have been investigated on other cancer cell models [12, 14]. It should be pointed out that the 4',7-dialkylquercetins reported in Ref. [14] should be corrected as 3,7-dialkylquercetins based on our HMBC data of **11** (Figure 1).

The anti-proliferative effect of eighteen synthetic quercetin derivatives (at 50  $\mu$ M) was evaluated against both androgen-sensitive (LNCaP) and androgen-refractory (DU145 and PC-3) human prostate cancer cell lines. WST-1 cell proliferation assay was selected for this evaluation because of its easy operation in a microtiter plate without washing steps. The procedure is described in the Experimental Section and the absorbance was measured using a microplate reader (Synergy HT) at a wavelength of 430 nm. As shown in Table 3, alkylation of the hydroxyl groups at C-3, C-4', and C-7 in quercetin with short, linear alkyl groups (such as methyl groups in **3** and ethyl groups in **6**) slightly increases the anti-proliferative activity towards three human prostate cancer cell lines. However, potency was rapidly diminished when either branched alkyl groups (isopropyl in **16** and isopentyl in **17**) or linear lengthy groups (propyl in **10**, butyl in **12**, pentyl in **14**, and hexyl in **15**) were introduced into these three positions. Incorporation of ethyl groups to 3,3',4'-hydroxyl groups in quercetin (**8**) led to neither gain nor loss in its inhibitory rate. Surprisingly, all four 3,7-*O*-dialkylquercetins (**2**, **5**, **9**, and **11**) exhibited significantly higher inhibitory rate than quercetin against the three prostate cancer cell lines at both concentrations. They were over 2-11 times more potent than quercetin in the cell culture based on the comparison of their  $IC_{50}$  values with that of quercetin (Table 3).

**Table 3:** Anti-proliferative activity of quercetin and its derivatives towards three cancer cell lines.

Comps	Inhibitory rate (%) @ 50 $\mu$ M			$IC_{50}$ ( $\mu$ M)		
	PC-3	DU145	LNCaP	PC-3	DU145	LNCaP
Quercetin	8	13	44	>100	>100	45.46 $\pm$ 1.31
<b>2</b>	64	40	80	22.27 $\pm$ 6.83	46.82 $\pm$ 3.69	13.23 $\pm$ 4.75
<b>3</b>	66	36	77	—	—	—
<b>4</b>	10	25	39	—	—	—
<b>5</b>	79	80	91	13.74 $\pm$ 1.62	12.59 $\pm$ 0.96	4.20 $\pm$ 0.96
<b>6</b>	25	32	50	—	—	—
<b>7</b>	0	16	9	—	—	—
<b>8</b>	6	8	48	—	—	—
<b>9</b>	95	91	96	17.68 $\pm$ 0.96	19.25 $\pm$ 2.21	6.42 $\pm$ 2.72
<b>10</b>	41	29	50	—	—	—
<b>11</b>	97	97	97	11.95 $\pm$ 1.12	18.63 $\pm$ 6.86	6.46 $\pm$ 1.10
<b>12</b>	0	0	0	—	—	—
<b>13</b>	0	0	0	—	—	—
<b>14</b>	0	19	7	—	—	—
<b>15</b>	0	5	0	—	—	—
<b>16</b>	25	30	40	—	—	—
<b>17</b>	3	13	0	—	—	—
<b>18</b>	0	19	11	—	—	—
<b>19</b>	0	3	16	—	—	—

Each experiment was performed at least three in duplicate.

In conclusion, eighteen alkylated derivatives of quercetin were prepared by a semi-synthesized approach. Their anti-proliferative activity was evaluated against three human prostate cancer cell lines using WST-1 cell proliferation assay. The structure-activity relationships acquired show i) that simultaneous introduction of three bulky or lengthy alkyl groups to C-3, C-4' and C-7 hydroxyl groups of quercetin led to a major loss of anti-proliferative activity in prostate cancer cells; and ii) that incorporation of two methyl groups in **2**, two ethyl groups in **5**, two propyl groups in **9**, and two butyl groups in **11** to C-3 and C-7 hydroxyl groups resulted in much more potent derivatives. Chemical manipulation of one phenolic hydroxyl group might be the best direction for the future.

## Experimental

**General:** NMR spectra were obtained on a Bruker Fourier 300 spectrometer in  $CDCl_3$  and  $CD_3COCD_3$ . The chemical shifts are given in  $\delta$  (ppm) referenced to the respective solvent peak, and coupling constants are reported in Hz. All reagents and solvents were purchased from commercial sources and were used without further purification. Column chromatography was performed using silica gel (32-63  $\mu$ m). Preparative thin-layer chromatography (PTLC) separations were carried out on 1000  $\mu$ m AnalTech thin layer chromatography plates (Lot No.13401).

**General procedure for the synthesis of alkylated quercetin derivatives:** To a solution of quercetin hydrate (1.0 equiv.) in DMF was added anhydrous  $K_2CO_3$  (3 equiv.), followed by the appropriate alkyl halide (3.0 equiv.). The reaction mixture was allowed to stir at room temperature for 12 to 48 h prior to being diluted with diethyl ether and ethyl acetate (300 mL in total, 1:1, v/v). The consequent mixture was rinsed with brine (30 mL  $\times$  5), and the organic layer was dried over anhydrous  $MgSO_4$ , filtered and concentrated *in vacuo* to give the corresponding crude product.

**Preparation of methylated derivatives of quercetin (2, 3, and 4):** These derivatives were prepared according to the general procedure from quercetin hydrate (320 mg, 1 mmol) in DMF (1 mL). PTLC purification of crude product, using 30% ethyl acetate in hexane as eluent, yielded compounds **2** (24 mg, 7% yield), **3** (83 mg, 24% yield), and **4** (21 mg, 6% yield).

### 3,7-*O*-Dimethylquercetin (2)

MP: 180-183°C.

IR (neat): 3244, 2923, 2852, 1653, 1605, 1559, 1506, 1456  $cm^{-1}$ .

$^1H$  NMR (300 MHz,  $CD_3COCD_3$ ): 12.77 (s, 1H), 7.73 (d,  $J$  = 2.1 Hz, 1H), 7.61 (dd,  $J$  = 8.4, 2.1 Hz, 1H), 7.00 (d,  $J$  = 8.4 Hz, 1H), 6.64 (d,  $J$  = 2.4 Hz, 1H), 6.31 (d,  $J$  = 2.4 Hz, 1H), 3.93 (s, 3H), 3.88 (s, 3H).

$^{13}C$  NMR (75 MHz,  $CDCl_3$ ): 178.7, 165.7, 161.9, 156.8, 156.1, 148.3, 145.0, 138.6, 122.1, 121.3, 115.5, 115.4, 105.7, 97.6, 91.9, 59.5, 55.5.

### 3,4',7-*O*-Trimethylquercetin (3)

MP: 151-153°C.

IR (neat): 3396, 2929, 1652, 1592, 1494  $cm^{-1}$ .

$^1H$  NMR (300 MHz,  $CDCl_3$ ): 12.64 (s, 1H), 7.73 (dd,  $J$  = 8.7, 2.1 Hz, 1H), 7.70 (d,  $J$  = 2.1 Hz, 1H), 6.98 (d,  $J$  = 8.7 Hz, 1H), 6.46 (d,  $J$  = 2.1 Hz, 1H), 6.37 (d,  $J$  = 2.1 Hz, 1H), 5.73 (s, 1H), 4.00 (s, 3H), 3.89 (s, 6H).

$^{13}C$  NMR (75 MHz,  $CDCl_3$ ): 179.5, 166.1, 162.6, 157.4, 156.2, 149.4, 146.2, 139.8, 124.3, 122.2, 115.0, 111.0, 106.7, 98.5, 92.8, 60.8, 56.7, 56.4.

### 3,3',4',7-*O*-Tetramethylquercetin (4)

MP: 136-137°C.

IR (KBr): 3580, 2933, 2839, 1652, 1589, 1496  $cm^{-1}$ .

$^1H$  NMR (300 MHz,  $CDCl_3$ ): 12.64 (s, 1H), 7.73 (dd,  $J$  = 8.7, 1.8 Hz, 1H), 7.70 (s, 1H), 7.00 (d,  $J$  = 8.7 Hz, 1H), 6.45 (d,  $J$  = 1.8 Hz, 1H), 6.36 (d,  $J$  = 1.8 Hz, 1H), 3.98 (s, 6H), 3.88 (s, 3H), 3.87 (s, 3H).

$^{13}C$  NMR (75 MHz,  $CDCl_3$ ): 179.4, 166.1, 162.7, 157.4, 156.5, 152.0, 149.4, 139.6, 123.6, 122.8, 111.9, 111.5, 106.7, 98.5, 92.9, 60.8, 56.7, 56.6, 56.5.

### Preparation of ethylated quercetin derivatives (5, 6, 7, and 8):

These derivatives were synthesized from quercetin hydrate (640 mg, 2 mmol) in DMF (2 mL). The crude product was purified through a

pad of silica gel, eluting with 33% ethyl acetate in hexanes, followed by repetitive PTLC, eluting with 33% ethyl acetate in hexanes and 5% methanol in DCM in hexane, to yield compounds **5** (56 mg, 8% yield), **6** (137 mg, 18% yield), **7** (57 mg, 7% yield), and **8** (15 mg, 2% yield).

### 3,7-*O*-Diethylquercetin (5)

MP: 155-156°C.

IR (KBr): 3210, 2980, 2929, 1654, 1590, 1496, 1339, 1206 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>COCD<sub>3</sub>): 12.76 (s, 1H), 8.50 (br.s, 2H), 7.76 (d, *J* = 2.1 Hz, 1H), 7.68 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.00 (d, *J* = 8.4 Hz, 1H), 6.55 (d, *J* = 2.1 Hz, 1H), 6.24 (d, *J* = 2.1 Hz, 1H), 4.15 (q, *J* = 6.9 Hz, 2H), 4.11 (q, *J* = 6.9 Hz, 2H), 1.40 (t, *J* = 6.9 Hz, 3H), 1.32 (t, *J* = 6.9 Hz, 3H).

<sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>COCD<sub>3</sub>): 181.4, 167.5, 164.5, 159.4, 158.8, 150.7, 147.5, 140.1, 124.9, 124.0, 118.2, 117.9, 108.1, 100.5, 94.8, 70.4, 66.7, 17.5, 16.6.

### 3,4',7-*O*-Triethylquercetin (6)

MP: 123-124°C.

IR (neat): 3397, 2980, 2931, 2887, 1654, 1592, 1497 cm<sup>-1</sup>.

<sup>1</sup>H and <sup>13</sup>C NMR: see Table 3.

HRMS-FAB: *m/z* [M + H]<sup>+</sup> calcd for C<sub>21</sub>H<sub>23</sub>O<sub>7</sub>: 387.1444; found: 387.1397; [M + Na]<sup>+</sup> calcd for C<sub>21</sub>H<sub>22</sub>O<sub>7</sub>Na: 409.1258; found: 409.1261.

### 3,3',4',7-*O*-Tetraethylquercetin (7)

MP: 121-122°C.

IR (neat): 2981, 2932, 1654, 1589, 1497 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 12.67 (s, 1H), 7.74 (d, *J* = 1.8 Hz, 1H), 7.71 (dd, *J* = 8.7, 1.8 Hz, 1H), 6.96 (d, *J* = 8.7 Hz, 1H), 6.40 (d, *J* = 2.1 Hz, 1H), 6.31 (d, *J* = 2.1 Hz, 1H), 4.18 (q, *J* = 6.9 Hz, 2H), 4.16 (q, *J* = 6.9 Hz, 2H), 4.08 (q, *J* = 6.9 Hz, 2H), 4.06 (q, *J* = 6.9 Hz, 2H), 1.500 (t, *J* = 6.9 Hz, 3 H), 1.498 (t, *J* = 6.9 Hz, 3 H), 1.44 (t, *J* = 6.9 Hz, 3H), 1.33 (t, *J* = 6.9 Hz, 3H).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 180.8, 166.7, 163.9, 158.6, 158.1, 153.0, 150.0, 139.8, 124.9, 124.0, 115.5, 114.1, 107.8, 100.0, 94.4, 70.4, 66.7, 66.4, 66.1, 17.6, 16.7, 16.6, 16.5.

### 3, 3',4'-*O*-Triethylquercetin (8)

MP: 170-172°C.

IR (neat): 3210, 3084, 2978, 2925, 1649, 1600, 1576, 1499, 1170 cm<sup>-1</sup>.

<sup>1</sup>H and <sup>13</sup>C NMR: see Table 3.

### Preparation of propylated derivatives of quercetin (9 and 10):

These derivatives were synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (0.6 mL). The crude product was purified through a pad of silica gel, eluting with 33% ethyl acetate in hexanes, followed by repetitive PTLC, eluting with 33% ethyl acetate in hexanes, to yield compounds **9** (9 mg, 4% yield) and **10** (9 mg, 3.6 % yield).

### 3,7-*O*-Dipropylquercetin (9)

MP: 143-144°C.

IR (neat): 3352, 2965, 2878, 1654, 1590, 1496, 1205 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>COCD<sub>3</sub>): 12.80 (s, 1H), 7.74 (d, *J* = 2.1 Hz, 1H), 7.61 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.00 (d, *J* = 8.4 Hz, 1H), 6.62 (d, *J* = 2.1 Hz, 1H), 6.30 (d, *J* = 2.1 Hz, 1H), 4.09 (t, *J* = 6.6 Hz, 2H), 4.00 (t, *J* = 6.6 Hz, 2H), 1.84-1.71 (m, 4H), 1.05 (t, *J* = 7.2 Hz, 3H), 0.97 (t, *J* = 7.2 Hz, 3H).

<sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>COCD<sub>3</sub>): 178.8, 165.1, 162.0, 156.8, 156.3, 148.2, 145.0, 137.7, 122.3, 121.4, 115.7, 115.2, 105.6, 97.9, 92.3, 73.8, 70.0, 23.1, 22.1, 9.9, 9.7.

### 3,4',7-*O*-Tripropylquercetin (10)

MP: 80-81°C.

IR (neat): 3320, 2965, 2877, 1654, 1597, 1497, 1207 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 12.69 (s, 1H), 7.73 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.72 (s, 1H), 6.95 (d, *J* = 8.7 Hz, 1H), 6.45 (d, *J* = 2.1 Hz, 1H), 6.42 (d, *J* = 2.1 Hz, 1H), 5.73 (s, 1H), 4.12 (t, *J* = 6.6 Hz, 2H), 4.00 (t, *J* = 6.6 Hz, 2H), 3.98 (t, *J* = 6.6 Hz, 2H), 1.98-1.72 (m, 6H), 1.10 (t, *J* = 7.5 Hz, 3 H), 1.07 (t, *J* = 7.5 Hz, 3H), 0.98 (t, *J* = 7.5 Hz, 3H).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 179.0, 165.0, 161.9, 156.8, 155.7, 148.1, 145.5, 138.3, 123.7, 121.7, 114.5, 110.9, 106.0, 98.2, 92.5, 74.5, 70.5, 70.1, 23.4, 22.5, 22.3, 10.5, 10.43, 10.41.

### Preparation of butylated derivatives of quercetin (11, 12, and 13):

These derivatives were synthesized from quercetin hydrate (426 mg, 1.26 mmol) in DMF (1.3 mL). The crude product was purified through a pad of silica gel, eluting with 33% ethyl acetate in hexanes, followed by repetitive PTLC, eluting with 33% ethyl acetate in hexanes, to yield compounds **11** (22 mg, 4% yield), **12** (42 mg, 7% yield), and **13** (16 mg, 2.4% yield).

### 3,7-*O*-Dibutylquercetin (11)

MP: 158-159°C.

IR (KBr): 3210, 2958, 2931, 2873, 1656, 1586, 1492, 1169 cm<sup>-1</sup>.

<sup>1</sup>H and <sup>13</sup>C NMR: see Table 3.

### 3,4',7-*O*-Tributylquercetin (12)

MP: 66-68°C.

IR (neat): 3431, 2958, 2933, 2872, 1655, 1597, 1497, 1206 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 12.69 (s, 1H), 7.71 (d, *J* = 9.3 Hz, 1H), 7.69 (s, 1H), 6.93 (d, *J* = 9.3 Hz, 1H), 6.42 (d, *J* = 1.8 Hz, 1H), 6.32 (d, *J* = 2.1 Hz, 1H), 5.81 (br.s, 1H), 4.14 (t, *J* = 6.6 Hz, 2H), 4.02 (t, *J* = 6.6 Hz, 2H), 4.00 (t, *J* = 6.6 Hz, 2H), 1.91-1.69 (m, 6H), 1.60-1.38 (m, 6H), 1.02 (t, *J* = 7.2 Hz, 3 H), 1.00 (t, *J* = 7.2 Hz, 3H), 0.93 (t, *J* = 7.2 Hz, 3H).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 178.9, 165.0, 161.9, 156.8, 155.8, 148.1, 145.5, 138.3, 123.6, 121.6, 114.5, 110.9, 105.9, 98.2, 92.4, 72.6, 68.8, 68.4, 32.1, 31.1, 31.0, 19.21, 19.16, 19.12, 13.84, 13.83, 13.79.

### 3,3',4',7-*O*-Tetrabutylquercetin (13)

MP: 59-60°C.

IR (neat): 2957, 2933, 2873, 1661, 1589, 1500 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 12.71 (s, 1H), 7.73 (d, *J* = 2.1 Hz, 1H), 7.69 (dd, *J* = 8.4, 2.1 Hz, 1H), 6.98 (d, *J* = 8.4 Hz, 1H), 6.44 (d, *J* = 2.1 Hz, 1H), 6.35 (d, *J* = 2.1 Hz, 1H), 4.11 (t, *J* = 6.6 Hz, 2H), 4.09 (t, *J* = 6.6 Hz, 2H), 4.04 (t, *J* = 6.6 Hz, 2H), 4.00 (t, *J* = 6.6 Hz, 2H), 1.92-1.68 (m, 8H), 1.63-1.40 (m, 8H), 1.02 (t, *J* = 7.2 Hz, 6 H), 1.00 (t, *J* = 7.2 Hz, 3 H), 0.92 (t, *J* = 7.2 Hz, 3H).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 178.9, 164.9, 162.0, 156.8, 156.2, 151.6, 148.5, 130.3, 123.0, 122.2, 114.1, 112.5, 105.9, 98.1, 92.6, 72.8, 69.2, 68.7, 68.4, 32.3, 31.3, 31.2, 31.0, 19.24, 19.17, 19.15, 13.89, 13.85, 13.79.

**Preparation of 3,4',7-*O*-tripentylquercetin (14):** This derivative was synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (10 mL). The crude product was subjected to PTLC purification eluting with 15% ethyl acetate in hexanes to yield compound **14** (19 mg, 7% yield).

### 3,4',7-*O*-Tripentylquercetin (14)

MP: 55-56°C.

IR (KBr): 3540, 2956, 2871, 1654, 1593, 1496 cm<sup>-1</sup>.

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 12.69 (s, 1H), 7.71 (dd,  $J = 9.3$ , 2.1 Hz, 1H), 7.70 (d,  $J = 2.1$  Hz, 1H), 6.94 (d,  $J = 9.3$  Hz, 1H), 6.43 (d,  $J = 2.1$  Hz, 1H), 6.33 (d,  $J = 2.1$  Hz, 1H), 5.77 (s, 1H), 4.14 (t,  $J = 6.6$  Hz, 2H), 4.02 (t,  $J = 6.6$  Hz, 2H), 4.00 (t,  $J = 6.6$  Hz, 2H), 1.91–1.73 (m, 6H), 1.51–1.31 (m, 12H), 0.99–0.93 (overlapped, 6H), 0.90 (t,  $J = 6.6$  Hz, 3H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 179.0, 165.0, 161.9, 156.8, 155.8, 148.1, 145.5, 138.3, 123.6, 121.6, 114.5, 110.9, 105.9, 98.2, 92.5, 73.0, 69.1, 68.7, 29.8, 28.8, 28.7, 28.12, 28.08, 28.06, 22.45, 22.43, 22.40, 13.4.

**Preparation of 3,4',7-O-trihexylquercetin (15):** This derivative was synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (10 mL). The crude product was subjected to PTLC purification eluting with 15% ethyl acetate in hexanes to yield compound **15** (20 mg, 6% yield).

### 3,4',7-O-Trihexylquercetin (15)

MP: 48–50°C.

IR (KBr): 3550, 2954, 2929, 2858, 1654, 1595, 1497  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 12.67 (s, 1H), 7.69 (d,  $J = 7.5$  Hz, 1H), 7.68 (s, 1H), 6.93 (d,  $J = 7.5$  Hz, 1H), 6.42 (d,  $J = 2.1$  Hz, 1H), 6.32 (d,  $J = 2.1$  Hz, 1H), 5.73 (s, 1H), 4.12 (t,  $J = 6.6$  Hz, 2H), 4.01 (t,  $J = 6.6$  Hz, 2H), 3.99 (t,  $J = 6.6$  Hz, 2H), 1.89–1.70 (m, 6H), 1.42–1.20 (m, 18H), 0.81–0.98 (m, 9H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 179.1, 165.1, 162.1, 156.9, 156.0, 148.2, 145.6, 138.4, 123.8, 121.8, 114.6, 111.0, 106.1, 98.4, 92.6, 73.2, 69.2, 68.8, 31.74, 31.65, 30.2, 29.2, 29.1, 25.7, 22.7, 14.2.

HRMS-FAB:  $m/z$   $[\text{M} + \text{Na}]^+$  calcd for  $\text{C}_{33}\text{H}_{46}\text{O}_7\text{Na}$ : 577.3136; found: 577.3127.

**Preparation of 3,4',7-O-triisopropylquercetin (16):** This derivative was synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (10 mL). PTLC purification of the crude product eluting with 15% ethyl acetate in hexanes generated compound **16** (18 mg, 7% yield).

### 3,4',7-O-Triisopropylquercetin (16)

MP: 62–64°C.

IR (KBr): 3428, 2976, 2932, 1652, 1589, 1492  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 12.73 (s, 1H), 6.76 (d,  $J = 8.6$  Hz, 1H), 7.74 (s, 1H), 6.95 (d,  $J = 8.6$  Hz, 1H), 6.42 (s, 1H), 6.33 (s, 1H), 5.78 (s, 1H), 4.79–4.69 (m, 1H), 4.69–4.54 (m, 2H), 1.45 (d,  $J = 7.0$  Hz, 6H), 1.40 (d,  $J = 7.0$  Hz, 6H), 1.23 (d,  $J = 7.0$  Hz, 6H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 179.4, 164.0, 162.1, 157.0, 156.5, 146.8, 146.1, 136.9, 124.0, 122.1, 115.0, 112.1, 105.8, 99.0, 93.4, 75.3, 71.8, 70.8, 22.5, 22.2, 22.0.

HRMS-FAB:  $m/z$   $[\text{M} + \text{Na}]^+$  calcd for  $\text{C}_{24}\text{H}_{28}\text{O}_7\text{Na}$ : 451.1733; found: 451.1727.

**Preparation of 3,4',7-O-triisopentylquercetin (17):** This derivative was synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (10 mL). The crude product was purified by repetitive PTLC eluting with 15% ethyl acetate in hexanes to yield compound **17** (24 mg, 8% yield).

### 3,4',7-O-Triisopentylquercetin (17)

MP: 75–76°C.

IR (KBr): 3540, 3054, 2957, 2871, 1653, 1594, 1464  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 12.67 (s, 1H), 7.69 (d,  $J = 8.6$  Hz, 1H), 7.68 (s, 1H), 6.93 (d,  $J = 8.6$  Hz, 1H), 6.42 (d,  $J = 2.4$  Hz, 1H), 6.33 (d,  $J = 2.4$  Hz, 1H), 5.72 (s, 1H), 4.15 (t,  $J = 6.4$  Hz, 2H), 4.05 (t,  $J = 6.4$  Hz, 2H), 4.02 (t,  $J = 6.4$  Hz, 2H), 1.88–1.79 (m, 3H), 1.78–1.75 (m, 2H), 1.72–1.68 (m, 2H), 1.65–1.62 (m, 2H),

1.00 (d,  $J = 6.8$  Hz, 6H), 0.97 (d,  $J = 6.8$  Hz, 6H), 0.90 (d,  $J = 6.8$  Hz, 6H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 179.1, 165.0, 162.0, 156.9, 155.9, 148.2, 145.6, 138.5, 123.7, 121.8, 114.6, 111.0, 106.1, 98.3, 92.6, 71.6, 67.6, 67.2, 39.0, 37.9, 37.7, 25.2, 25.1, 25.0, 22.7.

HRMS-FAB:  $m/z$   $[\text{M} + \text{Na}]^+$  calcd for  $\text{C}_{30}\text{H}_{40}\text{O}_7\text{Na}$ : 535.2666; found: 535.2678.

### Preparation of benzylated derivatives of quercetin (18 and 19):

These two derivatives were synthesized from quercetin hydrate (200 mg, 0.59 mmol) in DMF (10 mL). PTLC purification of the crude product, using 15% ethyl acetate in hexanes as eluent, generated **18** (73 mg, 22% yield) and **19** (58 mg, 15% yield).

### 3,4',7-O-Tribenzylquercetin (18)

MP: 147–148°C.

IR (KBr): 3651, 3089, 3064, 3032, 2926, 2870, 1653, 1592, 1494  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 12.74 (s, 1H), 7.66 (s, 1H), 7.65 (d,  $J = 8.4$  Hz, 1H), 7.45–7.28 (m, 15H), 6.96 (d,  $J = 8.4$  Hz, 1H), 6.51 (s, 1H), 6.46 (s, 1H), 5.88 (s, 1H), 5.20 (s, 2H), 5.14 (s, 2H), 5.10 (s, 2H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 178.8, 164.5, 162.0, 156.7, 156.3, 148.0, 145.7, 137.7, 136.5, 135.9, 135.8, 128.94, 128.89, 128.75, 128.65, 128.4, 128.3, 128.2, 127.9, 127.5, 123.9, 121.9, 115.0, 111.6, 106.2, 98.7, 93.0, 74.3, 71.1, 70.5.

### 3,3',4',7-O-Tetrabenzylquercetin (19)

MP: 135–136°C.

IR (KBr): 3034, 2930, 1654, 1595, 1495  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 12.70 (s, 1H), 7.71 (d,  $J = 2.0$  Hz, 1H), 7.55 (dd,  $J = 8.6$ , 2.0 Hz, 1H), 7.48–7.22 (m, 20H), 6.96 (d,  $J = 8.6$  Hz, 1H), 6.46 (d,  $J = 2.0$  Hz, 1H), 6.44 (d,  $J = 2.0$  Hz, 1H), 5.25 (s, 2H), 5.13 (s, 2H), 5.04 (s, 2H), 4.99 (s, 2H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 178.9, 164.6, 162.2, 156.8, 151.2, 148.4, 137.0, 136.8, 136.5, 135.9, 128.94, 128.89, 128.75, 128.65, 128.5, 128.4, 128.2, 128.1, 127.6, 127.5, 127.3, 123.6, 122.8, 115.4, 113.8, 106.3, 98.7, 93.2, 74.5, 71.2, 71.0, 70.6.

### Bioassay

**Cell culture:** All cell lines were initially purchased from American Type Culture Collection (ATCC<sup>TM</sup>). The PC-3 prostate cancer cell line and the LNCaP prostate cancer cell line were routinely cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cultures were maintained in 5% carbon dioxide at a temperature of 37°C. The DU-145 prostate cancer cells were routinely cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS and 1% penicillin/streptomycin.

**WST-1 cell proliferation assay:** PC-3, DU-145, or LNCaP cells were plated in 96-well plates at a density of 3200 in each well in 200  $\mu\text{L}$  of culture medium. The cells were then treated with either quercetin or synthesized quercetin derivatives separately at 2 different doses of 50  $\mu\text{M}$  for 3 days, while equal treatment volumes of DMSO (0.25%) were used as vehicle control. The cells were cultured in a  $\text{CO}_2$  incubator at 37°C for 3 days. Ten  $\mu\text{L}$  of the premixed WST-1 cell proliferation reagent (Clontech) was added to each well. After mixing gently for 1 min on an orbital shaker, the cells were incubated for an additional 3 h at 37°C. To ensure homogeneous distribution of color, it is important to mix gently on an orbital shaker for 1 min. The absorbance of each well was measured using a microplate reader (Synergy HT) at a wavelength of 430 nm. The  $\text{IC}_{50}$  value is the concentration of each compound that inhibits cell proliferation by 50% under the experimental

conditions and is the average from at least triplicate determinations that are reproducible and statistically significant. For calculating the IC<sub>50</sub> values, a linear proliferative inhibition was made based on at least 5 dosages for each compound.

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## Compounds with Antifouling Activities from the Roots of *Notopterygium franchetii*

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In antifouling screening, the extract of *Notopterygium franchetii* de Boiss showed obvious activity. Two new phenylpropanoids (**1-2**) and five known coumarins (**3-7**) were isolated from the methanol extract of the roots of this species. The structures of the isolated compounds were determined on the basis of spectroscopic analysis. Compounds **1-2** showed definite antifouling activity against larval settlement of *Bugula neritina*.

**Keywords:** *Notopterygium franchetii*, Umbelliferae, Chemical constituent, Phenylpropanoids, Antifouling.

*Notopterygium franchetii* de Boiss, family Umbelliferae, is a traditional Chinese medicine. Its main constituents include essential oils, coumarins, amino acids, and organic acids [1]. Extracts of *N. franchetii* and some other species of *Notopterygium* have been reported to possess anti-inflammatory [2], antibacterial [3], antiviral [4], anti-thrombus [5], anti-arrhythmia [6] and anti-allergy [7] properties. We have carried out a study of the chemical constituents and their activities on marine fouling resistance, and now report the isolation and structure elucidation of two new phenylpropanoids (**1-2**), together with five known coumarins (nodakenetin (**3**) [8], notopterol (**4**) [9], notoptol (**5**) [10], 5-[(2*E*,5*Z*)-7-hydroxy-3,7-dimethyl-2,5-octadienoxy]psoralen (**6**) [11] and 5-[(2,5)-epoxy-3-hydroxy-3,7-dimethyl-6-octadienoxy]psoralen (**7**) [11]. The structures were elucidated by NMR, IR and UV spectroscopy and MS.

The methanol extract of the roots of the title plant was subjected to column chromatography to give two new phenylpropanoids (**1-2**), and five known coumarins, nodakenetin (**3**) [8], notopterol (**4**) [9], notoptol (**5**) [10], 5-[(2*E*,5*Z*)-7-hydroxy-3,7-dimethyl-2,5-octadienoxy]psoralen (**6**) [11] and 5-[(2,5)-epoxy-3-hydroxy-3,7-dimethyl-6-octadienoxy]psoralen (**7**) [11]. The structures were elucidated by NMR, IR and UV spectroscopy and MS.

Compound **1** was isolated as a white amorphous powder with a molecular formula of C<sub>20</sub>H<sub>26</sub>O<sub>5</sub> (calcd. 346.1780) as determined by the [M]<sup>+</sup> ion at *m/z* 346.1773 in the HR-EI-MS, together with eight degrees of unsaturation [12]. The IR spectrum showed hydroxyl at 3432 cm<sup>-1</sup> and a double bond at 1596 cm<sup>-1</sup>, 1516 cm<sup>-1</sup>, while the UV spectrum showed absorption maxima at λ<sub>max</sub> 193, 217, 235 and 327 nm [13]. The <sup>1</sup>H NMR spectrum (Table 1) displayed three methyl singlet signals at δ<sub>H</sub> 1.15 (3H, s), 0.91 (3H, s) and 0.89 (3H, s), a tri-substituted benzene ring signals at δ<sub>H</sub> 7.35 (1H, d, *J* = 1.8), δ<sub>H</sub> 7.15 (1H, dd, *J* = 8.1, 1.8) and δ<sub>H</sub> 6.87 (1H, d, *J* = 8.1), a methoxyl group at δ<sub>H</sub> 3.91 (3H, s), and a double bond at δ<sub>H</sub> 7.59 (1H, d, *J* = 15.6), δ<sub>H</sub> 6.42 (1H, d, *J* = 15.6); these last two protons had a *trans* form from the coupling constant (*J* = 15.6). The <sup>13</sup>C NMR spectrum (Table 1) showed 20 carbon signals (four primary, two secondary, eight tertiary, and six quaternary), including one

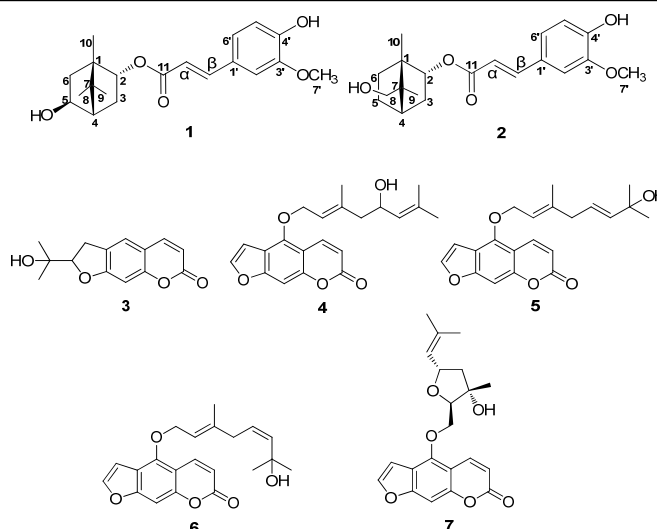


Figure 1: Isolated compounds from the roots of *Notopterygium franchetii*.

methoxyl carbon (δ<sub>C</sub> 56.2), one carboxylic carbon (δ<sub>C</sub> 167.6), eight aromatic carbons (δ<sub>C</sub> 116.1, 145.4, 127.4, 111.1, 148.7, 150.0, 116.0, and 124.0). The HMBC correlation of H-7' (δ<sub>H</sub> 3.91) with C-3' (δ<sub>C</sub> 148.7) indicated that this methoxy group was at C-3' [14]. The correlations of H-β (δ<sub>H</sub> 7.59) with C-2' (δ<sub>C</sub> 111.1), C-6' (δ<sub>C</sub> 124.0) and C-11 (δ<sub>C</sub> 167.6) indicated that C-β (δ<sub>C</sub> 145.4) was connected to C-1' (δ<sub>C</sub> 127.4), and C-α (δ<sub>C</sub> 116.1) with C-11 (δ<sub>C</sub> 167.6). Analysis of the NMR data (Tables 1 and 2) of compound **1** revealed nearly identical structural features to those of (-)-bornyl ferulate [15], except that a methylene at C-5 was replaced by an oxygen-substituted methine, indicating that compound **1** is an analogue of (-)-bornyl ferulate. This was further confirmed by relevant <sup>1</sup>H-<sup>1</sup>H COSY and HMBC data. The <sup>1</sup>H-<sup>1</sup>H COSY correlations of a proton signal at δ<sub>H</sub> 3.86 with H-4 (δ<sub>H</sub> 1.74) and H-6a (δ<sub>H</sub> 2.41) indicated this hydroxyl group at C-5 (δ<sub>C</sub> 75.0). The planar structure of compound **1** was, therefore, determined as shown in Figure 1.

**Table 1:** NMR spectral data of compound **1** in CD<sub>2</sub>COCD<sub>3</sub>.

Position	$\delta_H$ (mult, J Hz) (600MHz)	$\delta_C$ (150 MHz)	HMBC
1		48.1 (s)	
2	4.88 (m)	78.6 (d)	C-6, C-10, C-11
3a	2.35 (m)	35.0 (t)	C-5, C-7
3b	0.85 (dd, 14.0, 3.3)		C-1, C-5
4	1.74 (d, 5.1)	53.4 (d)	
5	3.86 (m)	75.0 (d)	
6a	2.41 (m)	40.3 (t)	
6b	1.45 (dt, 13.4, 2.2)		
7		50.2 (s)	
8	1.15 (s)	21.1 (q)	C-1, C-4, C-9
9	0.91 (s)	20.2 (q)	C-1, C-4, C-8
10	0.89 (s)	13.4 (q)	C-2, C-6, C-9
11		167.6 (s)	
$\alpha$	6.42 (d, 15.6)	116.1 (d)	
$\beta$	7.59 (d, 15.6)	145.4 (d)	C-2', C-6', C-11
1'		127.4 (s)	
2'	7.35 (d, 1.7)	111.1 (d)	
3'		148.7 (s)	
4'		150.0 (s)	
5'	6.87 (d, 8.1)	116.0 (d)	
6'	7.15 (dd, 1.8, 8.1)	124.0 (d)	C-2', C-4', C- $\beta$
7'	3.91 (s)	56.2 (q)	C-3'

**Table 2:** NMR spectral data of compound **2** and (-)-bornyl ferulate.

(2) (CD <sub>2</sub> COCD <sub>3</sub> )			(-)-bornyl ferulate (CDCl <sub>3</sub> )		
Position	$\delta_H$ (mult, J Hz) (600MHz)	$\delta_C$ (150 MHz)	Position	$\delta_H$ (mult, J Hz) (300 MHz)	$\delta_C$ (75 MHz)
1		50.3 (s)	1		48.9 (s)
2	4.00 (m)	76.3 (d)	2	5.02 (ddd, 10.0, 3.3, 1.8)	79.8 (d)
3a	2.23 (m)	39.3 (t)	3a	2.41 (dddd, 13.8, 10.0, 4.0, 3.5)	36.8 (t)
3b	0.97 (dd, 13.6, 3.6)		3b	1.05 (dd, 13.3, 3.3)	
4	1.89 (t, 4.5)	43.0 (d)	4	1.71 (m)	44.9 (d)
5a	1.77 (m)	28.4 (t)	5a	1.78 (m)	28.0 (d)
5b	1.30 (m)		5b	1.29 (m)	
6a	2.18 (dd, 9.1, 3.2)	26.6 (t)	6a	2.06 (m)	27.2 (t)
6b	1.27 (m)		6b	1.31 (m)	
7		51.8 (s)	7		47.8 (s)
8a	4.23 (d, 11.2)	68.0 (t)	8	0.90 (s)	19.7 (q)
8b	4.03 (d, 11.2)				
9	1.01 (s)	14.2 (q)	9	0.94 (s)	18.8 (q)
10	0.93 (s)	14.3 (q)	10	0.88 (s)	13.5 (q)
11		167.7 (s)	11		167.0 (s)
$\alpha$	6.43 (d, 15.9)	115.9 (d)	$\alpha$	6.31 (d, 15.9)	116.1 (d)
$\beta$	7.61 (d, 15.9)	145.5 (d)	$\beta$	7.59 (d, 15.9)	144.3 (d)
1'		127.4 (s)	1'		127.0 (s)
2'	7.35 (d, 1.7)	111.1 (d)	2'	7.05 (d, 1.5)	109.3 (d)
3'		148.7 (s)	3'		146.8 (s)
4'		150.0 (s)	4'		147.8 (s)
5'	6.86 (d, 8.2)	116.0 (d)	5'	6.91 (d, 8.2)	114.7 (d)
6'	7.15 (dd, 1.8, 8.2)	123.9 (d)	6'	7.08 (dd, 2.2, 8.2)	123.0 (d)
7'	3.91 (s)	56.2 (q)	7'	3.93 (s)	55.9 (q)

The ROESY correlations of H-2 ( $\delta_H$  4.88) with H-3a ( $\delta_H$  2.35) and H-9 ( $\delta_H$  0.91) indicated that H-2, H-3a and H-9 were  $\beta$ -oriented. The correlation of H-5 ( $\delta_H$  3.86) with H-3b ( $\delta_H$  0.85) indicated that H-5 and H-3b were  $\alpha$ -oriented. Herein, the OH at C-5 was  $\beta$ -oriented. Thus, the relative configuration of compound **1** was determined as shown in Figure 1.

Compound **2** was isolated as a white amorphous powder with a molecular formula of C<sub>20</sub>H<sub>26</sub>O<sub>5</sub> (calcd. 346.1780), as determined by the [M]<sup>+</sup> peak at *m/z* 346.1779 in the HR-EI-MS, together with eight degrees of unsaturation. The IR spectrum showed hydroxyl at 3442 cm<sup>-1</sup> and double bond at 1631 cm<sup>-1</sup>, 1516 cm<sup>-1</sup>, while the UV spectrum showed absorption maxima at  $\lambda_{max}$  202, 234 and 326 nm. Analysis of the NMR data (Table 2) of compound **2** revealed nearly identical structural features to those of (-)-bornyl ferulate [15], except that a methyl at C-8 was replaced by a hydroxymethyl,

indicating that compound **2** is an analogue of (-)-bornyl ferulate. This was further confirmed by relevant <sup>1</sup>H-<sup>1</sup>H COSY and HMBC data. The HMBC correlations of H-8a ( $\delta_H$  4.23) and H-8b ( $\delta_H$  4.03) with C-9 ( $\delta_C$  14.2) indicated that the hydroxymethyl was at C-8 ( $\delta_C$  68.0). The planar structure of compound **2** was, therefore, determined as shown (Figure 1).

The ROESY correlation of H-2 ( $\delta_H$  4.00) with H-9 ( $\delta_H$  1.01) indicated H-2 and H-9 to be  $\beta$ -oriented, while H-8 should be  $\alpha$ -oriented. Thus, the relative configuration of compound **2** was determined as shown in Figure 1.

Compounds **1**, **2** and **3-7** were evaluated for their antifouling activity against larval settlement of the barnacles *Balanus amphitrite* and *Bugula neritina*. Testing was performed in accordance with the method described by Kawahara [16]. Compounds **1** and **2** had activity against larval settlement of *Bugula neritina* with an EC<sub>50</sub> value of 25  $\mu$ g/mL, which indicated that the -OH group of the bornyl moiety had no effect on the larval settlement. The attachment rate of the negative control group was 100%. The test results were read 12 hours after dosing. Specific results are shown in Table 3.

## Experimental

**General:** The optical rotation data were measured on a JASCO P-1020 digital polarimeter (Jasco, Tokyo, Japan), UV-Vis spectra on a Shimadzu UV-2401 PC spectrophotometer, IR spectra on a Bruker Tensor-27 infrared spectrophotometer with a KBr disk, and ESI-MS and HR-EI-MS on Bruker HCT/E squire 3000 and Waters Autospec Premier P776 spectrometers, respectively. The NMR spectra were recorded on a Bruker Avance III-600 spectrometer with trimethylsilyl as internal standard. Column chromatography was performed on silica gel (200–300 and 300–400 mesh; Qingdao Marine Chemical Inc.), MCI gel CHP 20P (75–150  $\mu$ m; Mitsubishi Chemical Corporation), Sephadex LH-20 (40–70  $\mu$ m; Amersham Pharmacia Biotech AB), and Chromatorex Rp-C<sub>18</sub> gel (20–45  $\mu$ m; Merck). Compounds on thin layer chromatograms (TLC) plates; Qingdao Marine Chemical Inc.) were visualized under UV light and by dipping into 5% H<sub>2</sub>SO<sub>4</sub> in EtOH followed by heating.

**Plant material:** The dried roots of *Notopterygium franchetii* de Boiss were bought in Kunming Juhua market, Yunnan Province of China, in September 2012, and were identified by Mr Yu Chen (Kunming Institute of Botany, Chinese Academy of Sciences). A voucher specimen (H20120902) was deposited at the Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

**Extraction and isolation:** The air-dried roots (15.0 kg) of *N. franchetii* were powdered and extracted 3 times with MeOH under reflux. The combined MeOH extracts were concentrated using a rotary evaporator to give the crude extract, which was suspended in water and then partitioned successively with light petroleum, ethyl acetate and *n*-butyl alcohol, sequentially.

The ethyl acetate portion (1.08 kg) was subjected to silica gel CC and eluted with light petroleum–acetone mixtures of increasing polarity (from 10:0 to 4:6) to yield 5 fractions (Fr.1-5) on the basis of TLC. Fr. 3 (22.5 g) was then separated on a silica gel column (light petroleum/ethyl acetate from 7.5:2.5 to 7:3) to obtain 13 fractions (Fr.3A–3M). Fr.3H (4.0 g) was separated over a MCI-gel column (MeOH/H<sub>2</sub>O from 4:6 to 10:0) to obtain 11 fractions (Fr.3H1–3H11). Fr.3H4 (95.8 mg) was then separated on a silica gel column (light petroleum/ethyl acetate from 9.5:0.5) to obtain 7

(15.7 mg) [11]. Fr.3H6 (1.81 g) was then separated on a silica gel column (light petroleum/ethyl acetate, 9.5:0.5) to obtain 8 fractions (Fr.3H6A–3H6H). Fr.3H6B (200 mg) was purified by HPLC (MeOH/H<sub>2</sub>O, 55:45) to obtain **1** (7.3 mg) and **2** (5.2 mg). Fr.3H7 (491.2 mg) was then separated on a silica gel column (light petroleum/ethyl acetate, 9.5:0.5) to obtain 9 fractions (Fr.3H7A–3H7I). Fr.3H7E (70 mg) was purified by HPLC to obtain **5** (15.7 mg) [10] and **6** (4.8 mg) [11]. Fr.3H7G (120 mg) was purified by HPLC to yield **3** (65.9 mg) [8] and **4** (14.0 mg) [9].

#### Compound (1)

White amorphous powder.

$[\alpha]_D^{16}$ : -20.63 (*c* 0.34, MeOH).

UV (CH<sub>3</sub>OH)  $\lambda_{max}$  (log  $\epsilon$ ): 327 (0.39).

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

ESI-MS *m/z*: 369 [M+Na]<sup>+</sup>

HR-EI-MS *m/z*: 346.1773, C<sub>20</sub>H<sub>26</sub>O<sub>5</sub> (calcd. 346.1780).

#### Compound (2)

White amorphous powder.

$[\alpha]_D^{17}$ : -19.05 (*c* 0.175, MeOH).

UV (CH<sub>3</sub>OH)  $\lambda_{max}$  (log  $\epsilon$ ): 203 (0.43), 326 (0.40).

<sup>1</sup>H and <sup>13</sup>C NMR: Table 2.

ESI-MS *m/z*: 369 [M+Na]<sup>+</sup>

HR-EI-MS *m/z*: 346.1779, C<sub>20</sub>H<sub>26</sub>O<sub>5</sub> (calcd. 346.1780).

**Marine fouling resistance assay:** Activity testing was performed in accordance with the method described by Kawahara [16,17]. We used the larvae of the barnacles *Balanus amphitrite* and *Bugula neritina* as test larva. First, the larvae were cultured to the state of pre-attachment, and then the sample and a certain number of larvae were cultured together. Twelve h later, the number of non-attached and attached larvae was observed and counted under a microscope. Finally, the attachment rate of larvae was calculated. The experiment was conducted by Peiyuan Qian's research group at Hong Kong University of Science and Technology.

**Table 3:** Activities of resistance to marine fouling of compounds **1** and **2**.

Settlement bioassay of <i>Bugula neritina</i> larvae (EC <sub>50</sub> )	
Compounds	EC <sub>50</sub> (μg/mL)
<b>1</b>	<b>25</b>
<b>2</b>	<b>25</b>

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New Isochromane Derivatives from the Mangrove Fungus  
*Aspergillus ustus* 094102Peipei Liu<sup>a,1</sup>, Cong Wang<sup>a,1</sup>, Zhenyu Lu<sup>a,1</sup>, Tonghan Zhu<sup>a</sup>, Kui Hong<sup>b,\*</sup> and Weiming Zhu<sup>a,\*</sup><sup>a</sup>Key Laboratory of Marine Drugs, Ministry of Education of China, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, China<sup>b</sup>Key Laboratory of Combinatorial Biosynthesis and Drug Discovery, Ministry of Education of China, School of Pharmaceutical Sciences, Wuhan University, Wuhan 430071, China<sup>1</sup>The authors contributed equally to this paper

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Four new isochromane derivatives (**1–4**) along with the known peniciphenol (**5**) and (*R*)-2-(hydroxymethyl)-3-(2-hydroxypropyl)phenol (**6**) were isolated from the EtOAc extract of the fermentation broth of the mangrove fungus, *Aspergillus ustus* 094102. The structures of the new compounds including the absolute configuration were elucidated on the basis of spectroscopic analysis, CD and ECD calculation. Compounds **1** and **2** exhibited  $\alpha$ -glucosidase inhibition and anti-oxidation against DPPH radical with IC<sub>50</sub> values of 1.4 mM and 25.7  $\mu$ M, respectively.

**Keywords:** *Aspergillus ustus*, Isochromane derivatives, Anti-oxidation,  $\alpha$ -Glucosidase inhibition.

Microorganisms in special niches have proved to be an important source of bioactive compounds. The mangrove is a typically special ecosystem at the junction of land and sea, in which there are abundant microorganisms that may produce structurally new and bioactive natural products [1-6]. Previously we identified cytotoxic drimane sesquiterpenes and isochromane derivatives from *Aspergillus ustus* 094102 isolated from the mud around the roots of the mangrove plant *Bruguiera gymnorrhiza* [1]. Continuous study resulted in the isolation and identification of four new isocoumarin derivatives (**1–4**), as well as two known phenolic compounds, peniciphenol (**5**) [7] and (*R*)-2-(hydroxymethyl)-3-(2-hydroxypropyl) phenol (**6**) [8]. The new compound **1** exhibited  $\alpha$ -glucosidase inhibition while new compound **2** showed anti-oxidative activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical with IC<sub>50</sub> values of 1.41 mM and 25.7  $\mu$ M, respectively.

Compound **1** was obtained as a colorless solid with a molecular formula of C<sub>11</sub>H<sub>12</sub>O<sub>6</sub> from the HRESIMS peak at *m/z* 239.0558 [M-H]<sup>-</sup> (calcd 239.0556 for C<sub>11</sub>H<sub>11</sub>O<sub>6</sub>). The IR spectrum indicated the presence of hydroxyl (3226 cm<sup>-1</sup>), ester carbonyl groups (1751 cm<sup>-1</sup>) and an aromatic ring (1595, 1482 cm<sup>-1</sup>). The <sup>1</sup>H and <sup>13</sup>C NMR along with DEPT spectra (Table 1, Figures S1-S3) exhibited signals for a methoxyl ( $\delta_{\text{H/C}}$  3.89/56.1), two oxygenated methylenes ( $\delta_{\text{H/C}}$  4.36 & 4.52/72.2,  $\delta_{\text{H}}$  4.46/50.9), one oxygenated methine ( $\delta_{\text{H/C}}$  4.75/62.6), one aromatic methine ( $\delta_{\text{H/C}}$  6.72/101.0), six sp<sup>2</sup> quaternary carbons ( $\delta_{\text{C}}$  100.5, 144.0, 163.8, 115.7, 160.4, 169.0), and three exchangeable protons ( $\delta_{\text{H}}$  4.58, 5.95 & 11.36), indicating a penta-substituted benzopyrone skeleton in **1**. HMBC correlations (Figures 2 and S4) of H-3 ( $\delta_{\text{H}}$  4.36 & 4.52) to C-1 ( $\delta_{\text{C}}$  169.0), C-4 ( $\delta_{\text{C}}$  62.6) and C-4a ( $\delta_{\text{C}}$  144.0), along with HO-4 ( $\delta_{\text{H}}$  5.95) to C-3 ( $\delta_{\text{C}}$  72.2), C-4 and C-4a confirmed the skeleton as isochromane. In addition, the HMBC spectrum showed long distance <sup>1</sup>H-<sup>13</sup>C correlations from the methoxy proton ( $\delta_{\text{H}}$  3.89) to C-6 ( $\delta_{\text{C}}$  163.8), from H-5 ( $\delta_{\text{H}}$  6.72) to C-4, C-4a, C-1a ( $\delta_{\text{C}}$  100.5), C-6 ( $\delta_{\text{C}}$  163.8) and C-7 ( $\delta_{\text{C}}$  115.7), and from HO-9 ( $\delta_{\text{H}}$  4.58) to C-6, C-7 and C-8 ( $\delta_{\text{C}}$  160.4), locating the methoxy, hydroxymethyl and hydroxy groups at C-6,

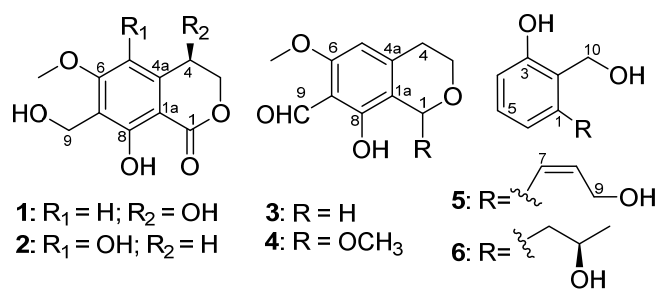
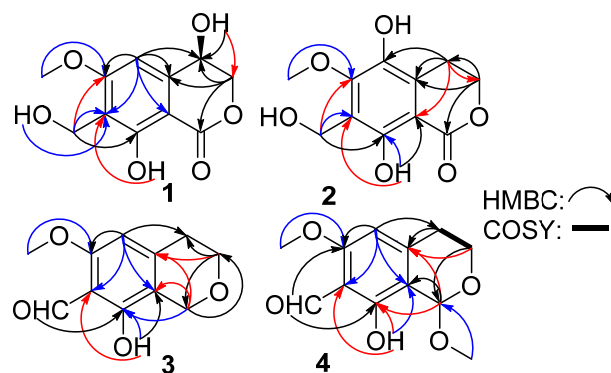


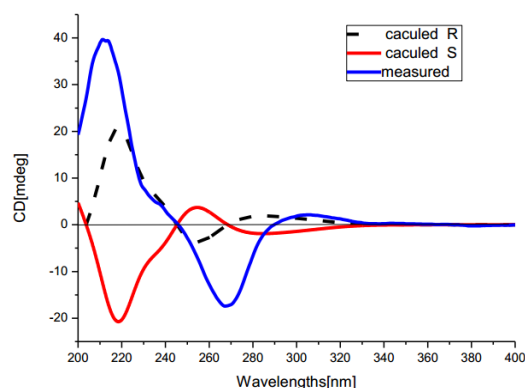
Figure 1: Chemical structures of compounds 1-6.

C-7 and C-8, respectively. The absolute configuration was determined as 4*R*- by ECD calculations using the time-dependent density functional theory (TD-DFT) method at the B3LYP/6-31G(d) level [9]. The results showed that the CD curve of **1** is matched well with the calculated ECD for *R*-**1** and opposite to that of *S*-**1** (Figure 3), indicating *R*-configuration. Thus, compound **1** was identified as (*R*)-4,8-dihydroxy-7-(hydroxymethyl)-6-methoxyisochroman-1-one.

Figure 2: HMBC and <sup>1</sup>H-<sup>1</sup>H COSY correlations of compounds 1-4.

**Table 1:**  $^1\text{H}$  (600 MHz) and  $^{13}\text{C}$  (150 MHz) NMR data for compounds **1–3** in  $\text{DMSO}-d_6$  and compound **4** in  $\text{CDCl}_3$  ( $\delta$ , ppm).

position	<b>1</b>		<b>2</b>		<b>3</b>		<b>4</b>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)
1	169.0, qC		169.6, qC		62.4, CH <sub>2</sub>	4.54, s	93.8, CH	5.55, s
1a	100.5, qC		103.6, qC		114.8, qC		115.6, qC	
3	72.2, CH <sub>2</sub>	4.52, dd (11.5, 3.8)	67.7, CH <sub>2</sub>	4.52, t (6.0)	63.5, CH <sub>2</sub>	3.83, t (5.5)	56.3, CH <sub>2</sub>	3.86, dd (15.4, 6.1)
4	62.6, CH	4.36, dd (11.5, 5.5)	21.5, CH <sub>2</sub>	2.95, t (6.0)	29.0, CH <sub>2</sub>	2.78, t (5.5)	29.1, CH <sub>2</sub>	4.18, ddd (15.4, 12.1, 3.3)
4a	144.0, qC		126.5, qC		146.0, qC		146.1, qC	2.58, dd (17.6, 3.3 Hz);
5	101.0, CH	6.72, s	138.3, qC		102.2, CH	6.45, s	100.7, CH	3.00, ddd (17.6, 12.2, 6.1)
6	163.8, qC		154.4, qC		160.0, qC		161.7, qC	
7	115.7, qC		120.8, qC		108.2, qC		109.2, qC	
8	160.4, qC		154.4, qC		158.3, qC		161.4, qC	
9	50.9, CH <sub>2</sub>	4.46, br d (3.5)	51.7, CH <sub>2</sub>	4.46, s	193.9, CH	10.18, s	193.7, CH	10.24, s
6-OMe	56.1, CH <sub>3</sub>	3.89, s	61.5, CH <sub>3</sub>	3.84, s	56.1, CH <sub>3</sub>	3.86, s	55.8, CH <sub>3</sub>	3.86, s
1-OMe							55.4, CH <sub>3</sub>	3.56, s
4-OH		5.95, d (5.3)						
5-OH			8.77, s					
8-OH		11.36, s	10.99, s			12.16, s		12.45, s
9-OH		4.58, t (3.5)	4.75, br s					

**Figure 3:** CD curve of **1** and the calculated ECD curves of *R*-**1** and *S*-**1**.

Compound **2** was obtained as a white solid with the same molecular formula as **1** from the HRESIMS peak at  $m/z$  239.0549 $[\text{M}-\text{H}]^-$ . The presence of hydroxyl, ester carbonyl groups and a benzene ring could be deduced from the IR absorptions at 3257, 1755, 1609, and 1465  $\text{cm}^{-1}$ , respectively. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data classified by DEPT experiments (Table 1, Figures S5–S7) were closely related to compound **1**, except that the oxygenated  $\text{sp}^3$ -methine and aromatic methine were replaced by a methylene ( $\delta_{\text{H/C}}$  2.95/21.5) and an aromatic quaternary carbon ( $\delta_{\text{C}}$  138.3), respectively. In addition, obvious up-field shifts for C-3, C-4a, C-6 and C-8 were observed while C-1a, C-7 and C-6-OMe shifted down-field. These data indicated that the HO- group at C-4 in **1** was moved to C-5 in compound **2**. This deduction was further supported by HMBC correlations of H-3 ( $\delta_{\text{H}}$  4.52) to C-1 ( $\delta_{\text{C}}$  169.6), C-4 ( $\delta_{\text{C}}$  21.5) and C-4a ( $\delta_{\text{C}}$  126.5), H-4 ( $\delta_{\text{H}}$  2.95) to C-1a ( $\delta_{\text{C}}$  103.6), C-3 ( $\delta_{\text{C}}$  67.7), C-4a and C-5 ( $\delta_{\text{C}}$  138.3), along with HO-9 ( $\delta_{\text{H}}$  4.75) to C-7 ( $\delta_{\text{C}}$  120.8), C-6 and C-8 ( $\delta_{\text{C}}$  154.4) (Figures 2 and S9). Thus, compound **2** was elucidated as 5,8-dihydroxy-7-(hydroxymethyl)-6-methoxyisochroman-1-one.

Compound **3** was obtained as a white solid with a molecular formula of  $\text{C}_{11}\text{H}_{12}\text{O}_4$  from the HRESIMS peak at  $m/z$  209.0839 $[\text{M}+\text{H}]^+$  (calcd 209.0830 for  $\text{C}_{11}\text{H}_{13}\text{O}_4$ ). The IR spectrum showed characteristic absorptions of  $-\text{CHO}$  at 2848, 2736 and 1695  $\text{cm}^{-1}$ . The  $^1\text{H}$  and  $^{13}\text{C}$  NMR along with the DEPT spectra (Table 1, Figures S10–S12) exhibited signals for one methoxyl ( $\delta_{\text{H/C}}$  3.86/56.1), one aldehyde group ( $\delta_{\text{H/C}}$  10.18/193.9), one penta-substituted benzene nucleus ( $\delta_{\text{H/C}}$  6.45/102.2, 114.8, 146.0, 160.0, 108.2, and 158.3), two oxygenated methylenes ( $\delta_{\text{H/C}}$  4.54/62.4, 3.83/63.5), one methylene ( $\delta_{\text{H/C}}$  2.78/29.0), and a phenolic hydroxyl ( $\delta_{\text{H}}$  12.16), probably suggesting an isochroman skeleton. This deduction was confirmed by the HMBC correlations (Figures 2 and

S14) from H-1 ( $\delta_{\text{H}}$  4.54) to C-3 ( $\delta_{\text{C}}$  63.5), C-1a ( $\delta_{\text{C}}$  114.8) and C-4a ( $\delta_{\text{C}}$  146.0), from H-3 ( $\delta_{\text{H}}$  3.83) to C-1 ( $\delta_{\text{C}}$  62.4), C-4 ( $\delta_{\text{C}}$  29.0) and C-4a, and from H-4 ( $\delta_{\text{H}}$  2.78) to C-1a, C-4a, C-3 and C-5 ( $\delta_{\text{C}}$  102.2). In addition, the HMBC spectrum also showed long-distance  $^1\text{H}$ - $^{13}\text{C}$  correlations from the aldehyde proton ( $\delta_{\text{H}}$  10.18) to C-7 ( $\delta_{\text{C}}$  108.2) and C-8 ( $\delta_{\text{C}}$  158.3), from the hydroxy proton ( $\delta_{\text{H}}$  12.16) to C-1a, C-7 and C-8, from the methoxy proton ( $\delta_{\text{H}}$  3.86) and C-6 ( $\delta_{\text{C}}$  160.0), and from H-5 ( $\delta_{\text{H}}$  6.45) to C-1a, C-4, C-6 and C-7, locating the methoxy, aldehyde and hydroxy protons at C-6, C-7 and C-8, respectively. Thus, compound **3** was determined as 8-hydroxy-6-methoxyisochroman-7-carbaldehyde.

Compound **4** was obtained as a white solid with a molecular formula of  $\text{C}_{12}\text{H}_{14}\text{O}_5$  according to a HRESIMS peak at  $m/z$  239.2445 $[\text{M}+\text{H}]^+$  (calcd 239.2437 for  $\text{C}_{12}\text{H}_{15}\text{O}_5$ ). The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data (Table 1 and Figures S16–S18) were closely related to compound **3**, except for an additional methoxy ( $\delta_{\text{H/C}}$  3.56/55.4) and the replacement of the oxygenated methylene in **3** by a hemiacetal methine ( $\delta_{\text{H/C}}$  5.55/93.8). The key HMBC correlation of the methoxy proton to the hemiacetal methine carbon (C-1) located the methoxy group at C-1, indicating that compound **4** was a C-1 methoxylated derivative of **3** that was further confirmed by  $^1\text{H}$ - $^1\text{H}$  COSY between H<sub>2</sub>-3 and H<sub>2</sub>-4 (Figures 2 and S20), along with almost the same HMBC pattern between **4** and **3** (Figures 2 and S21). The small  $[\alpha]_D^{20}$  value  $-5.6$  suggested that compound **4** might be a racemic mixture, which led us to carry out a chiral analysis. Unfortunately, compound **4** was not resolved by chiral HPLC analysis over chiralpak IA and chiralpak IC chiral columns (Figure S23). Thus, compound **4** was elucidated as ( $\pm$ )-8-hydroxy-1,6-dimethoxyisochroman-7-carbaldehyde.

Compounds **1–6** were assayed for their anti-oxidative activity against DPPH radical [10] and  $\alpha$ -glucosidase inhibitory effects using *p*-nitrophenyl- $\alpha$ -glucopyranoside (pNPG) as substrate [11]. The results showed that compound **2** displayed anti-oxidation with an  $\text{IC}_{50}$  value of 25.7  $\mu\text{M}$ , while compounds **1** and **3–6** were inactive ( $\text{IC}_{50} > 400 \mu\text{M}$ ) compared with L-ascorbic acid (positive control,  $\text{IC}_{50}$  16.5  $\mu\text{M}$ ) (Table 2, left). Compound **1** exhibited moderate  $\alpha$ -glucosidase inhibition with an  $\text{IC}_{50}$  value of 1.41 mM compared with acarbose (positive control,  $\text{IC}_{50}$  0.95 mM) (Table 2, right).

## Experimental

**General:** Optical rotations were obtained on a JASCO P-1020 digital polarimeter. CD data were collected on a JASCO J-715 spectropolarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer, and IR spectra on a Nicolet NEXUS 470 spectrophotometer as KBr discs.  $^1\text{H}/^{13}\text{C}$  NMR, DEPT, and

**Table 2:** DPPH radical scavenging activity (left) and  $\alpha$ -glucosidase inhibition (right) of compounds 1–6.

Compound	IC <sub>50</sub> , $\mu$ M	Compound	IC <sub>50</sub> , mM <sup>a</sup>
1	>417	1	1.4
2	25.7	2	6.9
3	>481	3	-
4	>420	4	7.0
5	>556	5	-
6	>549	6	12.7
ascorbic acid	16.5	acarbose	0.9

<sup>a</sup> - untested.

2D-NMR spectra were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard; chemical shifts were recorded as  $\delta$  values. ESI-MS were measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. HPLC was performed on an ODS RP-18 column [YMC-pack ODS-A, 10 $\times$ 250 mm, 5  $\mu$ m, 4 mL/min].

**Fungal material:** The fungus *Aspergillus ustus* 094102 was isolated and identified by Prof. Dr Kui Hong [1]. A reference culture of *A. ustus* 094102 is maintained at -80°C. Working stocks were prepared on Potato Dextrose agar slants and stored at 4°C.

**Fermentation and extraction:** *A. ustus* 094102 was incubated under static conditions at 30°C for 28 d in 100  $\times$  1000 mL Erlenmeyer flasks containing liquid medium (300 mL/flask) composed of maltose (20 g/L), mannitol (20 g/L), glucose (10 g/L), monosodium glutamate (10 g/L), yeast extract (3 g/L), corn steep liquor (1 g/L), KH<sub>2</sub>PO<sub>4</sub> (0.5 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.3 g/L), and seawater, after adjusting its pH to 7.0. The fermentation broth (30 L) was filtered through cheesecloth to be separated into supernatant and mycelium. The supernatant was concentrated under reduced pressure to about 25% of the original volume and then extracted 3 times with EtOAc to give an EtOAc solution, while the mycelium was extracted 3 times with acetone. After removing the acetone by evaporation under vacuum, the obtained aqueous acetone solution was extracted 3 times with equal volumes of EtOAc. Both EtOAc solutions were combined and dried under vacuum to give 70.0 g of extract.

**Purification:** The EtOAc extract (70.0 g) was separated into 8 fractions on a silica gel column eluting with a stepwise gradient of light petroleum-CHCl<sub>3</sub>-MeOH. Fraction 7 (2.2 g) was subjected to CC over an ODS column eluting with 30–80% MeOH/H<sub>2</sub>O to provide 5 sub-fractions (Fr7.1-Fr7.5). Fr7.5 (0.2 g) was further purified on an ODS column with 60% MeOH/H<sub>2</sub>O followed by semi-preparative HPLC to give compound 6 (7.7 mg, *t<sub>R</sub>* 4.7 min, 55% MeOH/H<sub>2</sub>O). Fraction 8 (5.3 g) was separated into 9 sub-fractions (Fr8.1-Fr8.9) on a silica gel column eluting with a stepwise gradient of CHCl<sub>3</sub>-MeOH. Fr8.1 (0.1 g) was further purified by semipreparative HPLC with 55% MeOH/H<sub>2</sub>O to give compound 5 (4.0 mg, *t<sub>R</sub>* 7.5 min). Fr8.2 (1.1 g) was further separated on a Sephadex LH-20 column eluting with MeOH followed by semipreparative HPLC to give compound 2 (27.7 mg, *t<sub>R</sub>* 4.4 min, 55% MeOH/H<sub>2</sub>O). Fr8.3 (2.7 g) was further separated into 5 sub-fractions (Fr8.3.1-Fr8.3.5) on a RP-18 column eluting with 60% MeOH/H<sub>2</sub>O. Fr8.3.4 (0.8 g) was then subjected to

chromatography over a Sephadex LH-20 column eluting with MeOH to afford 3 fractions (Fr8.3.4.1-Fr8.3.4.3). Fr8.3.4.1 (0.1 g) was further purified by semi-preparative HPLC with 55% MeOH/H<sub>2</sub>O to give compound 3 (5.2 mg, *t<sub>R</sub>* 14.6 min), while Fr8.3.4.2 (0.2 g) gave compounds 1 (12.6 mg, *t<sub>R</sub>* 12.2 min) and 4 (9.3 mg, *t<sub>R</sub>* 6.8 min) purified by semi-preparative HPLC with 55% MeOH/H<sub>2</sub>O.

**(R)-4,8-Dihydroxy-7-(hydroxymethyl)-6-methoxyisochroman-1-one (1)**

$[\alpha]_D^{20}$ : +9.7 (*c* 0.1, MeOH).

IR (KBr): 3226, 2910, 1751, 1595, 1482, 1359, 1195, 1004 cm<sup>-1</sup>.

UV/Vis  $\lambda_{max}$  (MeOH) nm (log  $\epsilon$ ): 305 (1.10), 270 (1.47), 219 (3.55).

CD  $\lambda_{max}$  (*c* 0.4, MeOH) ( $\Delta\epsilon$ ) nm: 212 (+4.8), 268 (-2.1) and 306 (+0.26).

<sup>1</sup>H and <sup>13</sup>C NMR; Table 1.

HRESIMS: *m/z* [M - H]<sup>-</sup> calcd for C<sub>11</sub>H<sub>11</sub>O<sub>6</sub>: 239.0556; found: 239.0558.

**5,8-Dihydroxy-7-(hydroxymethyl)-6-methoxyisochroman-1-one (2)**

IR (KBr): 3257, 2926, 1755, 1609, 1465, 1361, 1206 cm<sup>-1</sup>.

UV/Vis  $\lambda_{max}$  (MeOH) nm (log  $\epsilon$ ): 305 (1.21), 270 (1.47), 219 (3.65).

<sup>1</sup>H and <sup>13</sup>C NMR; Table 1.

HRESIMS: *m/z* [M - H]<sup>-</sup> calcd for C<sub>11</sub>H<sub>11</sub>O<sub>6</sub>: 239.0556; found: 239.0549.

**8-Hydroxy-6-methoxyisochroman-7-carbaldehyde (3)**

IR (KBr): 3254, 2925, 2848, 2736, 1695, 1602, 1458, 1323, 1193, 1085 cm<sup>-1</sup>.

UV/Vis  $\lambda_{max}$  (MeOH) nm (log  $\epsilon$ ): 349 (1.10), 284 (2.89), 206 (3.38).

<sup>1</sup>H and <sup>13</sup>C NMR; Table 1.

HRESIMS: *m/z* [M + H]<sup>+</sup> calcd for C<sub>11</sub>H<sub>13</sub>O<sub>4</sub>: 209.0830; found: 209.0839.

**(±)-8-Hydroxy-1,6-dimethoxy isochroman-7-carbaldehyde (4)**

$[\alpha]_D^{20}$  -5.6 (*c* 0.1, CDCl<sub>3</sub>).

IR (KBr): 3228, 2947, 2851, 2746, 1705, 1599, 1448, 1356, 1202, 997 cm<sup>-1</sup>.

UV/Vis  $\lambda_{max}$  (MeOH) nm (log  $\epsilon$ ): 349 (1.10), 284 (2.89), 206 (3.38).

<sup>1</sup>H and <sup>13</sup>C NMR; Table 1.

HRESIMS: *m/z* [M + H]<sup>+</sup> calcd for C<sub>12</sub>H<sub>15</sub>O<sub>5</sub>: 239.2447; found: 239.2445.

**Supporting information:** Bioassay protocols used, the NMR spectra of compounds 1–4, the chiral separation picture of compound 4. These materials are available free of charge via the Internet at <http://www.naturalproduct.us/>.

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Pericocins A–D, New Bioactive Compounds from *Periconia* sp.Yue-Hua Wu<sup>a,1</sup>, Gao-Keng Xiao<sup>a,1</sup>, Guo-Dong Chen<sup>a</sup>, Chuan-Xi Wang<sup>a</sup>, Dan Hu<sup>a</sup>, Yun-Yang Lian<sup>b</sup>, Feng Lin<sup>b</sup>, Liang-Dong Guo<sup>c</sup>, Xin-Sheng Yao<sup>a,\*</sup> and Hao Gao<sup>a,\*</sup><sup>a</sup>Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou 510632, China<sup>b</sup>Fujian Key Laboratory of Screening for Novel Microbial Products, Fujian Institute of Microbiology, Fuzhou 350007, China<sup>c</sup>State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100190, China

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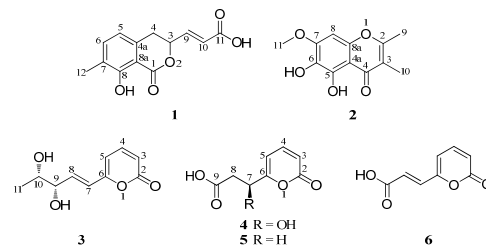
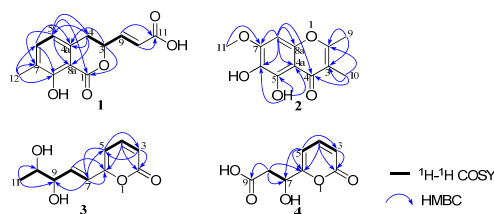
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One new dihydroisocoumarin, pericocin A (**1**), one new chromone, pericocin B (**2**), and two new  $\alpha$ -pyrone derivatives, pericocins C–D (**3**–**4**), together with two known compounds, 3-(2-oxo-2H-pyran-6-yl)propanoic acid (**5**) and (*E*)-3-(2-oxo-2H-pyran-6-yl)acrylic acid (**6**), were isolated from the culture of the endolichenic fungus *Periconia* sp.. Their structures were elucidated by spectroscopic methods. All these compounds are derived from the polyketone biosynthetic pathway. Compound **1** was obtained as a mixture of enantiomers. The antimicrobial activity of compounds **1**–**5** was tested against *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger*, and *Candida albicans*. Compounds **1**–**5** showed moderate antimicrobial activity against *A. niger* and weak activity against *C. albicans*.

**Keywords:** *Periconia* sp., Endolichenic fungus, Pericocin, Antimicrobial activity.

The *Periconia* genus is a rich source of bioactive compounds, such as cell-adhesion inhibitors (macrospheptides and peribysins) [1a–1d], antimicrobial agents (periconicins) [2a,2b], cytotoxic cytochalasans (periconiasins) [3a], and anti-inflammatory agents (periconianones) [3b]. In our early chemical investigation of a strain of this genus (19-4-2-1), some sesquiterpenes were isolated [3c,3d]. To search for more new bioactive secondary metabolites, a continuing chemical investigation of the strain (19-4-2-1) was carried out, which led to the isolation of four new compounds, pericocins A–D (**1**–**4**), along with two known compounds, 3-(2-oxo-2H-pyran-6-yl)propanoic acid (**5**) and (*E*)-3-(2-oxo-2H-pyran-6-yl)acrylic acid (**6**) (Figure 1). Herein, we report the isolation and structure elucidation, together with the antimicrobial activity of **1**–**5**.

Compound **1** was obtained as a white powder. The HR-ESI-MS showed a quasi-molecular ion peak at  $m/z$  271.0587 [ $M + Na$ ]<sup>+</sup>, which indicated that the molecular formula should be C<sub>13</sub>H<sub>12</sub>O<sub>5</sub> (eight degrees of unsaturation). The <sup>13</sup>C NMR spectrum showed 13 carbons signals, which was consistent with the deduction of the HRESIMS. Combined with the DEPT-135 experiment, the carbons can be classified into six sp<sup>2</sup> quaternary carbons [including two ester carbonyl/carboxyl carbons ( $\delta_C$  171.0, 169.6)], four sp<sup>2</sup> methine carbons, one oxygenated sp<sup>3</sup> methine carbon ( $\delta_C$  79.0), one sp<sup>3</sup> methylene carbon ( $\delta_C$  33.1), and one methyl carbon ( $\delta_C$  15.4). The <sup>1</sup>H NMR spectrum displayed four olefinic/aromatic proton signals [ $\delta_H$  7.35 (1H, d,  $J$  = 7.5 Hz), 6.95 (1H, dd,  $J$  = 15.7, 4.7 Hz), 6.72 (1H, d,  $J$  = 7.5 Hz), and 6.16 (1H, d,  $J$  = 15.6 Hz)], one oxymethine signal [ $\delta_H$  5.32 (1H, m)], one methylene signal [ $\delta_H$  3.17 (1H, dd,  $J$  = 16.3, 3.8 Hz), 3.02 (1H, dd,  $J$  = 16.3, 10.6 Hz)], and one methyl signal [ $\delta_H$  2.21 (3H, s)]. All the proton resonances were assigned to relevant carbon atoms through the HSQC experiment. An analysis of the <sup>1</sup>H–<sup>1</sup>H COSY spectrum revealed the presence of two isolated spin-systems corresponding to the C–4–C–3–C–9–C–10 and C–5–C–6 subunits. Combined with the <sup>1</sup>H–<sup>1</sup>H COSY analysis and

**Figure 1:** Chemical structures of **1**–**6**.**Figure 2:** <sup>1</sup>H–<sup>1</sup>H COSY and key HMBC correlations of **1**–**4**.

the molecular formula, the HMBC correlations from H-3 to C-1/C-4a, from Ha-4/Hb-4 to C-4a/C-5/C-8a, from H-5 to C-1/C-4/C-7/C-8a, from H-6 to C-8/C-4a, from H-9/H-10 to C-11, and from H<sub>3</sub>-12 to C-6/C-7/C-8 deduced the planar structure of **1**, as shown in Figure 2, and the assignments of all proton and carbon resonances are shown in Table 1. In an optical rotation experiment, compound **1** showed almost no optical activity. Compound **1** displayed a single peak in achiral analytical HPLC, but two peaks (20.8 min with relative peak area of 22.3% and 24.0 min with relative peak area of 77.7%) in chiral HPLC analysis, which indicated that **1** is a mixture of enantiomers. The geometry of the  $\Delta^9$  double bond was determined as 9*E* by the value of <sup>3</sup> $J_{H-9/H-10}$  (15.7 Hz) observed in the <sup>1</sup>H NMR spectrum. Based on the above analysis, the structure of **1** was established as (*E*)-3-(8-hydroxy-7-methyl-1-oxoisochroman-3-yl)acrylic acid, and named pericocin A.

Compound **2** was obtained as a white powder. The HR-ESI-MS showed a quasi-molecular ion peak at  $m/z$  237.0769  $[M + H]^+$ , which indicated that the molecular formula should be  $C_{12}H_{12}O_5$  (seven degrees of unsaturation). The  $^{13}C$  NMR spectrum showed 12 carbons signals, which was consistent with the deduction of the HRESIMS. Combined with the DEPT-135 experiment, the carbons can be classified into eight  $sp^2$  quaternary carbons [including one  $\alpha,\beta$ -conjugated keto carbonyl carbon ( $\delta_C$  181.4)], one  $sp^2$  methine carbon ( $\delta_C$  90.3), one methoxyl carbon ( $\delta_C$  56.2), and two methyl carbons ( $\delta_C$  18.3, 8.8). The  $^1H$  NMR spectrum displayed one olefinic/aromatic proton signal [ $\delta_H$  6.68 (1H, s)], one methoxyl signal [ $\delta_H$  3.87 (3H, s)], two methyl signals [ $\delta_H$  2.39 (3H, br s), 1.92 (3H, br s)], and two exchangeable hydrogen proton signals [ $\delta_H$  12.71 (1H, s), 8.61 (1H, s)]. All the non-exchangeable proton resonances were assigned to relevant carbon atoms through the HSQC experiment. Combined with the molecular formula, the HMBC correlations from H-8 to C-4/C-6/C-7/C-4a/C-8a, from H<sub>3</sub>-9 to C-2/C-3, from H<sub>3</sub>-10 to C-2/C-3/C-4, from H<sub>3</sub>-11 to C-7, and from 5-OH to C-5/C-6/C-4a deduced the structure of **2** as shown in Figure 2; the assignments of all proton and carbon resonances are shown in Table 1. Based on the above analyses, the structure of **2** was established as 5,6-dihydroxy-7-methoxy-2,3-dimethyl-4H-chromen-4-one, and named pericocin B.

Compound **3** was obtained as a brown oil. The HR-ESI-MS showed a quasi-molecular ion peak at  $m/z$  197.0809  $[M + H]^+$ , which indicated that the molecular formula should be  $C_{10}H_{12}O_4$  (five degrees of unsaturation). The  $^{13}C$  NMR spectrum showed 10 carbons signals, which was consistent with the deduction of the HRESIMS. Combined with the DEPT-135 experiment, the carbons can be classified into two  $sp^2$  quaternary carbons, five  $sp^2$  methine carbons, two  $sp^3$  oxygenated methine carbons ( $\delta_C$  76.6, 71.5), and one methyl carbon ( $\delta_C$  18.9). The  $^1H$  NMR spectrum displayed five olefinic/aromatic proton signals [ $\delta_H$  7.50 (1H, dd,  $J = 9.2, 6.7$  Hz), 6.74 (1H, dd,  $J = 15.7, 5.4$  Hz), 6.38 (1H, d,  $J = 15.7$  Hz), 6.30 (1H, d,  $J = 6.7$  Hz), and 6.22 (1H, d,  $J = 9.3$  Hz)], two oxymethine signals [ $\delta_H$  4.09 (1H, br t,  $J = 5.0$  Hz), 3.72 (1H, quint,  $J = 6.3$  Hz)], and one methyl signal [ $\delta_H$  1.18 (3H, d,  $J = 6.4$  Hz)]. All the proton resonances were assigned to relevant carbon atoms through the HSQC experiment. An analysis of the  $^1H$ - $^1H$  COSY spectrum revealed the presence of two isolated spin-systems corresponding to the C-3–C-4–C-5 and C-7–C-8–C-9–C-10–C-11 subunits. When **3** was tested in DMSO- $d_6$  (Table S4, Supporting Information), exchangeable protons were observed and two additional spin-systems (H-9 and 9-OH, H-10 and 10-OH) were revealed. Combined with the  $^1H$ - $^1H$  COSY analysis, the molecular formula, and the HMBC correlations from H-3 to C-2/C-5, from H-4 to C-2/C-6, from H-5 to C-3/C-7, from H-7 to C-5/C-6/C-9, from H-8 to C-6, and from H<sub>3</sub>-11 to C-9/C-10 deduced the planar structure of **3** as shown in Figure 2; the assignments of all proton and carbon resonances are shown in Table 1.

The geometry of the  $\Delta^7$  double bond was determined as 7*E* by the value of  $^3J_{H-7/H-8}$  (15.7 Hz) observed in the  $^1H$  NMR spectrum. In the ROESY experiment in DMSO- $d_6$  (Table S4, Supporting Information), the observed correlations between H-9 and 10-OH/H<sub>3</sub>-11, between H<sub>3</sub>-11 and H-8, and between H-10 and 9-OH/H-8 signified that the relative configuration of **3** was *threo* (Figure 3), combined with the value of  $^3J_{H-9/H-10}$  (6.1 Hz). The absolute configuration of the 9,10-diol moiety in **3** was determined by the *in situ* dimolybdenum CD method. On addition of  $Mo_2(OAc)_4$  to a solution of **3** in DMSO, a metal complex as an auxiliary chromophore was produced. The induced CD of the complex was obtained by subtracting the inherent CD. The observed sign of the

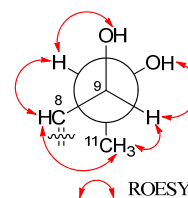


Figure 3: key ROESY correlations in DMSO- $d_6$  of **3**.

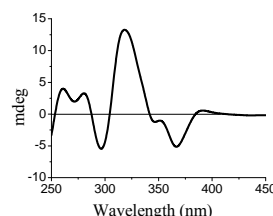


Figure 4: CD spectrum of **3** in DMSO containing  $Mo_2(OAc)_4$  with the inherent CD spectrum subtracted.

Cotton effect in the induced spectrum comes from the chirality of the vic-diol moiety expressed by the sign of the O-C-C-O torsion angle. In this study, positive Cotton effects were observed at 310 and 400 nm, respectively. The induced CD spectrum (Figure 4) permitted the assignment of 9*S* and 10*S* on the basis of the empirical rule proposed by Sneath [4a,b]. Thus, the structure of **3** was established as 6-((3*S*,4*S*,*E*)-3,4-dihydroxypent-1-en-1-yl)-2*H*-pyran-2-one, and named pericocin C.

Compound **4** was obtained as a brown oil. The HR-ESI-MS showed a quasi-molecular ion peak at  $m/z$  185.0447  $[M + H]^+$ , which indicated that the molecular formula should be  $C_8H_8O_5$  (five degrees of unsaturation). The  $^{13}C$  NMR spectrum showed 8 carbon signals, which was consistent with the deduction of the HRESIMS. Combined with the DEPT-135 experiment, the carbons can be classified into three  $sp^2$  quaternary carbons, three  $sp^2$  methine carbons, one oxygenated  $sp^2$  methine carbon ( $\delta_C$  68.3), and one  $sp^3$  methylene carbon ( $\delta_C$  40.9). The  $^1H$  NMR spectrum displayed three olefinic/aromatic proton signals [ $\delta_H$  7.52 (1H, dd,  $J = 9.3, 6.6$  Hz), 6.45 (1H, d,  $J = 6.6$  Hz), and 6.22 (1H, d,  $J = 9.3$  Hz)], one oxymethine signal [ $\delta_H$  4.80 (1H, dd,  $J = 8.6, 4.4$  Hz)], and one methylene signal [ $\delta_H$  2.82 (1H, dd,  $J = 15.7, 4.4$  Hz), 2.63 (1H, dd,  $J = 15.7, 8.6$  Hz)]. All the proton resonances were assigned to relevant carbon atoms through the HSQC experiment. An analysis of the  $^1H$ - $^1H$  COSY spectrum revealed the presence of two isolated spin-systems corresponding to the C-3–C-4–C-5 and C-7–C-8 subunits. Combined with the  $^1H$ - $^1H$  COSY analysis and the molecular formula, the HMBC correlations from H-3 to C-2, from H-4 to C-2/C-6, from H-5 to C-3/C-6/C-7, and from H<sub>3</sub>-8 to C-6/C-7/C-9 deduced the planar structure of **4** as shown in Figure 2; the assignments of all proton and carbon resonances are shown in Table 1.

The absolute configuration at C-7 of compound **4** was assigned by comparison of the Cotton effect and specific rotation with those of nodulisporipyrone A [5a], whose absolute configuration at C-7 was *R*. The CD spectrum of nodulisporipyrone A showed a positive Cotton effect at 280 nm, whereas a negative Cotton effect was seen in the CD spectrum of **4**. Likewise, nodulisporipyrone A showed a positive specific rotation ( $[\alpha]_D^{23} +67.6$ ), whereas **4** gave a negative value ( $[\alpha]_D^{27} -49.7$ ). Therefore, the absolute configuration of **4** was assigned as 7*S*. Based on the above analyses, the structure of **4** was established as (S)-3-hydroxy-3-(2-oxo-2*H*-pyran-6-yl)propanoic acid, and named pericocin D.

**Table 1:**  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR data of 1–4 ( $\delta$  in ppm,  $J$  in Hz).

No.	1 (CD <sub>3</sub> OD)		2 (DMSO- <i>d</i> <sub>6</sub> )		3 (CD <sub>3</sub> OD)		4 (CD <sub>3</sub> OD)	
	$\delta_{\text{C}}$ (150 MHz)	$\delta_{\text{H}}$ (600 MHz)	$\delta_{\text{C}}$ (100 MHz)	$\delta_{\text{H}}$ (400 MHz)	$\delta_{\text{C}}$ (100 MHz)	$\delta_{\text{H}}$ (400 MHz)	$\delta_{\text{C}}$ (100 MHz)	$\delta_{\text{H}}$ (400 MHz)
1	171.0							
2			163.3		164.1		164.1	
3	79.0	5.32, m	113.6		114.9	6.22, d (9.3)	114.9	6.22, d (9.3)
4	33.1	3.17, dd (16.3, 3.8), Ha 3.02, dd (16.3, 10.6), Hb	181.4		146.4	7.50, dd (9.2, 6.7)	146.1	7.52, dd (9.3, 6.6)
4a	137.7		104.0					
5	118.8	6.72, d (7.5)	146.0		106.1	6.30, d (6.7)	103.2	6.45, d (6.6)
6	138.4	7.35, d (7.5)	129.5		160.6		167.5	
7	126.3		154.2		123.1	6.38, d (15.7)	68.3	4.80, dd (8.6, 4.4)
8	161.5		90.3	6.68, s	138.7	6.74, dd (15.7, 5.4)	40.9	2.82, dd (15.7, 4.4), Ha 2.63, dd (15.7, 8.6), Hb
8a	108.6		149.6					
9	144.1	6.95, dd (15.7, 4.7)	18.3	2.39, br s	76.6	4.09, br t (5.0)	173.9	
10	124.9	6.16, d (15.6)	8.8	1.92, br s	71.5	3.72, quint (6.3)		
11	169.6		56.2	3.87, s	18.9	1.18, d (6.4)		
12	15.4	2.21, s						
5-OH				12.71, s				
6-OH				8.61, s				

The structures of 3-(2-oxo-2H-pyran-6-yl)propanoic acid (**5**), and (*E*)-3-(2-oxo-2H-pyran-6-yl)acrylic acid (**6**) were confirmed by comparison of their NMR data with literature values [5b,6a]. Compounds **1–5** were tested for antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger*, and *Candida albicans*, using tobramycin (bacteria) and cycloheximide (fungi) as the positive controls. Compounds **1–5** displayed moderate activities against *A. niger* and weak activities against *C. albicans* (Table 2).

**Table 2:** Antimicrobial activity of 1–5 (MIC,  $\mu\text{g/mL}$ ).

compounds	Bacteria		Fungi	
	<i>S. aureus</i>	<i>E. coli</i>	<i>A. niger</i>	<i>C. albicans</i>
<b>1</b>	>1000	>1000	31	500
<b>2</b>	>1000	>1000	31	500
<b>3</b>	>1000	>1000	31	500
<b>4</b>	>1000	>1000	31	500
<b>5</b>	>1000	>1000	31	500
Tobramycin <sup>a</sup>	<16	<16	nt	nt
Cycloheximide <sup>b</sup>	nt	nt	<16	<16

<sup>a</sup> Positive control (bacteria). <sup>b</sup> Positive control (fungi). nt: not tested

This work reports the isolation of six metabolites, including four new compounds {one dihydroisocoumarin (**1**), one chromone (**2**), and two  $\alpha$ -pyrone derivatives (**3–4**)}, and two known compounds ( $\alpha$ -pyrone derivatives (**5–6**)). To our best knowledge, compounds **3–6** are the first  $\alpha$ -pyrone derivatives from *Periconia* fungi.

## Experimental

**General:** UV, JASCO V-550 UV/Vis spectrophotometer; IR, JASCO FT/IR-480 plus Fourier transform infrared spectrometer; HR-ESI-MS, Waters Synapt G2 TOF mass spectrometer; NMR, Bruker AV-400 and Bruker AV-600 spectrometers; CC, Sephadex LH-20 (Pharmacia) and ODS (60–80 mm, YMC); TLC, precoated silica gel plates (SGF<sub>254</sub>, 0.2 mm, Yantai Chemical Industry Research Institute). The analytical HPLC was performed on a Shimadzu HPLC system equipped with a LC-20AB pump, and a SPD-20A diode array detector (Shimadzu) using a Phenomenex Gemini C18 column (5  $\mu\text{m}$ , 4.6 mm  $\times$  250 mm, Phenomenex Inc.). The chiral analytical HPLC was performed on a Shimadzu HPLC system equipped with a LC-20AB pump, and a SPD-20A diode array detector (Shimadzu) using a Phenomenex Lux Amylose-2 column (5  $\mu\text{m}$ , 4.6 mm  $\times$  250 mm, Phenomenex Inc.) at 1.0 mL/min under 60% MeOH–H<sub>2</sub>O (0.1% HCOOH). The semi-preparative HPLC was performed on a Shimadzu LC-6AD system equipped with an LC-6AD pump and a SPD-M20A detector (Shimadzu), using a YMC Park ODS-A column (5  $\mu\text{m}$ , 10 mm  $\times$  250 mm, YMC).

**Fungal material:** The strain of *Periconia* sp. (No.19-4-2-1) was isolated by one of the authors (L.D. Guo) from the lichen *Parmelia* sp. collected from Changbai Mountain, Jilin Province, China, in August 2006. The fungus strain was identified as *Periconia* sp. based on the morphological characteristics and sequence analysis of the internal transcribed spacer (ITS) regions ITS1-5.8S-ITS2 (GenBank accession No. KP873157). The strain was assigned the accession number 19-4-2-1 in the culture collection at the Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou. The fungus was cultured on slants of potato dextrose agar at 25°C for 5 days. Agar plugs were used to inoculate 4 Erlenmeyer flasks (250 mL), each containing 100 mL of potato dextrose broth. Four flasks of the inoculated media were incubated at 25°C on a rotary shaker at 200 rpm for 5 days to prepare the seed culture. Fermentation was carried out in 20 Erlenmeyer flasks (500 mL), each containing 70 g of rice. Distilled H<sub>2</sub>O (105 mL) was added to each flask, and the rice was soaked overnight before autoclaving at 120°C for 30 min. After cooling to room temperature, each flask was inoculated with 5.0 mL of the spore inoculum and incubated at room temperature for 45 days.

**Extraction and isolation:** The culture was extracted thrice with EtOAc, and the organic solvent was evaporated to dryness under vacuum to afford a crude extract (33.4 g). This was dissolved in 90%, v/v, aqueous MeOH (500 mL) and partitioned against the same volume of cyclohexane to afford a cyclohexane fraction (C, 24.5 g) and an aqueous MeOH fraction (W, 8.7 g). The aqueous MeOH fraction (W, 8.7 g) was separated by ODS CC eluting with MeOH–H<sub>2</sub>O (30:70, 50:50, 70:30, and 100:0, v/v) to afford 4 fractions (W1 to W4). Fraction W2 (2.3 g) was subjected to ODS CC with a gradient of MeOH–H<sub>2</sub>O (35:65, 40:60, 45:55, 50:50, 55:45, and 100:0 v/v) to give 8 sub-fractions (W2a–W2h). Sub-fraction W2e (533.6 mg) was separated by Sephadex LH-20 (MeOH) and semi-preparative HPLC (55% MeOH–H<sub>2</sub>O) to yield **1** (5.3 mg). Purification of sub-fraction W2d (199.9 mg) was carried out by Sephadex LH-20 (MeOH) and semi-preparative HPLC (45% MeOH–H<sub>2</sub>O) to yield **2** (6.9 mg). Fraction W1 (1.96 g) was subjected to ODS CC with a gradient of MeOH–H<sub>2</sub>O (10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60, 45:55, and 100:0 v/v) to give 6 sub-fractions (W1a–W1f). Sub-fraction W1a (392.2 mg) was purified by semi-preparative HPLC (15% MeOH–H<sub>2</sub>O) to yield **3** (29.9 mg), **4** (13.1 mg), **5** (35.5 mg), and **6** (3.5 mg).

## Pericocin A (1)

White powder.

IR (KBr): 1698, 1676, 1427, 1287, 1138, 974  $\text{cm}^{-1}$ .

UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 210 (4.20), 252 (3.58), 320 (3.36) nm.

$^1\text{H}$  and  $^{13}\text{C}$  NMR: Table 1.

HR-ESI-MS:  $m/z$  271.0587  $[\text{M} + \text{Na}]^+$  (calcd for  $\text{C}_{13}\text{H}_{12}\text{O}_5\text{Na}$ , 271.0582).

#### Pericocin B (2)

White powder.

IR (KBr): 3566, 3472, 1671, 1484, 1250, 1094, 803  $\text{cm}^{-1}$ .

UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 211 (4.27), 240 (4.05), 295 (3.75) nm.

$^1\text{H}$  and  $^{13}\text{C}$  NMR: Table 1.

HR-ESI-MS:  $m/z$  237.0769  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{12}\text{H}_{13}\text{O}_5$ , 237.0763).

#### Pericocin C (3)

Brown oil.

$[\alpha]_{\text{D}}^{27}$ : +9.9 ( $c$  1.0, MeOH).

IR (KBr): 3429, 2979, 1712, 1650, 1538, 1378, 1110, 804  $\text{cm}^{-1}$ .

UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 205 (3.98), 229 (3.96), 327 (3.90) nm.

$^1\text{H}$  and  $^{13}\text{C}$  NMR: Table 1.

HR-ESI-MS:  $m/z$  197.0809  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{10}\text{H}_{13}\text{O}_4$ , 197.0814).

#### Pericocin D (4)

Brown oil.

$[\alpha]_{\text{D}}^{27}$ : -49.7 ( $c$  1.0, MeOH).

IR (KBr): 3401, 1716, 1634, 1558, 1402, 813  $\text{cm}^{-1}$ .

UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 204 (3.22), 297 (3.41) nm.

CD ( $c$   $2.0 \times 10^{-4}$  M, MeOH)  $\lambda_{\text{max}}$  ( $\Delta\epsilon$ ): 231 (-0.85), 286 (-3.39).

$^1\text{H}$  and  $^{13}\text{C}$  NMR: Table 1.

HR-ESI-MS:  $m/z$  185.0447  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_8\text{H}_9\text{O}_5$ , 185.0450).

**Antimicrobial assay:** Compounds were tested for antimicrobial activity using a paper disk-diffusion assay [6b,c]. Seed cultures of two bacteria (*S. aureus* 209P, *E. coli* ATCC0111) and two fungi (*A. niger* R330, *C. albicans* FIM709) were prepared by incubating the organism for 12 h at either 37°C (bacteria) or 32°C (fungi). Aliquots of the overnight cultures (80  $\mu\text{L}$ ) were spread onto the surfaces of either nutrient agar (bacteria) or Sabouraud's dextrose agar (fungi). Sterile filter disks (6 mm diameter) infused with 6  $\mu\text{L}$  of test solution (in DMSO), positive control and vehicle only (DMSO) were added to the plates. The plates were left upright for 30 min at room temperature before being placed in an incubator at either 37°C (bacteria) or 32°C (fungi) for 12 h, and then the diameter of the zone of growth inhibition for each disk was recorded. The continuous twofold dilution methods were used to evaluate the minimal inhibitory concentrations (MICs). The MICs were defined as the lowest concentration at which no microbial growth could be observed.

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New Benzenoids from the Roots of *Lindera aggregata*Guo-Hao Ma<sup>a, #</sup>, Che-Wei Lin<sup>a, #</sup>, Hsin-Yi Hung<sup>b</sup>, Sheng-Yang Wang<sup>c</sup>, Po-Chuen Shieh<sup>d</sup> and Tian-Shung Wu<sup>b, d\*</sup><sup>a</sup>Department of Chemistry, National Cheng Kung University, Tainan, 701, Taiwan<sup>b</sup>School of Pharmacy, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, 701, Taiwan<sup>c</sup>Core Laboratory of Plant Metabolomics, Biotechnology Center and Department of Forestry, National Chung Hsing University, Kou Kung Road, Taichung 402, Taiwan<sup>d</sup>Department of Pharmacy, Tajen University, Pintung 907, Taiwan<sup>#</sup>The authors contributed equally to this work.

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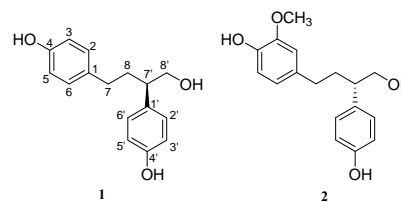
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Two new benzenoids, linderagatin A and B (**1-2**), were isolated from the roots of *Lindera aggregata*. Their structures were elucidated on the basis of 1D (<sup>1</sup>H, <sup>13</sup>C) and 2D NMR (COSY, NOESY, HSQC and HMBC) spectra. Moreover, their absolute configurations were established from ECD spectra compared with previous reports.

**Keywords:** *Lindera aggregata*, Lauraceae, Benzenoids, NMR spectroscopy, ECD.

*Lindera aggregata* (Sims) Kosterm (Lauraceae), also known as Wu Yao, a famous traditional Chinese medicine, has been used to treat abdominal distension and pain, acute asthma, and rheumatic diseases [1, 2]. In addition, recent studies showed the plant has other pharmacological effects, such as antioxidant [3], increased insulin sensitivity [3], anti-tumor [4] and anti-inflammatory [5]. Previous phytochemical studies on Wu Yao have led to the isolation of sesquiterpenes [6], alkaloids [7, 8], flavonoids [9] and lignans [10]. In this study, two new benzenoids, linderagatin-A (**1**) and linderagatin-B (**2**), were isolated from *L. aggregata*, as well as 27 known compounds, *N-trans*-feruloyltyramine (**3**) [11], *N-cis*-feruloyltyramine (**4**) [11], *N-trans*-feruloylmethoxytyramine (**5**) [12], thalifoline (**6**) [13], yuzirine (**7**) [14], linderaggrine-A (**8**) [8], northalifoline (**9**) [15], (+)-boldine (**10**) [16], (+)-*N*-methylaurotetanine (**11**) [17], (+)-isoboldine (**12**) [11], 3-hydroxy-1-(4-hydroxyphenyl)propan-1-one (**13**) [18], *p*-hydroxybenzoic acid (**14**) [12], 4-hydroxy-3-methoxy acetophenone (**15**) [19], methyl 3,5-dimethoxy-4-hydroxybenzoate (**16**) [20], vanillic acid (**17**) [12], tyrosol (**18**) [21], 2-(4-hydroxy-3-methoxyphenyl)ethanol (**19**) [22], 2-(4-hydroxy-3,5-dimethoxyphenyl)ethanol (**20**) [23], 3-hydroxy-1-(4-hydroxyphenyl)propan-1-one (**21**) [18], rel-(2 $\alpha$ , 3 $\beta$ )-7-*O*-methylcedrusin (**22**) [24], (-)-lyoniresinol (**23**) [25], hydroxylindrestrenolide (**24**) [6], 2,6-dimethoxy-*p*-quinone (**25**) [26], evofolin-B (**26**) [27], (-)-boscianin (**27**) [28], methyl dihydrophaseate (**28**) [29], and 6'-*O*-vanilloyltachioside (**29**) [30]. Here, we report the structure elucidation of the two new benzenoids.

Linderagatin-A (**1**) was obtained as a colorless powder. Its molecular formula was determined as C<sub>16</sub>H<sub>18</sub>O<sub>3</sub> on the basis of its positive HRESIMS (*m/z* 281.1153 [M + Na]<sup>+</sup>; calc. 281.1154). The complete <sup>1</sup>H and <sup>13</sup>C NMR data are shown in Table 1. The <sup>1</sup>H NMR spectrum showed two sets of AA'XX' systems at  $\delta_H$  7.07 (2H, d, *J* = 8.4 Hz, H-2', 6'), 6.79 (2H, d, *J* = 8.4 Hz, H-3', 5'), as well as signals at 6.94 (2H, d, *J* = 8.4 Hz, H-2, 6) and 6.72 (2H, d, *J* = 8.4 Hz, H-3, 5). Furthermore, two methylene groups at  $\delta_H$  3.60 (2H, m, H-8') and 2.37 (2H, m, H-7), and a methine group at  $\delta_H$  2.64 (1H, m,

Figure 1: Structure of compounds **1-2**.Table 1: <sup>1</sup>H and <sup>13</sup>C NMR spectroscopic data of compounds **1** (acetone-*d*<sub>6</sub>) and **2** (CDCl<sub>3</sub>).

position	<b>1</b> <sup>a</sup>		<b>2</b> <sup>b</sup>	
	$\delta_C$	$\delta_H$ (J in Hz)	$\delta_C$	$\delta_H$ (J in Hz)
1	134.1		133.9	
2	130.0	6.94 d (8.4)	111.0	6.59 br s
3	115.8	6.72 d (8.4)	146.3	
3-OCH <sub>3</sub>			55.9	3.85 s
4	156.2		143.6	
5	115.8	6.72 d (8.4)	114.2	6.80 d (8.0)
6	130.0	6.94 d (8.4)	120.9	6.60 dd (8.0, 1.6)
7	33.3	2.37 m	33.1	2.44 m
8a	35.2	2.11 m	33.9	1.97 m
8b		1.76 m		1.84 m
1'	134.7		134.0	
2'	129.8	7.07 d (8.4)	129.3	7.10 d (8.4)
3'	115.9	6.79 d (8.4)	115.6	6.83 d (8.4)
4'	156.6		154.4	
5'	115.9	6.79 d (8.4)	115.6	6.83 d (8.4)
6'	129.8	7.07 d (8.4)	129.3	7.10 d (8.4)
7'	48.3	2.64 m	47.1	2.74 m
8'	67.9	3.60 m	67.7	3.69 m

H-7') were found, while methylenes at  $\delta_H$  2.11 (1H, m, H-8a) and 1.76 (2H, m, H-8b) were observed in the <sup>1</sup>H NMR and HSQC spectra. Moreover, the COSY spectrum showed correlations that indicated the presence of the spin systems H-7, H-8, H-7' and H-8' with a partial -CH<sub>2</sub>CH<sub>2</sub>(CH)CH<sub>2</sub>OH structure. In the HMBC experiment, the signal at  $\delta_H$  6.94 (H-2, 6) correlated with the carbons at  $\delta_C$  33.3 (C-7), and the signal at  $\delta_H$  7.07 (H-2', 6') correlated with  $\delta_C$  48.3 (C-7'). The above results showed that C-7

and C-7' are substituted with a *p*-hydroxybenzyl group. Furthermore, the absolute configuration of **1** was determined from the ECD spectrum, in which positive Cotton effects at 206, 212 and 217 nm suggested that the absolute configuration at C-7' of **1** is opposite to that of *S*-(+)-2-phenyl-1-butanol. Therefore, the absolute configuration at C-7' were determined as 7'*R*. [31].

Linderagatin-B (**2**) was isolated as colorless syrup and its molecular formula was determined to be C<sub>17</sub>H<sub>20</sub>O<sub>4</sub> on the basis of its positive HRESIMS (*m/z* 311.1261 [M + Na]<sup>+</sup>; calc. 311.1259). The <sup>1</sup>H and <sup>13</sup>C NMR spectra of **2** (Table 1) were similar to those of **1**, except for the H-3 proton being replaced by a methoxyl group, the signal for which was observed at δ<sub>H</sub> 3.85 (s, 3-OCH<sub>3</sub>) in the <sup>1</sup>H NMR spectrum and at δ<sub>C</sub> 55.9 (3-OCH<sub>3</sub>) in the <sup>13</sup>C NMR spectrum. In the HMBC spectrum, the proton signal at δ<sub>H</sub> 6.59 (H-2) correlated with carbons at δ<sub>C</sub> 33.1 (C-7), and HMBC correlations of peaks at δ<sub>H</sub> 7.10 (H-2') with δ<sub>C</sub> 47.1 (C-7') were also found. The above results showed that C-7 and C-7' are substituted with a benzyl group. In addition, the proton signals at δ<sub>H</sub> 6.59 (H-2) and 6.60 (H-6) were correlated with carbon at δ<sub>C</sub> 143.6 (C-4) in the HMBC spectrum. And the proton signals at δ<sub>H</sub> 3.85 (3-OCH<sub>3</sub>) and 6.80 (H-5) were correlated with carbon at δ<sub>C</sub> 146.3 (C-3). The methoxy group was thus assigned at C-3 by the NOESY correlation between δ<sub>H</sub> 6.59 (H-2) and the methyl signal at δ<sub>H</sub> 3.85 (3H, s, 3-OCH<sub>3</sub>). The absolute configuration of **2** at C-7' was assigned from the ECD spectrum, which showed negative Cotton effects at 207, 212, and 219 nm similar to those of *S*-(+)-2-phenyl-1-butanol in ethanol, indicating a 7'*S* configuration. [31]

## Experimental

**General:** Optical rotations were measured with a JASCO P-2000 digital polarimeter in a 0.5 dm cell. UV spectra were obtained with a Hitachi UV-3210 spectrophotometer, and IR spectra with a Shimadzu FTIR Prestige-21 spectrometer. The ECD spectra were recorded on a JASCO J-720 spectrometer, and the <sup>1</sup>H and <sup>13</sup>C NMR spectra on a Bruker AVIII-400 with TMS as the internal reference, and chemical shifts expressed in δ (ppm). The ESIMS and HRESIMS were taken on a Bruker Daltonics APEX II 30e spectrometer. Silica gel (70–230 and 230–400 mesh; Merck) and Diaion HP-20 resin (Mitsubishi, Chemical, Tokyo, Japan) were used for column chromatography (CC), and silica gel 60 F<sub>254</sub> (Merck) for TLC.

**Plant material:** The roots of *Lindera aggregata* were collected from Huisun Forest Area, Ren Ai Township, Nantou County, Taiwan. The plant material was identified and authenticated by Assoc. Prof. Dr Chang-Sheng Kuoh, Department of Life Sciences, National Cheng Kung University. A voucher specimen (TSWu-20100601) was deposited in the Department of Chemistry, National Cheng Kung University, Tainan, Taiwan.

**Extraction and isolation:** The ground air-dried whole plants of *L. aggregata* (12.5 kg) were extracted with MeOH at 60°C by refluxing for 8 h. The MeOH extracts were combined and evaporated under reduced pressure to give ca. 1160 g residue.

The residue was suspended in water and then partitioned with EtOAc. After removing the solvent, the EtOAc-soluble portion (340 g) underwent partition extraction with 5% aqueous HCl solution. The HCl-aqueous solution was treated with 5% NH<sub>4</sub>OH<sub>(aq.)</sub> to obtain a phase with pH 9 and then extracted with EtOAc. The organic layer was evaporated under reduced pressure to yield an alkaloid fraction residue (40 g). This was subjected to CC over silica gel and eluted with *n*-hexane and acetone by step gradients to afford 16 fractions on the basis of TLC analysis. Fraction 11 was separated by CC with benzene–ethyl acetate (19:1) to afford **15** (1.4 mg), **16** (7.7 mg), **24** (4.9 mg), and **25** (4.9 mg). Separation of fraction 13 was performed by silica gel chromatography with CHCl<sub>3</sub>–MeOH (20:1) to give **1** (4.0 mg), **2** (2.3 mg), **3** (6.5 mg), **4** (2.2 mg), **5** (13.1 mg), **6** (1.4 mg), **7** (3.2 mg), **14** (0.8 mg), **17** (3.5 mg), **18** (5.3 mg), **19** (2.6 mg), **20** (2.7 mg), **21** (1.6 mg), **22** (8.6 mg), **26** (2.1 mg), **27** (14.9 mg), and **28** (3.0 mg). Fraction 14 was purified by CC with CHCl<sub>3</sub>–MeOH (15:1) to afford **9** (4.7 mg) and **23** (3.4 mg). Fraction 15 was purified by CC with CHCl<sub>3</sub>–MeOH (9:1) to give **10** (93.2 mg), **11** (5.4 mg), **12** (6.0 mg) and **29** (2.1 mg).

## Linderagatin-A (**1**)

Colorless powder;

[α]<sub>D</sub>: +99.0 (c 0.20, MeOH)

CD[θ]: [θ]<sub>196</sub>+405, [θ]<sub>199</sub>–61, [θ]<sub>203</sub>–91, [θ]<sub>206</sub> +71, [θ]<sub>210</sub>–413, [θ]<sub>212</sub> +329, [θ]<sub>215</sub> +423, [θ]<sub>217</sub>+337, [θ]<sub>224</sub>–133, [θ]<sub>231</sub> +175, [θ]<sub>236</sub>+163, [θ]<sub>240</sub>–113, [θ]<sub>251</sub>+123, [θ]<sub>267</sub>+153, [θ]<sub>280</sub>+1380, [θ]<sub>287</sub>+1602 (c 7.4 × 10<sup>–4</sup>, EtOH)

IR ν<sub>max</sub> (KBr) cm<sup>–1</sup>: 3356, 2924, 2854, 1652, 1458 cm<sup>–1</sup>.

UV (MeOH): λ<sub>max</sub> (log ε): 218 (3.6), 229 (3.5, sh), 273 (3.5), 282 (3.4, sh) nm.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

ESI-MS *m/z* (rel. int. %): 281 ([M+Na]<sup>+</sup>)

HR ESI-MS *m/z* (rel. int. %): 281.1153 ([M+Na]<sup>+</sup>) (calcd for C<sub>16</sub>H<sub>18</sub>O<sub>3</sub>Na, 281.1154).

## Linderagatin-B (**2**)

Colorless powder.

[α]<sub>D</sub>: –58.3 (c 0.11, MeOH).

CD[θ]: [θ]<sub>196</sub>+300, [θ]<sub>199</sub>–191, [θ]<sub>202</sub>+167, [θ]<sub>207</sub>–148, [θ]<sub>210</sub>–413, [θ]<sub>212</sub>–295, [θ]<sub>219</sub>–64, [θ]<sub>224</sub>+532, [θ]<sub>229</sub>+671, [θ]<sub>241</sub>–369, [θ]<sub>252</sub>+161, [θ]<sub>256</sub>+159, [θ]<sub>269</sub>+300, [θ]<sub>284</sub>+300, [θ]<sub>293</sub>+300 (c 4.9 × 10<sup>–4</sup>, EtOH)

IR ν<sub>max</sub> (KBr): 3333, 2928, 2855, 1608, 1454 cm<sup>–1</sup>

UV (MeOH): λ<sub>max</sub> (log ε): 217 (3.8), 232 (3.7, sh), 277 (3.6) nm.

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

ESI-MS *m/z* (rel. int. %): 331 ([M+Na]<sup>+</sup>)

HR ESI-MS *m/z* (rel. int. %): 311.1261 ([M+Na]<sup>+</sup>) (calcd for C<sub>17</sub>H<sub>20</sub>O<sub>4</sub>Na, 311.1259).

**Supplementary data:** NMR spectra (<sup>1</sup>H and <sup>13</sup>C NMR, COSY, NOESY, HSQC, and HMBC) for compounds **1** and **2**.

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## 12-Membered Resorcylic Acid Lactones Isolated from *Saccharicola bicolor*, an Endophytic Fungi from *Bergenia purpurascens*

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Two new resorcylic acid lactones, 13-hydroxyhidioresorcylicide (**1**) and 12-hydroxyhidioresorcylicide (**2**), along with four known congeners (**3-6**) were isolated from *Saccharicola bicolor*, an endophytic fungus from *Bergenia purpurascens*. Their structures were elucidated by interpretation of the spectroscopic evidence.

**Keywords:** *Saccharicola bicolor*, *Bergenia purpurascens*, Resorcylic acid lactone.

Resorcylic acid lactones (RALs) are a class of fungal polyketides that have a core structure of a  $\beta$ -resorcylic acid chromophore bearing a side-chain in the form of a lactone ring. The first isolated RAL is radicicol in 1953 [1]. Since then, more than 40 naturally occurring RALs have been reported. These have showed antifungal [2], antimalarial [3] and cytotoxic [4] activities. Due to their attractive biological activities, RALs had provoked interest in drug development. In the course of our ongoing research on the discovery of unique compounds from Tibetan plant endophytes, *Saccharicola bicolor*, an endophytic fungus, has been isolated from *Bergenia purpurascens*. The chemical investigation of its fermentation broth led to the isolation of two new resorcylic acid lactones namely 13-hydroxyhidioresorcylicide (**1**) and 12-hydroxyhidioresorcylicide (**2**), and four known congeners (**3-6**). Details of the isolation and structure elucidation of these compounds are discussed below.

Compound **1** was isolated as a yellow gum. Its molecular formula was assigned as  $C_{16}H_{20}O_6$  on the basis of HRESIMS by the pseudo-molecular ion peak at  $m/z$  309.1331  $[M+H]^+$  (calcd. 309.1333). The IR spectrum showed the presence of phenolic hydroxyl ( $3502\text{ cm}^{-1}$ ), methylene ( $2925.6, 2854.6\text{ cm}^{-1}$ ), and lactone ( $1640.6\text{ cm}^{-1}$ ) groups.  $^1\text{H}$ ,  $^{13}\text{C}$ , H-H COSY and HSQC NMR spectral data indicated the presence of a *m*-dihydroxybenzene unit [ $\delta_{\text{H}}$  6.25 (d,  $J = 2.5\text{ Hz}$ ), 6.11 (d,  $J = 2.5\text{ Hz}$ );  $\delta_{\text{C}}$  166.6, 163.9, 140.5, 114.0, 106.3, 102.9], a  $\text{CH}_3\text{CHCH}_2\text{CH}(\text{CH}_2)_3$  system [ $\delta_{\text{H}}$  5.08 (1H, m), 3.65 (1H, m), 2.85 (1H, m), 2.33 (1H, m), 2.08 (2H, m), 1.88 (1H, m), 1.62 (2H, m), 1.58 (1H, m), 1.34 (3H, d,  $J = 6.1\text{ Hz}$ );  $\delta_{\text{C}}$  73.3, 68.6, 44.1, 43.7, 38.2, 21.3, 20.7], a lactone carbonyl ( $\delta_{\text{C}}$  172.4), a methylene [ $\delta_{\text{H}}$  4.79 (1H, d,  $J = 18.6\text{ Hz}$ ), 3.60 (1H, d,  $J = 18.6\text{ Hz}$ );  $\delta_{\text{C}}$  50.7] and a ketone group ( $\delta_{\text{C}}$  211.6). HMBC correlations between H-10 and C-9 were observed and the aliphatic system was linked to the ketone. Both HMBC cross peaks between H-8 ( $\delta$  4.79, 3.60) and C-9 ( $\delta$  211.6), C-7 ( $\delta$  140.5), and between H-6 ( $\delta$  6.11) and C-8 ( $\delta$  50.7) placed the insular methylene between the aromatic ring and the ketone. HMBC correlations from H-6 ( $\delta$  6.11), H-4 ( $\delta$  6.25) and H-15 ( $\delta$  5.08) to the lactone carbonyl ( $\delta_{\text{C}}$  172.4) were observed and located the lactone between C-2 and C-15.

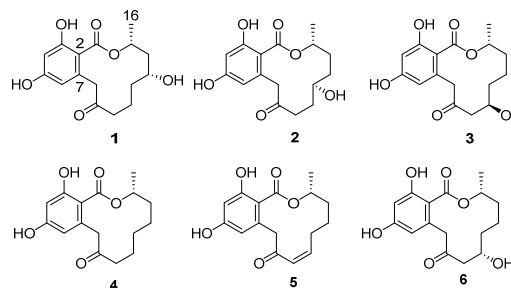


Figure 1: Compounds **1-6** isolated from *Saccharicola bicolor*.

The relative stereochemistry of **1** was established by NOESY correlations of relevant protons as in Figure 2. Both presence of the NOE interaction between H-15 ( $\delta$  5.08) and H-13 ( $\delta$  3.65) and the absence of the NOE correlation between 15-methyl ( $\delta$  1.34) and H-13 indicated that H-15 and H-13 were in the same orientation. Therefore, the structure of **1** (13-hydroxyhidioresorcylicide) was determined as shown in Figure 1.

Compound **2** was obtained as a yellow gum, and its molecular formula was determined as  $C_{16}H_{20}O_6$  from the  $[M+H]^+$  ion peak at  $m/z$  309.1333 (calcd. 309.1333) in the HRESIMS with seven degrees of unsaturation. The NMR data of **2** were similar to those of **1** with the difference of a hydroxyl located on the saturated chain system  $\text{CH}_3\text{CH}(\text{CH}_2)_2\text{CH}(\text{CH}_2)_2$  [ $\delta_{\text{H}}$  5.22 (1H, m), 3.73 (1H, m), 2.94 (1H, m), 2.20 (1H, m), 2.18 (1H, m), 1.94 (1H, ddd,  $J = 13.8, 9.0, 4.5\text{ Hz}$ ), 1.67 (2H, m), 1.49 (1H, m), 1.30 (3H, d,  $J = 6.3\text{ Hz}$ );  $\delta_{\text{C}}$  73.7, 70.5, 36.9, 30.3, 30.0, 29.9, 19.0]. The final structure was established by the 2D NMR experiments including HMQC and HMBC spectra. The relative stereochemistry of **2** was established by NOESY correlations of relevant protons as in Figure 2. Both the presence of a NOE interaction between H-15 and H-12 and the absence of the NOE correlation between 15-methyl and H-12 indicated that H-15 and H-12 were on the same side of the twelve-membered ring. The structure of **2** (12-hydroxyhidioresorcylicide) was thus elucidated as shown in Figure 1.

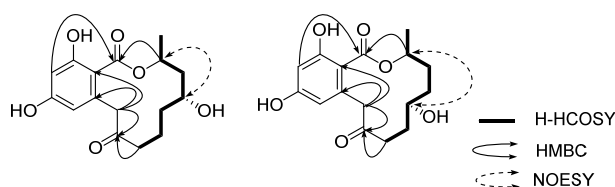


Figure 2: The H-H COSY, key HMBC, and NOESY correlations of **1** and **2**.

Table 1:  $^1\text{H}$  (400 MHz) and  $^{13}\text{C}$  (100 MHz) NMR data in  $\text{CD}_3\text{OD}$  of **1** and **2**.

Position	<b>1</b>		<b>2</b>	
	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$
1	-	172.4	-	172.3
2	-	106.3	-	106.6
3	-	166.6	-	166.6
4	6.25, d (2.5)	102.9	6.25, d (2.5)	103.0
5	-	163.9	-	163.9
6	6.11, d (2.5)	114.0	6.12, d (2.5)	113.7
7	-	140.5	-	140.0
8	4.79, d (18.6), 3.60, d (18.6)	50.7	4.73, d (18.8), 3.78, d (18.8)	52.3
9	-	211.6	-	211.0
10	2.08, 1.58 m	44.1	2.94, 2.20, m	36.9
11	1.62 (2H), m	38.2	2.18, m, 1.94, ddd (13.8, 9.0, 4.5)	29.9
12	2.08, 1.88, m	20.7	3.73, m	70.5
13	3.65, m	68.6	1.67 (2H), m	30.0
14	2.33, 2.85, m	43.7	1.49, 1.67, m	30.3
15	5.08, m	73.3	5.22, m	73.7
16	1.34, d (6.1)	21.3	1.30, d (6.3)	19.0

## Experimental

**General:** Optical rotations were determined on a JASCO P-1020 polarimeter at room temperature. UV spectra were recorded on a Perkin-Elmer Lambda 35 UV-VIS spectrophotometer, and IR spectra on a Perkin-Elmer FT-IR spectrometer (KBr). 1D and 2D NMR were carried out on a Bruker-Ascend-400 MHz instrument at 300 K, with TMS as internal standard. HRESIMS were recorded on a Bruker MicrO TOF-Q II mass spectrometer. Preparative HPLC was performed on a Waters 2545 equipped with a Waters 2489 detector on Kromasil RP-C18 column (10 × 250 mm). Column chromatography (CC) was performed with silica gel and Sephadex LH-20. All the solvents used were of analytical grade.

**Fungal material:** The title strain was isolated from the root of *Bergenia purpurascens*, collected from a suburb of Lhasa, Tibet Autonomous Region, People's Republic of China. The culture was grown on potato dextrose agar (PDA) and distinguished morphologically as *Saccharicola* sp., which was further reinforced by 18S rDNA sequence with a 99% identity to *Saccharicola bicolor*. The strain (GenBank accession no. KT367526) has been preserved at Chengdu Institute of Biology, Chinese Academy of Sciences, China.

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**Fungal culture and extraction:** This fungus was cultivated on a 4.8 L scale using 500 mL Erlenmeyer flasks containing 200 mL of the seed PDA medium for 3 days. The fermentations were carried out in 60 Fernbach flasks each containing 200 g rice. Distilled water (200 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 121°C for 30 min. The flasks were cooled to room temperature, inoculated with 3.0 mL of spore inoculum, and incubated for 21 days at 25°C. The fermented substrate in each flask was first fragmented with a spatula and then extracted 3 times with light petroleum, followed by EtOAc. The EtOAc solution was dried under vacuum and yielded 14 g extract.

**Fractionation and isolation:** The EtOAc residue (14 g) was separated into 4 fractions by CC on silica gel (300-400 mesh), eluting stepwise with a  $\text{CHCl}_3/\text{MeOH}$  gradient ( $\text{CHCl}_3$ ,  $\text{CHCl}_3/\text{MeOH}$ : 10:1 (v/v),  $\text{CHCl}_3/\text{MeOH}$ : 3:1 (v/v), MeOH). LC-MS analysis was performed on these fractions. The third fraction (eluted with MeOH) was selected. Sephadex LH-20 separation of this fraction ( $\text{CHCl}_3/\text{MeOH}$ : 1:1, v/v) afforded 3 sub-fractions (Fr.1-Fr.3). Fr.2 was further purified on a Waters preparative HPLC equipped with a Kromasil RP-C18 column (10×250 mm, ID×L; MeOH/ $\text{H}_2\text{O}$ : 55:45, v/v), which led to the separation of **1** (10.3 mg), **2** (4.5 mg), **3** (4.6 mg), **4** (11.3 mg), **5** (7.3mg) and **6** (4.7 mg).

### 13-Hydroxyhidroresorcylic (1)

$[\alpha]_{\text{D}}^{20}$ : -19.4 (c 0.1, MeOH).

IR (KBr): 3502.2, 2925.6, 1640.6, 1260.8  $\text{cm}^{-1}$ .

UV/Vis  $\lambda_{\text{max}}$  (MeOH) nm (log  $\epsilon$ ): 302.6 (3.48), 263.8 (3.74), 221.1(4.08).

$^1\text{H}$  and  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ): Table 1.

HRESIMS:  $m/z$  [ $\text{M} + \text{H}^+$ ] calcd. for  $\text{C}_{16}\text{H}_{20}\text{O}_6$ : 309.1333; found: 309.1331.

### 12-Hydroxyhidroresorcylic (2)

$[\alpha]_{\text{D}}^{20}$ : +25.1 (c 0.1, MeOH).

IR (KBr): 3383.9, 2936.3, 1645.9, 1622.1, 1261.8  $\text{cm}^{-1}$ .

UV/Vis  $\lambda_{\text{max}}$  (MeOH) nm (log  $\epsilon$ ): 302.6 (3.52), 263.8 (3.76), 221.1 (4.10).

$^1\text{H}$  and  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ): Table 1.

HRESIMS:  $m/z$  [ $\text{M} + \text{H}^+$ ] calcd. for  $\text{C}_{16}\text{H}_{20}\text{O}_6$ : 309.1333; found: 309.1333.

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Phenylpropanoid Glycosides from the Leaves of *Ananas comosus*

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Two new phenylpropanoid glycosides, named  $\beta$ -D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl  $\alpha$ -D-6'-*O*-acetylglucopyranoside (**1**) and  $\beta$ -D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl  $\alpha$ -D-glucopyranoside (**2**), along with two known analogues (**3**–**4**) and four glycerides (**5**–**8**), were isolated from the EtOAc extract of the leaves of *Ananas comosus*. Their structures were elucidated on the basis of 1D- and 2D-NMR analyses, as well as HR-ESI-MS experiments. Compounds **1**–**4** showed significant antibacterial activities against *Staphylococcus aureus* and *Escherichia coli*.

**Keywords:** *Ananas comosus*, Bromeliaceae, Phenylpropanoid glycosides, Antibacterial activities.

*Ananas comosus* L. (family Bromeliaceae) is a perennial herbaceous plant that is employed in a traditional Chinese medicinal preparation to treat stomach upsets and as an anti-diarrheal [1]. Modern pharmacological studies on *A. comosus* leaves indicated that they could have anti-oxidant, blood sugar lowering, blood lipid reducing and antibiotic activities [2]. Previous chemical studies on the leaves of *A. comosus* have led to the isolation of an array of structurally interesting compounds, including phenylpropanoids, glycerides, amides and triterpenes [3].

The ongoing investigation, seeking stronger antibacterial compounds from *A. comosus*, led to the isolation, from the EtOAc extract of the leaves, of two new phenylpropanoid glycosides, named  $\beta$ -D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl  $\alpha$ -D-6'-*O*-acetylglucopyranoside (**1**) and  $\beta$ -D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl  $\alpha$ -D-glucopyranoside (**2**), together with two known analogues and four glycerides. The known compounds were identified as 2-feruloyl-*O*- $\alpha$ -D-glucopyranosyl-(1'→2)-3,6-*O*-feruloyl- $\beta$ -D-fructofuranoside (**3**) [4],  $\beta$ -D-(1-*O*-acetyl-3,6-*O*-diferuloyl)fructofuranosyl  $\alpha$ -D-2',4',6'-triacylglucopyranoside (**4**) [5], 1-*O*-*p*-coumaroyl-3-*O*-caffeoylglycerol (**5**) [6], 1-*O*-feruloyl-3-*O*-*p*-coumaroylglycerol (**6**) [7], 1-*O*-feruloyl-3-*O*-caffeoylglycerol (**7**) [6], and 1,3-*O*-diferuloylglycerol (**8**) [7] (Figure 1). We present herein the isolation, structural elucidation, and antibacterial activity of these compounds.

Compound **1** was obtained as a colorless amorphous solid with the elemental composition C<sub>36</sub>H<sub>42</sub>O<sub>19</sub>, established by high-resolution MS. The <sup>1</sup>H NMR spectrum (Table 1) suggested that **1** contained two feruloyl moieties, represented by signals of two sets of *trans* olefinic protons [ $\delta_{\text{H}}$  6.40 (1H, d, *J* = 16.0 Hz), 7.66 (1H, d, *J* = 16.0 Hz); 6.46 (1H, d, *J* = 16.0 Hz), 7.72 (1H, d, *J* = 16.0 Hz)], two sets of 1,3,4-trisubstituted aromatic ring protons [ $\delta_{\text{H}}$  7.20 (1H, d, *J* = 1.8 Hz), 6.80 (1H, d, *J* = 8.0 Hz), 7.10 (1H, dd, *J* = 1.8, 8.0 Hz); 7.25 (1H, d, *J* = 1.8 Hz), 6.83 (1H, d, *J* = 8.0 Hz), 7.12 (1H, dd, *J* = 1.8, 8.0 Hz)], and two methoxy groups [ $\delta_{\text{H}}$  3.89 (3H, s), 3.90 (3H, s)]. Signals in the <sup>13</sup>C NMR spectrum (Table 1) at  $\delta_{\text{C}}$  90.2, 74.34, 72.03, 71.8, 71.9, 65.0; 65.2, 105.3, 81.1, 73.8, 78.3, and 64.8 suggested the presence of a disaccharide moiety [5]. Alkaline and

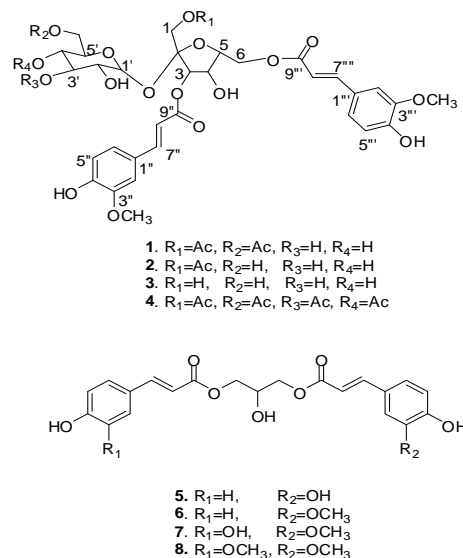


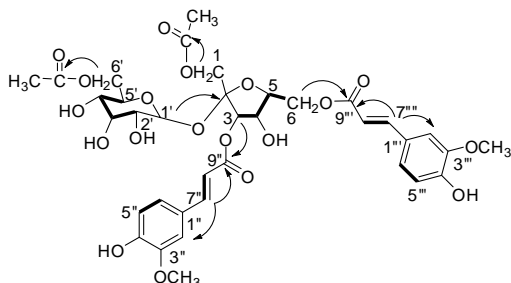
Figure 1: Structures of compounds **1**–**8**.

acid hydrolysis of **1** gave sucrose and a mixture of glucose and fructose, which were identified by direct comparison with authentic samples on TLC. A characteristic doublet signal with a small coupling constant at  $\delta_{\text{H}}$  5.66 (1H, d, *J* = 3.7 Hz) in the <sup>1</sup>H NMR spectrum that was ascribed to the anomeric proton in the  $\alpha$ -glucopyranose unit [8, 9] also supported the presence of a sucrose moiety in **1**. Furthermore, <sup>1</sup>H- and <sup>13</sup>C-NMR spectra (Table 1) revealed the presence of two acetyl groups [ $\delta_{\text{H}}$  2.07 (3H, s), 2.05 (3H, s);  $\delta_{\text{C}}$  20.9, 20.8] located in the sucrose moiety. The position of bond conjugation of the feruloyls and the acetyl groups on the sucrose was assigned by HMBC experiment. This HMBC spectrum (Figure 2) enabled the assignments of two feruloyl moieties located at positions 3 and 6 on the fructose unit of sucrose, since the methine proton ( $\delta_{\text{H}}$  5.56) of position 3 and one *trans* olefinic proton ( $\delta_{\text{H}}$  7.72) of position 7'' on a feruloyl moiety gave cross-peaks with the same carbonyl carbon ( $\delta_{\text{C}}$  168.3), and one set of the methylene protons ( $\delta_{\text{H}}$  4.50, 2H) of position 6 and another *trans* olefinic proton

**Table 1:**  $^1\text{H}$ - and  $^{13}\text{C}$  NMR data for compounds **1** and **2** in  $\text{CD}_3\text{OD}$  ( $\delta$  in ppm,  $J$  in Hz)<sup>a,b)</sup>.

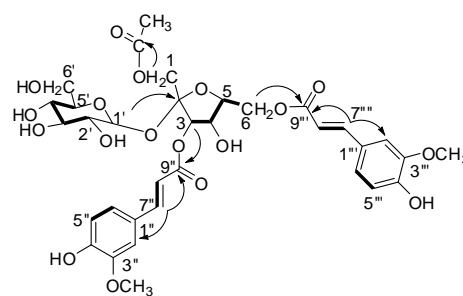
NO.	<b>1</b>		<b>2</b>	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$
1	4.54, 4.60 (m)	65.2, $\text{CH}_2$	4.19, 4.45 (m)	65.6, $\text{CH}_2$
2		105.2, C		104.9, C
3	5.56 (m)	78.3, CH	5.50 (d, 8.0)	78.9, CH
4	4.50 (m)	81.1, CH	4.50 (m)	81.2, CH
5	3.45 (m)	73.8, CH	3.45 (m)	74.3, CH
6	4.10, 4.50 (m)	64.8, $\text{CH}_2$	4.10, 4.50 (m)	65.7, $\text{CH}_2$
1'	5.66 (d, 3.6)	90.2, CH	5.49 (d, 3.6)	92.9, CH
2'				
3'	3.65 (m)	71.8, CH	3.65 (m)	71.9, CH
4'	4.16 (m)	74.3, CH	4.16 (m)	74.8, CH
5'	3.82 (dd, 9.0, 9.0)	72.0, CH	3.82 (dd, 9.0, 9.0)	73.0, CH
6'	3.34 (m)	71.9, CH	3.34 (m)	72.1, CH
6''	4.14, 4.20 (m)	65.0, $\text{CH}_2$	4.10, 4.19 (m)	65.5, $\text{CH}_2$
1''		127.5, C		127.5, C
2''	7.25 (d, 1.8)	111.8, CH	7.25 (d, 1.8)	111.9, CH
3''		149.3, C		149.4, C
4''		150.8, C		150.8, C
5''	6.82 (d, 8.2)	115.2, CH	6.82 (d, 8.2)	115.1, CH
6''	7.12 (dd, 1.6, 7.6)	124.4, CH	7.12 (dd, 1.6, 7.6)	124.3, CH
7''	7.72 (d, 16.0)	147.9, CH	7.72 (d, 16.0)	147.9, CH
8''	6.48 (d, 16.0)	116.4, CH	6.39 (d, 16.0)	116.5, CH
9''		168.3, C		168.3, C
1'''		127.7, C		127.6, C
2'''	7.19 (d, 1.8)	111.7, CH	7.19 (d, 1.8)	111.6, CH
3'''		149.3, C		149.3, C
4'''		150.6, C		150.7, C
5'''	6.80 (d, 8.2)	114.6, CH	6.80 (d, 8.2)	114.4, CH
6'''	7.08 (dd, 1.6, 7.6)	124.1, CH	7.08 (dd, 1.6, 7.6)	124.1, CH
7'''	7.67 (d, 16.0)	147.0, CH	7.67 (d, 16.0)	147.1, CH
8'''	6.41 (d, 16.0)	116.4, CH	6.41 (d, 16.0)	116.4, CH
9'''		168.8, C		168.8, C
3''-OCH <sub>3</sub>	3.90 (s)	56.5, $\text{CH}_3$	3.90 (s)	56.4, $\text{CH}_3$
3'''-OCH <sub>3</sub>	3.89 (s)	56.5, $\text{CH}_3$	3.89 (s)	56.4, $\text{CH}_3$
1-CO		172.4, C		173.0, C
6'-CO		172.8, C		
1-OAc	2.07 (s)	20.8, $\text{CH}_3$	2.08 (s)	20.9, $\text{CH}_3$
6'-OAc	2.05 (s)	20.9, $\text{CH}_3$		

a) The  $^1\text{H}$  NMR were measured at 400 MHz and  $^{13}\text{C}$  NMR at 100 MHz; b) The assignments were based upon DEPT135,  $^1\text{H}$ - $^1\text{H}$  COSY, HSQC and HMBC spectra.

**Figure 2:** Selected  $^1\text{H}$ - $^1\text{H}$  COSY (—) and HMBC (→) correlations of compound **1**.

( $\delta_{\text{H}}$  7.67) of position 7''' with the same carbonyl carbon ( $\delta_{\text{C}}$  168.8). Also, the methylene protons of position 6' ( $\delta_{\text{H}}$  4.14 and 4.20) on the glucose and methylene protons of position 1 ( $\delta_{\text{H}}$  4.54 and 4.60) on the fructose showed cross-peaks with respective acetyl carbonyl carbons ( $\delta_{\text{C}}$  172.8, 172.4). On the basis of these spectroscopic data and chemical evidence, the structure of compound **1** was determined to be  $\beta$ -D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl  $\alpha$ -D-6'-*O*-acetylglucopyranoside.

Compound **2**, obtained as a colorless amorphous solid with elemental composition  $\text{C}_{34}\text{H}_{40}\text{O}_{18}$ , was also a phenylpropanoid glycoside. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (Table 1) suggested that **2** possesses a structure similar to **1**, containing glucose (Glc) and fructose (Fru) units, and two feruloyl moieties [*trans* olefinic protons:  $\delta_{\text{H}}$  6.39 (1H, d,  $J$  = 15.6 Hz), 7.65 (1H, d,  $J$  = 15.6 Hz); 6.44 (1H, d,  $J$  = 16.0 Hz), 7.72 (1H, d,  $J$  = 16.0 Hz); 1,3,4-trisubstituted aromatic ring protons:  $\delta_{\text{H}}$  7.19 (1H, d,  $J$  = 1.6 Hz), 6.80 (1H, d,  $J$  = 8.0 Hz), 7.08 (1H, dd,  $J$  = 1.6, 8.0 Hz); 7.25 (1H, d,  $J$  = 1.6 Hz), 6.82 (1H, d,  $J$  = 8.0 Hz), 7.12 (1H, dd,  $J$  = 1.6, 8.0 Hz);

**Figure 3:** Selected  $^1\text{H}$ - $^1\text{H}$  COSY (—) and HMBC (→) correlations of compound **2**.

two methoxy groups:  $\delta_{\text{H}}$  3.89 (3H, s) and 3.90 (3H, s), but only one acetate group ( $\delta_{\text{H}}$  2.07, 3H, s) was present in **2**. Acid and alkaline hydrolysis, followed by TLC, indicated that **2** contained a sucrose moiety. The HMBC spectrum (Figure 3) of **2** revealed long range couplings between the oxygenated methine and methylene protons at positions 3 and 6 of the fructose, as well as the *trans* olefinic protons at positions 8'' and 8''' of two feruloyl groups, and their respective carbonyl carbons ( $^1\text{H}/^{13}\text{C}/^1\text{H}$ :  $\delta$  5.50/168.3/6.39 for the C-3 group), so these two feruloyl groups were located at C-3 and C-6, the same as **1** [10]. Analogously, couplings between the methyl protons of one acetyl groups, as well as methylene protons at 1 of the sucrose core and its carbonyl carbon ( $^1\text{H}/^{13}\text{C}/^1\text{H}$ : 2.08/173.0/4.19) indicated that the acetyl group was at C-1 [11]. These data confirmed that the substantial differences between **2** and **1** were in the interchange of the C-6' acetyl group and hydroxy group, so the structure of **2** was  $\beta$ -D-(1-*O*-acetyl-3,6-*O*-diferuloyl) fructofuranosyl  $\alpha$ -D-glucopyranoside.

The structures of compounds **3-8** were identified by comparison of their spectral data with those described in the literature. As stated before, the occurrence of natural phenolic diglycerides is restricted to species of Gramineae, Liliaceae, Sparganiaceae and Bromeliaceae [6]. The presence of the phenylpropanoid glycosides (**1-4**) and glycerides (**5-8**) could contribute to chemotaxonomic studies of the *Ananas* genus.

The antibacterial activities of all the isolates (**1-8**) from the leaves of *A. comosus* were evaluated using the 96-well plate method [13] and the results are given in terms of the concentration of the sample decreasing minimal inhibitory concentration (MIC). All the tested samples had different activity against different bacteria. Preliminary antibacterial activity screening revealed that four of the isolated phenylpropanoid glycosides (**1-4**) exhibited significant activity against *S. aureus* and *E. coli*, with MIC values ranging from 0.16 to 0.62  $\mu\text{g/mL}$ ; while **6** and **7** showed good inhibition of *S. aureus* and *M. luteus*, as shown in Table 2.

**Table 2:** Minimal inhibitory concentration (MIC) of compounds **1-8**.

Compds	MIC ( $\mu\text{g/mL}$ )				
	<i>S. aureus</i>	<i>S. albus</i>	<i>E. coli</i>	<i>B. subtilis</i>	<i>M. luteus</i>
<b>1</b>	0.16	5.00	0.31	0.62	2.50
<b>2</b>	0.16	10.00	0.16	0.31	1.25
<b>3</b>	0.16	2.50	0.62	10.00	1.25
<b>4</b>	0.16	1.25	0.16	10.00	5.00
<b>5</b>	0.62	0.62	10.0	10.00	N
<b>6</b>	0.31	1.25	5.00	2.50	0.62
<b>7</b>	0.62	2.50	5.00	1.25	0.62
<b>8</b>	0.62	0.62	1.25	10.00	N
CPFX <sup>a</sup>	0.16	0.31	0.16	0.16	0.31

<sup>a</sup> CPFX (ciprofloxacin) was used as positive control.

## Experimental

**General experimental procedure:** NMR spectra: Bruker AV 400 spectrometer {400 ( $^1\text{H}$ ) and 100 MHz ( $^{13}\text{C}$ )};  $\delta$  in ppm rel. to TMS

as internal standard,  $J$  in Hz. ESI-MS: Bruker Esquire 6000 Ion Trap mass spectrometer and HR-ESI-MS: Bruker Daltonics Apex-Ultra 7.0 T mass spectrometer; in  $m/z$ . Column chromatography (CC): silica gel (SiO<sub>2</sub>, 200–300 mesh; Qingdao Haiyang Chemical Group Co.), and sephadex LH-20 (Pharmacia). TLC: Precoated SiO<sub>2</sub> GF-254 (10–40  $\mu$ m) plates (Qingdao Haiyang Chemical Group Co.).

**Plant material:** *A. comosus* leaves were collected in Wanning, Hainan Province of China, and identified by Professor Qiong-xin Zhong of Hainan Normal University. A voucher specimen has been deposited at the Key Laboratory of Tropical Medicinal Plant Chemistry of the Ministry of Education, Hainan Normal University.

**Extraction and isolation:** Air-dried leaves (20.0 kg) of *A. comosus* were finely cut and extracted 3 times (each for 7 days) with 95% EtOH at room temperature. Evaporation of the solvent under reduced pressure produced the EtOH extract. This was dissolved in H<sub>2</sub>O and partitioned with light petroleum and EtOAc. The EtOAc soluble fraction (300 g) was initially subjected to column chromatography (CC) on silica gel, and then eluted with a step gradient of light petroleum/EtOAc (10/0-0/10, v/v) to give 10 fractions. Fraction 7 (15.6 g) was decolorized on a Sephadex LH-20 (CHCl<sub>3</sub>-MeOH, 2:3, v/v) column and then chromatographed on a silica gel column (CHCl<sub>3</sub>-MeOH, 6:1-1:1) to afford 6 fractions (fractions 7.1-7.6). Compounds **1** (7.0 mg) and **3** (7.4 mg) were obtained from fraction 7.2 by reversed-phase silica gel CC, and compounds **2** (7.2 mg) and **4** (6.8 mg) from fraction 7.4 by reversed-phase silica gel CC. Fraction 4 (14.6 g) was decolorized on Sephadex LH-20 (CHCl<sub>3</sub>-MeOH, 1:1, v/v) to obtain a sub-fraction, which was further isolated and purified by silica gel CC (CHCl<sub>3</sub>-Me<sub>2</sub>CO, 4:1-1:1, v/v), and Sephadex LH-20 (CHCl<sub>3</sub>-MeOH, 2:3, v/v) to afford compounds **5** (10.5 mg), **6** (12.1 mg), **7** (9.0 mg) and **8** (7.5 mg).

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### $\beta$ -D-(1-O-Acetyl-3,6-O-diferuloyl) fructofuranosyl $\alpha$ -D-6'-O-acetylglucopyranoside (**1**)

$[\alpha]_D$ : +16.9 (c 0.1, DMSO)

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

HRESI-MS:  $m/z$  777.2241 [M-H]<sup>+</sup>, calcd. for C<sub>36</sub>H<sub>41</sub>O<sub>19</sub>: 777.2248

### $\beta$ -D-(1-O-Acetyl-3,6-O-diferuloyl)fructofuranosyl- $\alpha$ -D-glucopyranoside (**2**)

$[\alpha]_D$ : +19.5 (c 0.1, DMSO)

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

HRESI-MS:  $m/z$  735.2149 [M-H]<sup>+</sup>, calcd. for C<sub>34</sub>H<sub>39</sub>O<sub>18</sub>: 735.2152

**Antibacterial activity assay:** Five bacterial strains, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus*, and *Staphylococcus albus*, were used for the assay using standard agar diffusion tests [13]. Ciprofloxacin (CPFX) was the positive control. The results are presented as minimum inhibitory concentrations (MIC) in Table 2.

**Supplementary data:** NMR (<sup>1</sup>H and <sup>13</sup>C NMR, DEPT135, HSQC, HMBC, <sup>1</sup>H-<sup>1</sup>H COSY and NOESY) and IR spectroscopic, and HR-ESI-MS data for the new compounds (**1-2**).

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## Tannins and Antioxidant Activities of the Walnut (*Juglans regia*) Pellicle

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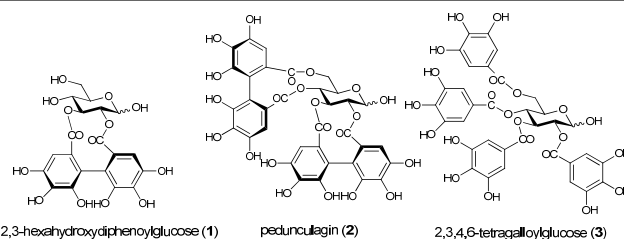
The total phenolic content and antioxidant activities of the acetone extract and derived fractions from the walnut (*Juglans regia*) pellicle were estimated. The BuOH fraction exhibited the strongest antioxidant activity with the highest phenolic content. A phytochemical investigation of this fraction led to the isolation of three tannins, 2,3-hexahydroxydiphenoylglucose (1), pedunculagin (2) and 2,3,4,6-tetragalloylglucose (3). Pedunculagin showed high content and powerful activity, which implied that this compound plays an important role in the antioxidant activity of the walnut pellicle.

**Keywords:** *Juglans regia*, Pellicle, Antioxidant, Phenolic, Tannin, Pedunculagin.

Walnut, the fruits of *Juglans regia* L. (Juglandaceae), is a highly nutritious food, which has been found to be an excellent source of multiple health-beneficial components, such as polyunsaturated fatty acids, tocopherols, phenolics, hormone and melatonin [1-3]. The increased consumption of walnuts has been correlated with reduced risk of cardiovascular disease, cancer and diabetes [4, 5]. The walnut kernel, which is officially listed in the Chinese Pharmacopoeia, has long been utilized to treat cough, stomachache and cancer in traditional Chinese medicine. Though rich in unsaturated fatty acids, walnuts are readily preserved from oxidation, which may be attributed to the protective property of the pellicle. Previous studies revealed that a large amount of phenolics possessing significant antioxidant activities exist in the walnut pellicle [6, 7]. In addition, the total phenolic content and antioxidant activity of the walnut pellicle were much higher than the kernel [8]. The antioxidant ingredients in the pellicle probably aid in stabilizing the walnut kernel against decomposition. Therefore, this investigation was conducted to elucidate the protective mechanism of the pellicle and to supply more scientific evidence of antioxidant activity for the further research and development of walnut.

Several studies concluded that walnuts possess higher antioxidant capacity than any other nuts [9], and this antioxidant capacity is presumably a product of phenolics, especially hydrolysable tannins located mainly in the pellicle [10]. The total phenolic content (TPC) of the fractions from the walnut pellicle followed the order BuOH fraction > EtOAc fraction > crude extract > light petroleum (PE) fraction > water fraction (Table 1). It revealed that phenolics in the walnut pellicle were more extractable with highly polar solvents [11, 12]. Combined with the previous studies, the major phenolics in the walnut pellicle were assigned to be tannins.

Plant phenolics, particularly phenolic acids, tannins and flavonoids, are known to be potent antioxidants, which could effectively scavenge free radicals, break radical chain reactions and chelate metal ions [13]. Three methods were utilized to evaluate the antioxidant activities of the fractions from the walnut pellicle.



**Figure 1:** Structures of tannins from the walnut pellicle.

2,2'-Diphenyl-1-picrylhydrazyl (DPPH) has been widely used to estimate the radical scavenging activity of natural antioxidants since it can accommodate a large number of samples in a short period and is sensitive enough to detect active principles at low concentrations. As observed in Table 1, the BuOH fraction of the walnut pellicle showed the highest radical scavenging activity, followed by the EtOAc fraction, crude extract, PE fraction and water fraction. The IC<sub>50</sub> value of the BuOH fraction ( $2.09 \pm 0.05$   $\mu\text{g/mL}$ ) was very close to that of the positive control, gallic acid ( $1.42 \pm 0.02$   $\mu\text{g/mL}$ ).

Tannins function as primary antioxidants and secondary antioxidants, which could chelate metal ions and interfere with one of the reaction steps in the Fenton reaction and thereby retard oxidation [14]. The Fe<sup>2+</sup> chelating activities decreased in the order BuOH fraction > EtOAc fraction > crude extract > water fraction > PE fraction, which were similar to those of the DPPH assay (Table 1). The result revealed that the BuOH fraction from the walnut pellicle could chelate Fe<sup>2+</sup> effectively, which was probably due to the presence of tannins.

The hydroxyl radical has been reported to be the most harmful reactive oxygen species that is responsible for the oxidative injury of biomolecules. It is generated from the Fenton reaction in the presence of transition metals such as Fe<sup>2+</sup> and Cu<sup>2+</sup> [15]. Similarly, the BuOH fraction showed the highest hydroxyl radical scavenging (HRS) activity, followed by the EtOAc fraction, crude extract, PE fraction and water fraction (Table 1). It can be concluded that the

extracts showed strong hydroxyl radical scavenging activity probably due to their  $\text{Fe}^{2+}$  chelating capability [16].

To demonstrate that the extracts' strong antioxidant activity was attributed to the large amount of phenolics, the correlation analysis between total phenolic content and antioxidant activity was carried out. The correlation between  $\text{IC}_{50}$  of DPPH and TPC was very strong ( $y = 63.8698 - 0.2029x$ ,  $R^2 = 0.8282$ ), and so was that between the  $\text{IC}_{50}$  of HRS and TPC ( $y = 418.7180 - 1.2384x$ ,  $R^2 = 0.7578$ ). The correlation between the  $\text{IC}_{50}$  of  $\text{Fe}^{2+}$  chelating activity and TPC was also remarkable ( $y = 77.6002 - 0.2538x$ ,  $R^2 = 0.6945$ ). The results suggested the strong antioxidant activity of walnut pellicle could be attributed to its high phenolic content.

**Table 1:** Total phenolic content and antioxidant activity of acetone extract and its derived soluble fractions from the walnut pellicle.

Sample	$\text{IC}_{50}$ ( $\mu\text{g/mL}$ )			
	TPC	DPPH	HRS	$\text{Fe}^{2+}$
Crude extract	122.5 $\pm$ 5.7	32.1 $\pm$ 1.7	146.7 $\pm$ 5.5	21.9 $\pm$ 0.8
PE fraction	19.7 $\pm$ 2.1	87.8 $\pm$ 2.9	453.1 $\pm$ 15.4	81.9 $\pm$ 1.3
EtOAc fraction	188.9 $\pm$ 8.7	14.9 $\pm$ 1.0	123.6 $\pm$ 3.5	15.5 $\pm$ 0.5
BuOH fraction	344.1 $\pm$ 13.5	2.09 $\pm$ 0.05	74.6 $\pm$ 2.6	6.42 $\pm$ 0.27
Water fraction	95.4 $\pm$ 7.9	55.6 $\pm$ 2.0	369.3 $\pm$ 9.8	37.3 $\pm$ 1.1
Gallic acid	—	1.42 $\pm$ 0.02	15.9 $\pm$ 2.3	—
EDTA	—	—	—	5.32 $\pm$ 0.19

To obtain the tannin compounds, which might be the main contributors to the high antioxidant activity of the walnut pellicle, a phytochemical investigation of the BuOH fraction was performed. Three hydrolyzable tannins were isolated and identified as 2,3-hexahydroxydiphenylglucose (**1**) [17], pedunculagin (**2**) [18], and 2,3,4,6-tetragalloylglucose (**3**) [19], by comparison of the MS,  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data with those reported in the literature (Figure 1). Compound **3** was isolated from this species for the first time. The isolated tannins exhibited significant high radical scavenging activity and had a decreasing order **1** ( $5.10 \pm 0.09 \mu\text{M}$ ) > **2** ( $5.69 \pm 0.10 \mu\text{M}$ ) > gallic acid ( $7.51 \pm 0.06 \mu\text{M}$ ) > **3** ( $11.35 \pm 0.14 \mu\text{M}$ ). Compounds **1** and **2** were more effective than gallic acid in terms of DPPH radical-scavenging activity, which was attributed to the galloyl groups present in the structure [20].

According to the UPLC chromatogram, pedunculagin (**2**) ( $6.97 \pm 0.09 \text{ mg/g}$  sample) is the major compound in walnut pellicle. The quantities of compounds **1** ( $0.57 \pm 0.03 \text{ mg/g}$  sample), **3** ( $0.96 \pm 0.03 \text{ mg/g}$  sample), and gallic acid ( $1.05 \pm 0.05 \text{ mg/g}$  sample) in walnut pellicle were also quantified. The result revealed that pedunculagin (**2**) may play a vital role in the antioxidant activity of the walnut pellicle, while the other tannins and gallic acid may also contribute to the enhanced activity. Additionally, the stabilizing or protective effect of the pellicle to the walnut kernel may mainly be attributed to the preferential oxidation of these easily oxidizable tannins [6].

## Experimental

**General experimental procedures:** A Shimadzu UV-Vis 2550 spectrometer (Shimadzu, Kyoto, Japan) was used for colorimetric measurements and collection of UV spectra. ESI-MS analyses were recorded with an Agilent G3250AA (Agilent, Santa Clara, USA) and Auto Spec Premier P776 spectrometer (Waters, Milford, USA). NMR spectra were acquired with a Bruker AM-500 spectrometer (Bruker, Karlsruhe, Germany) using TMS as the internal reference. A Waters semipreparative HPLC system equipped with a Waters 2487 dual  $\lambda$  absorbance detector, a Waters delta 600 quaternary pump, a Waters Spherisorb ODS2 ( $\text{C}_{18}$ ) Semi-Prep column (S10, 20 mm  $\times$  250 mm, 5  $\mu\text{m}$ , Waters, Milford, USA), and an Empower 3

HPLC workstation (Waters) was used for semipreparative HPLC. Silica gel (200-300 mesh and 300-400 mesh; Qingdao Marine, Qingdao, China), middle chromatogram isolated gel (MCI, Greenherbs, Beijing, China), and Sephadex LH-20 (GE Healthcare, Fairfield, USA) were used for column chromatography (CC). GF254 plates (Qingdao Marine, Qingdao, China) were used for thin layer chromatography, and compounds were visualized either under UV light or by spraying with 10%  $\text{H}_2\text{SO}_4$  in ethanol followed by heating. DPPH and ferrozine were obtained from J&K Scientific Ltd (Beijing, China), gallic acid from Aladdin-Reagent (Shanghai, China),  $\text{CD}_3\text{OH}$  from Sigma-Aldrich (Shanghai, China), and  $\text{CH}_3\text{CN}$  and  $\text{CH}_3\text{OH}$  (HPLC grade) from Fisher Chemicals (New Jersey, USA). Deionized water (resistivity  $\geq 18.25 \text{ M}\Omega\cdot\text{cm}$ ) was purified with a You Pu purity water system (You Pu, Chengdu, China).

**Plant materials:** The pellicle of *J. regia* was collected from Kunming City in Yunnan Province, China, in August 2011. The samples were identified by Professor Shu-gang Lu at the School of Life Sciences at Yunnan University in Kunming City, China. A voucher specimen (No. LCS-02) is deposited at the Key Laboratory of Medicinal Chemistry for Natural Resources, Ministry of Education in Kunming City of China.

**Extraction and isolation:** Air-dried and powdered pellicle of *J. regia* (1.8 kg) was extracted with 80% aqueous acetone at room temperature ( $3 \times 5 \text{ L}$ , each extraction 24 h). Removal of the solvent under reduced pressure afforded the crude extract (450 g), which was partitioned successively with PE, EtOAc, and BuOH to yield soluble fractions in PE (210 g), EtOAc (44 g), BuOH (144 g), and water (52 g), respectively. Five g samples were collected from each fraction for the antioxidant experiments. The remainders were used to study the fractions' chemical components.

The BuOH fraction was subjected to silica gel CC, elution with a  $\text{CHCl}_3$ - $\text{CH}_3\text{OH}$  gradient system (200:1 to 1:1, v/v) to give 8 fractions (FrC1-FrC8). FrC5 (1.2 g) was subjected to MCI gel CC ( $\text{CH}_3\text{OH}$ - $\text{H}_2\text{O}$ , 1:1, v/v), Sephadex LH-20 CC ( $\text{CH}_3\text{OH}$ ) and semipreparative HPLC ( $\text{CH}_3\text{OH}$ - $\text{H}_2\text{O}$ , 3:2, v/v) to provide **1** (40 mg). FrC6 (1.5 g) was subjected to silica gel CC (EtOAc- $\text{CH}_3\text{OH}$ , 10:1, v/v) and then purified by MCI CC ( $\text{CH}_3\text{OH}$ - $\text{H}_2\text{O}$ , 1:1, v/v) to provide **2** (252 mg) and **3** (28 mg).

**Total phenolic content:** The TPC was determined according to the Folin-Ciocalteu method [21]. Folin-Ciocalteu reagent was prepared according to a previously described method [22]. A total of 15  $\mu\text{L}$  of extract solution was added to 2.25 mL Folin-Ciocalteu reagent, which had been prediluted by a factor of 10 with deionized water. Five min later, 3.0 mL of  $\text{Na}_2\text{CO}_3$  (7.5%, w/v) solution was added and the mixture was allowed to stand for 30 min at room temperature. The absorbance was measured at 765 nm. TPC was expressed as mg gallic acid equivalents/g dry extract (mg GAE/g Ex) and calculated according to the calibration curve obtained from a standard solution of gallic acid at various concentrations.

**DPPH assay:** The DPPH assay was assessed by using a previously published method [23]. A total of 3.9 mL DPPH (0.075 mM) was mixed with 0.1 mL sample at various concentrations. The mixture was shaken and incubated at  $25^\circ\text{C}$  in the dark, and the absorbance of the reaction mixture was measured at 515 nm after 30 min. The DPPH radical scavenging activity was calculated by the following equation: inhibition =  $(1 - A_s/A_0) \times 100\%$ ; where  $A_0$  is the absorbance of DPPH without any sample, and  $A_s$  is the absorbance of a sample with DPPH in it. Inhibition was the scavenging effect, and the 50% absorbance reduction ( $\text{IC}_{50}$ ) was measured from a



curve relating concentration to the absorbance of a sample. Gallic acid was used as a reference standard.

**Fe<sup>2+</sup> chelating activity:** The Fe<sup>2+</sup> chelating assay was assessed by using a previously published method [24, 25]. Briefly, samples at various concentrations in CH<sub>3</sub>OH (0.4 mL) were added to a solution of 2 mM FeCl<sub>2</sub> (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), and the total volume was adjusted to 4 mL with CH<sub>3</sub>OH. The mixture was shaken vigorously and left standing at room temperature for 10 min; absorbance of the solution was then measured at 562 nm. The Fe<sup>2+</sup> chelating activity was calculated by the following equation: inhibition =  $(1 - A_s/A_o) \times 100\%$ ; where A<sub>o</sub> is the absorbance of the control which contains FeCl<sub>2</sub> and ferrozine, and A<sub>s</sub> is the absorbance in the presence of the sample or standard. EDTA was used as a reference standard.

**HRS assay:** The HRS assay was conducted by a Fenton reaction method [26]. Briefly, the reaction mixture containing 0.2 mL of acidic chrome blue K (0.2 mM), 1.2 mL of FeSO<sub>4</sub> (0.4 mM), 1.0 mL of H<sub>2</sub>O<sub>2</sub> (0.03%, v/v), 1.2 mL of phosphate buffer (0.1 mM, pH = 5.5) and 1.0 mL of samples at various concentrations was incubated at room temperature for 15 min, and the absorbance was measured at 520 nm. Hydroxyl radical scavenging activity was calculated by the following equation: inhibition =  $[(A_s - A_o) / (A - A_o)] \times 100\%$ ; where A<sub>s</sub> is the absorbance in the presence of the sample, A<sub>o</sub> is the absorbance of the control in the absence of the sample, and A is the absorbance without the sample and Fenton reaction system. Gallic acid was used as a reference standard.

**Tannins determined by UPLC:** Air-dried and powdered pellicle of *J. regia* (1.0000 g) was extracted 3 times with 80% aqueous acetone at room temperature, for 24 h each time. The resulting crude extract

and the authentic samples were prepared as solutions (10.00 mg/mL and 1.00 mg/mL, respectively). All sample solutions were filtered through a 0.45 µm filter before injection into the ACQUITY UPLC system equipped with a QSM (quaternary solvent manager), a UPL PDA (photodiode array) detector, a CHA column thermostat, a SDI sample manager, a reversed phase Symmetry1 C<sub>18</sub> column (T3, 2.1 mm × 100 mm, 1.8 µm; Waters, Milford, USA), and an Empower 3 UPLC workstation (Waters). The optimal mobile phase for the analysis was a gradient elution system consisting of solvent A (H<sub>2</sub>O, 1.0% acetic acid) and solvent B (CH<sub>3</sub>CN). The gradient program was as follows: 0-3 min, 0-5% solvent B; 3-6 min, 5-10% solvent B; 6-10 min, 10%-15% solvent B; 10-20 min, 15%-100% solvent B; 20-21 min, 100%-0 solvent B. The flow rate was 0.5 mL/min, the column temperature was set at 30 °C, and the injection volume was 5 µL. The UV detection wavelength was monitored at 280 nm. The identification of peaks was confirmed by the UV absorptions and retention times of authentic samples. The sample analyte content (C<sub>s</sub>, mg/g dry sample) was calculated according to the equation  $C_s = (C_a \times V) / M$ , where C<sub>a</sub> is the analyte content (mg/mL) based on the calibration curve, V (mL) is the final volume of sample, and M is the sample weight (g).

**Statistical analysis:** The results were determined as the mean ± SD for at least 3 replicates for each sample. Statistical analysis was performed using SPSS. A significant difference was evaluated at a level of  $p < 0.05$ .

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Chemical Constituents of *Cordyceps cicadae*Zhi-Bo Chu<sup>a</sup>, Jun Chang<sup>b</sup>, Ying Zhu<sup>a,\*</sup> and Xun Sun<sup>b,\*</sup><sup>a</sup>School of Pharmacy, Zhejiang Chinese Medical University, 548 Binwen Road, Hangzhou 310053, China<sup>b</sup>School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, China

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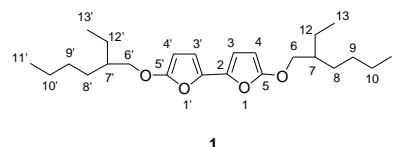
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One new bifuran derivative (**1**), together with fourteen known compounds, were isolated from *Cordyceps cicadae* X. Q. Shing. The known compounds included nine nucleosides, uracil (**2**), uridine (**3**), 2'-deoxyuridine (**4**), 2'-deoxyinosine (**5**), guanosine (**6**), 2'-deoxyguanosine (**7**), thymidine (**8**), adenosine (**9**), and 2'-deoxyadenosine (**10**); three amino acids tryptophan (**11**), phenylalanine (**12**), and tyrosine (**13**); and two dopamine analogues *N*-acetylnoradrenaline (**14**) and its dimer, *trans*-2-(3',4'-dihydroxyphenyl)-3-acetyl-amino-7-(*N*-acetyl-2''-amino-ethylene)-1,4-benzodioxane (**15**). Their structures were decisively elucidated by spectroscopic analysis, including 1D and 2D NMR techniques.

**Keywords:** *Cordyceps cicadae*, Bifuran derivative, Nucleoside, Amino acid, Dopamine analogue.

*Cordyceps cicadae* X. Q. Shing (Clavicipitaceae, Ascomycotina), named 'Chan Hua', an anamorph of *Isaria cicadae* Miq. is a major parasitic fungus growing on the nymphs of *Cicada flammata* Distant, *Platyleura kaempferi* Fabricius, *Cryptotympana pustulata* Fabricius, *Platylomia pili* Kato and *Oncotympana maculatioellis* Motsch [1]. As a traditional Chinese medicine used for thousands of years, 'Chan Hua' is reported to possess antitumor, antibacterial, immunoregulatory and sedative effects, and is widely used in the treatment of fever, infantile convulsion, palpitation and dizziness [2]. Some bioactive constituents, for example, polysaccharides, galactomannan, ISP-1 (myriocin) and cyclodepsipeptides have been isolated from *C. cicadae* [3]. Cordycepin, adenosine derivatives, aromatic 4-*O*-methylglucosides, cyclopentenone and furan derivative were reported from the cultivated mycelia of *C. cicadae* [4]. As part of our investigation on biologically active constituents from Chinese traditional medicines [5], *C. cicadae* was exhaustively studied. A crude extract of the drug was chromatographed repeatedly on the MCI gel CHP 20P, HW-40F, normal phase silica gel, Sephadex LH-20, and reversed phase silica gel. As a result, one new heterocyclic compound, 5,5'-di (2-ethyl-hexyloxy)-5,5'-bifuran (**1**) (Figure 1), was obtained, together with fourteen known compounds. Herein, we report the isolation and structural elucidation of compound **1**.

Compound **1** was obtained as colorless oil. The positive ESI-MS showed quasimolecular ion peaks at *m/z* 391 [*M* + *H*]<sup>+</sup> and 803 [*2M* + *Na*]<sup>+</sup>, indicating a molecular weight of 390. The molecular formula of **1** was determined to be C<sub>24</sub>H<sub>38</sub>O<sub>4</sub> from the HR-ESI-MS ion peak at *m/z* 413.2659 [*M* + *Na*]<sup>+</sup> (calcd 413.2662), and <sup>1</sup>H and <sup>13</sup>C NMR spectroscopic data. The low-field part of the <sup>1</sup>H NMR spectrum (Table 1) indicated the presence of four olefinic protons at δ 7.734, 7.726, 7.628, and 7.620 (d, *J* = 5.6 Hz). The high-field region of the spectrum showed two oxygenated methylenes at δ 4.229 (4H, m), two methines at δ 1.681 (2H, m), eight methylenes at δ 1.392-1.423 (8H, m), and four methyl groups at δ 0.961 (6H, t, *J* = 7.5 Hz) and 0.932 (6H, t, *J* = 7.5 Hz). The <sup>13</sup>C NMR spectrum showed two quaternary carbons at δ 169.23 and 133.59, and two methine carbons at δ 132.37 and 129.86, which suggested an oxygenated furan ring. There was evidence of an oxygenated methylene carbon at δ 69.02, one methine carbon at δ 40.13, four methylene carbons at δ 31.60, 30.12, 24.04, and 24.92, and two

Figure 1: The structure of compound **1**.Table 1: NMR data of compound **1**.

position	<sup>1</sup> H <sup>a</sup>	<sup>13</sup> C <sup>b</sup>	<sup>1</sup> H- <sup>1</sup> H COSY	HMBC (H→C)
2, 2'		133.59 s		
3, 3'	7.734, 7.726 d (5.6)	129.86 d	H-4	C-2, 4, 5
4, 4'	7.628, 7.620 d (5.6)	132.37 d	H-3	C-2, 3
5, 5'		169.23 s		
6, 6'	4.229 m	69.02 t	H-7	C-5, 7, 8, 12
7, 7'	1.681 m	40.13 d	H-6, 8, 12	C-6, 8, 9, 12, 13
8, 8'	1.421 m	31.60 t	H-7, 9	C-6, 7
9, 9'	1.392 m	30.12 t	H-8, 10	C-10
10, 10'	1.395 m	24.04 t	H-9, 11	C-9, 11
11, 11'	0.932 t (7.5)	14.45 q	H-10	C-9, 10
12, 12'	1.423 m	24.92 t	H-7, 13	C-6, 7, 8, 13
13, 13'	0.961 t (7.5)	11.44 q	H-12	C-7, 12

<sup>a</sup> 400 MHz, CD<sub>3</sub>OD; chemical shifts in ppm relative to TMS; coupling constant (*J*) in Hz. <sup>b</sup> 100 MHz, CD<sub>3</sub>OD; multiplicity was established from DEPT data.

terminal methyl carbons at δ 14.45 and 11.44, which were derived from the alkoxy moiety. Based on the ESI-MS, compound **1** was proved as a dimer of a furan derivative. The 2-ethyl-hexyloxy moiety was established unambiguously by <sup>1</sup>H-<sup>1</sup>H COSY, HMQC and HMBC experiments, in which long-range correlations were observed not only between H-7 (δ 1.681) and five carbons (C-6, C-8, C-9, C-12 and C-13), but also between H-11 (δ 0.932) and two carbons (C-9 and C-10). Moreover, the long-range correlations between the oxygenated methylene H-6, 6' (δ 4.229) and oxygenated quaternary olefin carbons C-5, 5' (δ 169.23) indicated that 2-ethyl-hexyloxy was substituted at C-5 and C-5' of each furan ring. The two furan rings were linked at C-2 and C-2' by a C-C bond, because the olefin methine signals at δ 7.734 and 7.726 (H-3, 3') showed cross peaks with a quaternary olefin carbon at δ 133.59 (C-2, 2') by *J*<sub>2</sub> and *J*<sub>3</sub> correlation. Consequently, compound **1** was identified as 5,5'-di (2-ethyl-hexyloxy)-5,5'-bifuran.

It is worth mentioned that Baek *et al.* reported a bifuran derivative from *Chrysanthemum coronarium* L. [6] and Guo *et al.* reported bifuran derivatives from *Cyathula officinalis* Kuan [7]. However, the peak of H-4 and H-4' of the furan was assigned as dd peaks with

coupling constants of either 5.6 Hz or 5.6 and 3.3 Hz. In fact, the peak of H-4 (H-3) was not completely overlapped by H-4' (H-3') of the furan because of a steric effect. Therefore, the aromatic protons of the furan should be assigned as four doublet peaks with a coupling constant of 5.6 Hz, respectively.

Fourteen known compounds were also isolated, including nine nucleosides, uracil (**2**), uridine (**3**), 2'-deoxyuridine (**4**), 2'-deoxyinosine (**5**), guanosine (**6**), 2'-deoxyguanosine (**7**), thymidine (**8**), adenosine (**9**), 2'-deoxyadenosine (**10**); three amino acid tryptophan (**11**), phenylalanine (**12**), and tyrosine (**13**); and two dopamine analogues, *N*-acetyl noradrenaline (**14**), and its dimer, *trans*-2-(3',4'-dihydroxyphenyl)-3-acetyl amino-7-(*N*-acetyl-2"-amino-ethylene)-1,4-benzodioxane (**15**) were isolated from *C. cicadae*.

## Experimental

**General:** Optical rotation, Perkin-Elmer 241 automatic digital polarimeter; UV, Shimadzu UV-260 instrument; IR, Perkin-Elmer 599B instrument; NMR, Bruker DRX-400 spectrometer; (<sup>1</sup>H 400 MHz and <sup>13</sup>C 100 MHz). ESI-MS, Quattro instrument. Reverse-phase chromatography utilized TSK gel Toyopearl HW-40F (30-60 μm, Toso Co., Ltd.), MCI gel CHP 20P (75-150 μm, Mitsubishi Chemical Industries Co., Ltd.) and Cosmosil 75 C18-OPN (42-105 μm, Nacalai Tesque Inc.) columns. TLC was performed using precoated silica gel 60 F254 plates (0.2 mm, Merck).

**Plant material:** *Cordyceps cicadae* X. Q. Shing was collected in Zhejiang Province, People's Republic of China in 2014, and authenticated by Dr Xie H. A voucher specimen has been deposited in the Herbarium of our lab (DNPC 2014006).

**Extraction and isolation:** *C. cicadae* (2 kg) was extracted 3 times with 70% aqueous acetone at room temperature (3 × 10 L). The solvent was evaporated under reduced pressure to 1 L and filtered through celite. The filtrate was concentrated *in vacuo* to yield 65 g of a gummy residue. This was dissolved in 400 mL water, and subjected to MCI gel CHP 20P (8 × 60 cm) eluting with a MeOH/H<sub>2</sub>O gradient with a flow rate of 15 mL/min to obtain fractions 1 [1.0 L, H<sub>2</sub>O], 2 [0.6 L, MeOH/H<sub>2</sub>O (10:90)], 3 [0.6 L, MeOH/H<sub>2</sub>O (30:70)], 4 [0.6 L, MeOH/H<sub>2</sub>O (50:50)], 5 [0.7 L,

MeOH/H<sub>2</sub>O (70:30)], 6 [1.0 L, MeOH], 7 [1.0 L, MeCOMe/H<sub>2</sub>O (60:40)], and 8 [1.0 L, MeCOMe]. Fraction 2 (6.4 g) was chromatographed on Toyopearl HW-40F (6 × 60 cm) using water as eluent to obtain 6 fractions 2A-2F (eluent volume: 200 mL/fraction). Fraction 2C (0.3 g) was further purified by MCI gel CHP 20P (5 × 40 cm, eluted with H<sub>2</sub>O→30% MeOH) and Cosmosil 75 C<sub>18</sub>-OPN (4 × 30 cm, eluted with H<sub>2</sub>O→30% MeOH) to give **13** (26 mg). Fraction 3 (5.8 g) was chromatographed on Toyopearl HW-40F (6 × 60 cm) using water as eluent to obtain 5 fractions 3A-3E (eluent volume: 200 mL/fraction). Fraction 3C (0.8 g) was purified by MCI gel CHP 20P (5 × 40 cm, eluted with 10-50% MeOH) and Cosmosil 75 C<sub>18</sub>-OPN (4 × 30 cm, eluted with H<sub>2</sub>O→50% MeOH) to give **3** (10 mg), **4** (15 mg), **5** (6 mg), **6** (11 mg), **7** (8 mg), and **9** (25 mg). Fraction 4 (6.2 g) was chromatographed on Toyopearl HW-40F (6 × 60 cm) using water as eluent to obtain 6 fractions 4A-4F (eluent volume: 200 mL/fraction). Fraction 4C (0.8 g) was further purified by MCI gel CHP 20P (5 × 40 cm, eluted with 20-60% MeOH) and Cosmosil 75 C<sub>18</sub>-OPN (4 × 30 cm, eluted with H<sub>2</sub>O→60% MeOH) to give **2** (6 mg), **8** (9 mg), **10** (18 mg), **11** (45 mg), **12** (30 mg) and **14** (6 mg). Fraction 8 (6.8 g) was chromatographed on LH-20 (4 × 200 cm) using methanol as eluent to obtain 6 fractions 8A-8F (eluent volume: 200 mL/fraction). Fraction 8C (0.4 g) was subjected to CC on silica gel to give **1** (15 mg) and **15** (11 mg).

## 5, 5'-Di (2-ethyl-hexyloxy)-5, 5'-bifuran (**1**)

Colorless oil.

[α]<sub>D</sub><sup>20</sup>: 0 (c 0.10, MeOH).

IR (KBr) ν<sub>max</sub>: 3100, 1660, 1245, 1230 cm<sup>-1</sup>.

UV (MeOH) λ<sub>max</sub> (log ε): 241 (3.89), 274 (3.14) nm;

<sup>1</sup>H and <sup>13</sup>C NMR: Table 1.

ESI-MS: *m/z* 391 [M + H]<sup>+</sup>, 803 [2M + Na]<sup>+</sup>.

HR-ESI-MS: *m/z* 413.2659 [M + Na]<sup>+</sup> (calcd. for C<sub>24</sub>H<sub>38</sub>O<sub>4</sub>Na, 413.2662).

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A New Bithiophene from the Root of *Echinops grijsii*Fang-Pin Chang<sup>a,f</sup>, Chien-Chih Chen<sup>b</sup>, Hui-Chi Huang<sup>c</sup>, Sheng-Yang Wang<sup>d</sup>, Jih-Jung Chen<sup>c</sup>, Chang-Syun Yang<sup>c</sup>, Chung-Yi Ou<sup>f</sup>, Jin-Bin Wu<sup>f,\*\*</sup>, Guan-Jhong Huang<sup>c,\*\*</sup> and Yueh-Hsiung Kuo<sup>c,g,\*\*</sup><sup>a</sup>The Ph.D. Program for Cancer Biology and Drug Discovery, China Medical University and Academia Sinica, Taichung 404, Taiwan<sup>b</sup>Department of Nursing and Department of Biotechnology, Hungkuang University, Taichung 443, Taiwan<sup>c</sup>Department of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung 404, Taiwan<sup>d</sup>Department of Forestry, National Chung Hsing University, Taichung 402, Taiwan<sup>e</sup>Department of Pharmacy, Tajen University, Pingtung, Taiwan 907<sup>f</sup>School of Pharmacy, China Medical University, Taichung 404, Taiwan<sup>g</sup>Department of Biotechnology, Asia University, Taichung 404, Taiwan

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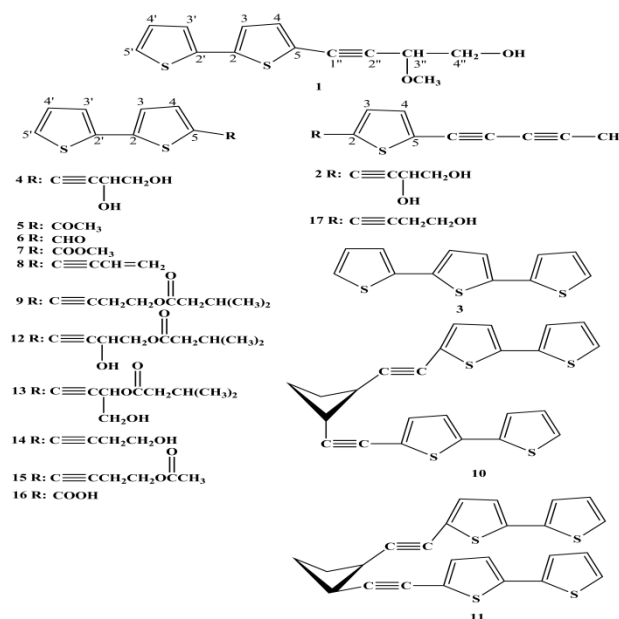
Received: August 19<sup>th</sup>, 2015; Accepted: November 5<sup>th</sup>, 2015

A new bithiophene, 5-(4-hydroxy-3-methoxy-1-butyne)-2,2'-bithiophene (**1**), and sixteen known thiophenes: 2-(3,4-dihydroxybut-1-ynyl)-5-(penta-1,3-diynyl)thiophene (**2**),  $\alpha$ -terthienyl (**3**), 5-(3,4-dihydroxybut-1-ynyl)-2,2'-bithiophene (**4**), 5-acetyl-2,2'-bithiophene (**5**), 5-formyl-2,2'-bithiophene (**6**), methyl 2,2'-bithiophene-5-carboxylate (**7**), 5-(but-3-en-1-ynyl)-2,2'-bithiophene (**8**), 5-(4-isovaleroyloxybut-1-ynyl)-2,2'-bithiophene (**9**), cardopatine (**10**), isocardopatine (**11**), 5-(3-hydroxy-4-isovaleroyloxybut-1-ynyl)-2,2'-bithiophene (**12**), 5-(3-hydroxymethyl-3-isovaleroyloxyprop-1-ynyl)-2,2'-bithiophene (**13**), 5-(4-hydroxy-1-butyne)-2,2'-bithiophene (**14**), 5-(4-acetoxy-1-butyne)-2,2'-bithiophene (**15**), 2,2'-bithiophene-5-carboxylic acid (**16**) and 2-(4-hydroxybut-1-ynyl)-5-(penta-1,3-diynyl)thiophene (**17**) were isolated from the roots of *Echinops grijsii* Hance. Among them, compounds **6**, **7** and **16** were isolated from a natural source for the first time. Compounds **2**, **4** and **14** exhibited significant anti-inflammatory activity against nitrite of LPS-stimulated production in the RAW 264.7 cell line.

**Keywords:** *Echinops grijsii*, Bithiophene, 5-(4-Hydroxy-3-methoxy-1-butyne)-bithiophene, Anti-inflammatory.

*Echinops grijsii* Hance (Compositae) is a perennial medicinal herb and the only native species of *Echinops* from Taiwan [1]. The roots of this plant have been used to clear heat, expel miasma and stimulate milk secretion. It was recorded in the Chinese Pharmacopoeia (2005 edition) as Yuzhou Loulu [2]. Previous chemical investigation of the roots of *E. grijsii* demonstrated the presence of thiophenes, steroids, triterpenes and essential oils [3]. Thiophenes have been proven to possess anti-inflammatory [2], anti-tumor [4] and anti-viral [5] activities. The MeOH extract of the roots showed a significant anti-inflammatory activity. Thus, the constituents of *E. grijsii* were investigated. This paper deals with the structure elucidation of a new compound and the anti-inflammatory activity of the isolates.

Silica gel column chromatography of the MeOH extract of dried roots of *E. grijsii* led to the isolation of a new compound: 5-(4-hydroxy-3-methoxy-1-butyne)-2,2'-bithiophene (**1**), as well as sixteen known compounds: 2-(3,4-dihydroxybut-1-ynyl)-5-(penta-1,3-diynyl)thiophene (**2**) [6],  $\alpha$ -terthienyl (**3**), 5-(3,4-dihydroxybut-1-ynyl)-2,2'-bithiophene (**4**), 5-acetyl-2,2'-bithiophene (**5**) [1], 5-formyl-2,2'-bithiophene (**6**) [7], methyl 2,2'-bithiophene-5-carboxylate (**7**) [8], 5-(but-3-en-1-ynyl)-2,2'-bithiophene (**8**), 5-(4-isovaleroyloxybut-1-ynyl)-2,2'-bithiophene (**9**), cardopatine (**10**), isocardopatine (**11**), 5-(3-hydroxy-4-isovaleroyloxybut-1-ynyl)-2,2'-bithiophene (**12**) [1], 5-(3-hydroxymethyl-3-isovaleroyloxyprop-1-ynyl)-2,2'-bithiophene (**13**) [9], 5-(4-hydroxy-1-butyne)-2,2'-bithiophene (**14**) [1], 5-(4-acetoxy-1-butyne)-2,2'-bithiophene (**15**)

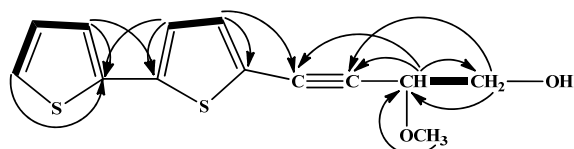
**Figure 1:** The chemical structures of compounds 1-17.

[10], 2,2'-bithiophene-5-carboxylic acid (**16**) [11] and 2-(4-hydroxybut-1-ynyl)-5-(penta-1,3-diynyl)thiophene (**17**) [12] (Figure 1).

**Table 1:**  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR data ( $\text{CDCl}_3$ ) of **1**.  $\delta$  in ppm,  $J$  in Hz.

Position	$^1\text{H}$ NMR (400 MHz, $\delta$ , $\text{CDCl}_3$ )	$^{13}\text{C}$ NMR (50 MHz, $\delta$ , $\text{CDCl}_3$ )
1		
2		139.5
3	7.13 (1H, d, $J=3.8$ Hz)	133.8
4	7.00 (1H, d, $J=3.8$ Hz)	128.2
5		122.3
1'		
2'		136.5
3'	7.17 (1H, d, $J=3.6$ Hz)	124.6
4'	7.02 (1H, dd, $J=5.1, 3.6$ Hz)	123.6
5'	7.24 (1H, d, $J=5.1$ Hz)	125.4
1''		90.0
2''		80.7
3''	4.31 (1H, t, $J=5.7$ Hz)	73.0
4''	3.82 (1H, d, $J=5.7$ Hz)	65.3
$\text{OCH}_3$	3.52 (3H, s)	57.3

The numbers in parentheses are  $J$  values in Hz

**Figure 2:**  $^1\text{H}$ - $^1\text{H}$  COSY (—) and key HMBC ( $^1\text{H} \rightarrow ^{13}\text{C}$ ) correlation of **1**.

Compound **1** was obtained as a yellow oil. Its molecular formula  $\text{C}_{13}\text{H}_{12}\text{O}_2\text{S}_2$  was determined by the pseudomolecular ion peak at  $m/z$  262.9758  $[\text{M}-2\text{H}]^+$  in the HR-ESIMS. The structure of **1** was established by analysis of  $^1\text{H}$  and  $^{13}\text{C}$  NMR, HMQC, COSY and HMBC spectral data. The UV absorption maximum at  $\lambda_{\text{max}}$  333 nm showed the presence of a bithiophene group conjugated with an acetylene functionality [13]. The IR spectrum showed the presence of hydroxy ( $3431\text{ cm}^{-1}$ ), alkyne ( $2214\text{ cm}^{-1}$ ), and bithiophene ( $802, 696\text{ cm}^{-1}$ ).

The  $^1\text{H}$  NMR spectrum showed characteristic signals of 5-substituted 2,2'-bithiophene protons at  $\delta$  7.24 (1H, d,  $J=5.1$  Hz, H-5'), 7.17 (1H, d,  $J=3.6$  Hz, H-3'), 7.13 (1H, d,  $J=3.8$  Hz, H-3), 7.02 (1H, dd,  $J=5.1, 3.6$  Hz, H-4') and 7.00 (1H, d,  $J=3.8$  Hz, H-4) [13]. Additionally, signals of  $\text{CH}_2\text{CH}$  protons appeared at  $\delta$  4.31 (1H, t,  $J=5.7$  Hz, H-3'') and  $\delta$  3.82 (2H, d,  $J=5.7$  Hz, H-4''); proton signals that appeared at  $\delta$  3.52 (3H, s) indicated the presence of a methoxy group. The  $^{13}\text{C}$  NMR spectrum of **1** exhibited 13 carbon signals including eight bithiophene carbons appearing at  $\delta$  139.5, 136.5, 133.8, 128.2, 125.4, 124.6, 123.6 and 122.3, along with the ynyl carbons at  $\delta$  90.0 and 80.7. Two oxygenated carbons appeared at  $\delta$  73.0 and 65.3, and one methoxy carbon at  $\delta$  57.3 (Table 1).

The COSY and HMBC experiments gave  $^1\text{H}$ - $^1\text{H}$  and  $^1\text{H}$ - $^{13}\text{C}$  direct correlations of **1** (Figure 2). The correlation between H-4 ( $\delta$  7.00) / C-5 ( $\delta$  122.3) and C-1'' ( $\delta$  90.0), H-3'' ( $\delta$  4.31) / C-1'' ( $\delta$  90.0), C-2'' ( $\delta$  80.7) and C-4'' ( $\delta$  65.3) indicated that the  $\text{CHCH}_2\text{OH}$  group was connected with the ynyl group, which was connected with the bithiophene at C-5. The  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectral data of compound **1** were very similar to those of **4**. The only difference was that the correlation between the methoxy group ( $\delta$  3.52) with C-3'' ( $\delta$  73.0) indicated that the methoxy group was connected at C-3''. From the above evidence, the structure of **1** was established as 5-(4-hydroxy-3-methoxy-1-butyne)-2,2'-bithiophene.

Compounds **1-17** were tested for anti-inflammatory activity against the RAW 264.7 cell line. Compounds **2**, **4** and **14** exhibited significant activity, with  $\text{IC}_{50}$  values of 2.5, 20.0 and 6.7  $\mu\text{g/mL}$  (Table 2).

**Table 2:** Cell viability and *in vitro* anti-inflammatory activities of compounds from *E. grijsii* against nitrite of LPS-stimulated production in RAW 264.7 cell.

Comps	$\text{IC}_{50}$ ( $\mu\text{g/mL}$ )	Cell viability (% of control)
<b>2</b>	2.5	$98.5 \pm 3.4$
<b>4</b>	20.0	$82.7 \pm 4.0$
<b>14</b>	6.7	$93.1 \pm 2.7$
indomethacin	65.4	

## Experimental

**Plant material:** The roots of *E. grijsii* were bought from the Shan You herb store in Nantou country in September 2010. The plant material was identified by assistant professor Shyh-Shyun Huang from the school of Pharmacy, China Medical University.

**Extraction and isolation:** The roots of *E. grijsii* (10 kg) were crushed into pieces and extracted with methanol ( $50\text{ L} \times 3$ ) at ambient temperature and concentrated under vacuum to yield the MeOH extract (2.5 kg). Part of this (1 kg) was partitioned between EtOAc- $\text{H}_2\text{O}$  to give EtOAc-soluble (100 g) and  $\text{H}_2\text{O}$ -soluble fractions. The EtOAc-soluble fraction was chromatographed over silica gel eluted with *n*-hexane and a gradient of *n*-hexane-EtOAc. The eluent was collected in constant volumes, and combined into 32 fractions based on TLC results. Fraction 10 (obtained with *n*-hexane: EtOAc = 90:10, amount 2 g) was re-separated by HPLC to yield **1** (1.46 mg), **5** (2.15 mg), **6** (13.75 mg), **7** (2.42 mg), **8** (24.74 mg), **9** (2.89 mg), **10** (4.53 mg), **11** (17.54 mg), **12** (3.82 mg), **13** (5.67 mg), 5-(4-hydroxy-1-butynyl)-2,2'-bithiophene (**14**) (3.1 mg), **15** (2.6 mg) and **17** (1.99 mg). Fraction 16 (obtained with *n*-hexane: EtOAc = 70:30, amount 1.2 g) was re-separated by Sephadex LH-20 and HPLC to yield **2** (63.6 mg), **3** (43.49 mg), **4** (77.64 mg) and **16** (112.07 mg).

**General:** UV spectra were obtained with a Shimadzu Pharmaspec-1700 UV-Visible spectrophotometer, optical rotations with a Jasco P-1020 polarimeter, and infrared spectra with a Shimadzu IR prestige-21 Fourier transform infrared spectrophotometer.  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra were recorded with Bruker DRX-400 and DRX-200 FT-NMR spectrometers. HRESIMS data were generated at the Mass Spectrometry Laboratory of the Chung Hsing University. Column chromatography was performed using LiChroCART Si gel (5  $\mu\text{m}$ ) and Sephadex<sup>TM</sup> LH-20. HPLC details: detector: IOTA 2, pump: P230 HPLC PUMP, column: Phenomenex  $250 \times 10.00\text{ mm}$  5 micron. TLC analysis was carried out using aluminum pre-coated Si plates and the compounds were visualized using a UV lamp at  $\lambda = 254\text{ nm}$  and  $\lambda = 365\text{ nm}$ .

**Cell viability:** Cells ( $2 \times 10^5$ ) were cultured in a 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with compounds **1-17** in the presence of 100 ng/mL LPS (lipopolysaccharide) for 24 h. After that, the cells were washed twice with DPBS and incubated with 100  $\mu\text{L}$  of 0.5 mg/mL MTT for 2 h at  $37^\circ\text{C}$ . The medium was then discarded and 100  $\mu\text{L}$  dimethyl sulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Measurement of nitric oxide/nitrite:** NO production was indirectly assessed by measuring the nitrite levels in the cultured media and serum determined by a colorimetric method based on the Griess reaction. The cells were incubated with compounds **1-17** (2.5, 5.0, 10.0 and 20  $\mu\text{g/mL}$ ) in the presence of LPS (100 ng/mL) at  $37^\circ\text{C}$  for 24 h. Then, cells were dispensed into 96-well plates, and 100  $\mu\text{L}$  of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 5% phosphoric acid) and incubated at room temperature for 10

min; the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices). Serum samples were diluted 4 times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at  $10,000 \times g$  for 5 min at room temperature, 100  $\mu$ L supernatant was applied to a microtiter plate well, followed by 100  $\mu$ L of Griess reagent. After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a Micro-Reader. By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured from the absorbance at 540 nm.

#### 5-(4-Hydroxy -3-methoxy-1-butylny)-2,2'-bithiophene (1)

Yellow oil

MP: 101-103°C.

$[\alpha]_D^{25}$ : +0.78 ( $c$  0.23,  $\text{CHCl}_3$ ).

IR (KBr): 3431, 2214, 802, 696  $\text{cm}^{-1}$ .

UV  $\lambda_{\text{max}}$  (MeOH) nm ( $\log \epsilon$ ): 234 (3.80), 280 (4.52), 324 (3.45).

$^1\text{H}$  and  $^{13}\text{C}$  NMR: Table 1.

HR-ESI-MS:  $m/z$  262.9758  $[\text{M}-2\text{H}]^+$ , calcd for  $\text{C}_{13}\text{H}_{12}\text{O}_2\text{S}_2$ .

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## Cyclic Lipopeptides with Herbicidal and Insecticidal Activities Produced by *Bacillus clausii* DTM1

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Seven cyclic lipopeptide biosurfactants (1-7) were isolated for the first time from the fermentation broth of endophytic *Bacillus clausii* DTM1 and were identified as anteisoC<sub>13</sub>[Val7] surfactin-(L-Glu)-O-methyl-ester (1), anteisoC<sub>12</sub>[Val7] surfactin (2), anteisoC<sub>15</sub>[Val7] surfactin (3), isoC<sub>14</sub>[Leu7] surfactin (4), anteisoC<sub>12</sub>[Leu7] surfactin (5), nC<sub>13</sub>[Leu7] surfactin (6), and anteisoC<sub>14</sub>[Leu7] surfactin-(L-Glu)-O-methyl-ester (7); 1 has not been isolated before as a natural product from any source. Plate-based herbicide and insecticide bioassays showed that all compounds exhibited interesting insecticidal and herbicidal activities.

**Keywords:** *Bacillus clausii*, Surfactin, Insecticidal, Herbicidal.

The secondary metabolites of *Bacillus* species are known for their ability to control plant diseases through various mechanisms [1]. It is also documented that *Bacillus* species have the potential to synthesize a wide variety of metabolites with antibacterial and/or antifungal activity, which is one determinant of their ability to control plant diseases when applied as a biological control agent [1-4]. For instance, *B. subtilis*, the most studied species, is known to have strong antifungal activity by producing fungicidal and fungistatic peptides [3]. However, to the best of our knowledge, there have been limited studies on the activity of these peptides for controlling insects and weeds [5-7]. *B. clausii*, as one of the *Bacillus* species, has rarely been studied.

In this study, seven cyclic lipopeptides were isolated from the fermentation broth of *B. clausii* DTM1, and were identified as surfactin isomers. Of these seven, compound 1 was isolated as a natural product for the first time (although it had been prepared before by esterification of valine-7 surfactin isomer with a chain length of 13 carbons, but its spectral data have not been previously reported [8]). The complete assignments of <sup>1</sup>H and <sup>13</sup>C NMR spectral data have been achieved by various 2D NMR experiments including <sup>1</sup>H-<sup>1</sup>H COSY, HSQC, HMBC and ROESY.

Compound 1 was obtained as a white gum. Its molecular formula was assigned as C<sub>51</sub>H<sub>89</sub>N<sub>7</sub>NaO<sub>13</sub> on the basis of the HRESIMS. In the <sup>1</sup>H NMR spectrum, seven NH and seven α-H signals showed the presence of seven amino acid units. A strong signal at δ1.21 indicated the presence of a fatty acid chain. The <sup>13</sup>C NMR spectrum showed 10 ester/amide carbonyl signals and 7α-C signals. Amino acid units were identified as glutamic acid (Glu×1), aspartic acid (Asp×1), valine (Val×2) and leucine (Leu×3) on the basis of <sup>1</sup>H-<sup>1</sup>H COSY analysis. The HMBC correlation between the signals at δ3.57 (the <sup>1</sup>H signal of OCH<sub>3</sub>) and 173.8 (the residual CO of Glu1) established that the carboxyl group of Glu 1 was esterified. The substitution type of the branching methyl in the hydroxyl fatty acid chain was identified as anteiso by a <sup>13</sup>C NMR experiment using the method described by Lin *et al.* [9]. The sequence of the amino acid units and the fatty acid unit were assigned by a ROSEY experiment.

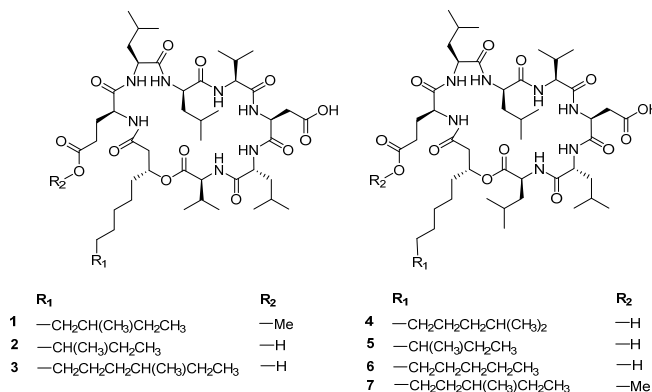


Figure 1: Structures of compounds 1-7.

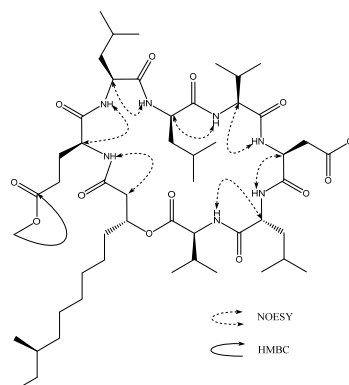


Figure2: Key HMBC and NOESY of compound 1.

Thus, the primary structure was established. The configurations of the amino acid units were confirmed as L-Glu (×1), L-Val (×2), L-Leu (×1), L-Asp (×1), and D-Leu (×2) by Marfey's reaction [10], and assigned as L-, L-, D-, L-, L-, D-, and L-, from N- to C-terminal according to previous biogenesis research. The absolute stereochemistry of the β-hydroxyl function of the fatty acid chain

**Table 1:**  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectral data of compound **1**<sup>a</sup>.

Name	Position	$\delta\text{H}$	$\delta\text{C}$	Name	Position	$\delta\text{H}$	$\delta\text{C}$
Glu1	NH	7.82 (d, 6.6)		$\alpha\text{-C}$	4.55(m)		49.6
	C=O		170.71	$\beta\text{-C}$	2.73(dd, 5.10, 16.6)	2.58(dd, 8.8, 16.6)	35.7
	$\alpha\text{-C}$	4.17(m)	52.39	$\delta\text{C=O}$			171.6
	$\beta\text{-C}$	1.79(m)/1.93(m)	27.1	Leu6	NH	7.62(d, 8.5)	
	$\gamma\text{-C}$	2.24(m)	29.7		C=O		171.5
	$\delta\text{-C=O}$		173.8		$\alpha\text{-C}$	4.51(m)	50.7
	$\delta\text{-OCH}_3$	3.57	51.3		$\beta\text{-C}$	1.52(m)	41.1
Leu2	NH	7.99(d, 5.7)			$\gamma\text{-C}$	1.52(m)	24.2
	C=O		172.8		$\delta\text{-C}$	0.84(m)	21.6/22.0
	$\alpha\text{-C}$	4.17(m)	51.9	Val7	NH	8.23(d, 8.2)	
	$\beta\text{-C}$	1.52(m)	39.4		C=O		171.7
	$\gamma\text{-C}$	1.52(m)	24.2		$\alpha\text{-C}$	4.09(m)	50.7
	$\delta\text{-C}$	0.84(m)	22.9/22.8		$\beta\text{-C}$	2.07(m)	29.4
Leu3	NH	8.29(d, 7.1)			$\gamma\text{-C}$	0.84(m)	17.8/19.0
	C=O		172.60	Fatty acid 1			169.9
	$\alpha\text{-C}$	4.17(m)	51.8	2	2.48(m)/2.33(m)		41.6
	$\beta\text{-C}$	1.52(m)	39.4	3	5.06(m)		71.4
	$\gamma\text{-C}$	1.52(m)	24.2	4	1.60(m)		33.5
	$\delta\text{-C}$	0.84(m)	22.8/22.6	5	1.22(m)		24.3
Val4	NH	7.74(d, 8.3)		6-8	1.21(m)		28.6-29.0
	C=O		171.0	9	1.05(m)/1.25(m)		35.9
	$\alpha\text{-C}$	4.02(m)	58.6	10	1.28(m)		33.6
	$\beta\text{-C}$	1.99(m)	30.1	11	1.21(m)		26.3
	$\gamma\text{-C}$	0.84(m)	17.8/19.0	12	0.82(m)		11.1
Asp5	NH	8.15(d, 7.4)		13	0.83(m)		19.0
	C=O		169.9				

<sup>a</sup>Measured in DMSO-*d*<sub>6</sub> at 400MHz for  $^1\text{H}$  NMR and at 100 MHz for  $^{13}\text{C}$  NMR.

was (**R**) according to previous biogenesis research [11] and confirmed by comparing the specific rotation with known compounds [12]. The structure of **1** was determined to be a methylated product of [Val7] surfactin isomer with a chain length of 13 carbons.

The structures of compounds **2-7** were identified by comparison of their spectral data with those described in the literature [12, 13].

The bioassay, arranged by Sygenta, has been described in earlier work [14, 15] and the results are given in Table 2. All the compounds showed activity against aphid species. It has been speculated that surfactin isomers interact with cuticle lipid molecules of aphids and induce cuticle membrane perturbation [5]. Compound **6** was also active against *Plutella xylostella*. Compounds **2-6** were active on *Poa annua*, and this is the first report of surfactin's activity against this plant species. Compounds **1** and **7** showed weaker activity against *P. annua*, which may be due to the methyl esterification of [Glu-1].

Due to the development of resistant mutants and new physiological races of pests and pathogens, many synthetic pesticides are gradually becoming ineffective. This creates a need to find alternative ways to control farm pests and pathogens. Natural products such as surfactins, which have activity against not only plant pathogens but also pests and weeds, make them an ideal biocontrol agent. *B. clausii* might be a valuable source which could be commercialized as mentioned above.

**Table 2:** Herbicidal and insecticidal activities of compounds **1-7**.

Compd.	Herbicidal activity		Insecticidal activity		
	<i>Poa annua</i>	<i>Arabidopsis thaliana</i>	Aphid species	<i>Plutella xylostella</i>	<i>Diabrotica balteata</i>
<b>1</b>	49 <sup>a</sup>	0	99	0	0
<b>2</b>	99	0	99	0	0
<b>3</b>	99	0	99	0	0
<b>4</b>	99	0	99	0	0
<b>5</b>	99	0	99	0	0
<b>6</b>	99	0	99	99	0
<b>7</b>	49	0	99	0	0

a. Data are presented as the means of the assessment scores across two replicates for the herbicide assays and three replicates for the insecticide assays.

## Experimental

**General:** Perkin-Elmer-241 polarimeter; Perkin-Elmer Lambda 35 UV-VIS spectrophotometer; Perkin-Elmer one FT-IR spectrometer (KBr); Bruker-Ascend-400 MHz instrument at 300 K, with TMS as

internal standard; preparative HPLC utilized a Waters equipped with a Kromasil RP-C18 column (10 × 250 mm, ID × L) and a UV detector; columns were of either silica gel (300-400 mesh) or Sephadex LH-20; all the solvents used were of analytical grade.

**Material and cultivation of *Bacillus* strain:** The bacterial strain was isolated using PDA from the plant *Dracocephalum tanguticum* Maxim., and was identified as *Bacillus clausii* by characterization and complete 16S rRNA gene sequence. The strain (Genbank accession number was KR632489) has been preserved at Chengdu Institute of Biology, Chinese Academy of Sciences, China. This bacterium was cultivated on a 30 L scale using 1L Erlenmeyer flasks containing 400 mL of the seed PDA medium for 3 days and fermentation medium (soluble starch 0.8%, peptone 0.5%, NaCl 0.2%, CaCO<sub>3</sub> 0.2%, MgSO<sub>4</sub>·7 H<sub>2</sub>O 0.05%, K<sub>2</sub>HPO<sub>4</sub> 0.05%) for 14 days at 28°C on a rotary shaker (250rpm).

**Extraction and isolation of compounds:** The fermentation broth (30 L) of *B. clausii* was filtered. The filtrate was extracted with EtOAc and the solvent was removed under vacuum. The EtOAc residue (18 g) was separated into 4 fractions by CC on silica gel (300-400 mesh), eluting stepwise with a CHCl<sub>3</sub>/MeOH gradient (CHCl<sub>3</sub>, CHCl<sub>3</sub>/MeOH: 10:1, v/v, CHCl<sub>3</sub>/MeOH: 3:1, v/v, MeOH). The fourth fraction (eluted with MeOH) was separated by Sephadex LH-20 (CHCl<sub>3</sub>/MeOH: 1:1, v/v) to afford 3 fractions (Fr.1-Fr.3). Fr.1 was further purified on a Waters preparative HPLC equipped with a Kromasil RP-C18 column (10 × 250 mm, ID × L; MeOH/H<sub>2</sub>O: 90:10, v/v) to afford **1** (8.3 mg), **2** (12.5 mg), **3** (82.6 mg), **4** (53.3 mg), **5** (34.7 mg), **6** (28.4 mg) and **7** (18.5 mg).

**Herbicide assays:** The compounds were tested for herbicidal activity against *Arabidopsis thaliana* at 10 ppm and *Poa annua* at 32 ppm. Test plates were stored for 7 days in a controlled environment cabinet. They were scored as 0 or 99, where 99 = herbicidal effect, and 0 = no effect.

**Insecticide assays:** The compounds were tested for activity against an aphid species at 1000 ppm on a leaf-piece based assay, and against *Plutella xylostella* and *Diabrotica balteata* at 500 ppm in artificial diet assays. Compounds were applied to feeding aphids, or prior to infestation with *P. xylostella* and *D. balteata* larvae.

Positive control compounds were included in each test: thiamethoxam and indoxacarb for insecticide assays and norflurazon for herbicide assays (S21 and S22 in supplementary information). Test plates were stored in a controlled environment cabinet for 6 days for the aphid species, 5 days for *D. balteata* and 9 days for *P. xylostella*. Mortality was assessed relative to control wells using a 2 band system (0 or 99 where 99 = significant mortality (≥ 70%), 0 = mortality not significant/no effect (≤ 70%)).

## Compound **1**

MP: 137-139°C.

[ $\alpha$ ]<sub>D</sub><sup>20</sup>: -14 (c 1.00, MeOH).

IR (KBr): 3421, 2957, 2927, 1657, 1534, 1205, 1018, 592 cm<sup>-1</sup>.

UV/Vis  $\lambda_{\text{max}}$  (MeOH) nm (log  $\epsilon$ ): 206 (1.38).

$^1\text{H}$  NMR and  $^{13}\text{C}$  NMR: Table 1.

HR-ESI-MS *m/z*: 1030.6359 (calcd. 1030.6411 for [C<sub>51</sub>H<sub>89</sub>N<sub>7</sub>NaO<sub>13</sub>]<sup>+</sup>)

**Appendix** - Supplementary data associated with this article including UV, IR, HR-ESIMS,  $^1\text{H}$ ,  $^{13}\text{C}$  NMR spectra and Positive control data can be found on line.

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# Synthesis of (6*R*,12*R*)-6,12-Dimethylpentadecan-2-one, the Female-Produced Sex Pheromone from Banded Cucumber Beetle *Diabrotica balteata*, Based on a Chiron Approach

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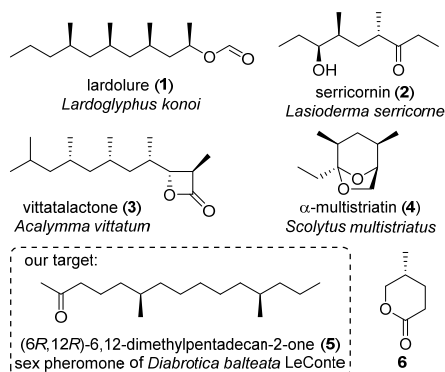
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Herein we describe a synthesis of (6*R*,12*R*)-6,12-dimethylpentadecan-2-one (**5**), the female produced sex pheromone of banded cucumber beetle *Diabrotica balteata* Le Conte, from (*R*)-4-methyl- $\delta$ -valerolactone, a methyl-branched chiron.

**Keywords:** Sex pheromone, (6*R*,12*R*)-6,12-dimethylpentadecan-2-one, Methyl-branched chiron, (*R*)-4-Methyl- $\delta$ -valerolactone, Synthesis.

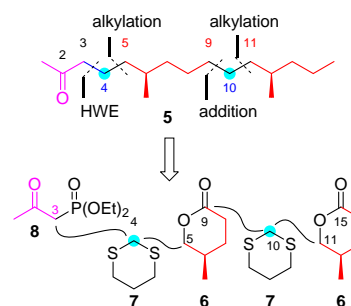
Pheromones are species-specific biofunctional molecules that are used for communication between individuals within the same species, and thus are useful tools for pest control. Among various pheromones, those with one or more chiral methyl-branched units [1]—such as compounds **1–5**, Figure 1—attract us most, for we have developed several methyl chirons from industrial waste and been interested in applying them to the syntheses of natural products [2]. Herein we report a synthesis of (6*R*,12*R*)-6,12-dimethylpentadecan-2-one (**5**) from (*R*)-4-methyl- $\delta$ -valerolactone (6).



**Figure 1:** Chemical structures of chiral methyl-branched pheromones **1–5**.

Ketone **5** is the female produced sex pheromone of banded cucumber beetle (BCB) *Diabrotica balteata* Le Conte, the larvae of which are serious pests of a variety of agricultural crops such as cabbages and sweet potatoes. The structure of **5** was elucidated by spectroscopic analyses and confirmed by synthesis [3]. The stereochemistry was provided by Mori's synthesis of all the four possible isomers and further affirmed by two other stereoselective syntheses [4].

Our synthetic plan is depicted in Scheme 1. Both lactone **6** and 1,3-dithiane **7** were to be used twice. The former was to form the C5–C9 and C11–C15 units in **5**, and the latter to act as linking point for fragments **6** and diethyl (2-oxopropyl)phosphonate (**8**).

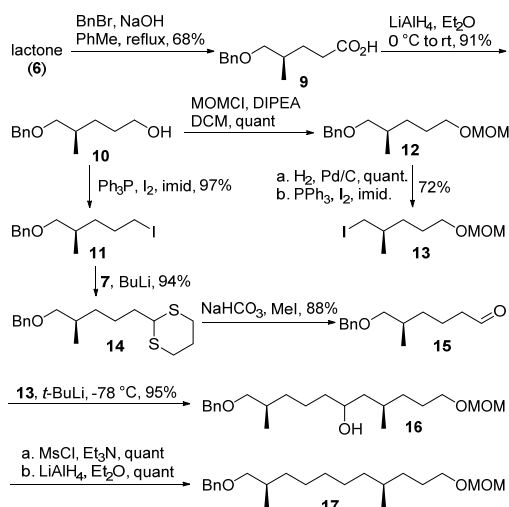


**Scheme 1:** Synthetic plan for (6*R*,12*R*)-6,12-dimethylpentadecan-2-one (**5**).

As shown in Scheme 2, our synthesis commenced with opening the lactone ring of **6**. Treatment of **6** with NaOH and BnBr in refluxing toluene successfully delivered acid **9** in 68% yield on a 0.2 mol scale. After reducing the acid with LiAlH<sub>4</sub>, removal of the exposed hydroxyl group in resultant **10** was investigated and found to be low-yielding due to the low boiling point of the product (*ca* 142°C). Therefore we decided to postpone this step to a later stage of the synthesis. Compound **10** was first protected as the MOM ether, then the benzyl group was removed by catalytic hydrogenation and the newly exposed OH was converted to iodide, giving **13** in 72% overall yield. In another direction, **10** was directly converted to iodide **11**, which was then treated with lithio dithiane to afford **14** in high yield.

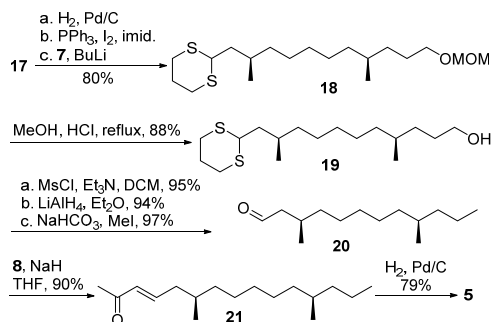
With **13** and **14** in hand, we first tried to couple them directly by preparing the anion of **14** and then reacting with **13**, but found this reaction to be low-yielding and irreproducible. Therefore, dithiane **14** was unmasked (NaHCO<sub>3</sub>, MeI, MeCN, water) [5] and the resultant aldehyde **15** was coupled with the lithio derivative of **13** [6] to give **16** in high yield as a mixture of epimers. The unneeded hydroxyl group on **16** was removed by transforming to mesylate and reducing it with LiAlH<sub>4</sub> [7], affording **17** in nearly quantitative yield.

Then the benzyl ether of **17** was cleaved and the exposed hydroxyl group was transformed to iodide and coupled with 1,3-dithiane anion again to deliver **18** in 80% overall yield. The unneeded hydroxyl group at the right side was again removed in good yield



Scheme 2: Synthesis of 17.

via cleaving the MOM ether with HCl, preparing the corresponding mesylate of **19**, and reducing it with LiAlH<sub>4</sub>. The dithiane moiety was unmasked to give aldehyde **20** in high yield and then underwent Horner–Wadsworth–Emmons olefination with **8** to give enone **21**, which was subjected to catalytic hydrogenation to deliver **5** in 79% yield. The spectroscopic properties of the synthetic **5** are consistent with those reported ( $[\alpha]_D^{24}$ :  $-0.395$  ( $c$  0.41, CHCl<sub>3</sub>); reported in reference 4d:  $[\alpha]_D^{23}$ :  $-0.40$  ( $c$  0.40, CHCl<sub>3</sub>)).

Scheme 3: Completion of the synthesis of (6*R*,12*R*)-6,12-dimethylpentadecan-2-one (**5**)

In conclusion, we have achieved a synthesis of (6*R*,12*R*)-6,12-dimethylpentadecan-2-one (**5**), the female produced sex pheromone of BCB *Diabrotica balteata* Le Conte, in 17 steps with an overall yield of 20% from (*R*)-4-methyl- $\delta$ -valerolactone (**6**). Although the synthesis is relatively longer than previous ones, the reactions used herein are simpler and easier to handle.

## Experimental

**General:** All reactions sensitive to air or moisture were performed in flame-dried round bottom flasks with rubber septum under a positive pressure of either argon or nitrogen atmosphere, unless otherwise noted. Air and moisture-sensitive liquids and solutions were transferred via syringe and stainless steel cannula. Reactions were monitored by thin-layer chromatography (TLC) carried out on silica gel plates using UV light as visualizing agent and an ethanolic solution of phosphomolybdic acid, and heat as developing agents. NMR spectra were recorded on a Bruker AMX-300 instrument and calibrated using TMS or residual undeuterated solvent as an internal reference [<sup>1</sup>H NMR: TMS (0.00); <sup>13</sup>C NMR: CDCl<sub>3</sub> (77.16)]. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, br = broad.

**(*R*)-5-(Benzyloxy)-4-methylpentanoic acid (**9**):** To a solution of lactone **6** (23.0 g, 0.20 mol) in toluene (400 mL) were added NaOH (24 g, 0.60 mol) and BnBr (37.6 g, 0.22 mol). The flask was equipped with a Dean–Stark trap, heated to reflux for 20 h, cooled, added water (200 mL), and acidified with HCl aqueous solution. The mixture was separated and the aqueous layer was extracted with diethylether (150 mL×2). The combined organic layers were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. Purification via flash column chromatography on silica gel (PE/EA: 3/1) afforded acid **10** (30.4 g, 68%) as a colorless liquid.

$[\alpha]_D^{27}$ :  $+1.68$  ( $c$  1.26, CHCl<sub>3</sub>).

IR (KBr): 3033, 2962, 1708, 1496, 1454, 1276, 1100, 938, 738, 714 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 7.26–7.35 (m, 5H), 4.50 (s, 2H), 3.31 (d, 2H,  $J$  = 5.7 Hz), 2.36–2.43 (m, 2H), 1.78–1.88 (m, 2H), 1.47–1.55 (m, 1H), 0.95 (d, 3H,  $J$  = 5.7 Hz).

MS (EI, 70 eV):  $m/z$  (%) = 222 [ $M^+$ ] (100).

Anal. Calcd for C<sub>13</sub>H<sub>18</sub>O<sub>3</sub>: C, 70.24; H, 8.16. Found C, 70.28; H, 8.27.

**(*R*)-5-(Benzyloxy)-4-methylpentan-1-ol (**10**):** To a suspension of LiAlH<sub>4</sub> (1.23 g, 32 mmol) in dry diethylether (50 mL) was added dropwise a solution of acid **9** (6.0 g, 27 mmol) in diethylether (20 mL) at 0°C. The mixture was then stirred at room temperature and quenched with Na<sub>2</sub>SO<sub>4</sub>·10H<sub>2</sub>O after which TLC showed complete consumption of **9**. The mixture was filtered and washed with diethylether. The filtrate was concentrated and purified via flash column chromatography on silica gel (PE/EA: 5/1) to give **10** (5.09 g, 91%) as a colorless liquid.

$[\alpha]_D^{28}$ :  $+2.03$  ( $c$  1.26, CHCl<sub>3</sub>).

IR (KBr): 2934, 2864, 1497, 1454, 1364, 1099, 1073, 1029, 736, 698 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 7.26–7.35 (m, 5H), 4.50 (s, 2H), 3.60–3.64 (m, 2H), 3.25–3.35 (m, 2H), 2.27 (bs, 1H), 1.47–1.81 (m, 4H), 1.16–1.21 (m, 1H), 0.93 (d, 3H,  $J$  = 6.6 Hz).

MS (EI, 70 eV):  $m/z$  (%) = 208 [ $M^+$ ] (100).

Anal. Calcd for C<sub>13</sub>H<sub>20</sub>O<sub>2</sub>: C, 74.96; H, 9.68. Found C, 74.92; H, 9.73.

**(2*R*)-1-Benzyloxy-5-iodo-2-methylpentane (**11**):** To a solution of PPh<sub>3</sub> (393 mg, 1.5 mmol) and imidazole (204 mg, 3.0 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (15 mL) was added I<sub>2</sub> (381 mg, 1.5 mmol) at 0°C. The mixture was stirred for 30 min before a solution of **10** (104 mg, 0.50 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (2 mL) was added. The mixture was stirred at ambient temperature and quenched with water after TLC showed complete consumption of **10**. The mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (50 mL), washed with a saturated solution of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (20 mL) and brine (20 mL) sequentially, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 80/1) afforded **11** (154 mg, 97%) as a colorless liquid.

$[\alpha]_D^{28}$ :  $+4.55$  ( $c$  1.03, CHCl<sub>3</sub>).

IR (KBr): 3064, 3030, 2957, 2854, 1496, 1454, 1363, 1222, 1179, 1099, 1029, 843, 736, 697, 611, 415 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 7.25–7.38 (m, 5H), 4.49 (s, 2H), 3.23–3.32 (m, 2H), 3.12–3.18 (m, 2H), 1.75–1.88 (m, 3H), 1.49–1.57 (m, 1H), 1.21–1.29 (m, 1H), 0.92 (d, 3H,  $J$  = 6.9 Hz).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 138.52, 128.24, 127.43, 127.39, 75.44, 72.90, 34.57, 32.69, 31.09, 17.05, 7.21.

HRMS-EI:  $m/z$  [ $M^+$ ] calcd for C<sub>13</sub>H<sub>19</sub>OI: 318.0481; found: 318.0480.

**(2*R*)-1-Benzyloxy-5-methoxymethoxy-2-methylpentane (**12**):** To a solution of **10** (15.0 g, 72 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (150 mL) were added *i*Pr<sub>2</sub>NEt (23.9 mL, 144 mmol) and MOMCl (11.3 mL, 144

mmol) at 0°C. The mixture was stirred at ambient temperature and quenched with water after TLC showed that **10** was completely consumed. The mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub>, separated, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 30/1) afforded **12** (18.8 g, quantitative) as a colorless liquid.

$[\alpha]_D^{24}$ : +0.24 (*c* 0.80, CHCl<sub>3</sub>).

IR (KBr): 2932, 2876, 1497, 1454, 1207, 1152, 1110, 1048, 919, 737, 698, 415, 410, 402 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 7.26-7.39 (m, 5H), 4.62 (s, 2H), 4.51 (s, 2H), 3.24-3.61 (m, 7H), 1.48-1.83 (m, 4H), 1.19-1.27 (m, 1H), 0.97 (d, 3H, *J* = 6.9 Hz).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 138.71, 128.24, 127.35, 96.33, 75.63, 72.91, 68.01, 65.80, 55.01, 33.30, 30.10, 27.15, 17.03.

MS (EI, 70 eV): *m/z* (%) = 252 [M<sup>+</sup>] (100).

HRMS-EI: *m/z* [M+Na<sup>+</sup>] calcd for C<sub>15</sub>H<sub>24</sub>O<sub>3</sub>: 275.1623; found: 275.1625.

**(*R*)-5-(Methoxymethoxy)-2-methylpentan-1-ol**: A reaction flask containing **12** (18.6 g, 74 mmol), Pd/C (5%, 3.0 g), and MeOH (150 mL) was evacuated and back-filled with hydrogen (1 atm). The reaction mixture was stirred at ambient temperature under hydrogen for 3 days and then filtered over a plug of silica gel topped with Celite (MeOH eluent). The filtrate was concentrated and purified by flash column chromatography on silica gel (PE/EA: 10/1) to give the title compound (11.3 g, quantitative) as a colorless liquid.

$[\alpha]_D^{24}$ : +10.5 (*c* 1.04, CHCl<sub>3</sub>).

IR (KBr): 2935, 2878, 1466, 1386, 1216, 1153, 1111, 1046, 920, 414, 402 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 4.62 (s, 2H), 3.38-3.55 (m, 4H), 3.36 (s, 3H), 2.71 (t, 1H, *J* = 5.4 Hz), 1.43-1.72 (m, 4H), 1.11-1.23 (m, 1H), 0.92 (d, 3H, *J* = 6.6 Hz).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 96.15, 67.92, 67.61, 54.90, 35.37, 29.43, 26.92, 16.40.

MS (ESI): *m/z* = 185 [M+Na<sup>+</sup>].

**(*R*)-1-Iodo-5-(methoxymethoxy)-2-methylpentane (13)**: To a solution of PPh<sub>3</sub> (19.65 g, 75 mmol) and imidazole (10.2 g, 75 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (100 mL) was added I<sub>2</sub> (19.1 g, 75 mmol) at 0°C. The mixture was stirred for 20 min before a solution of (*R*)-5-(methoxymethoxy)-2-methylpentan-1-ol (4.0 g, 24 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (15 mL) was added. The mixture was stirred at ambient temperature and quenched with water after TLC showed complete consumption of the starting material. The mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (150 mL), washed with a saturated solution of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (100 mL) and brine sequentially, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 20/1) afforded **13** (4.9 g, 72%) as a colorless liquid.

$[\alpha]_D^{24}$ : +0.18 (*c* 1.82, CHCl<sub>3</sub>).

IR (KBr): 2931, 2882, 1458, 1379, 1196, 1145, 1111, 1044, 919, 415, 409, 402 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 4.56 (s, 2H), 3.45-3.50 (m, 2H), 3.31 (s, 3H), 3.08-3.20 (m, 2H), 1.36-1.58 (m, 4H), 1.18-1.130 (m, 1H), 0.94 (d, 3H, *J* = 6.6 Hz).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 96.39, 67.64, 55.14, 36.18, 34.55, 27.13, 20.49, 17.40.

MS (EI, 70 eV): *m/z* (%) = 211 [M-C<sub>2</sub>H<sub>5</sub>O<sub>2</sub>]<sup>+</sup> (100).

**(*R*)-2-(5-(Benzyloxy)-4-methylpentyl)-1,3-dithiane (14)**: To a solution of 1,3-dithiane (**7**) (151 mg, 1.26 mmol) in anhydrous THF (8 mL) at 0°C under argon was added a solution of *n*-BuLi (1.5 M in pentane, 0.86 mL, 1.38 mmol). The mixture was stirred for 30 min and added to a solution of **11** (200 mg, 0.63 mmol) in THF (3.0 mL)

and warmed to room temperature. After **11** was fully consumed, the reaction was quenched with water, extracted with EtOAc (30 mL × 3), the extracts combined, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 50/1) afforded **14** (184 mg, 94%) as a colorless liquid.

$[\alpha]_D^{24}$ : +0.89 (*c* 0.92, CHCl<sub>3</sub>).

IR (KBr): 3030, 2934, 2901, 2856, 1496, 1454, 1423, 1363, 1276, 1180, 1100, 1028, 909, 845, 737, 698, 414 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 7.22-7.34 (m, 5H), 4.48 (s, 2H), 4.03 (t, 1H, *J* = 6.9 Hz), 3.19-3.31 (m, 2H), 2.72-2.89 (m, 4H), 2.05-2.17 (m, 1H), 1.68-1.92 (m, 4H), 1.40-1.61 (m, 3H), 1.04-1.19 (m, 1H), 0.92 (d, 3H, *J* = 6.3 Hz).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 138.60, 128.17, 127.37, 127.27, 75.60, 72.80, 47.44, 35.54, 33.15, 32.99, 30.33, 25.89, 23.87, 16.90.

MS (ESI): *m/z* = 349 [M+K<sup>+</sup>].

HRMS-MALDI: *m/z* [M+Na<sup>+</sup>] calcd for C<sub>17</sub>H<sub>26</sub>OS<sub>2</sub>: 333.1323; found: 333.1316.

**(*R*)-6-(Benzyloxy)-5-methylhexanal (15)**: To a solution of **14** (8.01 g, 25.8 mmol) in MeCN/water (80 mL/24 mL) were added MeI (16 mL, 258 mmol) and NaHCO<sub>3</sub> (21.7 g, 258 mmol) at room temperature. The mixture was stirred for 12 h, concentrated under reduced pressure, dissolved in water, extracted with EtOAc (100 mL × 3), the extracts combined, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 20/1) afforded **15** (4.98 g, 88%) as a colorless liquid.

$[\alpha]_D^{24}$ : +3.54 (*c* 1.08, CHCl<sub>3</sub>).

IR (KBr): 3035, 2856, 2721, 1725, 1496, 1455, 1363, 1100, 1029, 738, 698, 463, 451, 415 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 9.74 (t, 1H, *J* = 1.5 Hz), 7.25-7.35 (m, 5H), 4.49 (s, 2H), 3.23-3.33 (m, 2H), 2.39-2.44 (m, 2H), 1.44-1.81 (m, 4H), 1.14-1.18 (m, 1H), 0.93 (d, 3H, *J* = 6.6 Hz).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 202.60, 138.61, 128.26, 127.46, 127.40, 75.52, 72.96, 44.06, 33.31, 33.10, 19.47, 16.88.

MS (EI, 70 eV): *m/z* (%) = 221 [M+H<sup>+</sup>] (100).

HRMS-MALDI: *m/z* [M+Na<sup>+</sup>] calcd for C<sub>14</sub>H<sub>20</sub>O<sub>2</sub>: 243.1361; found: 243.1360.

**(2*R*,8*R*)-1-(Benzyloxy)-11-(methoxymethoxy)-2,8-dimethylundecan-6-ol (16)**: Under argon, to a solution of **13** (2.48 g, 9.10 mmol) in dry diethylether/pentane (20 mL/30 mL) was added a solution of *t*-BuLi (1.5 M in pentane, 12.7 mL, 19.1 mmol) at -78°C. The mixture was stirred at -78°C for 5 min and then at room temperature for 1 h. At -78°C, to the resultant solution was added a solution of **15** (800 mg, 3.64 mmol) in diethylether/pentane (8 mL/12 mL). The mixture was stirred at room temperature and quenched with water after **15** was consumed completely. The mixture was separated and the aqueous layer was extracted with diethylether (60 mL × 3). The combined organic layers were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 5/1) gave **16** (1.27 g, 95%) as an inseparable mixture of epimers.

IR (KBr): 3031, 2930, 2873, 1497, 1455, 1213, 1153, 1111, 1045, 919, 736, 698, 434, 416, 403 cm<sup>-1</sup>.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): 7.12-7.20 (m, 5H), 4.48 (s, 2H), 4.36 (s, 2H), 3.46-3.51 (m, 1H), 3.35-3.40 (m, 1H), 3.22 (s, 3H), 3.08-3.19 (m, 2H), 0.98-1.64 (m, 15H), 0.76-0.80 (m, 6H).

<sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): 138.74, 128.26, 127.49, 127.38, 96.37, 75.84, 72.92, 69.43, 55.12, 45.14, 38.61, 35.55, 35.41, 29.59, 28.42, 27.02, 22.91, 19.20, 17.05, 16.53.

HRMS-MALDI: *m/z* [M+Na<sup>+</sup>] calcd for C<sub>22</sub>H<sub>38</sub>O<sub>4</sub>: 389.2668; found: 389.2670.

**(2R,8R)-1-(Benzyloxy)-11-(methoxymethoxy)-2,8-dimethylundecan-6-yl methanesulfonate:** To a solution of **16** (290 mg, 0.79 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (5 mL) were added  $\text{MsCl}$  (0.10 mL, 1.2 mmol) and  $\text{Et}_3\text{N}$  (0.17 mL, 1.2 mmol) at  $0^\circ\text{C}$ . Then the mixture was stirred at room temperature for 3 h and diluted with  $\text{CH}_2\text{Cl}_2$  (50 mL). The solution was washed with water and brine sequentially, dried over  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 20/1) afforded the title compound (355 mg, inseparable mixture of epimers, 100%) as a colorless liquid.

$[\alpha]_{\text{D}}^{24}$ : +1.68 ( $c$  1.26,  $\text{CHCl}_3$ ).

IR (KBr): 3031, 2935, 2872, 1497, 1455, 1356, 1336, 1175, 1110, 1030, 971, 906, 792, 739, 699, 610, 528, 415, 409  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 7.26-7.39 (m, 5H), 4.79-4.82 (m, 1H), 4.61 (s, 2H), 4.49 (s, 2H), 3.49-3.56 (m, 2H), 3.35 (s, 3H), 3.24-3.30 (m, 2H), 2.98 (t, 3H,  $J = 2.1$  Hz), 1.14-1.80 (m, 14H), 0.92-0.98 (m, 6H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 138.67, 128.27, 127.48, 127.41, 96.36, 82.42, 82.35, 75.41, 72.96, 67.89, 67.86, 65.48, 65.28, 55.14, 55.08, 41.82, 41.72, 38.72, 35.55, 34.90, 33.69, 33.39, 33.31, 33.05, 29.28, 28.92, 27.00, 26.95, 22.22, 19.69, 19.24, 17.00.

MS (EI, 70 eV):  $m/z$  (%) = 467 [ $\text{M} + \text{Na}^+$ ] (100).

**(2R,8S)-1-Benzyloxy-11-methoxymethoxy-2,8-dimethylundecane (17):** To a suspension of  $\text{LiAlH}_4$  (600 mg, 1.58 mmol) in dry diethylether (10 mL) was added dropwise a solution of the above prepared mesylate (350 mg, 0.79 mmol) in diethylether (5 mL) at  $0^\circ\text{C}$ . The mixture was then stirred at room temperature for 12 h and quenched with  $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$  after TLC showed complete consumption of **9**. The mixture was filtered and washed with diethylether. The filtrate was concentrated and purified via flash column chromatography on silica gel (with hexane) to give **17** (285 mg, 100%) as a colorless oil.

$[\alpha]_{\text{D}}^{24}$ : -1.20 ( $c$  0.82,  $\text{CHCl}_3$ ).

IR (KBr): 3032, 2957, 2928, 2856, 1497, 1455, 1378, 1261, 1208, 1111, 1047, 920, 801, 736, 697, 417, 404  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 7.25-7.38 (m, 5H), 4.62 (t, 2H,  $J = 1.5$  Hz), 4.50 (s, 2H), 3.48-3.55 (m, 2H), 3.37 (s, 3H), 3.20-3.35 (m, 2H), 1.72-1.76 (m, 1H), 1.54-1.64 (m, 2H), 1.08-1.19 (m, 13H), 0.85-0.91 (m, 6H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 138.84, 128.27, 127.48, 127.36, 96.38, 76.02, 72.93, 68.25, 55.07, 36.96, 33.66, 33.45, 33.39, 32.62, 30.25, 27.29, 27.01, 26.96, 19.59, 17.15.

MS (EI, 70 eV):  $m/z$  (%) = 305 [ $\text{M} - \text{C}_2\text{H}_5\text{O}^+$ ] (100)

HRMS-MALDI:  $m/z$  [ $\text{M} - \text{C}_2\text{H}_5\text{O}^+$ ] calcd for  $\text{C}_{22}\text{H}_{38}\text{O}_3$ : 305.2481; found: 305.2487.

**(2R,8S)-11-(Methoxymethoxy)-2,8-dimethylundecan-1-ol:** A reaction flask containing **17** (2.30 g, 6.57 mmol),  $\text{Pd/C}$  (10%, 300 mg), and  $\text{MeOH}$  (30 mL) was evacuated and back-filled with hydrogen (1 atm). The reaction mixture was stirred at ambient temperature under hydrogen for 24 h and then filtered over a plug of silica gel topped with Celite ( $\text{MeOH}$  eluent). The filtrate was concentrated and purified by flash column chromatography on silica gel (PE/EA: 5/1) to give the title compound (1.67 g, 98%) as a colorless liquid.

$[\alpha]_{\text{D}}^{28}$ : +6.54 ( $c$  0.99,  $\text{CHCl}_3$ ).

IR (KBr): 2928, 2857, 1465, 1379, 1214, 1153, 1112, 1046, 921, 418, 410  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 4.62 (d, 2H,  $J = 0.9$  Hz), 3.38-3.56 (m, 4H), 3.36 (s, 3H), 1.73-1.78 (m, 1H), 1.52-1.66 (m, 3H), 1.07-1.19 (m, 12H), 0.86-0.92 (m, 6H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 96.33, 68.30, 68.23, 66.07, 55.04, 36.90, 35.72, 33.33, 33.11, 32.56, 30.19, 27.25, 26.94, 19.56, 16.56.

MS (EI, 70 eV):  $m/z$  (%) = 215 [ $\text{M} - \text{C}_2\text{H}_5\text{O}^+$ ] (100).

HRMS-MALDI:  $m/z$  [ $\text{M} - \text{MeO}^+$ ] calcd for  $\text{C}_{15}\text{H}_{32}\text{O}_3$ : 229.2168; found: 229.2164.

**(2R,8S)-1-Iodo-11-(methoxymethoxy)-2,8-dimethylundecane:** To a solution of  $\text{PPh}_3$  (487 mg, 1.86 mmol) and imidazole (253 mg, 3.72 mmol) in dry  $\text{CH}_2\text{Cl}_2$  (10 mL) was added  $\text{I}_2$  (473 mg, 1.86 mmol) at  $0^\circ\text{C}$ . The mixture was stirred for 20 min before a solution of alcohol (160 mg, 0.62 mmol) in  $\text{CH}_2\text{Cl}_2$  (2 mL) was added. The mixture was stirred at ambient temperature and quenched with water after TLC showed complete consumption of the starting material. The mixture was diluted with  $\text{CH}_2\text{Cl}_2$  (40 mL), washed with a saturated solution of  $\text{Na}_2\text{S}_2\text{O}_3$  (20 mL) and brine sequentially, dried over  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 20/1) afforded iodide (210 mg, 92%) as a colorless liquid.

$[\alpha]_{\text{D}}^{24}$ : -2.76 ( $c$  0.77,  $\text{CHCl}_3$ ).

IR (KBr): 2928, 2855, 1460, 1378, 1195, 1153, 1112, 1046, 920, 417, 404  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 4.63 (s, 2H), 3.51 (d, 2H,  $J = 7.2$  Hz), 3.36 (s, 3H), 3.13-3.26 (m, 2H), 1.49-1.61 (m, 2H), 1.12-1.43 (m, 14H), 0.97 (d, 3H,  $J = 6.3$  Hz), 0.86 (d, 3H,  $J = 6.6$  Hz).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 96.37, 68.22, 66.04, 55.06, 36.88, 36.42, 34.71, 33.37, 32.58, 29.94, 27.28, 26.82, 20.59, 19.58, 17.95.

MS (EI, 70 eV):  $m/z$  (%) = 325 [ $\text{M} - \text{MOM}^+$ ] (100).

**2-((2R,8S)-11-(Methoxymethoxy)-2,8-dimethylundecyl)-1,3-dithiane (18):** To a solution of 1,3-dithiane **7** (2.27 g, 18.9 mmol) in anhydrous THF (40 mL) at  $0^\circ\text{C}$  under argon was added a solution of  $n\text{-BuLi}$  (2.5 M in hexane, 7.56 mL, 18.9 mmol). The mixture was stirred for 30 min and added to a solution of previously prepared iodide (1.40 g, 3.78 mmol) in THF (10 mL) and warmed to room temperature. After the starting material was fully consumed, the reaction was quenched with water, extracted with  $\text{EtOAc}$  (30 mL  $\times$  3), combined, washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 50/1) afforded **18** (1.22 g, 89%) as a pale yellow liquid.

$[\alpha]_{\text{D}}^{24}$ : -6.49 ( $c$  0.55,  $\text{CHCl}_3$ ).

IR (KBr): 2928, 2856, 1465, 1423, 1378, 1276, 1243, 1153, 1111, 1046, 919, 417, 404  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 4.63 (s, 2H), 4.08-4.13 (m, 1H), 3.51 (t, 2H,  $J = 6.9$  Hz), 3.37 (s, 3H), 2.78-2.92 (m, 4H), 2.08-2.17 (m, 1H), 1.43-1.92 (m, 6H), 1.11-1.39 (m, 13H), 0.85-0.92 (m, 6H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 96.36, 68.24, 55.06, 45.57, 42.55, 36.93, 36.75, 33.37, 32.60, 30.57, 30.36, 30.13, 29.57, 27.28, 26.99, 26.75, 26.12, 19.58, 19.39.

MS (EI, 70 eV):  $m/z$  (%) = 362 [ $\text{M}^+$ ] (100)

HRMS-EI:  $m/z$  [ $\text{M}^+$ ] calcd for  $\text{C}_{19}\text{H}_{38}\text{O}_2\text{S}_2$ : 362.2313; found: 362.2322.

**(4S,10R)-11-(1,3-Dithian-2-yl)-4,10-dimethylundecan-1-ol (19):** A solution of **18** (90 mg, 0.25 mmol) in acidified  $\text{MeOH}$  (5 mL, two drops of  $\text{HCl}$ ) was heated at reflux for 5 h and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (PE/EA: 10/1) to give **19** (70 mg, 88%) as a colorless liquid.

$[\alpha]_{\text{D}}^{24}$ : -9.59 ( $c$  0.45,  $\text{CHCl}_3$ ).

IR (KBr): 2927, 2855, 1461, 1423, 1378, 1276, 1057, 908, 411  $\text{cm}^{-1}$ .

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ): 4.05-4.10 (m, 1H), 3.60 (t, 2H,  $J = 6.6$  Hz), 2.74-2.88 (m, 4H), 2.07-2.13 (m, 1H), 1.07-1.83 (m, 20H), 0.80-0.92 (m, 6H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ): 63.37, 45.55, 42.51, 36.88, 36.72, 32.87, 32.55, 30.56, 30.34, 30.30, 30.07, 29.52, 26.93, 26.85, 26.71, 26.10, 19.60, 19.38.



MS (EI, 70 eV):  $m/z$  (%) = 318 [ $M^+$ ] (100).

HRMS-EI:  $m/z$  [ $M^+$ ] calcd for  $C_{17}H_{34}OS_2$ : 318.2051; found: 318.2053.

**(4*S*,10*R*)-11-(1,3-Dithian-2-yl)-4,10-dimethylundecyl methane-sulfonate:** To a solution of **19** (770 mg, 2.42 mmol) in dry  $CH_2Cl_2$  (30 mL) were added  $MsCl$  (0.28 mL, 3.63 mmol) and  $Et_3N$  (0.50 mL, 3.63 mmol) at 0°C. Then the mixture was stirred at room temperature for 5 h and diluted with  $CH_2Cl_2$  (40 mL). The solution was washed with water and brine sequentially, dried over  $Na_2SO_4$ , filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 10/1) afforded the title compound (910 mg, 95%) as a colorless liquid.

$[\alpha]_D^{24}$ : -5.81 ( $c$  0.43,  $CHCl_3$ ).

IR (KBr): 2928, 2854, 1465, 1423, 1356, 1276, 1176, 919, 823, 528, 410  $cm^{-1}$ .

$^1H$  NMR (300 MHz,  $CDCl_3$ ): 4.22 (t, 2H,  $J$  = 6.6 Hz), 4.08-4.13 (m, 1H), 3.02 (s, 3H), 2.79-2.92 (m, 4H), 2.09-2.18 (m, 1H), 1.63-1.88 (m, 5H), 1.12-52 (m, 14H), 0.83-0.92 (m, 6H).

$^{13}C$  NMR (75 MHz,  $CDCl_3$ ): 70.50, 45.54, 42.52, 37.34, 36.72, 35.93, 32.50, 32.32, 30.57, 30.35, 30.03, 29.93, 29.53, 29.31, 26.90, 26.72, 26.11, 19.43, 19.38, 19.24.

MS (EI, 70 eV):  $m/z$  (%) = 396 [ $M^+$ ] (100).

HRMS-EI:  $m/z$  [ $M^+$ ] calcd for  $C_{18}H_{36}O_3S_3$ : 396.1827; found: 396.1821.

**2-((2*R*,8*R*)-2,8-Dimethylundecyl)-1,3-dithiane:** To a suspension of  $LiAlH_4$  (239 mg, 6.3 mmol) in dry diethylether (30 mL) was added, dropwise, a solution of mesylate (830 mg, 2.1 mmol) in diethylether (10 mL) at 0°C. The mixture was then stirred at room temperature and quenched with  $Na_2SO_4 \cdot 10H_2O$  after TLC showed complete consumption of **9**. The mixture was filtered and washed with diethylether. The filtrate was concentrated and purified via flash column chromatography on silica gel (with hexane) to give the title compound (592 mg, 94%) as a colorless oil.

$[\alpha]_D^{24}$ : -9.10 ( $c$  0.63,  $CHCl_3$ ).

IR (KBr): 2956, 2927, 2855, 1465, 1423, 1378, 1275, 1243, 1185, 909, 418  $cm^{-1}$ .

$^1H$  NMR (300 MHz,  $CDCl_3$ ): 4.08-4.13 (m, 1H), 2.79-2.92 (m, 4H), 2.10-2.16 (m, 1H), 1.67-1.89 (m, 3H), 1.47-1.52 (m, 1H), 1.05-1.36 (m, 15H), 0.82-0.93 (m, 9H).

$^{13}C$  NMR (75 MHz,  $CDCl_3$ ): 45.59, 42.58, 39.39, 37.04, 36.78, 32.46, 30.59, 30.39, 30.18, 29.60, 27.03, 26.78, 26.15, 20.12, 19.66, 19.41, 14.40.

MS (EI, 70 eV):  $m/z$  (%) = 302 [ $M^+$ ] (100).

HRMS-EI:  $m/z$  [ $M^+$ ] calcd for  $C_{17}H_{34}S_2$ : 302.2102; found: 302.2099.

**(3*R*,9*R*)-3,9-Dimethyldodecanal (20):** To a solution of dithiane (44 mg, 0.15 mmol) in MeCN/water (3 mL/1 mL) were added  $MeI$  (0.19 mL, 3 mmol) and  $NaHCO_3$  (252 mg, 3 mmol) at room temperature. The mixture was stirred for 10 h, concentrated under

reduced pressure, dissolved in water, extracted with diethylether (20 mL  $\times$  3), the extracts combined, washed with brine, dried over  $Na_2SO_4$ , filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 10/1) afforded **20** (30 mg, 97%) as a colorless liquid.

$[\alpha]_D^{24}$ : +5.54 ( $c$  0.81,  $CHCl_3$ ).

IR (KBr): 2985, 2928, 2857, 2711, 1729, 1464, 1379, 1261, 1018, 804, 471, 430, 413  $cm^{-1}$ .

$^1H$  NMR (300 MHz,  $CDCl_3$ ): 9.75 (t, 1H,  $J$  = 2.1 Hz), 2.17-2.44 (m, 2H), 2.03-2.07 (m, 1H), 1.02-1.38 (m, 15H), 0.95 (d, 3H,  $J$  = 6.6 Hz), 0.82-0.89 (m, 6H).

$^{13}C$  NMR (75 MHz,  $CDCl_3$ ): 203.14, 51.09, 39.40, 37.03, 36.94, 32.46, 30.07, 28.20, 27.00, 20.13, 19.99, 19.65, 14.40.

MS (EI, 70 eV):  $m/z$  (%) = 212 [ $M^+$ ] (100).

HRMS-EI:  $m/z$  [ $M^+$ ] calcd for  $C_{14}H_{28}O$ : 212.2140; found: 212.2143.

**(6*R*,12*R*,*E*)-6,12-Dimethylpentadec-3-en-2-one (21):** To a suspension of  $NaH$  (60%, 9.0 mg, 0.22 mmol) in anhydrous THF (2 mL) was added a solution of phosphonate **8** (42 mg, 0.22 mmol) in THF (1 mL) at 0°C. The mixture was stirred for 10 min and added to a solution of aldehyde **20** (30 mg, 0.14 mmol) in THF (1 mL). The solution was stirred at room temperature for 6 h, quenched with water, extracted with  $EtOAc$  (20 mL  $\times$  3), combined, washed with brine, dried over  $Na_2SO_4$ , filtered, and concentrated under reduced pressure. Flash column chromatography on silica gel (PE/EA: 30/1) afforded enone **21** (32 mg, 90%) as a colorless oil.

$[\alpha]_D^{24}$ : -3.41 ( $c$  0.42,  $CHCl_3$ ).

IR (KBr): 2958, 2928, 2872, 2856, 1701, 1678, 1629, 1465, 1379, 1361, 1252, 1180, 980, 616, 543  $cm^{-1}$ .

$^1H$  NMR (300 MHz,  $CDCl_3$ ): 6.74-6.82 (m, 1H), 6.05 (d, 1H,  $J$  = 15.9 Hz), 1.98-2.23 (m, 5H), 1.03-1.35 (m, 16H), 0.80-0.89 (m, 9H).

$^{13}C$  NMR (75 MHz,  $CDCl_3$ ): 198.61, 147.52, 132.39, 39.98, 39.41, 37.05, 36.73, 32.64, 32.48, 30.17, 27.06, 26.89, 20.14, 19.66, 14.41.

MS (EI, 70 eV):  $m/z$  (%) = 253 [ $M+H^+$ ] (100).

**(6*R*,12*R*)-6,12-Dimethylpentadecan-2-one (5):** A reaction flask containing **21** (30 mg, 0.13 mmol),  $Pd/C$  (10%, 30 mg), and  $MeOH$  (5 mL) was evacuated and back-filled with  $H_2$  (1 atm). The reaction mixture was stirred at ambient temperature under hydrogen for 2 days and then filtered over a plug of silica gel topped with Celite ( $MeOH$  eluent). The filtrate was concentrated and purified by flash column chromatography on silica gel (PE/EA: 100/1) to give **5** (24 mg, 79%) as a colorless liquid.

$[\alpha]_D^{24}$ : -0.395 ( $c$  0.41,  $CHCl_3$ ).

IR (KBr): 2957, 2928, 2856, 1720, 1464, 1409, 1378, 1362, 1164, 726, 435, 419  $cm^{-1}$ .

$^1H$  NMR (300 MHz,  $CDCl_3$ ): 2.38 (t, 2H,  $J$  = 7.8 Hz), 2.12 (s, 3H), 1.43-1.62 (m, 2H), 1.01-1.40 (m, 18H), 0.80-0.89 (m, 9H).

$^{13}C$  NMR (75 MHz,  $CDCl_3$ ): 209.36, 44.14, 39.41, 37.08, 36.90, 36.64, 36.51, 32.65, 32.47, 30.33, 29.84, 29.48, 27.09, 27.06, 21.43, 20.13, 19.67, 19.54, 19.22, 14.40.

MS (EI, 70 eV):  $m/z$  (%) = 254 [ $M^+$ ] (100).

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## A Rapid Study of Botanical Drug–Drug Interaction with Protein by Re-ligand Fishing using Human Serum Albumin–Functionalized Magnetic Nanoparticles

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A great many active constituents of botanical drugs bind to human serum albumin (HSA) reversibly with a dynamic balance between the free- and bound-forms in blood. The curative or side effect of a drug depends on its free-form level, which is always influenced by other drugs, combined dosed or multi-constituents of botanical drugs. This paper presented a rapid and convenient methodology to investigate the drug-drug interactions with HSA. The interaction of two steroidal saponins, dioscin and *pseudo*-protodioscin, from a botanical drug was studied for their equilibrium time and equilibrium amount by re-ligand fishing using HSA functionalized magnetic nanoparticles. A clear competitive situation was obtained by this method. The equilibrium was reached soon about 15 s at a ratio of 0.44: 1. Furthermore, the interaction of *pseudo*-protodioscin to total steroidal saponins from DAXXX was also studied. The operation procedures of this method were faster and more convenient compared with other methods reported.

**Keywords:** Drug–drug interaction, Re-ligand fishing, Dioscin, *Pseudo*-protodioscin, Human serum albumin–functionalized magnetic nanoparticles.

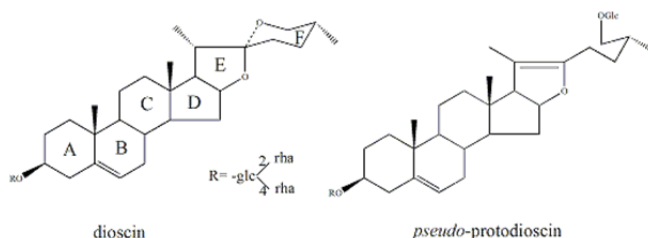
Botanical drugs are often mixtures of many active ingredients that could bind to human serum albumin (HSA) reversibly with a dynamic balance of the free- and bound-form in blood. The drug distribution of the free- or bound-form will probably be affected by the competitive binding with HSA, which is directly related to the curative or side effect. Therefore, it is of great importance to study botanical drug–drug interactions.

There has been much research on interactions between drugs and HSA using classical methods such as equilibrium dialysis [1], ultrafiltration [2], X-ray crystallography [3], and mass spectrometry [4], as well as some spectroscopic methods [5]. However, these methods are only suitable for pure compound study, not being able to detect multiple ingredients in a complex matrix. As for herb-drug interaction, the present studies concentrate mainly on the change in the curative effect of the drug, as well as the activity of CPY450 [6-8], while very few studies are involved in the interaction between each single component. Herein we demonstrate that re-ligand fishing could be applied to study the drug-drug interactions, which is based on ligand fishing experiments with HSA functionalized magnetic nanoparticles (HSA–MNPs) used in our previous research [9-12]. Ligand fishing based on receptor theory had been proposed as a new method for drug-protein interaction using a protein immobilized on the surface of MNPs for the isolation of ligands from a mixture of compound extracts [13]. Correspondingly, re-ligand fishing was defined as HSA–MNPs saturated binding with one drug in advance and then ligand fish other drugs in the second ligand fishing experiment.

“Di Ao Xin Xue Kang” capsule (DAXXX), manufactured from the total steroid saponins extract of *Dioscorea nipponica* and *D. panthaica*, is a popular botanical medicine in China for prevention and treatment of coronary heart disease by inhibiting platelet aggregation [14]. It is an interesting phenomenon that a single saponin either inhibits or induces platelet aggregation [15, 16], while the total steroid saponins has an obvious platelet aggregation

inhibition activity due to multicomponent effective ingredients synergy and/or antagonistic action [17, 18]. There has been some research on the interaction between certain proteins with saponins from *D. nipponica* [19-21]. However, the interactions with HSA by individual steroid saponins have never been reported. Thus, in the present work, the drug–drug interaction with HSA between the two main constituents of DAXXX, dioscin and *pseudo*-protodioscin (ppd), were studied by re-ligand fishing using *pseudo*-protodioscin pre-saturated HSA–MNPs (ppd-HSA–MNPs). Mass spectrometry (MS) using an electrospray ionization source (ESI) was chosen for detection.

Different drugs, even with few structural differences, have different binding capacity to HSA. As shown in Fig 1, dioscin is an isoprostanol type steroid saponin with F ring closed, and *pseudo*-protodioscin (ppd) is a furostanol type steroid saponin with F ring opened. It was reported that dioscin is the inhibitor of platelet aggregation while *pseudo*-protodioscin is not. Dioscin and *pseudo*-protodioscin are two main ingredients of DAXXX. Thus, it is of great interest to investigate the dioscin–*pseudo*-protodioscin interaction with HSA by re-ligand fishing experiment.



**Figure 1:** Chemical structures of dioscin and *pseudo*-protodioscin.

Our previous study revealed that dioscin had a greater affinity to HSA than *pseudo*-protodioscin [12]. Thus, HSA–MNPs pre-saturated with *pseudo*-protodioscin (ppd-HSA–MNPs) was used to ligand fish dioscin in this study. An excessive amount of *pseudo*-

protodioscin was used to ensure that all the HSA binding sites were saturated with *pseudo*-protodioscin. The de-binding wash of the ppd-HSA-MNPs was performed in 1 mL buffer containing 50% acetonitrile by vigorous shaking. The supernatant was carefully removed and saved for MS analysis. The existence of *pseudo*-protodioscin de-bound from ppd-HSA-MNPs was revealed by the peak at  $m/z$  1029  $[M-H]^-$ , which could easily be assigned as *pseudo*-protodioscin; this was confirmed by MS<sup>2</sup>.

Dioscin and *pseudo*-protodioscin were competitively bound with HSA at the same site. The dynamic interaction process and equilibrium amount of these two constituents could be rapidly reached using the ppd-HSA-MNPs re-ligand fish dioscin. It was anticipated that the ratio of dioscin/*pseudo*-protodioscin would be increased as the re-ligand fishing time increased, until the equilibrium point arrived. The re-ligand fishing time in five experiments, 3 s, 5 s, 10 s, 15 s and 25 s, was set as the different interaction time points for these two drug constituents. Their de-binding wash solutions were named as A2-1, A2-2, A2-3, A2-4 and A2-5, respectively. The peak at  $m/z$  867  $[M-H]^-$  in the mass spectrum could be easily assigned to dioscin, which was confirmed by MS<sup>2</sup>. The mass spectra of A2-1, A2-2, A2-3, A2-4 and A2-5 showed the peaks of dioscin and *pseudo*-protodioscin, the height ratios of which correspond to their competitive binding; the results are summarized in Table 1. It was obvious that the height ratio of dioscin/*pseudo*-protodioscin was gradually increased with the re-ligand fishing time.

To calculate the amount of dioscin and *pseudo*-protodioscin, an eleven-point calibration curve was obtained with standard solutions at concentration ratios ranging from 8 : 1 to 1 : 128. Peak heights were used for the calculation. Linear regression analysis of the results yielded the following equations for the ratio of dioscin/*pseudo*-protodioscin:  $Y = 8.4247 X + 0.0157$ ,  $r^2 = 0.998$ , where  $Y$  was the peak height ratio of dioscin/*pseudo*-protodioscin,  $X$  was the concentration ratio of dioscin/*pseudo*-protodioscin, and  $r^2$  was the correlation coefficient. The concentration ratio of dioscin/*pseudo*-protodioscin calculated by peak heights was determined in solutions A2-1, A2-2, A2-3, A2-4 and A2-5, as shown in Table 1, which reflected the dynamic interaction process intuitively. The equilibrium was reached after about 15 s at a ratio of 0.44 : 1.

Among the steroidal saponins exhibiting activity for the prevention and treatment of coronary heart disease in DAXXK, there are two categories, the furostanol type and the isospirostanol type. Dioscin is the most important isospirostanol type and *pseudo*-protodioscin the most important furostanol type; both are determined in the Chinese Pharmacopoeia assay [14]. In this current study, further studies of the total steroidal saponins from DAXXK were conducted by comparison of ligand fishing results (de-binding wash solution B1) using HSA-MNPs and re-ligand fishing using ppd-HSA-MNPs (de-binding wash solution B2).

**Table 1:** Height ratios and concentration ratios of dioscin/*pseudo*-protodioscin at the different interaction times.

Solution	Re-ligand fishing time(s)	Height Ratio (dioscin/ <i>pseudo</i> -protodioscin)	Con. Ratio (dioscin/ <i>pseudo</i> -protodioscin)
A 1	0	0	0
A 2-1	3	1.03	0.12
A 2-2	5	1.72	0.20
A 2-3	10	3.57	0.42
A 2-4	15	3.70	0.44
A 2-5	25	3.70	0.44

Although dozens of steroidal saponins are present in DAXXK, only three, i.e. dioscin ( $m/z$  687  $[M-H]^-$ ), *pseudo*-protodioscin ( $m/z$  1029  $[M-H]^-$ ) and gracillin ( $m/z$  883  $[M-H]^-$ ) were bound to HSA significantly in the ligand fishing experiment. The ratio of dioscin/*pseudo*-protodioscin in the B1 solution was 0.54: 1, calculated by peak heights. By contrast, the ratio changed to 0.07: 1 in the re-ligand fishing experiment (B2 solution). This result indicated that the equilibrium was changed significantly in the presence of some outside source of *pseudo*-protodioscin and the drug-drug interaction of each constituent in DAXXK was serious. Further, it was reported that both dioscin and *pseudo*-protodioscin were degradation products of 26-*O*- $\beta$ -D-glucopyranosyl-(25*R*)-furost-5-en-3 $\beta$ ,22 $\xi$ ,26-triol-3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-[ $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucopyranoside in *D. panthaica* [22]. The assay standard for DAXXK in the Chinese Pharmacopoeia is the content of *pseudo*-protodioscin and total saponins [14]. However, unlike modern therapeutic drugs that are single active pure compounds, DAXXK is an herbal medicine that contains many active ingredients, and the chemical compositions always vary depending on the variety, origin, harvesting time and processing technique. Hence, the strong binding ingredients such as dioscin should be integrated into the quality control standard. It is necessary to monitor the drug blood concentration in clinical use, especially with combined use with narrow therapeutic index drugs (for example warfarin, digoxin). Furthermore, this work can be important for the evaluation of the pharmaceutical effect of a herb medicine containing multi-components.

In summary, a rapid and convenient methodology was presented to determine the balance ratio and balance time of dioscin/*pseudo*-protodioscin interaction with HSA by re-ligand fishing using human serum albumin-functionalized magnetic nanoparticles. The equilibrium was reached soon after 15 s at the amount ratio of 0.44: 1. Furthermore, the interaction of *pseudo*-protodioscin to total steroidal saponins from DAXXK was also studied. The procedures with the aid of mass spectrometry detection were very sensitive, quick and convenient. These results revealed that re-ligand fishing coupled with MS detection could be applied to investigate the botanical drug-drug interactions.

## Experimental

**Preparation of HSA-MNPs:** HSA functionalized MNPs (HSA-MNPs) were prepared following the procedures reported previously [9-12]. Briefly, magnetic nanoparticles were prepared by coprecipitation with a molar ratio of  $Fe^{2+} : Fe^{3+} = 1 : 2$  at pH 10. MNPs were first coated with SiO<sub>2</sub> using TEOS. Secondly, the particles were dispersed in APTMS solution to add -NH<sub>2</sub> to the SiO<sub>2</sub>; the resultant particles were then dispensed in the GD solution to provide -CHO to the surface. Finally, the -CHO coated MNPs were incubated with HSA to obtain HSA functionalized MNPs. HSA-MNPs were suspended in NH<sub>4</sub>Ac solution and kept at 4°C before use.

## Re-ligand fishing experiments

**Saturated binding *pseudo*-protodioscin to HSA-MNPs (ppd-HSA-MNPs):** A 225  $\mu$ g/mL solution of *pseudo*-protodioscin was prepared using ammonium acetate buffer solution (10 mM/L, pH 7.4). One mL *pseudo*-protodioscin solution and 100  $\mu$ L HSA-MNPs were added to a 4 mL Eppendorf tube. The tube was vigorously shaken for 2 min using a vortex oscillator, and then put aside for magnetic separation for 1 min. The supernatant was removed. The HSA-MNPs were washed 3 times with 1 mL of buffer solution, each by vigorously shaking for 1 min, and then the supernatants

were removed after magnetic separation. Hence, the pseudo-protodioscin saturated binding HSA-MNPs (ppd-HSA-MNPs) were obtained.

**Detection binding amount of pseudo-protodioscin in ppd-HSA-MNPs:** The de-binding wash of the ppd-HSA-MNPs was performed in 1 mL buffer containing 50% acetonitrile by vigorous shaking for 2 min. The supernatant was carefully removed and saved (A1) for mass spectrometric analysis.

**Re-ligand fish dioscin using ppd-HSA-MNPs for different times:** A 0.55 µg/mL solution of dioscin was prepared using ammonium acetate buffer solution (10 mM/L, pH 7.4). One mL dioscin solution was added to a 4 mL Eppendorf tube containing 100 µL ppd-HSA-MNPs. The tube was vigorously shaken for 3 s (re-ligand fish time investigated) using a vortex oscillator, and then put aside for magnetic separation for 1 min. The supernatant was removed. The ppd-HSA-MNPs were washed 3 times with 1 mL of buffer solution each by vigorous shaking for 1 min, and then the supernatants were removed, after magnetic separation. Finally, the de-binding wash was performed in 1 mL buffer containing 50% acetonitrile by vigorous shaking for 2 min. The supernatant was carefully removed and saved (A2-1).

Another 4 experiments were undertaken using the parallel procedures above as well. Four de-binding solutions A2-2, A2-3,

A2-4 and A2-5 were obtained for mass spectrometric analysis by prolonging the re-ligand fishing time from 3 s (A2-1) to 5 s (A2-2), 10 s (A2-3), 15 s (A2-4) and 25 s (A2-5), respectively.

**Re-ligand fish total steroidal saponins using ppd-HSA-MNPs:** First, a comparative ligand fishing experiment was made using HSA-MNPs. One mL DAXXX solution and 100 µL HSA-MNPs were added to a 4 mL Eppendorf tube. After vigorous shaken, magnetic separation, and buffer wash, the 50% acetonitrile de-binding wash solution (B1, ligand fishing) was obtained for comparison with B2 below.

Then, a 52 µg/mL solution of the DAXXX solution was prepared using ammonium acetate buffer solution (10 mM/L, pH 7.4). One mL dioscin solution was added to a 4 mL Eppendorf tube containing 100 µL ppd-HSA-MNPs. The 50% acetonitrile de-binding wash solution (B2, re-ligand fishing) was obtained following the same procedure. All the experiments above were repeated 3 times.

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## Serum Metabolomic Profiling of Rats by Intervention of *Aconitum soongaricum*

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To understand the toxic mechanism and to find the changes in the endogenous metabolites of *Aconitum soongaricum* Stapf for clinical detection, a combination of <sup>1</sup>H NMR spectroscopy and multivariate statistical analysis was applied to examine the metabolic profiles of the blood serum samples collected from the rat model. In total, thirteen biomarkers of *A. soongaricum* were found and identified. It turned out that *A. soongaricum* treatment may partially disorder the metabolism. The study has shown the potential application of NMR-based metabolomic analysis in providing further insights into the toxicity caused by *A. soongaricum*.

**Keywords:** Ranunculaceae, *Aconitum soongaricum*, Metabolomic Profiling, Biomarkers, Nuclear Magnetic Resonance.

*Aconitum soongaricum* Stapf, family Ranunculaceae, is well known for its anti-inflammatory and analgesic effect, and is widely used in the north of China as an analgesic for neuralgia, toothache, arthritis and other pains [1a-d]. However, as this plant contains *Aconitum* alkaloids, it has been reported that it has resulted in several poisoning accidents [2a,b]. To understand the toxic mechanism and to find the changes in endogenous metabolites for clinical detection, NMR based metabolomics was conducted to investigate the intervention effects of *A. soongaricum* on mice. Metabolomics analysis was carried out on serum samples, while orthogonal partial least squares-discriminant analysis (OPLS-DA) was employed to investigate the toxic effects of *A. soongaricum* and to detect related potential biomarkers [3a-c].

In this study, rats were fed with *A. soongaricum* and the biochemical variations in the serum from these rats were investigated by high resolution <sup>1</sup>H NMR spectroscopy. This procedure has provided an insight into the systematic metabolism after use of *A. soongaricum*. Results of this study have validated the applicability of NMR based metabolomics in the study of toxicity and biomarkers detection. In total, thirteen biomarkers were found and identified (Table 1). It turned out that *A. soongaricum* treatment may partially disorder the metabolism. Besides, NMR based metabolomics proved to be a powerful tool for investigating the pharmacodynamic effects of natural products and underlying mechanisms.

OPLS-DA analysis of endogenous metabolites in rat serum at different times after administration showed that there were significant differences in serum metabolites in each time group. It can be seen from the OPLS-DA analysis of different time groups that the 2d group is far away from the others in the space, but the 2d and 5d groups were closer in the plane and space, while the 2d group can be separated completely from the 9d and 13d groups in the score plot. The 9d and 13d groups are gradually approaching the blank group, the results showing that the effects of *A. soongaricum* on rats occurred within two days after the administration, but then they gradually recovered.

**Table 1:** Potential biomarkers of *A. soongaricum* detected by NMR.

No.	Metabolites	Reference value, <sup>1</sup> H NMR, δ	Measured value, <sup>1</sup> H NMR, δ
1	β-Glucose	3.24(dd)	3.24(dd)
		3.40(t)	3.40(t)
		4.64(d)	4.64(d)
2	α-Glucose	3.53(dd)	3.53(dd)
		3.72(dd)	3.72(dd)
		3.83(dd)	3.83(dd)
3	VLDL	5.23(d)	5.23(d)
		0.85(m)	0.85(m)
		0.88(m)	0.88(m)
4	LDL	1.27(m)	1.27(m)
		1.26(m)	1.26(m)
		4.25(m)	4.25(m)
5	Lactic acid	1.33(d)	1.32(d)
		4.11(q)	4.11(q)
6	Creatine	3.03(s)	3.03(s)
7	Trimethylamine oxide	3.93(s)	3.92(s)
8	β-Hydroxybutyric acid	3.27(s)	3.26(s)
9	Glycine	1.19(d)	1.19(d)
10	Alanine	3.57(s)	3.55(s)
		1.47(d)	1.47(d)
11	Glutamic acid	3.76(q)	3.76(q)
12	Pyruvic acid	2.13(m)	2.13(m)
13	Acetoacetic acid	2.38(s)	2.36(s)
		2.27(s)	2.27(s)

The variable metabolites in the serum of different time groups were VLDL, LDL, glucose, lactic acid, creatine, trimethylamine oxide, glycine, glutamic acid, alanine, β-hydroxybutyric acid, pyruvic acid and acetoacetic acid. The levels of glucose, glycine, and glutamic acid were decreased in the 2 d, 5 d and 9 d groups comparing with the blank group; these metabolites were obviously decreased in the day after administration, but were restored to normal levels in 13 d. The levels of VLDL, LDL and lactic acid were decreased 2 d after administration, but increased significantly in 9 d. The levels of creatine and trimethylamine oxide were increased compared with the blank group and trimethylamine oxide was obviously increased in 9 d. The levels of β-hydroxybutyric acid and acetoacetic acid

Table 2: The change of metabolites at different times.

No.	Metabolites	2d	5d	9d	13d
1	β-Glucose	↓↓	↓	↓	—
2	α-Glucose	↓↓	↓	↓	—
3	VLDL	↓	↓	↑↑	↑
4	LDL	↓	↓	↑↑	↑
5	Lactic acid	↓	↓	↑↑	↑
6	Creatine	↑	↑	↑	↑
7	Trimethylamine oxide	↑	↑	↑↑	↑
8	β-Hydroxybutyric acid	↑↑	↑	↑	—
9	Glycine	↓↓	↓	↓	—
10	Alanine	↓↓	↓	↓	↓
11	Glutamic acid	↓↓	↓	↓	—
12	Pyruvic acid	—	—	↓	—
13	Acetoacetic acid	↑↑	↑	↑	—

Concentration compared with the blank group was not obvious; ↑ and ↓ concentration increase or decrease compared with the blank group; ↑↑ and ↓↓ concentration increase or decrease compared with the blank group was significant.

were increased obviously in 2d and higher than the blank group, while levels of pyruvic acid were decreased in 9d compared with the blank group (Table 2).

In this study, metabolomics have been used to highlight the metabolic effect of *A. soongaricum* treatment in the rat model. NMR based metabolomics has been shown to be an efficient technique to investigate the metabolic perturbation due to *A. soongaricum* poisoning. The changes in endogenous metabolites in serum samples from models of different groups of rats were identified by OPLS-DA. The toxicity of *A. soongaricum* was proven to be selective and affected a number of metabolic pathways. To complement the current study, future proteomic and immunohistochemistry studies may provide further insights into the metabolic mechanism and discover more effective treatments for toxicity detection. In summary, the current results indicate that <sup>1</sup>H NMR based metabolomics have the potential to uncover the metabolic response of *A. soongaricum* toxicity that could provide better understanding of the metabolic biomarkers.

Experimental

**Plant material:**The fresh roots of *A. soongaricum* were collected in Narat Grassland, Xinjiang Province of China in July 2012 and were identified by Prof. Fan Zhang (North Sichuan Medical University, Sichuan, P. R. China) [4a]. Avoucher specimen (ZGEWT20120801) was deposited at the Xinjiang Museum of Traditional Chinese Medicine and Ethnomedicine.

**Animal handling and sample collection:** Sixty-six healthy SPF Sprague-Dawley rats (200±20g weight) were provided by North Sichuan Medical University. All rats were housed individually in metabolic cages. The animal room was maintained under controlled condition (a 12 h light-dark cycle with constant temperature and humidity). All efforts were made to minimize animal suffering. After acclimatization for one week, the rats were randomly separated into 11 groups, i.e. 2 h group, 6 h group, 2 d group, 3 d group, 5 d group, 7 d group, 9 d group, 11 d group, 13 d group, 14 d group and blank group (n=6). The blank group was given normal diet and clean water throughout the experiment, while the model groups were fed with the dose of 170 mg/kg, which was confirmed byacute toxicity test[4b]. After 2 h, 6 h, 2 d, 3 d, 5 d, 7 d, 9 d, 11 d, 13 d and14 d, respectively, the blood was drawn from orbit using a syringe and the blood was left to clot at room temperature for 30 min. Serum samples were centrifuged (12,000 rpm) for 15 min at 4°C to remove particulate contaminants and then stored at -80°C until further analysis.

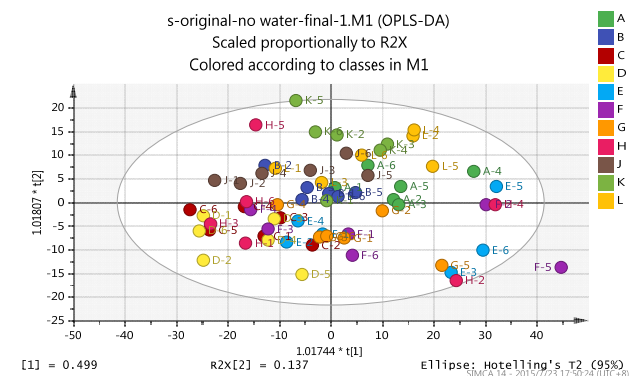


Figure 1: Scores plot of OPLS-DA of <sup>1</sup>H NMR data of serum samples.

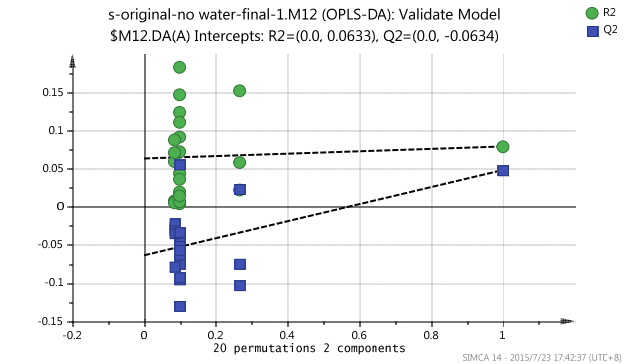
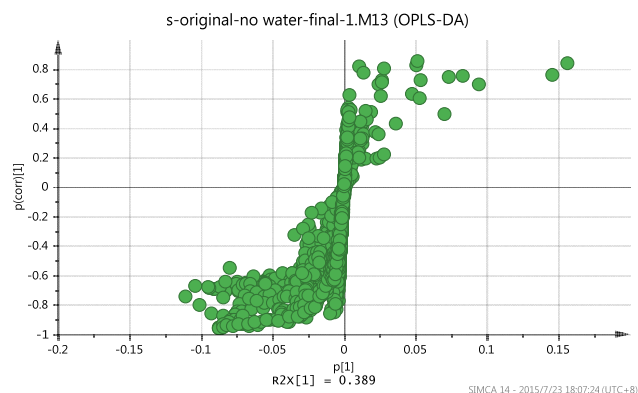


Figure 2: Permutations plot of OPLS-DA of <sup>1</sup>H-NMR data of serum samples.

**Sample preparation and <sup>1</sup>H NMR spectroscopy:** For <sup>1</sup>H NMR analysis, 500 μL of serum samples was mixed with 200 μL phosphate buffer solution (0.2M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 20% D<sub>2</sub>O) to reduce pH variation across samples. In addition, 1×10<sup>-3</sup> mol/L TSP (3-(trimethylsilyl)propionic-2,2,3,3-d<sub>4</sub> acid sodium salt) was used as an internal reference standard at δ 0.0. The mixture was then transferred into a 5 mm NMR tube. The <sup>1</sup>H NMR measurements of the serum samples were performed using a Varian NMR system 600 MHz spectrometer equipped with a triple resonance probe. The experimental temperature was set to 298 K and the 90° pulse length was calibrated individually for each sample. For the serum samples, an additional Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse train was incorporated into the NOESYPR sequence with a relaxation delay-90°-(τ-180°-τ) n-acquire. A total of 128 scans with a spectral width of 10 kHz were collected for all NMR experiments. The acquired signals were zero filled to 32k before Fourier transformation. For data analysis, metabolites in the Metabolomic Responses to *A. soongaricum* rat serum <sup>1</sup>H NMR spectra were assigned with reference to published data and HMDB database (<http://www.hmdb.ca/>).

**Data preprocessing of NMR spectra and pattern recognition:** The collected NMR spectra were phased and baseline corrected using the software Topspin 2.0 (Bruker, Germany)(Figure 1). All NMR spectra were also peak-aligned manually using in-house software to minimize variation due to peak shift. The chemical shift regions of δ 4.6-6.0 (water resonances), δ 0.0-0.2 (TSP resonance) and the regions which are peak-free for all spectra were excluded from further analysis. Table S1 shows the raw spectral data of serum samples. Then each spectrum was binned into segments by adaptive binning method. To account for variations in sample concentration, the spectra were normalized using group aggregating normalization technique. The NMR spectral data were converted to Microsoft Excel format and imported into SIMCA software (version 14.0, Umetrics, Sweden) for multivariate analysis.





**Figure 3:** S-plots of OPLS-DA of  $^1\text{H}$ -NMR data of serum samples.

Orthogonal partial least squares discriminant analysis (OPLS-DA) is a regression method that uses binary variables  $Y$  to indicate class membership. The OPLS-DA projects the original variables  $X$  (metabolic concentrations) to latent variables that focus on class separation, thus obtaining a better classification. The quality of the

models is described by  $R^2X$  and  $Q^2$  values.  $R^2X$  is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and  $Q^2$  is defined as the proportion of variance in the data predictable by the model and indicates predictability. The metabolites with high variable importance in the projection values in each OPLS-DA models are selected to investigate further their quantitative changes in all groups.

**OPLS-DA of blood serum samples:** OPLS-DA analysis of blood serum samples showed that the samples clustered based on studied groups (Figure 1). To investigate further the metabolic patterns and identify potential characteristic metabolites, group comparisons were carried out using OPLS-DA (Figures 2 and 3).

**Supplementary data:** S1 Table. Raw spectral data of serum samples.

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## Re-evaluation of ABTS•<sup>+</sup> Assay for Total Antioxidant Capacity of Natural Products

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2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS•<sup>+</sup>) is a stable free radical frequently used for estimating the total antioxidant capacity (TAC) of natural products. The existing methods for ABTS•<sup>+</sup> radical-scavenging activity assays are diverse in pre-diluting solvents and reaction time, which lead to errors in the TAC estimations. To develop an effective and universal method for estimating the ABTS•<sup>+</sup> capacity accurately and reasonably, five pre-dilution solvents [methanol, ethanol, phosphate buffer (Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>, 200 mM, pH = 7.4), PBS buffer (Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>-NaCl, 200 mM, pH = 7.4), and distilled water] and different reaction times were investigated in ABTS•<sup>+</sup> assays of five typical antioxidants. The results showed that the solvent effects were very significant. When using different pre-diluting solvents, different detection wavelengths should be selected. ABTS•<sup>+</sup> assay could be measured within 2-10 min to obtain a rough result, which was mostly 6 min in the literature. However, full and accurate evaluation of antioxidant reactivity rather than capacity requires recording ABTS•<sup>+</sup> loss continuously during the whole reaction period. The present study makes a recommendation for estimating the ABTS•<sup>+</sup> capacity accurately and reasonably.

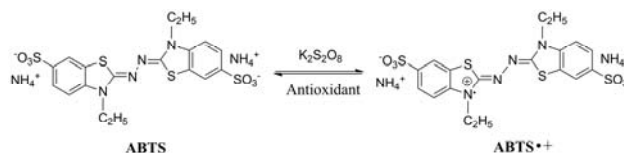
**Keywords:** ABTS, Solvent, Reaction time, Phenolic compound, Recommendation.

Reactive oxygen species (ROS), a collective term often used to include oxygen radicals [superoxide (O<sub>2</sub><sup>•-</sup>), hydroxyl (OH<sup>•</sup>), peroxyl (RO<sub>2</sub><sup>•</sup>), and alkoxyl (RO<sup>•</sup>)] and certain nonradicals that either are oxidizing agents and/or are easily converted into radicals, such as HOCl, ozone (O<sub>3</sub>), peroxyntirite (ONOO<sup>-</sup>), singlet oxygen (<sup>1</sup>O<sub>2</sub>), and H<sub>2</sub>O<sub>2</sub> [1], may cause cell aging, cardiovascular diseases, mutagenic changes, and cancerous tumor growth [2]. Antioxidants are compounds that can delay the oxidation of other molecules by inhibiting either the initiation or propagation of oxidizing chain reactions caused by free radicals and thereby may reduce oxidative damage to the human body [3]. Phenolic substances, which exist in plant tissues as secondary metabolites, can be categorized into simple phenols, phenolic acids (both hydroxybenzoic acids and hydroxycinnamic acids), flavonoids, lignins and tannins. These substances are responsible for color, bitterness, acerbic taste, flavour, odor and antioxidant properties [4].

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS•<sup>+</sup>), a stable free radical, is frequently used for estimating the total antioxidant capacity (TAC) of natural products, including crude extracts [5-9], polyphenols [10], phenolic acids [11], flavonoids [12], and others [13]. The original ABTS•<sup>+</sup> radical-scavenging assay was measured according to the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants [14]. With the improving technology for determination of the blue ABTS•<sup>+</sup> chromophore produced through the reaction between ABTS and potassium persulfate (Figure 1), a feasible method was established according to a maximal absorption of ABTS•<sup>+</sup> at wavelengths of 645 nm, 734 nm, and 815 nm [15, 16]; the original method measured at 415 nm [17, 18].

Until 1999, the applicable and universal ABTS•<sup>+</sup> method for the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants, was

proposed by Re *et al.* [14]. This ABTS•<sup>+</sup> assay was measured by a colorimetric method at 734 nm, and the extent of decolorization as a percentage inhibition of the ABTS•<sup>+</sup> radical cation was determined as a function of concentration and time and calculated relative to the reactivity of Trolox, as a standard, under the same conditions. Recently, the LC-ABTS•<sup>+</sup> assay is frequently used for ABTS•<sup>+</sup> radical-scavenging activity. He *et al.* [19, 20] identified the phenolic compounds from pomegranate (*Punica granatum* L.) seed residues and investigated their antioxidant capacities by HPLC-ABTS•<sup>+</sup> assay; Kalili *et al.* [21] reported that the combination of LC × LC separation with an on-line radical scavenging assay increased the likelihood of identifying individual radical scavenging species in comparison with the conventional LC-ABTS•<sup>+</sup> assay.



**Figure 1:** Reaction mechanism of ABTS and the oxygen radical (ABTS•<sup>+</sup>).

However, in the course of the application of the ABTS•<sup>+</sup> method, some problems appeared. First, the pre-diluting solvents in ABTS•<sup>+</sup> assay are various in the existing literature. For example, Bangoura *et al.* [22] and Shao *et al.* [23] selected PBS buffer solution (Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>-NaCl, 200 mM, pH = 7.4) as the solvent and the absorbance of ABTS•<sup>+</sup> assay measured at 734 nm. Gursoy *et al.* [24] reported that ABTS•<sup>+</sup> free radical-scavenging assay was measured after ABTS•<sup>+</sup> dilution with phosphate buffer (0.1M, pH 7.4). Luo *et al.* [10] diluted the ABTS•<sup>+</sup> with 95% ethanol, and determined the absorbance at 734 nm. Siddhuraju [25] reported the ABTS•<sup>+</sup> solution diluted in ethanol (1:89, v/v) and used an absorbance at 734 nm. However, our previous research revealed that the ABTS•<sup>+</sup> solution diluted with ethanol had maximal absorption at 753 nm [26]. It is inaccurate to measure the absorbance at 734 nm in either ethanol or the other solvents.

Although Dawidowicz and Olszowy discussed the importance of solvent type in estimating antioxidant properties of phenolic compounds by ABTS<sup>•+</sup> assay [27], only three solvents (methanol, ethanol and propanol) were discussed in detail. The solvents frequently-used in ABTS<sup>•+</sup> assay, including PBS and phosphate buffer, were not reported and discussed to our knowledge. Secondly, the reaction time is an important factor causing errors in estimating the ABTS<sup>•+</sup> free radical-scavenging activity. ABTS<sup>•+</sup> can be quenched by both electron (fast) and hydrogen atom transfer (slow) like DPPH [28]. That is to say, the reaction time of different antioxidants with ABTS<sup>•+</sup> is not same. In the literature, 6 min of reaction time were employed universally, which could cause error in the ABTS<sup>•+</sup> radical-scavenging, especially for complex samples (e.g. crude extracts). Therefore, it is necessary to investigate the proper reaction time for the ABTS<sup>•+</sup> capacity determination of different antioxidants.

This paper deals with the effects of different solvents and different reaction times on ABTS<sup>•+</sup> assay. In this study, five pre-diluting solvents [methanol, ethanol, phosphate buffer (PB, Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>, 200 mM, pH = 7.4), PBS buffer, and distilled water] and different reaction times (0-30 min) were investigated in ABTS<sup>•+</sup> assays for five antioxidants.

**Effects of the solvents:** ABTS<sup>•+</sup> has a maximal absorption at 734 nm according to the literature [14, 22]. However, the maximal absorption wavelengths of ABTS<sup>•+</sup> in different solvents are not the same, which might be ascribed to a red or blue shift caused by solvent effects. Although Dawidowicz and Olszowy [27] have reported the methanol, ethanol, and propanol effects, other solvents are frequently used to pre-dilute ABTS<sup>•+</sup>. In the present study, ABTS<sup>•+</sup> solution was diluted with PBS buffer, PB, H<sub>2</sub>O, MeOH, and EtOH, respectively. The UV-Vis spectra of pre-diluting ABTS<sup>•+</sup> solutions were scanned in the range of 500-1000 nm. Figure 2 shows that the ABTS<sup>•+</sup> solutions diluted with PBS buffer, PB, and H<sub>2</sub>O have the same maximal absorption at 734 nm, but MeOH pre-dilution of ABTS<sup>•+</sup> has a maximal absorption at 745 nm, and the EtOH pre-dilution, a maximal absorption at 753 nm. Therefore, it is unsuitable for the literature methods [10, 24] to measure ABTS<sup>•+</sup> capacity at the same wavelength (734 nm) without regard to the solvent effects. Additionally, the linearity coefficients between absorptions and concentrations were analyzed and are shown in Table 1. The results reveal that all the above pre-diluting ABTS<sup>•+</sup> have good linearity coefficients ( $r^2 \geq 0.998$ ), indicating that all of the above solvents could be used for pre-diluting ABTS<sup>•+</sup>. However, when using different pre-diluting solvents, the corresponding maximal absorption wavelengths for detection were varied.

**Effects of reaction time:** The same as with the DPPH free radical, ABTS<sup>•+</sup> could be quenched by both electron (fast) and hydrogen atom transfer (slow) [28, 29]. That is to say, the time of various antioxidants quenching ABTS<sup>•+</sup> is different. However, most existing methods used for ABTS<sup>•+</sup> assay are measured after 6 min

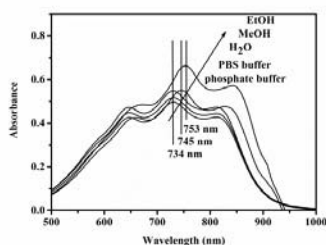


Figure 2: UV-Vis spectra (500-1000 nm) of ABTS<sup>•+</sup> solution diluted with different solvents.

Table 1: Linearity analysis of ABTS<sup>•+</sup> solutions at different concentrations (2.2-140  $\mu$ M).<sup>a</sup>

Pre-diluting solvents	Linear equation (correlation coefficient)
PBS	$A = -0.00717 + 0.00748 C$ ( $r^2 = 0.99999$ )
PB	$A = -0.01641 + 0.00769 C$ ( $r^2 = 0.99848$ )
H <sub>2</sub> O	$A = -0.00477 + 0.00781 C$ ( $r^2 = 0.99987$ )
MeOH	$A = -0.01316 + 0.00806 C$ ( $r^2 = 0.99985$ )
EtOH	$A = 0.00261 + 0.00936 C$ ( $r^2 = 0.99962$ )

<sup>a</sup> Abbreviation: *A*, the absorbance of ABTS<sup>•+</sup> solution at different concentrations; *C*, the concentration of ABTS<sup>•+</sup> solution.

of reaction time, which provide little chemical information about the antioxidants. In the present study, the ABTS<sup>•+</sup> scavenging activity of five familiar antioxidants, gallic acid, ferulic acid, caffeic acid, phenol, and glutathione were determined under different reaction times, as shown in Figure 3A. The reaction rate ( $k_i$ ) at different times is shown in Figure 3B. The results reveal that ABTS<sup>•+</sup> could be quenched very rapidly by phenol, but very slowly by glutathione. Gallic acid, ferulic acid, and caffeic acid also quench ABTS<sup>•+</sup> very rapidly, but it takes them a longer time to reach a steady state. The ABTS<sup>•+</sup> scavenging activity of glutathione is very weak, especially within a short reaction time, indicating that glutathione quenches ABTS<sup>•+</sup> according to hydrogen atom transfer. That is to say, the antioxidants like glutathione need longer time to accomplish the reaction. According to our experiments, the six minutes of reaction time used in the literature is too long for reaching a slow reaction state, and it is difficult for the majority of compounds to obtain a steady state within a limited time. It can be concluded from Figure 3 that ABTS assay could be measured within 2-10 min to obtain a rough result. To get more accurate and reliable results, a time-varying measurement is recommended.

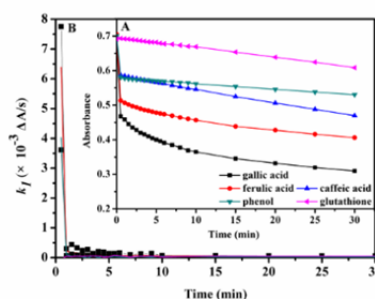


Figure 3: Absorbance (A) and reaction rate ( $k_i$ ) (B) of ABTS<sup>•+</sup> solution mixed with antioxidants (5  $\mu$ M) at different time.

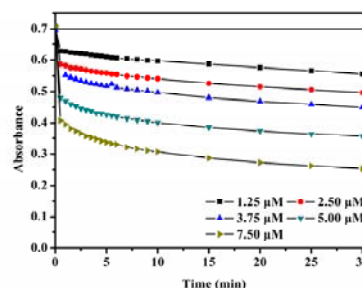


Figure 4: Absorbance of ABTS<sup>•+</sup> solution mixed with gallic acid at different concentrations.

**Effects of concentration of antioxidants:** The reaction needs more time when the antioxidants have a high concentration, according to a previous report by Xie and Schaich [28]. In the present study, gallic acid was selected to determine the effects of the concentration of the antioxidant on the reaction time. The concentrations of gallic acid ranged from 1.25  $\mu$ M to 7.50  $\mu$ M. The results in Figure 4 show that the time of reaching a stationary state becomes longer with higher concentrations. The stationary state could not be reached within a stationary time for different concentrations of antioxidants.

Therefore, the absorbance of the ABTS<sup>•+</sup> assay could not be measured at a stationary reaction time, and a time-varying measurement is encouraged to obtain accurate results.

**Antioxidant assay:** The ABTS<sup>•+</sup> scavenging activity of five antioxidants, gallic acid, ferulic acid, caffeic acid, phenol, and glutathione were measured using five different solvents (PBS buffer solution, PB, H<sub>2</sub>O, MeOH, and EtOH) to pre-dilute ABTS<sup>•+</sup> radical solution, respectively. The absorbance was recorded at the corresponding maximum absorption wavelength. The results in Table 2 (IC<sub>50</sub> [APR] and I<sub>7.5</sub>) reveal that phenolic acids and phenol exhibit similar ABTS<sup>•+</sup> radical-scavenging capacity in the five solvents. Additionally, good linear relationships between inhibition and antioxidant concentrations of three phenolic acids and phenol could be obtained at an inhibition of about 50%. For a limited range of concentrations, a reliable linear relationship does exist between antioxidant concentration and percentage inhibition according to the literature [30, 31]. Carmona-Jiménez *et al.* [32] reported that the IC<sub>50</sub> can be obtained accurately when a reliable linear relationship exists. Therefore, the five different solvents might be alternative solvents for pre-diluting ABTS<sup>•+</sup> radical solution in the ABTS<sup>•+</sup> radical-scavenging assay.

**Table 2:** The compounds' properties and their ABTS<sup>•+</sup> radical-scavenging capacity.

Compound	gallic acid	ferulic acid	caffeic acid	phenol	glutathione
# -OH <sup>a</sup>	3	1	2	1	0
E <sub>7</sub> (V) <sup>b</sup>	0.55	0.60	0.54	0.90	0.24
IC <sub>50</sub> (APR) of ABTS <sup>•+</sup>					
PBS	4.5 (0.22)	5.7 (0.18)	7.9 (0.13)	8.9 (0.11)	nd <sup>g</sup>
PB	3.9 (0.26)	5.3 (0.19)	7.7 (0.13)	8.7 (0.12)	nd <sup>g</sup>
H <sub>2</sub> O	4.5 (0.22)	4.2 (0.24)	9.7 (0.10)	11.6 (0.09)	nd <sup>g</sup>
MeOH	4.9 (0.20)	5.7 (0.17)	8.1 (0.12)	nd <sup>g</sup>	nd <sup>g</sup>
EtOH	5.6 (0.18)	5.5 (0.39)	8.4 (0.12)	10.0 (0.10)	nd <sup>g</sup>
IC <sub>50</sub> (APR) of DPPH <sup>d</sup>	-	9.5 (0.10)	84 (0.012)	21 (0.05)	nr <sup>f</sup>
I <sub>7.5</sub> (%) <sup>e</sup>					
PBS	78.5	64.1	49.6	42.3	14.6
PB	86.3	67.1	49.3	42.9	11.6
H <sub>2</sub> O	76.5	73.6	40.6	32.0	2.4
MeOH	75.6	60.1	47.4	36.5	1.9
EtOH	69.4	65.3	44.9	38.0	1.1

<sup>a</sup> Number of reactive -OH groups; <sup>b</sup> Redox potential at pH 7; <sup>c</sup> IC<sub>50</sub> of ABTS<sup>•+</sup> is expressed as μM, APR=1/IC<sub>50</sub>; <sup>d</sup> IC<sub>50</sub> of DPPH is obtained from the literature [28] and expressed as μM, APR=1/IC<sub>50</sub>; <sup>e</sup> Inhibition of ABTS<sup>•+</sup> at the concentration of 7.5 μM. <sup>f</sup> no reaction; <sup>g</sup> response too low to determine stoichiometry accurately.

It is interesting that phenol (monohydric phenol) exhibited moderate ABTS<sup>•+</sup> radical-scavenging activity, but exhibited no DPPH radical-scavenging activity (Table 2). Ferulic acid has stronger APR than caffeic acid in the ABTS<sup>•+</sup> assay, which might be caused by the higher redox potential of ferulic acid (E<sub>7</sub>: ferulic acid > caffeic acid); this further supported the view that ABTS<sup>•+</sup> can be quenched by both electron and hydrogen atom transfer. The above results imply that accurate ABTS<sup>•+</sup> antioxidant capacity could not be obtained from a stationary time measurement because of the existence of diverse samples, especially for mixture samples and crude extracts of plants.

**Recommendations for the use of ABTS<sup>•+</sup> assay in the evaluation of total antioxidant activity:** The ABTS<sup>•+</sup> assay has been used extensively to compare and rank the antioxidant effectiveness of a wide range of natural extracts in thousands of studies. Nonetheless, results reported here raise serious questions about its validity for quantitating and comparing the activity of antioxidants with different sizes and structures, and especially for ranking their relative reactivity. Hence, some recommendations for the use of the ABTS<sup>•+</sup> assay according to the results of the present study are listed as follow: (1) When using different pre-diluting solvents, the corresponding maximal absorption wavelengths for detection should be selected; (2) Although ABTS<sup>•+</sup> antioxidant capacity could be measured within 2-10 min to obtain a rough result, to obtain more accurate and reliable results, a time-varying measurement is recommended, with calculation of reaction kinetics from early response.

## Experimental

**Reagents and instruments:** 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from J&K Scientific Ltd. (Beijing, China), gallic acid and ferulic acid from Sigma-Aldrich Co. LLC (Shanghai, China), and caffeic acid, phenol, and glutathione from Aladdin Chemistry Co. Ltd (Shanghai, China). The water (resistivity ≥ 18.25 MΩ/cm) used was purified with a purity water system (Chengdu, China). A Shimadzu UV-Vis 2401 PC spectrophotometer was used for recording the UV-Vis spectra and absorbance in colorimetric methods.

**Reagent preparation:** ABTS (38.4 mg) was dissolved in 10 mL 2.5 mM potassium persulfate and the mixture was reacted in the dark at room temperature for 12-16 h to prepare ABTS<sup>•+</sup> solution. This was diluted with methanol, ethanol, PB (Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>, 200 mM, pH = 7.4), PBS buffer (Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>-NaCl, 200 mM, pH = 7.4), and distilled water, respectively, to obtain an absorbance of 0.70 ± 0.02 for ABTS<sup>•+</sup> colorimetric method.

**Sample preparation:** Stock solutions (2 mM) were prepared in methanol, and then diluted with methanol to obtain serial concentration solutions. In the present study, all antioxidant sample concentrations noted are final reaction concentrations.

**Effects of the solvents:** The ABTS<sup>•+</sup> was diluted 50 times with methanol, ethanol, PB (Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>, 200 mM, pH = 7.4), PBS buffer (Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub>-NaCl, 200 mM, pH = 7.4), and distilled water, respectively. The absorbance of the ABTS<sup>•+</sup> solutions pre-diluted to different concentrations with the above solvents was measured in the wavelength range from 500 nm to 1000 nm.

**Effects of reaction time:** ABTS<sup>•+</sup> was diluted with PBS buffer solution to obtain an absorbance in the range of 0.70 ± 0.02. Ten μL of 2 mM antioxidant samples (5 μM, final concentration) and 90 μL distilled water were added to the tube, then 3.9 mL of diluting ABTS<sup>•+</sup> solution was added and vibrated vigorously. The mixture was transferred to the cuvette, and the absorbance at 734 nm was measured rapidly. The absorbance was recorded successively every 30 s (0-6 min), 1 min (6-10 min), and 5 min (10-30 min), respectively. The initial absorbance was recorded by replacing the sample with methanol. The reaction rate (*k<sub>t</sub>*) was obtained from the absorbance drop (Δ*A<sub>t</sub>*) for every time unit (Δ*t*) and calculated as follows: *k<sub>t</sub>* = Δ*A<sub>t</sub>*/Δ*t* (Δ*A*/s)

**Effects of concentration of the antioxidants:** Gallic acid solutions at different concentrations were selected to determine the effects of the concentration of the antioxidants on ABTS<sup>•+</sup> assay. The absorbance was measured as above.

**ABTS free radical-scavenging activity assays:** One hundred μL of each sample in methanol at various concentrations (2.5, 5.0, 7.5, 10.0, and 15.0 μM, final concentration) was added to 3.9 mL of pre-diluting ABTS<sup>•+</sup> (absorbance at 0.70 ± 0.02) and vibrated vigorously. Then the reaction mixture was incubated at room temperature for 2-10 min, and the absorbance was recorded at the corresponding maximum absorption wavelength (734 nm for PBS, PB solution, and H<sub>2</sub>O, 745 nm for MeOH, and 753 nm for EtOH). The ABTS free radical-scavenging activity was expressed by inhibition (*I*%), calculated as follows: *I*% = (*A*<sub>blank</sub> - *A*<sub>sample</sub>)/*A*<sub>blank</sub> × 100; where *A*<sub>sample</sub> was the absorbance of ABTS solution mixed with sample and *A*<sub>blank</sub> was the absorbance of ABTS solution mixed with solvent. The IC<sub>50</sub> value was defined as the effective concentration at which the DPPH radical was scavenged by 50% and the antiradical power (ARP) was calculated as follow: ARP = 1/IC<sub>50</sub>.

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## Chemical Synthesis of the Echinopine Sesquiterpenoids

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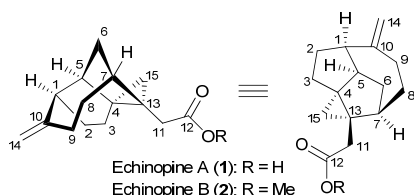
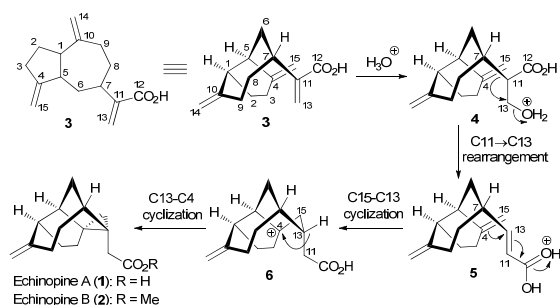
As new members of the sesquiterpenoid family, echinopines A and B were found to possess an unprecedented [3/5/5/7] carbon framework, which has stimulated considerable interest among the chemistry community since their isolation. This review article documents chronologically the steps towards chemical synthesis of the echinopine sesquiterpenoids, showcasing different strategies by resorting to the toolkit of organic chemistry.

**Keywords:** Echinopine, Natural product, Sesquiterpenoid, Total synthesis.

## 1. Introduction

Throughout the long research history of natural products, the terpenoids constitute the largest family, with the most diversified structures created by Mother Nature. Notable features associated with their chemical structures and therapeutic applications have made them a permanently pursued theme among the synthetic chemists worldwide [1,2].

Echinopines A (**1**) and B (**2**) (Figure 1), two closely related sesquiterpenoids with remarkable architectural novelties, were isolated from the roots of *Echinops spinosus* by Shi, Kiyota, and co-workers in 2008 [3]. Based upon spectroscopic analyses, the structures of echinopines A (**1**) and B (**2**) were established as shown in Figure 1, which represents the first example of a unique [3/5/5/7] carbon framework. Biogenetically, the unprecedented skeleton of echinopine sesquiterpenoids was proposed to originate from a guaiane-type precursor **3** (Scheme 1) [3]. Key steps concerning their biosynthetic pathways involved a C11→C13 rearrangement followed by successive C15–C13 and C13–C4 bond formations.

Figure 1: Structures of echinopines A (**1**) and B (**2**).

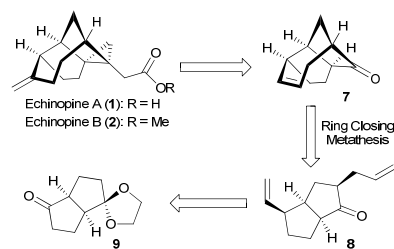
Scheme 1: Proposed biosynthesis of the echinopine sesquiterpenoids.

In spite of the lack of reported biological activities, echinopines attracted many synthetic interests immediately by their novel structures. Thus far, five successful total syntheses [4–8], together with two interesting formal syntheses [9,10], have been achieved. To the best of our knowledge, a logical summary concerning this topic is missing in the literature. This review aims to highlight these accomplishments described to date towards the fascinating echinopine sesquiterpenoids.

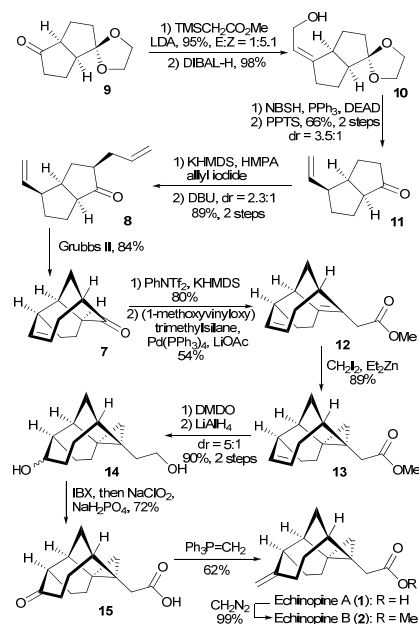
## 2. Chemical synthesis of echinopines

## 2.1 Magauer-Tiefenbacher's total synthesis of (+)-echinopines A and B

The first total synthesis of (+)-echinopines A and B was reported by Magauer, Mulzer, and Tiefenbacher in 2009 [4]. Their approach features an intermolecular cyclopropanation to form the cyclopropane, as well as a ring closing metathesis (RCM) to construct the seven-membered ring. As illustrated in Scheme 2, the precursor **8** could be synthesized from the known optically pure bicyclic intermediate **9**.

Scheme 2: Retrosynthetic analysis by Magauer *et al.*

The synthesis commenced with the preparation of bicycle **9** from 1,5-cyclooctadiene based on the literature method [11]. Subsequent Peterson olefination [12] and reduction of the ester afforded allylic alcohol **10** (Scheme 3). Transformation of **10** to the requisite terminal alkene was secured by a Myers' [3,3]-rearrangement protocol [13], which was followed by removal of acetal protection, resulting in the unmasked ketone as a separable diastereomeric mixture (3.5:1). The major and desired isomer **11** was subjected to allylation and epimerization to furnish the key intermediate **8**. Ring closing metathesis of the latter under the conditions of Grubbs II catalyst generated the [5/5/7]-tricyclic framework **7** (84%).

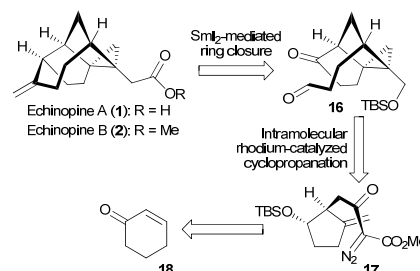


Scheme 3: The first total synthesis of (+)-echinopines A and B.

The remaining task was to introduce the side chain and cyclopropane unit. To this end, the authors developed two routes to the advanced intermediate **14**; the shorter one is shown in Scheme 3. Specifically, palladium-catalyzed coupling reaction of the corresponding vinyl triflate derived from **7** with (1-methoxyvinyl)oxytrimethylsilane [14] provided ester **12**. Regioselective cyclopropanation [15,16], followed by epoxidation and  $\text{LiAlH}_4$  reduction, delivered the tetracyclic diol **14**. After global oxidation, Wittig methylenation of the resulting ketone ultimately led to the production of (+)-echinopine A (**1**), which could be converted to (+)-echinopine B (**2**) on exposure to diazomethane. The absolute configuration of echinopines was determined through the synthesis, together with a single crystal X-ray diffraction analysis of **1**. Thus, the first synthesis of enantiomerically pure echinopine sesquiterpenoids was achieved starting from the known bicyclic compound **9**. The highlight of this pioneering approach was embodied in forging of the seven-membered ring by RCM to access the bridged core structure.

## 2.2 Nicolaou-Chen's total synthesis of echinopines A and B

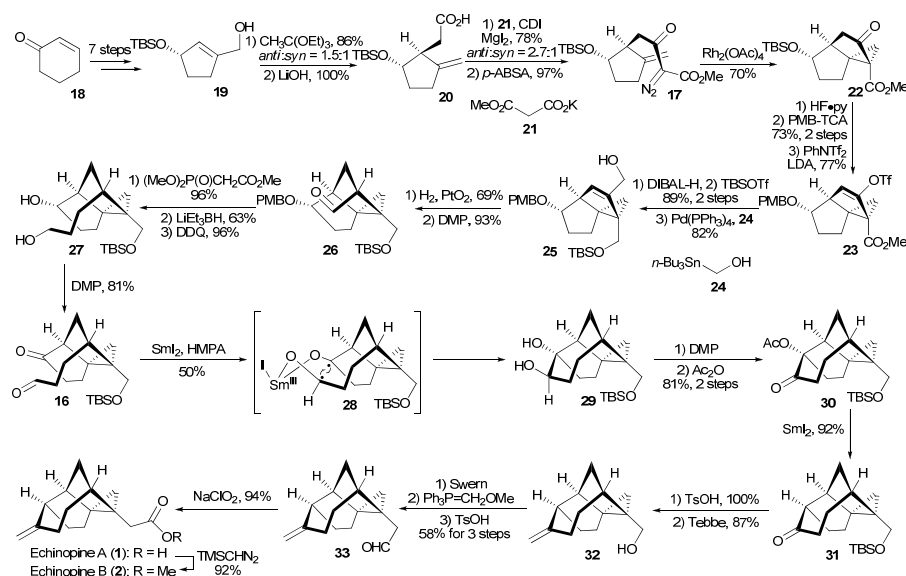
Shortly after the first total synthesis, Nicolaou, Chen and co-workers reported their endeavors on echinopine synthesis [5]. The retrosynthetic analysis is outlined in Scheme 4, which relied on an intramolecular cyclopropanation and a  $\text{SmI}_2$ -mediated [17] ring closure to establish the crucial cyclopropane and cycloheptane, respectively.



Scheme 4: Nicolaou-Chen's bond disconnection towards echinopines.

The functionalized cyclopentyl allylic alcohol **19** was prepared by a ring contraction process from the commercial cyclohexenone (**18**) at the beginning stage of the synthesis (Scheme 5). Compound **19** could be further transformed into the unsaturated carboxylic acid **20** through Jonson-Claisen rearrangement [18], followed by saponification. The latter was then subjected to homologation and subsequently reacted with *p*-ABSA to give the key intermediate **17**. As anticipated, the intramolecular cyclopropanation of diazo **17** took place smoothly with catalytic  $\text{Rh}_2(\text{OAc})_4$ , affording the [3/5/5] tricyclic system (**22**) as a single isomer in 70% yield.

Further functional group manipulations of **22**, including Stille coupling of enol triflate with hydroxymethylstannane **24** and a stereocontrolled hydrogenation, furnished aldehyde **26**. Next, Horner-Wadsworth-Emmons reaction of the aldehyde followed by reduction of the resultant  $\alpha,\beta$ -unsaturated ester and deprotection of PMB afforded diol **27**. After oxidation of diol to keto aldehyde **16**, the stage was set for the intramolecular pinacol coupling. Consequently, the core structure **29** was successfully established (as a single diastereoisomer) under the conditions of  $\text{SmI}_2$ -HMPA, through the proposed transition state **28**.



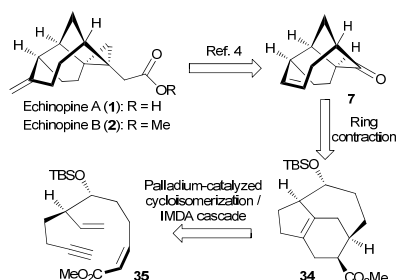
Scheme 5: Total synthesis of echinopines by Nicolaou, Chen and co-workers.



Having the tetracyclic framework **29** secured, the final stage of the synthesis was relatively easy. Oxidation of the secondary alcohol followed by removal of the tertiary acetate mediated by  $\text{SmI}_2$  gave rise to ketone **31**. Prior to homologation of the side chain, an exocyclic olefin was installed through Tebbe olefination. A routine procedure was then applied to convert alcohol **32** to aldehyde **33** with homologation of one more carbon. Finally, echinopine A (**1**) was obtained after oxidation of the aldehyde to acid by using  $\text{NaClO}_2$ , while echinopine B (**2**) was realized by further esterification. The synthesis was achieved in both forms employing enantiomeric or racemic intermediate **19** [5].

### 2.3 Chen's synthesis of echinopines A and B

Continuous studies by Chen and co-workers led to a conceptually different approach [9] to the intriguing sesquiterpenoids compared with the aforementioned synthesis. Inspired by the biosynthetic proposal [3], a [5/6/7] carbocyclic framework (i.e., the hypothetical intermediate **6**, Scheme 1) was associated with the planned chemical synthesis of echinopines A (**1**) and B (**2**). In this context, a ring contraction event could enable the central [5/5/7] core of the natural products. As shown in Scheme 6, the desired [5/6/7] tricyclic system **34** was envisioned to be constructed from the linear substrate **35** by a palladium-mediated cycloisomerization/intramolecular Diels-Alder cycloaddition sequence.



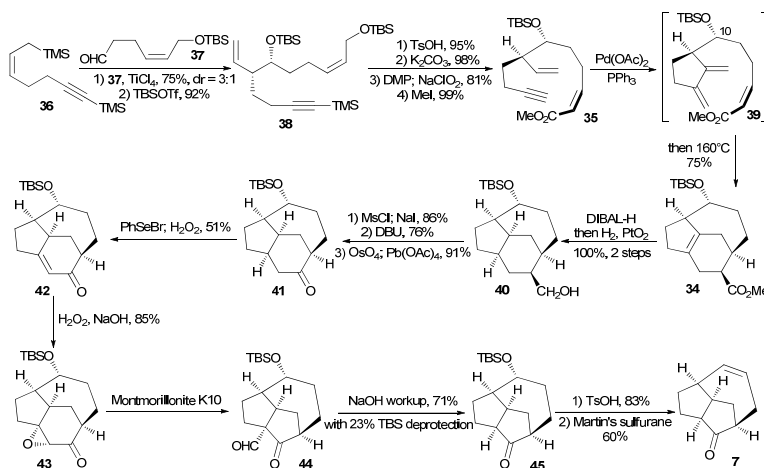
Scheme 6: Palladium-catalyzed cascade strategy from Chen group.

To access the acyclic precursor **35**, a crucial Hosomi-Sakurai reaction [19] was realized between allylsilane **36** and aldehyde **37** in the presence of  $\text{TiCl}_4$ ; and the resulting alcohol (dr = 3:1) was silylated to afford **38** (Scheme 7). Subsequent functional group manipulations could smoothly give rise to the enyne **35**. Upon treatment of **35** with  $\text{Pd}(\text{OAc})_2/\text{PPh}_3$  at  $80^\circ\text{C}$ , the diene enoate **39** was detected, which could be further converted into the tricycle **34**

(75%) in one-pot with prolonged heating at  $160^\circ\text{C}$  by intramolecular Diels-Alder cycloaddition. A selective *endo*-cycloaddition was observed, while the configuration at C10 proved to be insignificant for the stereochemical outcome of the sequence.

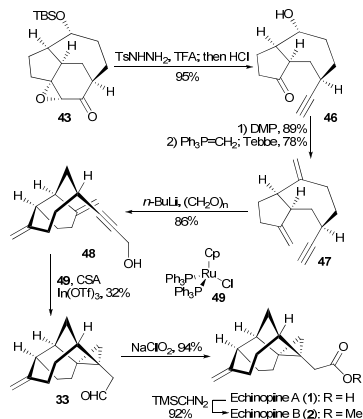
Further elaboration commenced with a two-step reduction (DIBAL-H;  $\text{H}_2/\text{PtO}_2$ ) of **34** with high efficiency. The hydroxymethyl group in compound **40** was converted to ketone **41** through the sequence of iodination, elimination, and oxidative cleavage. Preparation of an epoxy ketone would be the next task, which was envisaged to serve as the appropriate substrate for the corresponding ring contraction process. After introduction of enone *via* the well-established  $\alpha$ -selenation/oxidative elimination sequence, **42** underwent epoxidation by treatment with  $\text{H}_2\text{O}_2/\text{NaOH}$ , furnishing epoxy ketone **43**. Based on the method of Sheldon [20], keto aldehyde **44** was obtained from **43** in the presence of montmorillonite K10, which after basic aqueous workup, resulted in deformylation to provide tricyclic ketone **45** (71%) together with the TBS deprotection product (23%). Finally, global desilylation ( $\text{TsOH}$ , 83%) followed by dehydration using Martin's sulfurane delivered the known intermediate **7** [4], constituting a formal synthesis of echinopines A (**1**) and B (**2**). In addition, an auxiliary-based aldol reaction strategy provided the same intermediate **38** in its optically active form, thus enabling an asymmetric version of this synthetic approach [9].

Alternatively, Chen's group developed a novel endgame towards echinopines starting from intermediate **43** [6], which for the first time showcased the [5/7]→[3/5/5/7] ring-forming sequence by a bio-inspired late-stage intramolecular cyclopropanation strategy (Scheme 8). With an improved synthesis of epoxy ketone **43**, an Eschenmoser fragmentation [21] was envisioned to realize the bond cleavage. As such, alkynyl ketone **46** was obtained *via* treatment with  $\text{TsNHNH}_2$  followed by acidic workup, setting the stage for [5/7]→[3/5/5/7] skeletal conversion. Prior to that, oxidation of the hydroxyl group followed by subsequent double methylenation of the diketone afforded diene-yne **47**. The cycloisomerization precursor **48** was produced by a lithium chemistry to introduce the terminal hydroxymethyl group. After extensive investigations, the conditions reported by the group of Trost [ $\text{CpRu}(\text{PPh}_3)_2\text{Cl}$ , CSA,  $\text{In}(\text{OTf})_3$ ] [22] was revealed to be successful to furnish the tetracycle **33** with a modest yield. Lastly, echinopines A (**1**) and B (**2**) could be formed through the same procedures as described before.



Scheme 7: Chen's formal synthesis of echinopines.

Central to Chen's novel approach towards echinopines was the use of two transition-metal-catalyzed enyne cycloisomerizations. Especially the ruthenium-mediated intramolecular cyclopropanation was successfully demonstrated in a complex molecular system, which resembled the late-stage bond formations in the proposed biosynthetic pathway for the echinopine framework.



Scheme 8: Synthesis of echinopines by a bio-inspired strategy.

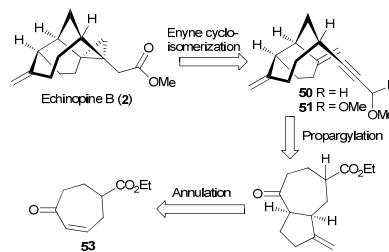
## 2.4 Vanderwal's synthesis of echinopine B

Another synthesis of echinopine B (**2**), employing a bio-inspired conversion of a *cis*-guaiane precursor bearing a [3/5]-bicycle into the [3/5/5/7] skeleton, was achieved by the team of Vanderwal in 2012 [7]. While the core of echinopines was established through an enyne cycloisomerization [23] from **50/51**, the bicyclic **52** was accessible by an annulation of cycloheptenone **53** (Scheme 9).

Starting from ketone **54**, a standard ring expansion process was carried out to generate the  $\delta$ -substituted cycloheptenone **53** with a 46% overall yield (Scheme 10). Since the one-step annulation by Piers protocol [24] was unsuccessful, the group adopted a stepwise procedure, beginning with installation of a vinyl chain through conjugate addition of cuprate **55** (dr = 6.5:1). The relative stereochemical outcome between the two centers could not be fully determined by NMR spectroscopy at this stage. The synthesis was continued by transforming the silyl ether into tosylate ester **56** before the annulation took place. Under the condition of LDA, the *cis*-fused bicyclo[5.3.0]decane **52** was prepared in 25–40% yield, along with a near 30% recovery of the starting material. After Wittig olefination of compound **52**, the resultant ester was subjected to DIBAL-H reduction, followed by treatment with Ohira-Bestmann reagent [25], leading to the diene-yne **58** as a mixture of diastereomers (4.5:1). A control experiment indicated epimerization occurred under the Ohira-Bestmann conditions.

With bicyclic **58** in hand, the terminal alkyne was then functionalized to propargylic ether **50** upon exposure to *n*-BuLi/MOMCl. The key cycloisomerization was realized in the

presence of  $\text{PtCl}_2$  [26], delivering the tetracyclic core of echinopine in 56% yield. On the basis of the successful cyclopropanation, it could be concluded that the conjugate addition (**53**→**56**) might give the undesired stereoisomer predominantly, which was corrected during the alkylation to enable the desired cycloisomerization. Since the two-carbon chain was attached to the core as an enol ether, a direct oxidation using PCC formed the methyl carboxylate [27],



Scheme 9: Retrosynthetic analysis of echinopine B by Vanderwal *et al.*

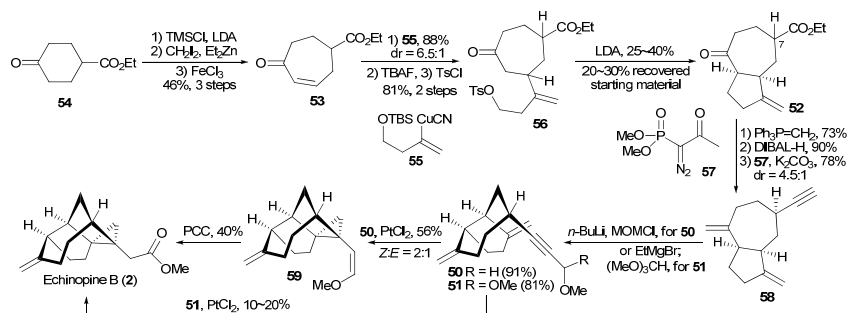
providing racemic echinopine B. Alternatively, a propargylacetal **51** was introduced from **58**, and its cycloisomerization could straightforwardly afford echinopine B, although with less efficiency (10–20% yield).

By use of the enyne cycloisomerization, together with a facile synthesis of [5/7]-bicyclic system **52**, Vanderwal's accomplishments provided a very concise solution to the synthetic issue of echinopines.

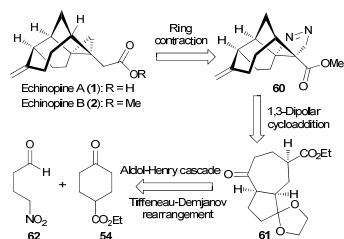
## 2.5 Liang's synthesis of echinopines

Liang and co-workers described a new synthesis of echinopines in 2013 [8], taking advantage of an intramolecular 1,3-dipolar cycloaddition and ring contraction sequence for the core construction. From a retrosynthetic viewpoint, the *cis*-fused bicyclo[5.3.0]decane system (**61**) could be formed through a tandem aldol-Henry reaction, followed by a Tiffeneau-Demjanov rearrangement (Scheme 11) [28].

In the forward synthesis, an aldol-Henry cascade between 4-nitrobutanal **62** and ketone **54** was employed as the launch point, with subsequent oxidation of the resulting secondary alcohol, giving access to *trans*-decaline **63a** and **63b** as a pair of isomers (Scheme 12). The undesired **63a** could be epimerized to **63b** upon treatment with  $\text{Et}_3\text{N}$ , which offered a scalable and efficient preparation of **63b** from the commercially available **54**. After ketone protection and nitro reduction, subjecting amine **64** to the Tiffeneau-Demjanov rearrangement conditions [29] delivered *cis*-fused [5/7]-bicycle **61** (77%) through the diazo intermediate **65**. The short sequence for supplying the bicyclo[5.3.0]decane system set the stage for the following chemistry.

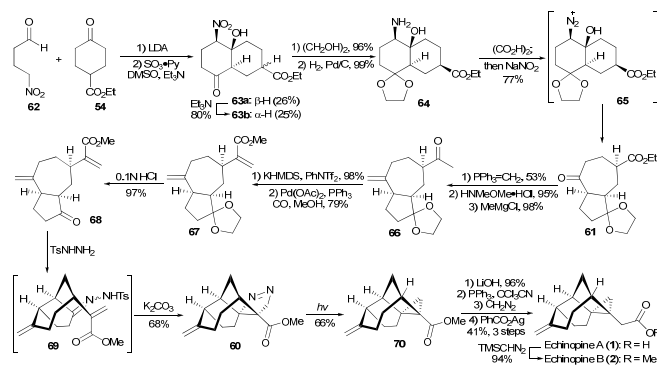


Scheme 10: A concise synthesis of echinopine B via a cycloisomerization strategy.



Scheme 11: Liang's bond disconnection towards echinopines.

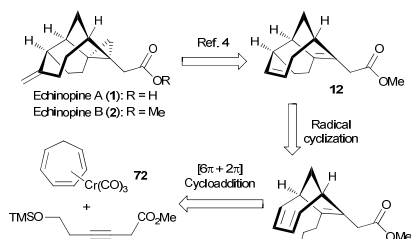
Several conventional transformations were involved before the designed 1,3-dipolar cycloaddition took place. Specifically, Wittig methylenation of **61** followed by a Weinreb ketone synthesis [30] furnished the olefinic ketone **66**. The latter was converted into unsaturated ester **67** efficiently *via* a palladium-catalyzed carbonylation of the vinyl triflate. After cleavage of the dioxolane, tosylhydrazone **69** was formed and ready for the 1,3-dipolar cycloaddition. Gratifyingly, upon exposure to  $K_2CO_3$ , **69** underwent the cycloaddition smoothly to give the advanced intermediate **60** [31]. The anticipated ring contraction was realized by a light-induced decomposition of pyrazoline to cyclopropane [32], leading to the echinopine framework **70** in 66% yield. After hydrolysis of the ester to carboxylic acid in **70**, the synthesis of echinopine A (**1**) was completed through an Arndt-Eistert homologation process [33], while echinopine B (**2**) was obtained by further methylation.

Scheme 12: Total synthesis of echinopines by Liang *et al.*

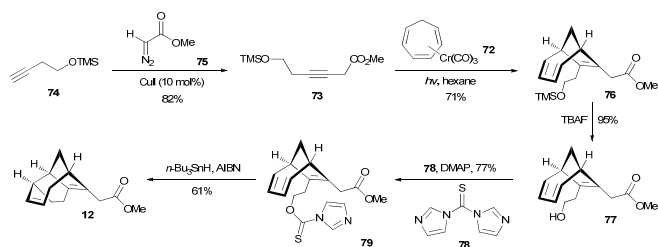
The Liang group demonstrated a new entry to the *cis*-fused bicyclo[5.3.0]decane system from an aldol-Henry cascade reaction followed by a Tiffeneau-Demjanov rearrangement. This critical building block was ultimately elaborated to echinopines by a 1,3-dipolar cycloaddition with subsequent light-induced ring contraction. The crucial 1,3-dipolar cycloaddition between diazo alkane and alkene functionalities established the structural complexity rapidly, representing its second application in natural product synthesis [34].

## 2.6 Misra's formal synthesis of echinopines

For a given molecule, there can be various ways for bond disconnection. In the case of echinopines, aside from the aforementioned synthesis, Misra *et al* disclosed a distinct strategy very recently [10]. The target sesquiterpenoids could be accessed from intermediate **12** according to Magauer *et al* [4], in which the five-membered ring could be forged by a radical cyclization of compound **71** (Scheme 13). The crucial bicyclo[4.2.1]nonane core could in turn be assembled via a  $[6\pi + 2\pi]$  cycloaddition of cycloheptatriene tricarbonyl chromium complex **72** and alkyne **73** [35].



Scheme 13: Retrosynthetic analysis of echinopines by Misra and co-workers.



Scheme 14: Formal synthesis of echinopines by Misra and co-workers.

After a successful model study showing the feasibility of the  $[6\pi + 2\pi]$  cycloaddition/radical cyclization sequence, the authors initiated their efforts towards echinopines A and B. As depicted in Scheme 14, the substituted alkynoate **73** was easily prepared on a multigram scale from alkyne **74** and diazoester **75**, by harnessing Fu's C–H insertion method in the presence of catalytic CuI [36]. Under photolytic conditions, the key  $[6\pi + 2\pi]$  cycloaddition between **72** and **73** proceeded with excellent stereo- and regioselectivity, leading to the bicyclic intermediate **76** in 71% yield. Next, desilylation followed by treatment with 1,1'-(thiocarbonyl)diimidazole (**78**) afforded thioxoester **79** smoothly. Thus, the stage was set for the radical cyclization. After screening of the conditions, it was found that slow addition of the *n*-Bu<sub>3</sub>SnH/AIBN mixture to the refluxing solution of **79** ultimately gave the desired product **12** in 61% yield. Synthesis of the known tricyclic intermediate **12**, which was previously prepared by Magauer and co-workers, represents a new formal synthesis of the echinopine sesquiterpenoids.

As mentioned above, Misra *et al.* have accomplished the formal synthesis of echinopines A and B in an extremely concise manner, by reaching the known intermediate **12** in only five steps. Remarkably, the utility of a photo-induced  $[6\pi + 2\pi]$  cycloaddition to construct the bicyclo[4.2.1]nonane system was of great significance to this synthesis.

## 3 Conclusions

The echinopine sesquiterpenoids have attracted the attention of synthetic laboratories worldwide, being reflected in the chemical synthesis published almost each year since the report of their isolation. The major achievements have been highlighted in this review, which illustrate the evolution of creative strategies and applications of modern chemical methodologies. We hope the endeavors in this field will not only serve to enrich our education in synthesis design and execution, but also as the launching point for a better understanding of the biological activities and medicinal profiles of these intriguing compounds.

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## Synergistic Effects of Dietary Natural Products as Anti-Prostate Cancer Agents

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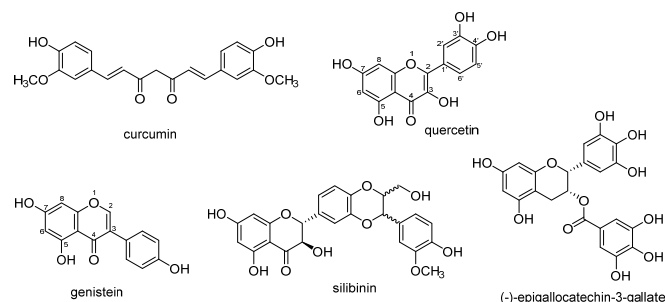
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This review is to describe synergistic effects of various combinations of dietary natural products including curcumin, quercetin, soybean isoflavones, silibinin, and EGCG that have potential for the treatment of prostate cancer. These data can provide valuable insights into the future rational design and development of synergistic and/or hybrid agents for potential treatment of prostate cancer.

**Keywords:** Dietary natural product, Synergy, Combination therapy, Prostate cancer.

### 1. Introduction

Dietary habit has been identified as one of the risk factors for prostate cancer as evidenced by several epidemiological studies. For example, Asian traditional food has been demonstrated to be correlated with the low incidence of prostate cancer in Asian men in contrast to soaring risk of prostate cancer in the Asian immigrants who live in the United States [1]. A variety of dietary agents (e.g. curcumin, quercetin, and silibinin) with diverse chemical structures (Figure 1) have been verified by *in vitro* cell-based and *in vivo* animal studies to possess the potential for preventing and treating prostate cancer [2-3]. The safety profiles enable these dietary natural products to be qualified as drug candidates. However, their moderate potency and poor bioavailability impede them in becoming effective chemotherapeutics when taken alone [4-5]. Combination therapy might be an important strategy to overcome this problem in order to achieve enhanced overall clinical efficacy with acceptable clinical toxicity. Indeed, synergistic inhibition of growth, proliferation, and apoptosis of prostate cancer cells has been evident when using combinations of one dietary natural product with another natural product or with well-established chemotherapeutics. This review summarizes the synergistic effects of dietary anti-prostate cancer agents including curcumin, quercetin, soybean isoflavones, silibinin, and (-)-epigallocatechin-3-gallate (EGCG, Figure 1). These data can provide valuable insights into future rational design and development of synergistic and/or hybrid agents for potential treatment of prostate cancer.



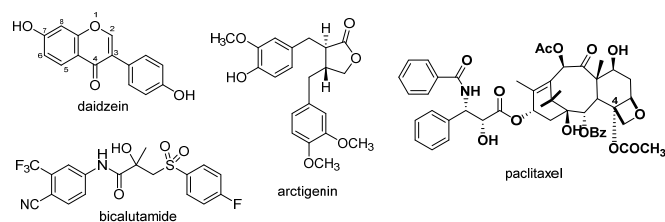
**Figure 1:** Structures of curcumin, quercetin, genistein, silibinin, and EGCG.

### 2. Synergistic effects of curcumin as anti-prostate cancer agents

Curcumin (Figure 1), derived from *Curcuma longa* (turmeric) that has long been used for food flavoring and Ayurvedic medicine, represents a dietary natural product possessing potential in preventing and treating prostate cancer [2]. As summarized below, several studies have established the synergistic effects of curcumin in combination with either other natural products or clinical used chemotherapeutics as anti-prostate cancer agents.

**2.1 Synergistic effects of curcumin with isoflavones:** Genistein (4',5,7-trihydroxyisoflavone, Figure 1) and daidzein (4',7-dihydroxyisoflavone, Figure 2) are two chief isoflavones that were exclusively obtained from soybeans. When they were used for the respective single agent treatment, soybean milk, genistein, and daidzein were capable of inhibiting prostate cancer cell growth *in vitro* and tumor xenograft growth *in vivo* [6].

Combined ingestion of soy isoflavones and curcumin has been unveiled by Ide and co-workers to substantially downregulate serum prostate-specific antigen levels for men who received biopsies in one clinical study [7]. Further investigation by the same research group indicated that the combination treatment of isoflavones and curcumin led to additive suppression of cell proliferation through activation of DNA damage response in LNCaP prostate cancer cells [8]. The synergism was also observed recently between curcumin and genistein in growth suppression of androgen-insensitive human prostate cancer cells [9]. Additionally, combined administration of curcumin and genistein exhibited more noticeable of the capability to inhibit cell proliferation and to induce apoptosis in androgen-insensitive PC-3 human prostate cancer cells than the respective single-agent treatment [10].



**Figure 2:** Structures of daidzein, bicalutamide, arctigenin, and paclitaxel.

**2.2 Synergistic effects of curcumin with EGCG:** EGCG stands for (-)-epigallocatechin-3-gallate (Figure 1) and is the most abundant flavan-3-ol in green tea. EGCG has been shown by various *in vitro* and animal studies to possess potential for preventing and treating prostate cancer [11]. The combination of 5-10  $\mu$ M curcumin and 40  $\mu$ M EGCG has been revealed by one study to improve synergistically the *in vitro* antiproliferative effects by 40% in androgen-sensitive LNCaP prostate cancer cells as compared with their individual treatments [12]. The synergism between curcumin and EGCG was linked to enhanced cell apoptosis induction and cell cycle perturbation through upregulation of multiple critical carcinogenesis-involved signaling pathways (e.g. PI3k/Akt and nuclear factor kappa B).

**2.3 Synergistic effects of curcumin with arctigenin:** Arctigenin (Figure 2) is a plant lignan derived mainly from *Arctium lappa* of the Asteraceae family that is commonly used in traditional Chinese medicine for the treatment of inflammation-associated diseases. The *in vitro* antiproliferative property of arctigenin has been evidenced in several cancer cell lines [12]. The cell proliferation inhibition and cell apoptosis induction of the combination treatment of curcumin and arctigenin towards androgen-sensitive LNCaP human prostate cancer cells was appreciably enhanced as compared with their individual treatment [12]. Additionally, the effect of curcumin and arctigenin in combination and alone on several vital signaling pathways relevant to cell proliferation and apoptosis has been examined. The data showed that combination treatment was capable of enhancing the ratio of Bax (pro-apoptotic protein) to Bcl-2 (anti-apoptotic protein), increasing the phosphorylation suppression of nuclear factor kappa B, and reducing the level of p-I $\kappa$ B.

**2.4 Synergistic effects of curcumin with TRAIL:** TRAIL, also designated as Apo2L, is a cytokine member of the tumor necrosis factor family and plays as an extracellular signal that activates apoptosis in a variety of cancer cell lines, but exhibits little toxicity to normal cells [13]. In combination with its *in vivo* antitumor efficacy towards human tumor xenografts in nude mice, TRAIL is regarded as an appealing cytokine for the treatment of advanced cancers, such as advanced, metastatic prostate cancer [14]. However, TRAIL alone is not efficient enough for the treatment due to its drug resistance and moderate potency. The combination of TRAIL and dietary natural products with safety profiles might provide an alternative to chemotoxic agents to treat prostate cancer.

Curcumin has been shown in both *in vitro* and *in vivo* studies to sensitize both androgen-sensitive (LNCaP) and androgen-insensitive (PC-3) human prostate cancer cells to TRAIL-induced apoptosis [15-16]. This sensitization was demonstrated by a follow-up study to be associated with downregulation of Akt-regulated nuclear factor- $\kappa$ B (NF- $\kappa$ B) and NF- $\kappa$ B dependent anti-apoptotic proteins, including Bcl-2, Bcl-xL, and XIAP [17]. The in-depth *in vivo* animal experiment verified that curcumin and TRAIL combination appreciably reduced the growth of PC-3 prostate tumor xenografts in Balb/c nude mice by reducing the tumor growth rate to 139% and 147% for TRAIL-treated and curcumin-treated group, respectively, to 59% for the combined treatment group [16].

**2.5 Synergistic effects of curcumin with adenoviral mutants:** Adenoviruses have been identified as oncolytic virotherapeutic agents that can selectively replicate in and destroy cancer cells, but causing no harm to normal cells. Numerous viral mutants have been established as potential anti-prostate cancer agents, featuring clinical safety profiles. However, the clinical efficacy when administered alone needs to be improved due to its moderate potency. Combination administration of curcumin and tumor

selective adenoviral mutant (Ad5) can amplify the cytotoxicity three to eight times towards both virus-sensitive 22Rv1 and virus-insensitive PC-3 human prostate cancer lines. This suggests that curcumin and Ad5 served as a potential combination of chemotherapy and oncolytic virotherapy [18].

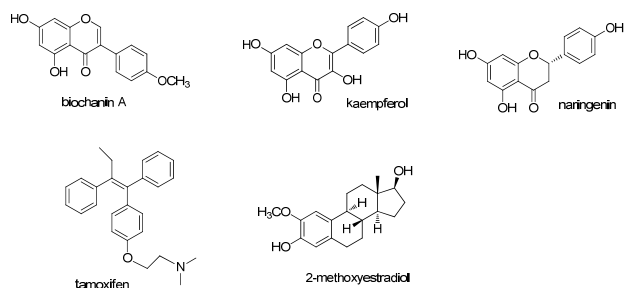
**2.6 Synergistic effects of curcumin with bicalutamide:** Treatment of androgen-sensitive LNCaP prostate cancer cells with bicalutamide (Figure 2), a nonsteroidal androgen receptor antagonist, at 10  $\mu$ M for 24 hours resulted in notable suppression of cell proliferation. Significant antiproliferative effects of bicalutamide towards androgen-insensitive human prostate cancer cell lines (PC-3 and DU145) can only be observed at higher concentration and prolonged exposure time (30  $\mu$ M, 72 hours). Intriguingly, a profound synergy has been observed between curcumin at low doses (10-20  $\mu$ M) and bicalutamide in both androgen-sensitive and androgen-insensitive prostate cancer cell lines [19]. The potential underlying mechanism was correlated with phosphorylation escalation of extracellular signal-regulated kinase 1/2 (ERK1/2) and stress-activated protein kinase (SAPK)/Jun amino-terminal kinase (JNK).

**2.7 Synergistic effects of curcumin with paclitaxel:** Curcumin has been demonstrated *in vitro* to considerably potentiate the cytotoxicity of paclitaxel (Figure 2), a naturally occurring microtubule stabilizer and well-known clinically used anticancer drug, towards two androgen-insensitive DU145 and PC-3 human prostate cancer cell lines [20]. Intriguingly, this synergy can be affected by the treatment schedule. Sequential treatment with curcumin followed by paclitaxel yielded more significant synergism than concurrent treatment with curcumin/paclitaxel and sequential treatment with paclitaxel followed by curcumin [21]. The synergism between curcumin and paclitaxel in PC-3 prostate cancer cells was evidenced to be correlated with upregulation of p21WAF1/CIP1 expression and downregulation of paclitaxel-induced nuclear factor kappa B.

**2.8 Synergistic effects of curcumin with radiotherapy:** The limitation of radiotherapy, a frequent therapy for prostate cancer, is radio resistance. Radiotherapy failure has been more often observed in the prostate cancer patients with abnormal p53. Sensitization of curcumin on radiotherapy has been examined in one study [22]. It was concluded that the combination of curcumin and radiation can synergistically reduce cell viability and proliferation relative to either curcumin or radiation alone in both wild type p53-containing LNCaP and mutant p53-containing PC-3 human prostate cancer cell lines. Additionally, a synergistic effect independent of p53 status was also observed between curcumin and radiation towards clonogenic cell death in both LNCaP and PC-3 human prostate cancer cell lines.

### 3. Synergistic effects of quercetin as anti-prostate cancer agents

Quercetin, 3,3',4',5,7-pentahydroxyflavone (Figure 1), is an ubiquitous flavonol widely occurring in a variety of edible vegetables and fruits. Several *in vitro* cell based evaluations indicate that quercetin processes cytotoxic and antiproliferative effects against a panel of prostate cancer cell lines, but not obvious toxicity towards normal prostate epithelial cells [3a]. The *in vivo* anti-prostate tumor efficacy of quercetin has also been attested to by several animal experiments [23]. Combination treatment of quercetin with either other natural products or well-known chemotherapeutics was believed to be one good strategy to overcome its moderate potency and poor bioavailability.



**Figure 3:** Structures of biochanin A, kaempferol, naringenin, tamoxifen, and 2-methoxyestradiol.

**3.1 Synergistic effects of quercetin with genistein:** One clinical trial (ClinicalTrials.gov Identifier: NCT01538316) is investigating the effectiveness of the combination of genistein and quercetin in reducing the level of prostate-specific antigen (PSA) in patients with rising PSA.

**3.2 Synergistic effects of quercetin with genistein and biochanin A:** It is believed that combinations of different subclasses of flavonoids might provide either additive or synergistic activity *in vitro* and *in vivo* when compared with single-flavonoid treatment. Kumar *et al.* systematically investigated the synergistic effects of two-flavonoid and three-flavonoid combinations among quercetin, genistein, and biochanin A (Figure 3) on suppressing cell proliferation of three prostate cancer cell lines (PC-3, DU145, and LNCaP). The findings of this study showed (i) the two-flavonoid combinations at 25  $\mu$ M (12.5  $\mu$ M of each flavonoid) only exhibited additive effects in inhibiting prostate cancer cell proliferation; and (ii) the three-flavonoid combination (8.33  $\mu$ M of quercetin, genistein, and biochanin A, respectively) can synergistically repress cell proliferation in three prostate cancer cell lines [24]. The androgen-insensitive PC-3 and DU145 human prostate cancer cell lines are more responsive to the combination treatments than the androgen-sensitive LNCaP cell line. Both two-flavonoid and three-flavonoid combinations can cause synergistic apoptosis and cell cycle arrest at the G<sub>1</sub> phase of PC-3 prostate cancer cells as evidenced by flow cytometric analysis [24]. Further investigation of the molecular mechanism revealed that the synergistic effects in PC-3 cells are associated with (i) the downregulation of c-myc, PCNA, mitogen activated protein kinase (MAPK)/ERK-1/2 signaling, and PI3K/AKT signaling pathway, and (ii) upregulation of ER- $\beta$  gene and MAPK/c-Jun N-terminal kinase signaling. The synergistic inhibition of cell-cycle in PC-3 cells is correlated with gene expression downregulation of cyclins D1 and E. The synergistic apoptosis contributed by the flavonoid combinations is related to the cooperative upregulation of caspase-3 and pro-apoptotic protein Bax, and to the collaborative downregulation of anti-apoptotic protein Bcl-2 in PC-3 cells [24].

**3.3 Synergistic effects of quercetin with green tea catechins:** The combination of quercetin with epigallocatechin gallate (EGCG, Figure 1) only exerts additive instead of synergistic effects in controlling proliferation of CWR22Rv1 and PC-3 prostate cancer cells [25-26]. Intriguingly, a three-flavonoid combination consisting of quercetin, EGCG, and genistein can synergistically inhibit the CWR22Rv1 prostate cancer cell proliferation [25]. In contrast, androgen-sensitive LNCaP prostate cancer cells are more responsive to the treatment of quercetin combined with EGCG. The combination treatment acts synergistically in inhibiting proliferation, apoptosis, and cell cycle regulation of LNCaP cells [26]. Combination treatment of PC-3 and LNCaP prostate cancer cells with quercetin plus EGCG led to synergistically increased cellular absorption and decreased methylation of both EGCG and

quercetin [26]. Additionally, the combination of quercetin and EGCG synergistically suppresses the self-renewal activities of prostate cancer stem cells, activates apoptosis, and prevents cancer stem cells' migration and invasion [27].

The *in vivo* antitumor efficacy of green tea catechins has been shown to be improved by quercetin. A Phase I clinical study (ClinicalTrials.gov Identifier: NCT01912820) is designated to translate these preclinical results into a clinical setting by assessing the possible bioavailability improvement of quercetin and green tea catechins in combination.

**3.4 Synergistic effects of quercetin with kaempferol:** The only structural difference between kaempferol (3,4',5,7-tetrahydroxyflavone, Figure 3) and quercetin is that kaempferol lacks the 3'-OH group in quercetin. Kaempferol has been revealed to have antiproliferative effects towards prostate cancer cells with IC<sub>50</sub> values ranging from 10 to 55  $\mu$ M [28]. Quercetin and kaempferol have been demonstrated to inhibit synergistically proliferation of androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate cancer cells [29].

**3.5 Synergistic effects of quercetin with naringenin:** Naringenin (Figure 3) is a naturally occurring flavonoid belonging to the flavanone subclass, which only exhibits weak antiproliferative effects towards prostate cancer cells [29b]. Its synergistic effect on cell proliferation suppression towards the androgen-sensitive LNCaP prostate cancer cell line has been identified [29a].

**3.6 Synergistic effects of quercetin with arctigenin:** As mentioned in section 2.3, arctigenin is a naturally occurring lignan with promising antiproliferative activity towards prostate cancer cells. Arctigenin has been demonstrated to exhibit 10- to 20-fold stronger antiproliferative potency than quercetin in both LAPC-4 and LNCaP prostate cancer cell lines. LNCaP cells were more refractory to arctigenin than LAPC-4 cells. The treatment with arctigenin in combination with quercetin synergistically inhibits the cell proliferation in both prostate cancer cell lines. Cell proliferation suppression was enhanced by 30% in both cell lines by a combination treatment with arctigenin (1  $\mu$ M) and quercetin (20  $\mu$ M) as compared with the treatments with the individual compounds. The combination index values were in a range of 0.5-0.7 for LAPC-4 cells, and 0.2-0.8 for LNCaP cells [30]. The combination of quercetin plus arctigenin also led to the synergistic phosphorylation inhibition of multiple signaling molecules in LAPC-4 prostate cancer cells as determined by the intracellular signaling array analysis, as well as by Western blot analysis [30]. The synergistic downregulation of androgen receptor and prostate specific antigen in LAPC-4 cells was generated by the combination treatment as examined by qRT-PCR analysis and Western blot analysis. Additionally, the synergistic migration suppression of LAPC-4 and LNCaP prostate cancer cells was noticeably enhanced by the combination treatment [30].

**3.7 Synergistic effects of quercetin with tamoxifen:** Tamoxifen (Figure 3), a specific antagonist of the estrogen receptor, has been demonstrated to be capable of potentiating the cytotoxic effect of chemotherapeutic agents in prostate cancer cell lines [31]. The *in vivo* synergistic efficacy of combined tamoxifen (10 mg/kg/week) plus quercetin (200 mg/kg/day) has been examined in CWR22 prostate tumor xenografts in severe combined immune deficient (SCID) mice. The results showed that combined tamoxifen with quercetin appreciably prolonged the appearance time of tumors by four days, reduced the final tumor volume by 73%, and lowered the tumor weight at the endpoint by 67%. As compared with the

individual agent treatment, a statistically significant synergism between quercetin and tamoxifen was established. The synergistic antitumor efficacy is linked with the modulation of angiogenesis [32].

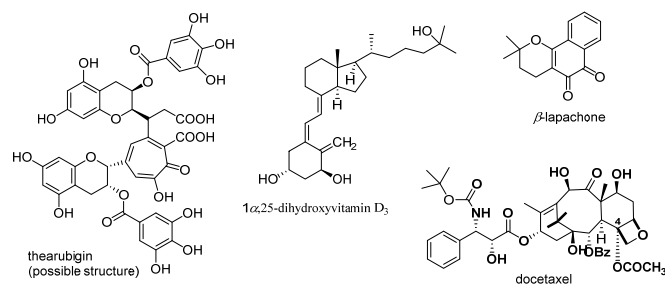
**3.8 Synergistic effects of quercetin with 2-methoxyestradiol:** 2-Methoxyestradiol (Figure 3), an endogenous metabolite of 17 $\beta$ -estradiol, has been revealed to inhibit growth and activate apoptosis of both androgen-sensitive and androgen-insensitive human prostate cancer cells by arresting cell cycle regulation in the G2/M phase [33]. One clinical study identified that 2-methoxyestradiol has a good safety profile for humans, but poor bioavailability as a drug candidate [34]. Wang *et al.* examined the growth inhibitory effects of sixteen combinations of quercetin (5, 10, 20, 40  $\mu$ M) and 2-methoxyestradiol (0.5, 1, 3, 5  $\mu$ M) towards androgen-sensitive LNCaP and androgen-insensitive PC-3 human prostate cancer cell lines. Combination index values were in a range of 0.36-2.18 for LNCaP cells, and 0.32-1.77 for PC-3 cells. The combination index values indicated that synergistic effects can only be achieved when combining lower doses of quercetin (5 and 10  $\mu$ M) with higher doses of 2-methoxyestradiol (3 and 5  $\mu$ M) for LNCaP cells, and mixing higher doses of quercetin (20 and 40  $\mu$ M) with higher doses of 2-methoxyestradiol (3 and 5  $\mu$ M) for PC-3 cells [35]. The combination treatment for 48 hours significantly enhanced the apoptosis induction through regulation of anti-apoptotic Bcl-2 and proapoptotic Bax as compared with two individual treatments [35]. Additionally, the synergistic cell cycle regulation in the G2/M phase was also observed by treating LNCaP and PC-3 cells with quercetin plus 2-methoxyestradiol.

**3.9 Synergistic effects of quercetin with TRAIL:** Combination treatment of quercetin (100  $\mu$ M) and the death ligand TRAIL (10-100 ng/mL) for 24 hours generated strong synergistic cytotoxicity and apoptosis induction in androgen-insensitive DU-145 and PC-3 human prostate cancer cell lines, as determined by Annexin V and PI staining assays. The PC-3 cell line was illustrated to be more sensitive to the synergy of quercetin with TRAIL than the DU145 cell line even though these two cell lines are both tumorigenic and independent of wild-type p53. This disparity was assumed to be caused by an inherent dissimilarity in TRAIL sensitivity [36]. Further Western blot analysis displayed that the quercetin-induced sensitization to TRAIL-mediated DU145 cell apoptosis is linked to the upregulation of caspases -3, -8, and -9 [36-38]. Downregulation of survivin (anti-apoptotic protein) gene expression in both DU145 and PC-3 cell lines in an ERK-MSK1 dependent pathway was suggested to be a vital molecular mechanism associated with the apoptosis synergism between quercetin and TRAIL. Upregulation of DR5 (decoy receptor 5) protein expression by quercetin through enhancing transcription and protein stability has also been demonstrated by Jung *et al.* to be associated with its potentiating effect on the apoptosis of prostate cancer cells induced by TRAIL [37]. However, Kim and Lee did not observe any alteration of DR5 in their study when treating prostate cancer cells with quercetin in combination with TRAIL [38]. The combination of quercetin plus TRAIL has been revealed to exhibit a stronger synergistic cytostatic effect than synergistic cytotoxicity. The overriding feature of the combination is that the synergistic apoptosis is triggered by a mixture of an apoptotic “sensitizer” (quercetin) and an apoptotic “inducer” (TRAIL) [36].

## 2. Synergistic effects of soybean isoflavones as anti-prostate cancer agents

**4.1 Synergistic effects of genistein with daidzein:** As indicated previously, genistein and daidzein are two isoflavones exclusively isolated from soybeans [6]. The only structural difference between

genistein (Figure 1) and daidzein (Figure 2) is that daidzein lacks the hydroxyl group at C-5 of genistein. Genistein has been elicited to be more potent than daidzein in suppressing cell growth and proliferation of various prostate cancer cells [39]. Interestingly, the combination of daidzein and genistein exhibited greater anti-proliferative and apoptotic effects than the respective single agent treatment towards androgen-sensitive LNCaP and its derived C4-2B human prostate cancer cell lines [40]. It is especially worth noting that an apparent apoptosis induction in C4-2B prostate cancer cells can be caused by the combination of genistein (50  $\mu$ M) and daidzein (25-50  $\mu$ M), but not individual genistein or daidzein. The synergism between genistein and daidzein might be contributed by their shared molecular mechanism of actions through modulating cell cycle and angiogenesis related genes and by the multi-target mechanism underlying the anti-proliferative effect of genistein.



**Figure 4:** Structures of thearubigin, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>,  $\beta$ -lapachone, and docetaxel.

**4.2 Synergistic effects of soybean isoflavones with tea:** In one *in vivo* animal study carried out by Zhou and co-workers in 2003, it was concluded that prostate tumor progression and metastasis can be synergistically suppressed by soy isoflavones (with genistein as its major chemical component) and tea (both green and black tea) in androgen-sensitive LNCaP human prostate tumor xenografts in severe combined immune deficient (SCID) mice [41]. They observed that (i) treatment of mice with soy phytochemicals and black tea in combination led to a 1.46-times greater effect on tumorigenicity rate than their additive effect; and (ii) combination treatment of soy phytochemicals and tea (both black and green tea) synergistically suppressed tumor metastasis to lymph nodes with ratios of 2.13 and 1.87. The synergistic effects might be linked to effective reduction of both testosterone and DHT in serum.

**4.3 Synergistic effects of genistein with thearubigin:** Thearubigin (Figure 4) is synonymous with black tea polyphenols, which are transformed from green tea catechins (flavan-3-ols) catalyzed by polyphenol oxidase during tea leaf fermentation [42]. Thearubigin alone (up to 25  $\mu$ g) neither showed any apparent cell growth inhibition nor DNA distribution change of cell cycle modulation in an androgen-insensitive prostate cancer cell line. However, a combination of thearubigin and genistein (1:40) appreciably enhanced cell growth suppression and cell cycle arrest at the G2/M phase as compared with the genistein-treated group [43]. Additionally, exposure of PC-3 prostate cancer cells to a combination of 2.5  $\mu$ g/mL of genistein and 0.0625  $\mu$ g/mL of thearubigin resulted in cell growth suppression by 33%. No cell growth inhibition was observed from either thearubigin-treated (0.0625  $\mu$ g/mL) or genistein-treated (2.5  $\mu$ g/mL) groups. This result thus confirmed the synergism between thearubigin and genistein. The molecular mechanism underlying the synergism is still elusive. Sakamoto *et al.* pointed out that genistein, but not thearubigin, can directly regulate the cell cycle. However, thearubigin in conjugation with genistein can synergistically cause cell cycle arrest at the G2/M phase and therefore repress cell growth.



#### 4.4 Synergistic effects of genistein with 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>:

Vitamin D deficiency has been hypothesized to be one of the primary risks for prostate cancer [44]. This hypothesis was not only corroborated by the correlation between the low incidence of prostate cancer and the vitamin D-rich Asian diet, but also confirmed by the antiproliferative effect of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (the most bioactive metabolite of vitamin D, Figure 4) against androgen-sensitive LNCaP human prostate cancer cells [45]. The inhibition of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> on prostate cancer cell proliferation is linked to the nuclear vitamin D receptor. The soy isoflavone genistein (0.1 to 1  $\mu$ M) and 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (0.1 to 0.5 nM) have been demonstrated by Cramer and co-workers to possess synergistic effects on suppressing the growth of primary human prostatic epithelial cells and androgen-sensitive LNCaP human prostate cancer cells through cell cycle arrest [46]. The further mechanism exploration from the same research group demonstrated that 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> and genistein can act jointly to enhance the stability and subsequently the protein level of vitamin D receptor; and to escalate the levels of cell cycle inhibitor p21 that is essential to the anti-proliferative effects of both genistein and 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. Accordingly, modulation of nuclear vitamin D signaling was concluded as one mechanism for the synergism between genistein and 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> [47].

#### 4.5 Synergistic effects of genistein with $\beta$ -lapachone:

$\beta$ -Lapachone (Figure 4) is an abundant naturally occurring quinone and can be readily obtained from *Tabebuia avellanedae*, the lapacho tree native to South America.  $\beta$ -Lapachone has been shown to be a distinct Topo I inhibitor that suppresses the relevant enzymatic activity through directly binding to Topo I. Additionally,  $\beta$ -lapachone has been revealed to be capable of suppressing cell growth and promoting cell apoptosis in both androgen-sensitive and insensitive human prostate cancer cells [48]. The androgen-insensitive PC-3 human prostate cancer cells have been shown to be significantly more responsive to the combined administration of genistein and  $\beta$ -lapachone when compared with the respective single-agent treatment [49]. Additionally, genistein and  $\beta$ -lapachone have been demonstrated to act synergistically to induce apoptosis in the PC-3 cell line.

#### 4.6 Synergistic effects of genistein with sodium selenite:

The anti-prostate cancer potential of selenite has been established based on various *in vitro* cell-based assays, *in vivo* animal experiments, and human clinical studies [50]. In search of a combination of anti-prostate cancer agents with different underlying mechanisms, Zhao *et al.* evaluated the synergistic effects of genistein and selenite. The data revealed that the combination of genistein and selenite can synergistically induce cell apoptosis, cause cell cycle arrest at the G<sub>2</sub>/M phase, and modulate relevant signaling pathways in both androgen-sensitive LNCaP (p53-positive) and insensitive PC-3 (p53-negative) human prostate cancer cells [50]. The synergism was assumed to be partly due to their distinct molecular mechanisms. For example, selenite was shown to activate cell apoptosis but was not involved in cell cycle modulation. In contrast, genistein can cause both apoptosis activation and cell cycle modulation at the G<sub>2</sub>/M phase, which was contributed in part by downregulation of AKT [50].

**4.7 Synergistic effects of GCP with docetaxel:** GCP, with the isoflavones genistein and daidzein as the main chemical components, stands for a genistein combined polysaccharide that is marketed as a nutritional supplement. GCP with an enhanced absorption rate of genistein in the small intestine is generated from soybean extract catalyzed by mycelia. Docetaxel (Figure 4),

working by mainly promoting the polymerization of tubulin to microtubules and microtubule stabilization, is the current FDA-approved gold standard, first-line treatment for castration-resistant prostate cancer. The limitation of docetaxel's clinical efficacy is that it can barely prolong a maximum 2-3 years of survival for a prostate cancer patient [51]. Even worse, approximately 30,000 US men die each year of castration-resistant prostate cancer due to the inevitable progression of resistance to first-line treatment with docetaxel [52]. To explore the possible advantage of GCP in combination with docetaxel for the treatment of castration-resistant prostate cancer, the *in vitro* antiproliferative and apoptotic effects of the combination of GCP and docetaxel were examined by Burich *et al.* in one androgen-sensitive LNCaP prostate cancer cell line and three androgen-insensitive CWR22Rv1, PC-3, and LNCaP-R273H prostate cancer cell lines. The results showed that GCP can potentiate docetaxel in inhibiting cell proliferation and promoting cell apoptosis in the four prostate cancer cell lines [53]. However, the degree of synergism between GCP and docetaxel is dependent on the administration schedule. Much greater synergism was observed in the LNCaP cell model when docetaxel rather than GCP was administered first in the sequential treatment.

**4.8 Synergistic effects of GCP with bicalutamide:** GCP could also synergistically enhance the *in vitro* antiproliferative effects of androgen receptor antagonist bicalutamide (Figure 2) in androgen-dependent LNCaP and androgen-independent LNCaP-R273H prostate cancer cell lines [53]. The LNCaP-R273H model, established from its parental LNCaP cell line by constitutive expression of the p53 gain-of-function mutation R273H, may better represent patient prostate tumor cells after progression to androgen insensitive. Parental LNCaP prostate cancer cells have been demonstrated to be far more responsive than the variant LNCaP-R273H subline to individual treatment with either GCP or bicalutamide. Intriguingly, both prostate cancer cell lines were well-sensitized to the combination of GCP and bicalutamide.

### 3. Synergistic effects of silibinin as anti-prostate cancer agents

Silymarin is a commercially available herb preparation that is extracted from the seeds of *Silybum marianum* (L.) Gaertn. Seven flavonolignans compose the critical chemical components of silymarin, with silibinin (a 1:1 mixture of diastereomers silybin A and silybin B, Figure 1) as the most abundant one [54]. The medicinal values of silymarin and silibinin as chemotherapeutics to treat hepatotoxicity have been extensively explored, especially in Europe and Asia [55]. Since 1998, the anti-prostate cancer potential of silymarin and silibinin has been evidenced by several *in vitro* cell-based evaluations and *in vivo* animal studies [56]. Their liver protective function, antiproliferative effects, and antitumor efficacy suggest the potential of silymarin and silibinin for clinical use, along with currently established cancer therapies, to improve synergistically the efficacy while alleviating toxicity for prostate cancer patient treatment [57]. Silymarin has already been used by cancer patients to eliminate the hepatotoxicity caused by several chemotherapies [58]. The synergistic effects of silibinin and chemotherapeutic drugs on cell proliferation, cell cycle modulation, and apoptosis activation in various cancer cell lines have been reviewed by Raina and Agarwal [59]. As described in the following subsections, silymarin and silibinin have been reported to be synergized with well-established chemotherapeutic agents including cisplatin, carboplatin, doxorubicin, docetaxel, and mitoxantrone as anti-prostate cancer agents.

#### 5.1 Synergistic effects of silibinin with cisplatin/carboplatin:

Cisplatin and carboplatin belong to two platinum complexes that act as cytotoxic chemotherapeutics with molecular mechanism

underlying cytotoxicity similar to that of alkylating agents. Cisplatin [*cis*-diamminedichloroplatinum(II)] is a well-known and commonly used cytotoxic chemotherapy drug that was introduced into clinical trials in 1972. Its capability to form DNA adducts mainly contributes to the cytotoxicity of cisplatin [60]. However, its clinical efficacy is largely compromised by its high toxicity [61]. Carboplatin is another cytotoxic platinum complex with its full name as *cis*-diammine[1,1-cyclobutane-dicarboxylato]platinum. Compared with cisplatin, carboplatin has a better safety profile but no considerable effect towards castration-resistant prostate cancer [62]. Silibinin has been reported to potentiate cisplatin and carboplatin in inhibiting cell proliferation and activating cell apoptosis in the androgen-insensitive DU145 human prostate cancer cells. The synergistic effects on apoptosis were further confirmed by the results that the cleaved levels of PARP and Caspases 3, 7, and 9 were also synergistically upregulated by the combination regimen. The combination of silibinin (50-100  $\mu$ M) with either cisplatin (2  $\mu$ g/mL) or carboplatin (20  $\mu$ g/L) has also been reported to cause synergistic cell cycle arrest at the G<sub>2</sub>-M phase and downregulate the relevant proteins of Cdc2, cyclin B1, and Cdc25C. It is worth noting that exposure of DU145 cells to cisplatin, carboplatin, and silibinin alone resulted in cell cycle arrest at G<sub>2</sub>-M, S, and G<sub>1</sub> phases, respectively. Both immunocytochemical and Western immunoblotting analyses indicated that combination treatment with silibinin and platinum complex led to a noticeable increase in the levels of cytosolic cytochrome *c* [63].

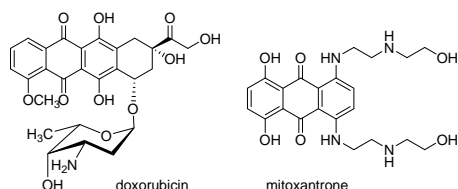


Figure 5: Structures of doxorubicin and mitoxantrone.

**5.2 Synergistic effects of silibinin with doxorubicin:** Doxorubicin (Figure 5) is a well-known chemotherapeutic for a variety of cancers targeting topoisomerase II; its clinical efficacy, however, is limited by its high systemic toxicity. The synergism between silibinin and doxorubicin has been examined against both androgen-insensitive DU145 and androgen-sensitive LNCaP human prostate cancer cell lines [64]. The results showed that treatment with silibinin (25-100  $\mu$ M) and doxorubicin (15-25 nM) alone or in combination led to considerable cell growth suppression in a dose-dependent manner in both prostate cancer cell lines. However, DU145 cells are much more sensitive to the synergistic effects of silibinin and doxorubicin than LNCaP cells. It was established that the combination treatment of silibinin (100  $\mu$ M) and doxorubicin (25 nM) generated a profound synergistic effect on inhibition of DU145 cell growth with combination index values in a range of 0.23-0.59. In contrast, almost no synergistic effect of silibinin (25  $\mu$ M) and doxorubicin (15 nM) was observed on suppression of LNCaP cell growth with a combination index of 0.929. The perceived synergistic effects on growth suppression of DU145 cells were linked to intense cell cycle perturbation at the G<sub>2</sub>-M phase. The combination treatment caused up to 88% cell arrest in the G<sub>2</sub>-M phase while silibinin treatment alone caused G<sub>1</sub> arrest, and doxorubicin alone only caused moderate G<sub>2</sub>-M arrest (41%). Mechanistically, the combination of silibinin and doxorubicin can synergistically downregulate the G<sub>2</sub>-M cell cycle related protein expression, including cdc25C, cdc2/p34 and cyclin B1, and cdc2/p34 kinase activity affiliated with histone H1. Additionally, the combination treatment can also act synergistically in promoting DU145 cell apoptosis [65].

### 5.3 Synergistic effects of silibinin with docetaxel/mitoxantrone:

Docetaxel (Figure 4) is a well-established anticancer drug and inhibits cell division mainly through binding to and stabilizing microtubules. Docetaxel represents the first cytotoxic chemotherapeutic that offers survival benefit for patients with castration-resistant prostate cancer. For this reason, the US Food and Drug Administration (FDA) approved docetaxel in combination with prednisone in 2004 for the first-line treatment in patients with castration-resistant prostate cancer [66]. However, an average 2.4 years of survival improvement and drug resistance make docetaxel less effective in the clinical use [67]. Chemotherapy with mitoxantrone (Figure 5), an inhibitor of topoisomerase II, has been shown to alleviate symptoms, but not prolong survival rate of patients with advanced castration-resistant prostate cancer [68]. In one study, only additive or even antagonistic effects on cell proliferation inhibition were observed when treating three human prostate cancer cell lines with silibinin in combination with docetaxel, with combination index values of 0.898-2.54 for DU145 cells, 0.921-2.32 for LNCaP cells, and 0.895-4.47 for PC-3 cells [69]. In contrast, the combination treatment of silibinin and mitoxantrone has been demonstrated to have noticeable synergy with combination index values in a range of 0.515-0.929 for DU145 cells, 0.521-0.967 for LNCaP cells, and 0.413-2.65 for PC-3 cells. The only exception is that antagonism was observed in the treatment of PC-3 cells with a low dose of mitoxantrone (e.g. 25 nM) in combined with silibinin [69]. To explore the molecular mechanism underlying the synergy between silibinin and mitoxantrone, the levels of caspase-3 and caspase-7 were evaluated. The results revealed that the combination treatment synergistically and significantly induced the apoptosis of three prostate cancer cell lines.

### 4. Synergistic effects of green tea catechins as anti-prostate cancer agents

Green tea crude extracts have been designated as green tea catechins that consist of a group of naturally occurring flavan-3-ols, with (-)-epigallocatechin-3-gallate (EGCG) as the most abundant one. Green tea catechins and EGCG have been demonstrated by several *in vitro* cell-based assays and *in vivo* animal studies to have potential in preventing and treating prostate cancer [11]. Several studies as shown in the following subsections have suggested that either green tea catechins or EGCG might be even more effective as anti-prostate agents when they synergistically act with other natural or non-natural compounds. The synergistic apoptosis induced by combinations of EGCG with anti-cancer drugs in various cancer cells (including prostate cancer cells) has been systemically reviewed [70].

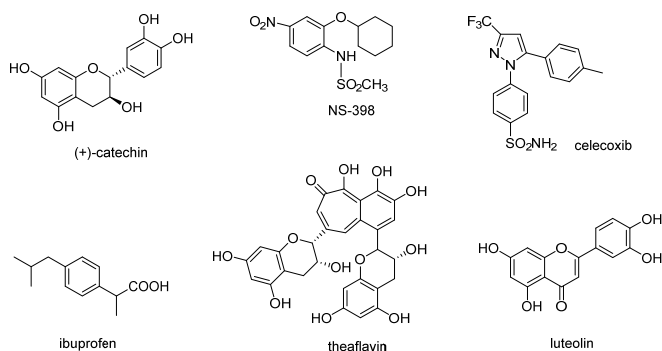


Figure 6: Structures of catechin, NS-398, celecoxib, ibuprofen, theaflavin, and luteolin.

### 6.1 Synergistic effects of EGCG with selective COX-2 inhibitors [71]:

Cyclooxygenase (COX) is a critical enzyme that catalyzes the

transformation of arachidonic acid to prostaglandins. Highly expressed in prostate cancer tissue, prostaglandins are believed to serve as an essential role in the cell proliferation of prostate cancer. COX-2 is an inductive isoform and has been revealed to affect the proliferation and apoptosis of prostate cancer cells [72]. Consequently, COX-2 inhibitors have been proposed to be useful for potential chemotherapy for prostate cancer. (+)-Catechin (Figure 6) is the simplest flava-3-ol derived from green tea, which showed certain cell proliferation suppression at a high concentration (100  $\mu$ M) toward LNCaP and DU145 (but not PC-3) prostate cancer cell lines. Zaslau and co-workers reported that catechin and NS398 (a selective COX-2 inhibitor, Figure 6) would act synergistically to inhibit prostate cancer cell proliferation [72a]. Adhami and co-workers also explored the combinatorial effect of (-)-epigallocatechin-3-gallate (EGCG) and selective COX-2 inhibitors (NS-398 and celecoxib, Figure 6) in both *in vitro* human prostate cancer cell models and *in vivo* prostate cancer CWR22Rv1 xenografts in athymic nude mice [71]. The *in vitro* synergism between EGCG and NS-398 was established because the combination treatment enhanced cell growth suppression and apoptosis activation, which was also supported by increased expression of Bax, pro-caspase-6, and pro-caspase-9, and poly (ADP)ribose polymerase cleavage. Additionally, the suppression of proliferator activated receptor  $\gamma$  and nuclear factor- $\kappa$ B were also enhanced as compared with their additive effects. Combination administration of EGCG and celecoxib in *in vivo* tumor xenografts led to improved anti-tumor efficacy, and decreased PSA levels and insulin-like growth factor-1 levels.

**6.2 Synergistic effects of EGCG with ibuprofen:** Ibuprofen (Figure 6) is a well-established non-steroidal anti-inflammatory drug (NASID) that functions *via* inhibiting cyclooxygenase (COX) enzymes. Administration of ibuprofen was associated with low incidence of prostate cancer probably due to its ability to suppress COX enzymes [73]. The *in vitro* study has revealed that ibuprofen can inhibit cell proliferation and activate cell apoptosis in LNCaP prostate cancer cells [74]. The synergism between EGCG and ibuprofen has been established by *in vitro* cell-based assays, in which they can act synergistically in inhibiting cell proliferation and inducing cell apoptosis in both androgen-insensitive and sensitive (PC-3, DU145, and LNCaP) prostate cancer cell lines [75]. The further underlying mechanism investigation indicated that combined treatment of PC-3 cells can synergistically upregulate protein phosphatase 1 activity and subsequently activate alternative splicing of anti-apoptotic proteins Bcl-X and Mcl-1 [75b].

**6.3 Synergistic effects of green tea catechins with black tea theaflavin:** As mentioned previously, EGCG represents the most copious flavan-3-ol in green tea while theaflavin (Figure 6) is the major flavonoid polymer in black tea. Interestingly, EGCG and theaflavin can function synergistically to decrease the growth rate of prostate cancer as compared with either EGCG or theaflavin alone. This synergism may be caused by functionally supplemented mechanisms of EGCG and theaflavin [76].

**6.4 Synergistic effects of green tea catechins with luteolin:** It has been demonstrated that prostate cancer progression can be driven by the existence of myofibroblasts and that EGCG may suppress the myofibroblast differentiation relevant to prostate cancer [77]. Luteolin, 3',4',5,7-tetrahydroxyflavone (Figure 6), is another ordinary flavone and can be readily obtained from a variety of vegetables and fruits, as well as traditional Chinese medicines [78]. Several *in vitro* and *in vivo* studies have shown its potential in preventing and treating prostate cancer [79]. Luteolin can potentiate EGCG to upregulate fibronectin expression in prostate fibroblast

cell lines [77]. Consequently, myofibroblast phenotypes and extracellular matrix contraction (a promoter of cancer cell invasion) mediated by TGF- $\beta$  can be synergistically suppressed by combinations of EGCG and luteolin at micromolar concentrations. Mechanistically, EGCG and luteolin can individually downregulate ERK and Rho that are downstream signalings induced by TGF- $\beta$  and are essential for fibronectin expression [77]. Additionally, EGCG and luteolin have been shown to reverse synergistically fibronectin expression induced by TGF- $\beta$ . These findings suggested that the combination of EGCG and luteolin might be clinically useful in preventing and reversing prostate cancer progression *via* targeting the tumor microenvironment rather than the tumor itself.

**6.5 Synergistic effects of EGCG with TRAIL:** TRAIL is known to inhibit cell proliferation in PC-3 instead of LNCaP prostate cancer cells. The synergism between EGCG and TRAIL has been identified according to the following experimental findings: (i) combination treatment of EGCG and TRAIL enhanced antiproliferative effects towards TRAIL refractory LNCaP cells as compared with the single-agent treatments; (ii) the combination treatment led to escalated apoptosis of LNCaP cells relative to effects caused by the single treatments; and (iii) the combination treatment resulted in a synergistic suppression of the invasion and migration of LNCaP cells [80]. The improved apoptosis contributed by the combined treatment is linked to both intrinsic and extrinsic pathways through several molecular mechanisms. For example, the apoptosis was found to be accompanied by the increased poly(ADP-ribose)polymerase cleavage and pro-apoptotic proteins, together with reduced anti-apoptotic proteins.

**6.6 Synergistic effects of EGCG with taxanes:** The combination of EGCG and either paclitaxel (Figure 2) or docetaxel (Figure 4) generated synergistic efficacy *in vitro* and *in vivo* [81]. The *in vitro* cell-based studies have unveiled that EGCG (30  $\mu$ M) in combination with either paclitaxel (6.25 nM) or docetaxel (3.12 nM) exhibited synergistic effects in inhibiting proliferation and activating apoptosis of PC-3ML human prostate cancer cells. The *in vivo* animal studies indicated that the synergistic effects in suppressing PC-3ML tumor xenograft growth were generated for the combined treatment with EGCG and docetaxel in severe combined immunodeficient mice. In addition to attenuating the preexisting PC-3ML tumor xenografts, the combined treatment increased disease-free survival rates to greater than 90%. EGCG combined with either paclitaxel or docetaxel through intraperitoneal injection (i.p.) promoted a considerable enhancement in apoptosis rates, as determined by TUNEL assays. The *in vitro* cell-based assays and *in vivo* mechanism studies in mice have also revealed that EGCG and either paclitaxel or docetaxel can act synergistically to upregulate certain apoptotic genes [81].

**6.7 Synergistic effects of EGCG with doxorubicin:** Doxorubicin is a well-known chemotherapeutic for a variety of cancers. Low doses of doxorubicin have been demonstrated by *in vitro* studies to act synergistically with EGCG in inhibiting cell proliferation and colony forming of PC-3ML human prostate cancer cells. The synergism between EGCG and doxorubicin was also verified by *in vivo* animal studies in mice implanted with advanced, metastatic PC-3ML human prostate cancer cells. The data showed that the combined treatment led to synergistic suppression of tumor xenograft growth, elimination of tumors, and enhancement of mouse survival rates [82]. Additionally, EGCG was also demonstrated to escalate doxorubicin retention by the prostate cancer cells *in vitro* and by the metastatic tumor xenografts *in vivo*.

**6.8 Synergistic effects of EGCG with cisplatin:** As described in section 5.1, cisplatin is a well-known and commonly used cytotoxic chemotherapy drug that processes promising efficacy but high toxicity. EGCG (25  $\mu$ M) has been evidenced to induce synergistically apoptosis of PC-3 prostate cancer cells in combination with cisplatin (2.5  $\mu$ M) as compared with the effects caused by treating the individual agent alone [83].

**6.9 Synergistic effects of EGCG with free  $Zn^{2+}$ :** Zinc is an essential trace element. The elevated level of intracellular zinc has been demonstrated to be linked with decreased proliferation and increased apoptosis of prostate cancer cells [84]. The antiproliferative activities of  $Zn^{2+}$ /EGCG mixture and zinc-EGCG complex were examined on an androgen-insensitive PC-3 human prostate cancer cell line. It was revealed that free  $Zn^{2+}$  can potentiate the antiproliferative effect of EGCG on PC-3 cells [85].

## 5. Conclusion

These data can provide valuable insights into future design and development of multi-targeting and synergistic agents for potential treatment of prostate cancer. However, most currently available data are only derived from *in vitro* evaluations. The molecular mechanism of the underlying synergism is still elusive. The in-depth mechanism investigation of the synergism of dietary natural products and the rational application of the synergism to the design of hybrid molecules would thus be a good future direction for the development of potential anti-prostate cancer agents.

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## *Ligustrum lucidum* and its Constituents : A Mini-Review on the Anti-Osteoporosis Potential

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Osteoporosis is a metabolic bone disorder commonly occurred in aging populations, particularly postmenopausal women and patients who undergo long-term steroid or anti-estrogen therapies. Given the rapid growth of the aging population, the prevalence of bone loss, and the huge medical and healthcare cost involved, demand for alternative approaches for the promotion of bone health is pressing. With the advent of global interest in complementary and alternative medicine and natural products, Chinese medicine serves as a viable source that offers benefits to improve and maintain bone health. This review summarizes the scientific information on the Chinese medicinal herb *Ligustrum lucidum* and its chemical components as potential therapy for osteoporosis.

**Keywords:** *Ligustrum lucidum*, Ligustri Lucidi Fructus, Nu-Zhen-Zi, Osteoporosis, Anti-osteoporotic activity, Bone health.

Osteopenia (low bone density) and osteoporosis (“porous bone”) are metabolic bone disorders commonly occurred in aging populations, particularly postmenopausal women and patients who undergo long-term steroid or anti-estrogen therapies. Although postmenopausal women are at greater risk, osteopenia/osteoporosis can strike at any age of both genders. The disease is characterized by thinning of bones, with reduction in bone mass and bone mineral density, as well as microarchitectural deterioration of the bone tissue due to depletion of calcium and bone protein. The clinical result is loss of bone strength, thus making bone more fragile and vulnerable to fracture, which often happens in the hip, spine, and wrist. Worldwide, osteoporosis is estimated to affect 200 million women, approximately one-tenth of women aged 60 and one-fifth of women aged 70 [1]. Although the overall prevalence of fragility fractures is higher in women, men generally have higher rates of fracture related mortality. The National Osteoporosis Foundation projected that by 2020, 14 million Americans over the age of 50 are expected to have osteoporosis and another 47 million to have low bone mass, accounting for 55% of the population 50 years of age and older [2].

A comprehensive bone health management plan usually includes non-pharmacologic measures such as balanced diet, adequate calcium and vitamin D intake, exercise and fall prevention, together with pharmacologic therapy [3]. For osteoporosis prevention and treatment, less than ten FDA-approved drugs are currently available. They fall into two major classes, the anti-resorptive and the anabolic drugs. Anti-resorptive medications such as the bisphosphonates (alendronate, ibandronate, risedronate, and zoledronic acid), calcitonin, denosumab (a receptor activator of nuclear factor- $\kappa$ B ligand [RANKL] inhibitor), selective estrogen receptor modulator (raloxifene), and estrogen (with or without progesterone) slow down the process of bone loss; and the anabolic drug such as the recombinant form of parathyroid hormone (teriparatide) enhances bone formation [4-6]. However, the long-term safety of many of these anti-osteoporosis drugs has posted some concerns; for example, potentially serious adverse effects of bisphosphonate therapy have been reported [7-12]. Only a small number of investigational drugs are currently in the pipeline of development [13, 14].

Apart from drug therapy, hormone replacement therapy is effective in increasing bone density and reducing the risk of fracture, and it is an effective regimen for the prevention and treatment of postmenopausal osteoporosis. Nevertheless, the use of estrogenic hormones has been severely limited by concerns for the increased risk of breast, endometrial and ovarian cancers, heart attack, and stroke [15]. Dietary supplementation of calcium and vitamin D is often included as part of the treatment plan, yet calcium and vitamin D alone or in combination are ineffective in reducing fractures in the absence of pharmacologic agents [16, 17].

Studies in Canada [18] and Australia [19] indicated that over 50% of osteoporosis patients go for complementary and alternative medicines, with up to 10% seeking treatment from herbal therapy or Chinese medicine. Soy isoflavone [20-23], red clover [24] and black cohosh [25] are commonly used by postmenopausal women with or without osteoporosis. Indeed, botanicals prepared as dietary supplements hold promise for use and there is considerable interest because the general population is receptive to the use of natural products for long-term treatment, and the cost for dietary supplements is more affordable to the elderly population.

The dried fruit of *Ligustrum lucidum* Ait. (Oleaceae), known in herbal medicine as “Ligustri Lucidi Fructus” and in Chinese medicine as “Lu-Zhen-Zi”, is a tonic drug (“good-for-health”) often included in herbal prescriptions for vitalizing the “liver and kidney” functions as well as nourishing the “Yin component” in these organs. It is indicated for “weakness of the loin and knees” [26], which may represent a symptom of bone deterioration. Indeed, the Chinese medical theory states that “the kidney serves to strengthen the bone” [27] and, therefore, the use of *Lucidum* fruits as a kidney-tonifying agent among the elderly to improve skeletal strength is supported by substantial ethnomedical evidence.

To the best of our knowledge, the first reports on the osteoprotective activity of *L. lucidum* were published by Zhang *et al.* [28, 29]. In the studies, an extract of *L. lucidum* fruits was given to three-month-old ovariectomized rats four weeks after surgical operation and continued for fourteen weeks. Results showed that

both bone turnover and calcium balance were improved. Thus, treatment with the plant extract suppressed bone turnover markers such as serum osteocalcin and urinary deoxypyridinoline. It also decreased urinary and fecal calcium excretion as well as increased the bone calcium content. In addition, the mRNA expressions of renal calbindin-D9K and calbindin-D28K were up-regulated. These findings suggested the involvement of both vitamin D metabolism and calcium reabsorption. Indeed, in aged (eleven-month old) female rats treated with the ethanol extract of *L. lucidum* fruits, serum levels of 1,25-dihydroxyvitamin D<sub>3</sub> and the mRNA expression of duodenal calcium binding protein CaBP-9k were increased; renal CaBP-28K mRNA expression was also up-regulated [30]. In a study to investigate whether the plant extract could improve bone properties in aged animals, both sham- and ovariectomized ten-month-old female rats were treated with the extract for 12 weeks, at three levels of dietary calcium contents. The *L. lucidum* extract significantly improved bone mineral density and content at tibial and femoral diaphysis as well as the lumbar vertebrae (LV-2) in rats fed with diets containing either low calcium (0.1%) or medium calcium (0.6%) level; the biomechanical strength of the tibial diaphysis was also improved [31]. In a similar experiment, eight-week treatment in ovariectomized aged rats also prevented the loss of bone mineral density [32].

In a retinoic acid-induced osteoporosis rat model, treatment with *L. lucidum* led to an increase of serum calcium and phosphorus contents, together with lowered alkaline phosphatase and tartrate-resistant acid phosphatase levels, as well as improved bone microarchitecture [33].

When the osteoblast-like UMR-106 cells were treated with the plant extract, increased formation of calcified matrix and increased extracellular calcium and phosphorus deposition were observed. These results suggested that *L. lucidum* extract could improve bone properties possibly via the enhancement of differentiation and mineralization of osteoblasts [31]. Indeed, *in vitro* study using human bone marrow mesenchymal stem cells treated with an ethanol extract of *L. lucidum* displayed stimulatory activity on osteogenic differentiation, evidenced by an increase in alkaline phosphatase activity and shortening of time needed for mineralization. The mRNA expressions of osteoblast differentiation regulatory genes involved in the Wnt signaling, such as  $\beta$ -catenin, bone morphogenetic protein (BMP)-2, cyclin D1, MT1-MMP, osteoprotegerin (OPG) and TBX3, were up-regulated, thus enhancing osteogenesis [34]. The aqueous extract of the same plant materials also demonstrated promoting effects on both human and rat mesenchymal stem cells as shown by enhanced alkaline phosphatase activity, mineralization level, and the expression of osteopontin [35]. In osteoblast-like MC3T3-E1 cells, an extract of *L. lucidum* increased cell viability and promoted cell differentiation by up-regulating the OPG and RANKL mRNA and protein expressions [36].

The improved calcium balance after treatment was found to associate, at least in part, with an increase of circulating levels of 1,25-dihydroxyvitamin D<sub>3</sub> via up-regulating the 25-hydroxyvitamin D 1 $\alpha$ -hydroxylase activity [37]. At the same time, the renal mRNA expression of 25-hydroxyvitamin D 24-hydroxylase was suppressed [38]. These findings suggested that the protective effects of the ethanol extract on bone could be accounted for by its direct actions on the vitamin D system, including the biosynthesis of 1,25-dihydroxyvitamin D<sub>3</sub> and the expression of vitamin D-dependent calcium transport protein expression. In a subsequent study, the plant extract was separated into ethyl acetate-soluble and water-soluble fractions by solvent extraction. The results indicated that the

**Table 1:** Secondary metabolites in the fruit of *Ligustrum lucidum* known prior to 2011 (Adopted and translated based on the information available in [44])

Compound
<b>PHENYLETHANOIDS</b>
Salidroside
<b>TRITERPENOIDS</b>
$\alpha$ -Amyrin
$\beta$ -Amyrin
Betulin
3- <i>O</i> -Acetyl-12-hydroxydammar-24-ene
(20 <i>S</i> )-3- <i>O</i> -Acetyl-dammar-24-en-3 $\beta$ ,20-diol
3- <i>O</i> -Acetyl-20,25-epoxydammaran-3 $\beta$ ,24 $\alpha$ -diol
(20 <i>S</i> ,24 <i>R</i> )-3- <i>O</i> -Acetyl-24-hydroperoxydammar-25-en-3 $\beta$ ,20-diol
3 $\beta$ ,12-Dihydroxydammar-24-ene
20,25-Epoxydammaran-3 $\beta$ ,24 $\alpha$ -diol
(20 <i>S</i> )-25-Hydroperoxydammar-23( <i>E</i> )-en-3 $\beta$ ,20-diol
3 $\beta$ - <i>O</i> -Palmitoyl-dammar-24-en-12-ol
Fouquierol
Ligustrin
Lupeol
Ocotillol II 3- <i>O</i> -palmitate
Oleanolic acid
Oleanolic acid 3-acetate
Oleanolic acid ethyl acetate
2 $\alpha$ -Hydroxyoleanolic acid
2 $\alpha$ -Hydroxy-3 $\beta$ - <i>O</i> -(trans- <i>p</i> -coumaroyl)-oleanolic acid
Oliganthas A
Tormentic acid
Ursolic acid
Ursolic acid 3-acetate
3- <i>O</i> -Acetyl-19-hydroxyursolic acid
2 $\alpha$ -Hydroxyursolic acid
3 $\beta$ -Hydroxyursolic acid ethyl ester
$\alpha$ -Ursolic acid methyl ester
<b>SECOIRIDOIDS</b>
Ligustroside
10-Hydroxyligustroside
Ligustrosidic acid
Ligustaloid A
Ligustaloid B
Lucidumoside A
Lucidumoside B
Nu(e)zhenide
10-Hydroxynuezhenide
Nuezhenidic acid
Nuezhengaloid
Isonuezhenide
Neonuezhenide
Oleoside dimethyl ester
Oleoside 7- $\beta$ -D-glucosyl-11-methyl diester
10-Hydroxyoleoside 7,11-dimethyl ester
Oleuropein
10-Hydroxyoleuropein
Oleuropeinic acid
Specnuezhenide
<b>FLAVONOIDS</b>
Apigenin
Apigenin 7- <i>O</i> - $\beta$ -D-glucopyranoside
Apigenin 7- <i>O</i> -(6'-acetyl)- $\beta$ -D-glucopyranoside
Apigenin-7- <i>O</i> - $\beta$ -D-rutinoside
Cosmosiin
Eriodictyol
Kaemoterol
Luteolin
Luteolin 7- <i>O</i> - $\beta$ -D-glucopyranoside
Quercetin
Taxifolin

water fraction inhibited urinary and fecal calcium excretion in mature female rats. However, it is noteworthy that the enhancement of calcium absorption by the water fraction was not associated with an increase in serum 1,25-dihydroxyvitamin D<sub>3</sub> levels, but serum parathyroid hormone levels [39].

Apart from the aged and/or ovariectomized animals, the extract of *L. lucidum* was found to benefit bone mass acquisition during early life of the animals. Thus, when an ethanol extract of *L. lucidum* was given to one-month old male and female rats for 4 months, improvements in bone mineral density, bone microarchitecture and bone mechanical properties were observed (compared with control).



**Table 2:** Additional secondary metabolites found in the fruit of *Ligustrum lucidum*

Compound	Reference
<b>PHENYLETHANOIDS</b>	
Acteoside (verbacoside)	[59]
Cimidarhinine	[60]
2-(3,4-Dihydroxyphenyl)-ethanol	[60]
2-(3,4-Dihydroxyphenyl)-ethyl- <i>O</i> - $\beta$ -D-glucopyranoside	[60]
Osmanthuside H	[60]
Tyrosol	[59, 61]
Tyrosol acetate	[61]
Hydroxytyrosol	[59, 61]
<b>TRITERPENOIDS</b>	
Crataegolic acid	[60]
Dammarendiol-II	[60]
Dammarendiol-II 3- <i>O</i> -palmitate	[60]
(20 <i>S</i> )-Dammar-23-en-3 $\beta$ ,20,25-triol	[62]
(20 <i>S</i> ,24 <i>R</i> ) 24-Hydroperoxydammar-25-en-3 $\beta$ ,20-diol	[62]
(20 <i>S</i> ,24 <i>R</i> ) 3- <i>O</i> -Acetyl-24-hydroperoxydammar-25-en-3 $\beta$ ,20-diol	[62]
(20 <i>S</i> ,24 <i>R</i> )-24-Hydroperoxydammar-25-en-3 $\beta$ ,20-diol	[60]
3- <i>O</i> - <i>cis</i> - <i>p</i> -Coumaroylmaslinic acid	[63]
3- <i>O</i> - <i>trans</i> - <i>p</i> -Coumaroylmaslinic acid	[63]
Oleanolic acid methyl ester	[63]
3-Keto-oleanolic acid	[63]
Oliganthas A	[60]
19 $\alpha$ -Hydroxyursolic acid	[63]
19 $\alpha$ -Hydroxyursolic acid 3-acetate	[63]
<b>SECOIRIDOIDS</b>	
<i>p</i> -Hydroxyphenethyl 7- $\beta$ -D-glycosylelenolic acid ester	[64]
1'''- <i>O</i> - $\beta$ -D-glucosylformoside	[65]
GI 3	[59, 61, 65, 66]
Jaspolyside methyl ester	[66]
6'- <i>O</i> - <i>trans</i> -Cinnamoyl-8-epikingsidic acid	[67]
6'- <i>O</i> - <i>cis</i> -Cinnamoyl-8-epikingsidic acid	[67]
Ligustide A	[65]
Ligustide B	[65]
Isoligustosidic acid	[67]
8( <i>Z</i> )-Nuezhenide A	[65]
Nuzhenal A	[67]
Nuzhenal B	[67]
Oleonuezhenide	[65, 66]
Oleopolynuezhenide	[67]
Iso-oleonuezhenide	[66]
Nicotiflorine	[64]
6'''-Acetylnicotiflorine	[64]
6'-Elenolynicotiflorine	[64]
Oleolin	[68]
Methyloleoside 7-ethyl ester	[66]
Oleoside 11-methyl ester	[64]
Oleoside7-ethyl-11-methyl diester	[64]

In addition, *L. lucidum* treatment increased bone formation marker osteocalcin (OCN), decreased the bone-resorbing marker CTX-1, while increasing serum 25-hydroxyvitamin D<sub>3</sub>. The vitamin D metabolism- and calcium absorption-related gene expressions (1 $\alpha$ -vitamin D hydroxylase, vitamin D receptor, calcium transporter calbindin-D9K, and transient receptor potential vanilloid 6) were up-regulated. These results suggested that the extract had beneficial effects on peak bone mass acquisition during early life of the animals and the improvement of bone mechanical properties by favoring calcium metabolism [40, 41].

In diabetic mice, feeding with an aqueous extract of *L. lucidum* was found to protect against excessive urinary calcium excretion and trabecular bone deterioration. The underlying mechanism might be attributed to the regulation of duodenal calcium transporting proteins and renal calcium-sensing receptor [42].

The volatile oil obtained from *L. lucidum* fruits exhibited *in vitro* osteoblastic activity by stimulating the proliferation and alkaline phosphatase activity of rat calvarial osteoblasts [43]. The main components in the volatile oil included (*Z,Z*)-9,12-octadecadienoic acid, *n*-hexadecanoic acid, (*E*)-9-octadecenoic acid,  $\alpha$ -cadinol, 4-hexyl-2,5-dihydro-2,5-dioxo-3-furanacetic acid, and (*E*)-8-octadecenoic acid methyl ester [43]. A number of phenylethanoids, secoiridoids, flavonoids and triterpenoids have been identified from

**Table 3:** Osteo-active secondary metabolites in the fruit of *Ligustrum lucidum*.

Compound	Biological activity	Reference
<b>PHENYLETHANOIDS</b>		
Tyrosol (3)	Protect UMR-106 cells against H <sub>2</sub> O <sub>2</sub> -induced injury	[59]
Hydroxytyrosol (4)	Increase alkaline phosphatase activity in UMR-106 cells; Protect UMR-106 cells against H <sub>2</sub> O <sub>2</sub> -induced injury	[59, 61]
Salidroside (5)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59, 61]
Acteoside (6)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59]
<b>SECOIRIDOIDS</b>		
Oleoside dimethyl ester (7)	Promote UMR-106 cell proliferation	[61]
Oleoside7-ethyl-11-methyl diester (8)	Promote UMR-106 cell proliferation	[61]
Oleuropein (9)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59]
Nu(e)zhenide (10)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[61]
Specnuezhenide (11)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59]
G13 (12)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59, 61]
<b>FLAVONOIDS</b>		
Luteolin 7- <i>O</i> - $\beta$ -D-glucopyranoside (13)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59]
Apigenin (14)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells; Protect UMR-106 cells against H <sub>2</sub> O <sub>2</sub> -induced injury	[59]
Apigenin 7- <i>O</i> - $\beta$ -D-glucopyranoside (15)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells; Protect UMR-106 cells against H <sub>2</sub> O <sub>2</sub> -induced injury	[59]
Apigenin 7- <i>O</i> -acetyl- $\beta$ -D-glucopyranoside (16)	Promote UMR-106 cell proliferation; Increase alkaline phosphatase activity in UMR-106 cells	[59]

the fruit of *L. lucidum* and they were summarized in several review articles [44-47]. Since most of these review articles were published in the Chinese language, the chemical ingredients as highlighted by Huang and Wang [44] are listed in Table 1.

In recent years, further phenylethanoids, triterpenoids and secoiridoids have been reported and they are included in Table 2. Several ingredients of *L. lucidum* fruits have been demonstrated to display potential anti-osteoporosis activities in cell-based and/or animal models. Thus, oleanolic acid (1) and its glycosidic and synthetic derivatives have been known to be inhibitors of osteoclast formation [48-51]. They inhibited the formation of osteoclast-like multinucleated cells induced by 1 $\alpha$ , 25-dihydroxyvitamin D<sub>3</sub>. Oleanolic acid acetate was demonstrated to inhibit receptor activator of nuclear factor- $\kappa$ B (RANKL)-induced osteoclast differentiation; and it attenuated lipopolysaccharide-induced bone erosion in mice [52]. Apart from the anti-osteoclastogenic activity, oleanolic acid was found to be able to promote osteoblastic differentiation and change the gene expression profile of bone marrow stromal cells obtained from rats with corticosterone-induced osteoporosis [53]. In ovariectomized rats, oleanolic acid exerted osteoprotective effect by increasing the population of osteoblasts as well as the levels of osteocalcin and the Runt-related protein-2. It also stimulated the osteoblastic differentiation of bone mesenchymal stem cells *in vitro*. Gene expression profile analysis suggested that the effect might be related to the Notch signaling pathway [54].

Another triterpene ingredient of *L. lucidum*, ursolic acid (**2**), has been found to stimulate osteoblast differentiation and mineralization by activating osteoblast-specific genes such as mitogen-activated protein kinases, nuclear factor- $\kappa$ B, and activator protein-1 [55]. It also promoted bone formation in a mouse calvarial bone formation model [55]. In addition, recent studies showed that ursolic acid was able to inhibit RANKL-induced osteoclast differentiation [56-58] and down-regulate the NFATc1-regulated osteoclast marker genes [58]. Using a mouse model of titanium particle-induced osteolysis, ursolic acid protected calvarial bone loss and decreased the population of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts [58].

Two screening and bioactivity-guided isolation reports on the osteoprotective activity of *L. lucidum* have identified fourteen active compounds [59, 61]. While the screening results are considered to be preliminary at this time, the active compounds were found to promote the proliferation of osteoblast-like UMR-106 cells, increase the alkaline phosphatase activity, and/or protect the cells from hydrogen peroxide-induced damage (Table 3). The chemical structures of these osteo-active compounds are shown in Figure 1.

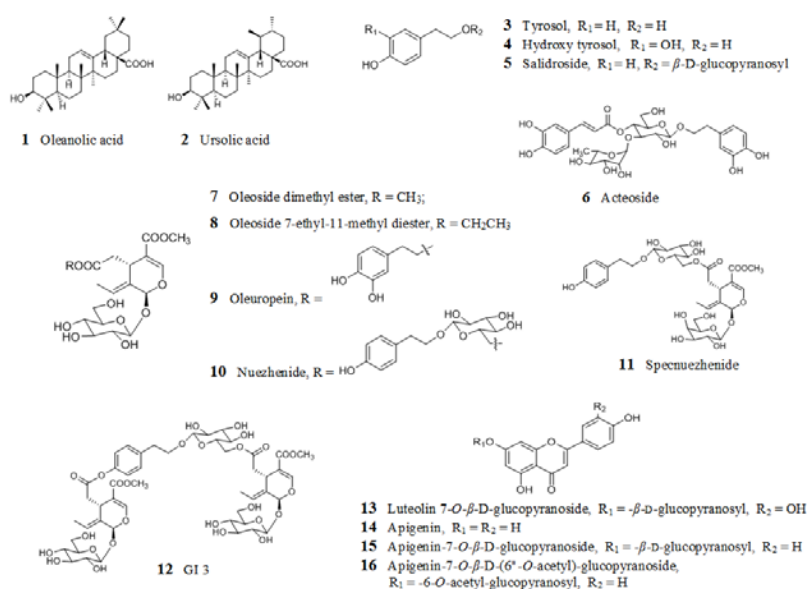
Among the four active phenylethanoids, tyrosol (**3**) and hydroxytyrosol (**4**) are also present in olive oil [69-71], which has been reported to possess anti-osteoporosis property when tested in ovariectomized rats [72]. Salidroside (**5**) is a major bioactive ingredient of *Rhodiola* plants and is well known for its adaptogenic potential for use in high-altitude environment [73, 74]. The inhibitory effect of this compound on diabetes-related osteoporosis has been described [75]. Acteoside (**6**), also known as verbascoside, is a phenylethanoid glycoside possessing pharmacologically beneficial properties such as antioxidant, anti-inflammatory, antitumor, and neuroprotective activities [76, 77].

For the secoiridoids, oleuropein (**9**) is also present in olive oil [69-71]. Together with the phenylethanoids, it is considered to be the active principle. The structure of specnuezhenide (**11**) differs from nu(e)zhenide (**10**) by replacing the glucose with galactose. Both compounds were demonstrated to possess proliferative activity in the osteoblast-like UMR-106 cells and they enhanced the alkaline phosphatase activity [59, 61]. Compound GI 3 (**12**) can be considered as a dimeric derivative of nu(e)zhenide; it displayed similar *in vitro* activities. It is noteworthy that nu(e)zhenide (**10**), specnuezhenide (**11**) and GI 3 (**12**) contain the salidroside moiety in their structures. It will, therefore, be of interest to determine whether the salidroside portion of the molecule acts as a pharmacophore for the osteoprotective activity.

The flavonoids apigenin (**14**) and two glucoside derivatives (**15** and **16**), as well as luteolin 7-*O*- $\beta$ -D-glucopyranoside (**13**), were found to be active in UMR-106 cells [59, 61].

All in all, available information strongly suggests that the fruit of *Ligustrum lucidum* possesses osteoprotective property and it may be useful in promoting bone health. The beneficial effects seem to be associated with improved calcium balance, modulation of vitamin D metabolism, as well as osteoblastogenic and anti-osteoclastogenic activities. Yet the exact mechanisms remain to be elucidated. Further studies on the potentials of this plant drug and its chemical ingredients for use in the improvement of age-related changes in calcium homeostasis and development of anti-osteoporosis remedies are warranted.

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**Figure 1:** Chemical structures of osteo-active compounds from *Ligustrum lucidum* fruits.

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## Alice, Benzene, and Coffee: The ABCs of Ecopharmacognosy

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The sesquicentennial celebrations of the publication of “Alice’s Adventures in Wonderland” and the structure of benzene offer a unique opportunity to develop a contemporary interpretation of aspects of Alice’s adventures, illuminate the symbolism of benzene, and contextualize both with the globalization of coffee, transitioning to how the philosophy and sustainable practices of ecopharmacognosy may be applied to modulating approaches to the quality, safety, efficacy, and consistency (QSEC) of traditional medicines and dietary supplements through technology integration, thereby improving patient-centered health care.

**Keywords:** Alice in Wonderland, Benzene, Coffee, Ecopharmacognosy, Traditional medicines, Quality control, Technology integration, Global health care.

“Everything’s got a moral, if only you can find it.” said the Duchess to Alice.

We begin our allegoric thoughts with Alice and Benzene. The year 2015 marks the sesquicentennial of two, apparently disparate, events. In 1865, August Kekulé published an iconic paper in French (for he was then a Professor in Ghent, Belgium) suggesting that the structure of benzene contained a six-membered ring of carbon atoms with alternating single and double bonds, each with an attached hydrogen [1, 2]. Describing the origin of his idea twenty-five years later, Kekulé indicated that the benzene ring shape had come to him from a dream of a snake seizing its own tail, mimicking the ancient Egyptian symbol, the ouroboros.

Coincidentally, at about the same time, across the North Sea in England, the Oxford University mathematician Charles Lutwidge Dodgson, writing under the pseudonym Lewis Carroll, was publishing the most famous and widely-translated “children’s” books, “Alice’s Adventures in Wonderland” and “Through the Looking Glass” [3]. The audacities of Kekulé’s and Carroll’s vivid imaginations have survived and thrived, and in 2015 are being celebrated following 150 years of global impact. Molecular architecture, rabbit holes, and dreams..... What can their connections be with our contemporary challenges in traditional medicine on this disappearing Earth? This brief contribution attempts to tie these two “events” together in an allegoric manner, offering a contemporary interpretation of aspects of Alice’s adventures, and illuminating the symbolism of benzene, with a relationship to the globalization of coffee, and transitioning to the global need for the philosophy and practices of ecopharmacognosy as integral to a patient-centered approach for advancing the status of traditional medicines (TMs) in integrated health care systems.

Alice’s story begins with curiosity after she meets the White Rabbit wearing a waistcoat and looking at a pocket watch (“...this watch is exactly two days slow.”). As if on a shamanistic journey, she shrinks in size and follows him deep down a hidden hole into a world where her orderly, mundane, upper-class existence becomes completely discombobulated. Since the beginning of the industrial revolution about 260 years ago in England, we have inexorably descended our own “rabbit-hole”, accelerating in the past 150 years, to our contemporary life on Earth. Aspects of that incredible

progress make abundant sense, and the continuously evolving scientific realities have dramatically and spectacularly extended our human and technological capacities throughout all aspects of society, including the extension of life expectancy which has risen from 47 years in 1950-1955 to 69 years in 2005-2010 [4].

Alice in her “Wonderland” of endless surprises and quirks represents us in ours; the wholeness of the single human race in a continuously changing, almost unpredictable world. In that wondrous and alien world, she almost completely loses both her orientation and her power of independent decision-making. Her leader in the unexpected and bizarre environment of Wonderland is the White Rabbit; for us it is burgeoning technology. We are challenged to ask whether we have now lost control of the applicability of our technology over the past 150 years [5], and the recent movie “Ex Machina”, directed by Garland, provides a fascinatingly evocative and dramatic cautionary tale of creating truly humanistic, artificial intelligence systems as our pathway progresses. How did Alice get out of the deep rabbit hole and of Wonderland and return to her mundane and structured upper-class English life? She was woken up by a whirling pack of cards.....it was all a dream. Can we be awakened from our dream-like stupor and take charge of global health care for the majority? Or are we already too deeply somnolent and transfixed by our technological prowess to consider serving the minority? One situational difference is that the rabbit-hole into which Alice fell was animal-created, “zoogenic”, whereas *our* “hole” is anthropogenic, a human-created, altered environment. Alice didn’t have to change her habits to restore balance to her environment; we must.

Benzene, a distillate of coal tar [6], results from the decay, over hundreds of millions of years, of Earth’s primordial forests. The availability and continued use of benzene as a synthetic precursor, and in gasoline fuels (at the 0.62% level in the US), epitomizes human civilization taking from the Earth, and not returning to the Earth. It recognizes our deep societal dependence on depleting Earth’s finite gifts, such as coal and oil. Benzene is the synthetic base for styrene, phenol, nylon fibers, lubricants, dyes, detergents, drugs, and pesticides, etc. It is an integral (now essential) aspect of an evolving global lifestyle; one which has turned into a continuing destructive saga of the very source of everything that supports us. Ironically, like the ouroboros, we are continuously “eating” our own

tails! As such, benzene represents the antithesis of sustainability; for its primary source, bituminous coal, may only last another 100 years [7]. Ironically though, in the obverse context, we could equally now deploy the ouruboros symbol, as it was originally used in Egypt, to represent the reincarnation (recycling) of the human state. We can extend that concept to the recycling of waste to reduce the rate of depletion of our planetary resources for future generations.

A similar dichotomy of philosophy and practice exists with coffee. The coffee bean (berry) is derived from either *Coffea arabica* L. or *C. canephora* Pierre ex A. Froehner (Rubiaceae). Beginning in Ethiopia in the 15<sup>th</sup> century, and moving into Yemen [8], and then to Arabia and Persia, coffee as a consumed beverage spread to Venice, where it was deemed a Christian drink by Pope Clement VIII in 1600. The first coffee house in England appeared in 1650, and within 25 years, 3,000 existed [9]. Brazil, where cultivation began in 1822, is by far the world's largest producer (33.1%). Coffee is now grown in more than 70 countries, and engages over 100 million people [8]. It is the second-most valuable commodity exported by developing countries [10].

Coffee serves as a representative example of the philosophical culmination of the concept of transitioning a plant from the forest into a global commodity (globalization). The continued, expanding daily consumption of coffee by billions of people illuminates the underlying assumptions of the global evolution of coffee as *the* marketed beverage in four ways: i) that the coffee contents are of good quality and not contaminated; ii) that they are safe when taken on a chronic basis, iii) that they are effective on continued use, and iv) that they are consistent on a day-to-day basis. These are the fundamental principles of quality, safety, efficacy, and consistency (QSEC) which should, at the global level, underpin the sciences of patient-centered traditional medicines. In addition, because it has been globalized as a stimulant beverage over hundreds of years [9], coffee is produced in a sustainable, and in many places organic, manner, through widespread cultivation, at a level of approximately 12 billion pounds per year.

Coffee also serves as a classic example of a non-sustainable and controversial commodity. Because of issues related to the susceptibility of *C. arabica* to infestation, particularly by coffee leaf rust *Hemileia vastatrix* and the coffee berry borer beetle (*Hypothenemus hampei*), a variety of pesticides is required for cultivation. Growing in open-fields compared with shaded, under canopy, cultivation [11], the need for extensive deforestation, as well as the extreme water requirements (est. 140 L) to produce the beans for one cup of coffee [12], provide important lessons through which to lessen the environmental impact as traditional medicines are transitioned into cultivated global commodities.

Alice, Benzene, and Coffee, each provide important background concepts as we look at the future development of traditional medicines. The last aspect to be introduced is the term “ecopharmacognosy”, a word 25 years in development [13] over the course of numerous articles [13-34], including some on that precise topic [30, 32, 34]. “Pharmacognosy” is defined as “the study of biologically active natural products” [17]. Therein, the constituent sciences of pharmacognosy are unlimited by the source material (plant, marine, microbial, insect, mammalian, etc.), or the research area (information systems, botanical, analytical, chemical, biosynthetic, biological, pharmacological, clinical, economic, legal, regulatory, etc.). This inherently suggests that to be successful in bringing focus to the implications for, and outcomes of, pharmacognosy research, a highly integrated and collaborative approach is needed.

In London, in March 2012, the “State of the Planet Declaration” was promulgated by the “Planet under Pressure” Conference [35]. It sought support for a societal contract to encompass: i) global sustainability analyses based in science, ii) integrated, international, and solutions-oriented research, implemented and involving government, society, scientists, and the private sector, and iii) enhanced dialog on issues of global sustainability. More recently, in July 2015, the Rockefeller Foundation–*Lancet* Commission on planetary health released a report on Planetary Health [4], calling for societies to “address the drivers of environmental change by promoting sustainable and equitable patterns of consumption, reducing population growth, and harnessing the power of technology for change.”

As a series of global sciences requiring, as a fundamental aspect of success, the continuing availability and judicious use of natural resources, it was astounding and distressing to acknowledge that pharmacognosy and its practices had barely considered “sustainability” as a conscious and evident component in our daily scientific research ideas and practices. There was a discussion sponsored by the World Health Organization on the conservation of medicinal plants in 1988 [36] and more recently aspects of “sustainable medicines” were discussed [25-28]; the need for awareness is much greater. Based on this situation, it was deduced that the profile and consciousness of the environmental and sustainability issues surrounding TMs needed to be raised, and hence a new term to integrate philosophy and practice was developed.

Thus “ecopharmacognosy” was born and introduced at a scientific meeting in Lublin, Poland in May, 2012. It is defined as “the study of sustainable, biologically active natural products” [30,32,34]. It offers a philosophical approach and a system of practices which stresses environmental concerns in natural product research from the perspective of the types of materials which should be studied, to opportunities for changing the practices used for their botanical, chemical, biological, and clinical assessment, to assuring a high QSEC product for the patient and the practitioner. It also embraces aspects of naturally-resourced agricultural practices, and the development of marine, bacterial, and fungal systems for scientific and economic development.

This brief and somewhat droll essay will attempt to coalesce some of the most well-known quotes and concepts from *Alice's Adventures in Wonderland* (AAIW) as a personal allegory for the considered use of our natural resources, with a more eco-centric view of developing Earth's natural medicinal resources, considering the patient-centered view of coffee as a pathway to develop a broad vision for traditional medicine enhancement within more integrated health care systems. Perhaps for some readers who are not familiar with “Alice's Adventures in Wonderland” this may be initially confusing. ““Then you should say what you mean,” the March Hare went on.” I will.

We all are aware of the Doomsday clock.....since 1947 a philosophical construct which “indicates” how close Earth is to nuclear disaster, based on the levels of war and conflict in its various forms around the world, and since 2007 to the impending disaster from climate change. It is presently set at 11.57 pm [37]. When Alice first meets the White Rabbit in her real world, he is concerned about how late it is as he scurries off down the hole. The White Rabbit is OUR timepiece for getting things done in time (his watch is two days slow remember!), suggesting that we look now at how late it is becoming to restore our world towards a semblance of sanity. For the truth is, as the grinning Cheshire cat states, “*We are all mad here*”, in the way we use Earth's resources.

Since 2000, the World Health Organization has been promoting the evidence-based assessment of traditional medicine practices [38], and the Beijing Declaration of 2008 encouraged countries to: i) respect, preserve, and promote knowledge and practices of TM; ii) formulate national policies, regulations and standards, as part of integrated health systems to ensure the appropriate, safe and effective use of TMs, and iii) promote TM research and innovation [39]. Further support has come from “The Regional Strategy for Traditional Medicine in the Western Pacific (2011-2020)” [40] and the “WHO traditional medicine strategy: 2014-2023” [41], which have also placed significant emphasis on the sustainability aspects of developing traditional medicines. These strategy documents challenge countries to introduce policies and practices which will ensure the availability and improve the status of TM in their respective health-care systems, based on a continuum of evidence-based approaches, in order to narrow the overt gaps in health care practices.

The economic and health care outcome gaps between traditional medicine and allopathic medicine globally are vast [30, 42-47]. The additional challenges for enhancing TM based on an evidence-based, patient-centered approach entail an exploration from a scientific perspective of the many myths which are associated with, and in some respects inhibit, unbiased TM research and development [32-34], to address the desperate need for quality control systems for TMs [13, 29, 31, 33], and to examine the profound necessity to assure the supply of critically-needed traditional medicines through sustainable sourcing [25, 26, 29, 32, 33] for the majority of patients in the world. That those situations even exist in the 21<sup>st</sup> century reflect another deep-seated “madness”, a failure to bring even a semblance of equality to our health-care priorities globally, and an irrational view of natural resource sustainability, namely, the enduring “myth” that the plants will always be there.

When we speak of “sustainable medicines”, the reference is to both natural and synthetic medicinal agents [25-28]. For the patient, accessibility to treatment is critical for healing, and must embrace both affordability and sustainability. In addition, the patient requirement is for a safe and effective regimen for healing; the source of the medicine is irrelevant. The costs of allopathic medicines have risen well beyond the index of inflation [48]. Stunningly, there are a number of medical treatment protocols which have been approved by the USFDA in recent years where the annual patient/insurance company cost is in excess of \$200,000 [49]. Meanwhile, at the global level, we are unable to deliver the simple drug aspirin (at less than 5 cents per dose) to those in pain. Drug resistance against cancer chemotherapeutics, antibiotics, antimalarials, anti-AIDS, and other infectious diseases is increasing steadily, but is not being addressed as a priority by the global pharmaceutical drug companies for economic reasons. Similarly, systematic drug discovery for the global major killer diseases and for tropical (orphan) diseases is not on the radar of Big Pharma, or even WHO; although the Gates Foundation and a recent Drugs for Neglected Diseases Initiative ([www.dndi.org](http://www.dndi.org)) will hopefully be impactful over time. Typically, and quite absurdly, these health care issues have been deemed to be addressed by the countries where the disease is prevalent; a truly inhumane and unethical decision with unrealistic expectations when only a handful of countries has the capacity to “discover” new medicinal agents for therapeutic use. Their typical resource is their plants and the medicinal knowledge associated with them, accumulated over time.

Let us return to our allegoric tale and to the sustainability of plant-based traditional medicine systems. It is fascinating that the dodo is one of the animals mentioned and depicted in *AAIW*; speculated to

be because of Do-do-Dodgson’s stammer [3]. The dodo, endemic to Mauritius, in 1662 became the first recorded extinction of a species caused by the actions of humans. It serves here as a profound reminder of the fragility of nature, and of the facile detrimental outcomes of the irrational urge for human dominance over nature. Today, there is a global system in place actively reviewing and recording the status of disappearing animals, plants, and other species, under the auspices of the International Union for Conservation of Nature. On these Red Lists of threatened and endangered species are (as of 2011) an estimated 9,568 plants of 61,900 recognized medicinal plant species. One of the priority activities recommended by WHO [40, 41, 50] is for countries to invest in assessing the availability and projected future access and supply chain for the needed TMs for their respective systems; some have done so [51, 52]. In the former instance, 39 of the most widely-used medicinal plant species were classified as threatened. The European Union has also recently issued a Red List of medicinal plants [53]. It was estimated that 31% of the medicinal plant species were in decline, principally due to wild-crafting.

The most powerful biological effects in *AAIW* are those produced by the mushroom, which is introduced to Alice by the caterpillar, who sits atop it, smoking a hookah. For us, Caterpillar is the local medicine practitioner, curandero, hakim, shaman, etc. The left part of the mushroom causes Alice to grow larger, while the right part causes her to diminish in size. She eats these parts in turn to “control” her height. The mushroom represents what in pharmacognosy are known to be the very powerful attributes of biologically active natural products; on one hand to promote disease, to cause profound toxicity, even death, and on the other to overcome toxicity, treat disease, and restore homeostasis.

The mushroom is also an allegory for the impact of what we ingest (physically and metaphorically) on global population dynamics. Over the past two centuries, humanity has been taking the left part of the mushroom, which has allowed the global population to grow larger (to 7.4 billion in 2015), stay healthier, have more mothers and children surviving, and to live longer. Disease, famine, war, natural events, and some naturally-derived products for male and female fertility regulation, and social and personal reassessments of family size have caused a modulated population growth, or even a diminishing population, as seen in countries like Russia, Japan, Germany, and Bulgaria [54].

Those are effects derived from the right part of the mushroom. The mushroom also suggests that, through nature, an acceptable balance for Earth’s population can be found. It is distressingly clear that such a balanced view of humanity is not on the agenda of any of the major international agencies or religions at this time. Maybe their leaders have not yet met any White Rabbits with watches indicating how behind the current time they are! For the dream seems to be of an Earth filled beyond sustainable capacity with healthy people. In this regard, Caterpillar asks Alice, “*What size do you want to be?*” As a human species, what is our answer for Earth’s population? We have already passed, or are close to passing, several tipping points for a sustainable population of Earth [55-57], such is the measure of our “overshoot”. This occurs when the population exceeds the carrying capacity that an environment can maintain indefinitely. Even optimistic estimates for what population Earth could support for extended periods are low (ca. 4 billion; passed in July, 1974) based on an ecological footprint, and much lower (ca. 1 billion; passed in August, 1804) based on a thermodynamic footprint [58].

Alice takes the left part of the mushroom that makes her grow taller to a point where she completely fills the little room she is in and then can’t move. “*There’s no room to grow up any more here*” she

cries out. Some of the major cities in the world (Beijing, Manila, Delhi, Sao Paulo, Dhaka) are like that now, houses are cramped, streets are thronged with people, cars cause endless traffic jams, and combined with industrialization, produce stunning levels of pollution, and in war zones, refugees live with many families to a single tent. Like Alice, we too are losing our freedom of movement. Earth's population is projected to rise to 10 billion by 2045. The oceans are rising, and are becoming distinctly more acidic, as a result of climate modulation (global warming) [59-61]. Major cities, such as Bangkok [62] and Venice [63], are sinking at alarming rates, and the future fate of needed medicinal plants in low-lying regions of the world is poorly studied [64, 65]. We can all shed tears, buckets of them, as we travel and observe what has happened to the state of our planet in our lifetimes. So when Alice swims in the pool of her tears with the dormouse (now an endangered species in England, by the way) she does so reflecting our tears for our environmental and biodiversity losses, now, and for the future generations. In terms of health care and medicinal agents, there are no solutions at present for delivering traditional and synthetic medicines in a sustainable manner to this exploding patient base.

Interestingly, and as a slight diversion from the main discussion, there are a number of direct associations with pharmacognosy present in the original drawings for *AAIW* by John Tenniel. There is for example a foxglove depicted in the meeting with the Cheshire Cat, and also when Alice is carrying the piglet. Then there is the powerful mushroom upon which the caterpillar sits, and chamomile is also cited. There is also the reference to the children down a well living on treacle, which was originally an extract of medicinal plants. And there is the warning on the first bottle that Alice encounters which is labeled "*Drink me*". "*Better read it first, for if one drinks too much from a bottle marked 'Poison', it's almost certain to disagree with one sooner or later*", says Alice. This is an admirable admonition (read the label!) to the patient taking any medicine, particularly one where the plant material is known to have toxic effects at higher doses. It is a reminder too that the notion of "more is better", does not apply to the dosing of traditional medicines, a topic in itself of great controversy. With the differential effects of the mushroom evident, the important relationship between stereochemistry (handedness) and biological activity is also raised, where one enantiomer may have a completely different, even opposite biological effect, than the other enantiomer [66-68].

At one point in the narrative, Alice ponders the question, "*What is a 'mustard'?*" Is it a mineral, an animal, or a vegetable? A reasonable question for a young, sheltered girl from an "upstairs" environment, for which the answer is actually not trivial, beyond being "vegetable" [69]. Her question though is a reminder to consider, and be grateful for, the items in our daily lives which we take for granted, and which are derived from nature without us thinking about them. In a contemporary context, consider, for example, how many readers of this article are aware of the origins and processing of cochineal and its uses or its' potential for sustainable development to provide the natural rouges and lipsticks for the faces of an expanding world? [70, 71].

Speaking of red, Alice witnesses the presaging of GMOs, for there in the garden are three playing cards (spades, of course) painting the white roses red at the behest of the Queen. Biotechnology has revealed approaches to a new scenario, where colors and biologically useful metabolites can be modulated and potentiated in biosynthetic terms, and deleterious compounds minimized or eliminated in content; a highly effective demonstration of the

principles of ecopharmacognosy, since expensive methods of separation and purification can be reduced.

Gryphons (griffins) are, according to legend, half eagle, and half lion. They are characterized by their power, extreme alertness and being stirred into rapid and fatally aggressive action at the slightest sound or movement, so as to alert the community. In *AAIW*, Carroll's gryphon is dozing, in spite of everything going on around. In our allegory, this is a clear reference to our lack of awareness and active concern, of not seeing and hearing the long standing, and increasingly loud indications from Nature itself, and those humanistic scientists concerned about nature, that aggressive corrective action in our behavior is needed.

Perhaps the most relevant scene to Earth's contemporary situation in *AAIW* is the Mad Hatters' Tea Party, where the time never changes (it is always six o'clock, and thus the time for tea). At the tea party, the participants (the Mad Hatter, the March Hare, and Dormouse) move around the table at each tea time, and always a new tea service is used. It is a classic example of our profligate consumption, as we move around our substantially disposable, non-reusable world, and of how, only very slowly, is global thinking at the political level concerning the sustainability and re-using of our resources (washing the dishes in *AAIW*) evolving. This scene can also be viewed as illustrating that changing time, actually moving through time as the world turns, requires the continuous reassessment of the *status quo*, whereas if time stands still, so does the *status quo*. For traditional medicines, it is this very *status quo* situation, which must shift to a patient-centered perspective for the benefit of humanity; we are already two days too slow (!), and need to move to a different time where the product and science and technology coalesce for the benefit of the patient. We, in consideration of future generations, have to wake up, "smell the coffee", and change our habits and practices to improve the role of traditional medicine in global health care.

The Preamble to the Constitution of the World Health Organization [72] states that "the highest attainable standard of health is one of the fundamental rights of every human being without distinction of race, religion, political belief, economic or social condition". Only when a population is healthy and not ravaged by disease, whether acute (Ebola) or chronic (malaria), can social and economic development be linked to effective and appropriate resource use [73]. About 80% of the plant resources used for traditional medicines in commerce are wild-crafted, and many are typically offered in an open manner in a local market, with little or no quality control, marginal concerns for safety, and almost no consideration afforded as to the age of the plant, and its changing efficacy and consistency. For most of the population of the world, this scene *is* their primary health care *status quo*, and has been for at least 4,000 years since the times of the early Chinese and Persian physicians until the present day [28]. As a global natural product health care community, we should be deeply embarrassed and ashamed by this appalling patient care situation.

In developed countries, the status of a patient-centric system for dietary supplements and traditional medicines is no better (and in some respects is even worse). A recent action by the Attorney General for New York State offers a glimmer of hope that even in the United States, where dietary supplement quality control is notoriously poor, and that of traditional medicines even worse, positive movement is possible [74]. In this instance, four large companies, GNC, Walgreen's, Walmart, and Target, were determined (through some not particularly well-conducted science) to have sold fraudulent dietary supplements; indeed 80% of the



package contents were not as labeled. Stunningly, several industry-related groups [75], not the retailers, went on the defensive, rather than acknowledging that, irrespective of the quality of the science, this is a huge, long-standing, industry-wide issue. Numerous instances of fraud in dietary supplements have been identified recently [76-83]. When products are recalled by the FDA, they may remain on the market for over four years and still contain synthetic adulterants, including those products from US manufacturers [84]. However, it seems unlikely that, absent US Congressional action, concerted industry reform in terms of research investment and an enhanced commitment to real quality control (QSEC) will follow voluntarily. The present system is based entirely on the trust between the manufacturer and the patient; a trust which is now essentially dissipated. From the patient perspective, the opportunity for the dietary supplement manufacturing industry to change effectively the *status quo* as an evidence-based commitment to US patient health care has been lost. Similarly to when Alice is “lost” in Wonderland, the situation begs the very core question for the global traditional medicine and the dietary supplement industry:

*“Would you tell me, please, which way I ought to go from here?” asked Alice. “That depends a good deal on where you want to get to,” said the Cat.*

Louis Sullivan, perhaps the most famous early American architect, who was based in Chicago, wrote, in an essay in 1896 concerning the design of tall buildings, that “....form ever follows function. That is the law.” [85]. In practical terms, therefore, know what the building is for, and then design its form to meet the functional requirements. So what then is to be the perceived function of (eco)pharmacognosy? For what, as a conglomeration of diverse and integrated scientific applications, will it accept responsibility in terms of meeting the needs of those patients who choose to take natural products for their health care? Will it redefine itself, as this author has urged for more than 25 years, or will it forever be engulfed at the junction of *status quo* and societal health care responsibility; frozen at, indeed by, the abyss of change?

Traditional medicines, TM practitioners, and their patients around the world are in a state of deep flux, moving down the long pathway of information and experimentation from an inherently knowledge-based system, to an experience-based system, and eventually to an evidence-based system. It is well-known that the existing frameworks for the development, regulation, quality control, and sale of traditional medicines, dietary supplements and phytotherapeutics, in spite of burgeoning sales, are not scientifically or societally acceptable because they are not patient-centered. The patient, who we as individuals all are, should be assured of the continued availability of quality, safe, effective, consistent (QSEC), and affordable traditional medicine products based on the results of evidence-based research and sustainability.

In the mid-1950s, before the global Japanese brands were renowned for their product quality, W. Edwards Deming, an American management expert, introduced a focus on continuous quality improvement to enhance the performance and product quality of Japanese industry [86]. One of his aphorisms from that time is “Quality begins with intent”. Another dictum is that “We have lived in a world..... of defective products. It is time to adopt a new philosophy.” [86]. In terms of the global quality control of traditional medicines and dietary supplements, we are still stuck living in a time of defective products. Moreover, the manufacturing industry, as the New York case showed, cannot grasp that it is their responsibility to change voluntarily their practices and their products to lead the improvements that are anticipated by the

patient. The *status quo* places the patient in the unacceptable position of “buyer beware”, with no reliable resources to consult. Now is indeed the time for a new philosophy, a new intent for the quality of traditional medicines and dietary supplements; the existing paradigm must change [13, 28, 29, 31, 32, 33]. The patient must be assured of QSEC in a trustworthy product as an essential moral and ethical responsibility. Less than that is an ethical misstep by the manufacturer, the provider, and a moral dilemma for the practitioner. Therefore, evidence-based research, focused on the development of sustainably-resourced, standardized traditional medicines and dietary supplements, as well as those essential oils used in aromatherapy and cosmeceutical products, is fundamental. In this way, appropriate biological and clinical studies can be conducted for the health benefit of the patient, and the assurances of the practitioner. There is a need also to label products, in an appropriate manner, to reflect the standards being applied to placing the product in the market place, and how sustainable is its sourcing.

There are numerous practical examples of ecopharmacognosy. Long-term resourcing of traditional medicines (and those for drug discovery) is an early stage development question. Consideration of a bark or a root material offers very different long-term challenges for sustainability than the corresponding development of a leaf or fruit. Following the principles of “green chemistry”, ecopharmacognosy could embrace six applicable and practical principles [26, 87], so that the global use of plants in traditional medicine can transition a “forest economy” to a “field economy”, just as coffee has done [88-95]. Some other examples of the practical application of ecopharmacognosy include: i) the improved use of information systems to delineate what has been done and what needs to be done (not wasting resources by reinventing the wheel!); ii) reducing the energy requirements for plant material extraction; iii) reducing the reliance on non-recyclable chromatographic supports and solvents; iv) determining the significance of individual plants in complex plant matrices to conserve species use; v) using network pharmacology [96, 97] to develop new uses for established and sustainable plants, vi) studying the waste products of the industrial processing of plants for new applications; vii) developing natural pesticides and insecticides from renewable resources, viii) assessing whole organisms, plants, and microbial systems as renewable catalytic reagents showing high yield and high enantioselectivity for organic chemical processes [98-101]; ix) re-energizing the development of natural dyestuffs from sustainable resources; and x) investigating well-established commercial crops, such as turmeric, ginger, oregano, garlic, cinnamon, caraway, etc. for new biological responses. One recent study found that an aqueous extract of caraway seed could have an obesity-reducing effect [102, 103]. Finally, microfluidic, lab-on-a-chip approaches to the chemical and biological assessment of plant samples in the field (previously referred to as “pharmacognosy in a suitcase”) [25-27, 29, 30, 34] would save significantly on the drying, transportation, and macro-analytic processes required to evaluate the optimal time for accessing plant materials for therapeutic purposes based on the levels of their bioactive agents. Similarly, hyperspectral imaging using drone technology and/or remote sensing devices based on Raman technology could play a significant role in the field determination of important biological molecules, as has already been achieved with collagen [104, 105].

## Conclusions

Using the allegories based on *Alice's Adventures in Wonderland*, the dreamt discovery of the structure of benzene, and the globalization of coffee, new insights regarding the future, evidence-based directions of traditional medicine, are presented from the perspective of patient needs and expectations.

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## Additions and Corrections

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**Title:** Cytotoxic and Antimalarial Alkaloids from the Twigs of *Dasymaschalon obtusipetalum*

**Authors:** Atchara Jaidee, Thanika Promchai, Kongkiat Trisuwan, Surat Laphookhieo, Roonglawan Rattanajak, Sumalee Kamchonwongpaisan, Stephen G. Pyne and Thunwadee Ritthiwigrom\*

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- After publishing the above paper we became aware of the following reference indicating that the correct name for the plant species that we studied is *Dasymaschalon yunnanense* and the name *D. obtusipetalum* is now regarded as a heterotypic synonym.

Guo, X., Wang, J., Xue, B., Thomas, D.C., Su, Y. C. F., Tan, Y. H., Saunders, R. M. K., "Reassessing the taxonomic status of two enigmatic *Desmos* species (Annonaceae): Morphological and molecular phylogenetic support for a new genus, *Wangia*," *Journal of Systematics and Evolution*, **52**, 1-15 (2014).

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### Natural Products from Endophytes

(Guest Editors: John A. Johnson and Christopher A. Gray)

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