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Lignans from the Stem of *Cinnamomum camphora*

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(+)-Diasesamin (**1**), (+)-sesamin (**2**), (+)-episesamin, stearic acid, palmitic acid, a mixture of β-sitosterol and stigmasterol, and a mixture of β-sitosterol-D-glucoside and stigmasterol-D-glucoside were isolated from the stems of *Cinnamomum camphora* (L.) Presl.. Among these compounds, **1** is obtained for the first time from a natural source. The cytotoxicity of **2** on Hep G2 and HeLa cell cancer lines was also investigated. The flow cytometric assay results indicated that **2** elicited a decrease in the percentage of the S phase of the cell cycle in Hep G2 cells after incubation for 24 h. These results reflect that **2** inhibited the DNA synthetic event in Hep G2 cells.

Keywords: *Cinnamomum camphora*, Lauraceae, (+)-diasesamin, (+)-sesamin, cytotoxicity.

Cinnamomum camphora (L.) Presl. (Lauraceae) is the source of natural camphor, which is extensively employed in the treatment of muscular strain, inflammation and rheumatism. *C. camphora* has a reputation for possessing stimulant, diaphoretic, anthelmintic and anodyne properties [1]. In previous studies, the stems were found to contain a novel cyclopentenone, 5-dodecanyl-4-hydroxy-4-methyl-2-cyclopentenone [2], glycerols, flavanols, lactones and acyclic monoterpene diols [1, 3]. As part of our continuing investigation of Formosan Lauraceous plants, a new lignan, (+)-diasesamin (**1**), together with eight known compounds, (+)-sesamin (**2**) [4], (+)-episesamin (**3**) [5], stearic acid (**4**) [6], palmitic acid (**5**) [7], a mixture of β-sitosterol (**6**) and stigmasterol (**7**) [8], and a mixture of β-sitosterol-D-glucoside (**8**) and stigmasterol-D-glucoside (**9**) [9] were isolated from the stem extracts of this plant. Compounds **3-9** were reported for the first time from this species. The cytotoxic effect of **2** on cell cycle events of the human hepatoma Hep G2 and HeLa cell lines are also reported herein.

Diasesamin, mp 165-168°C, $[\alpha]_D^{21}$ +214° (*c* 0.3, $CHCl_3$) has a molecular formula $C_{20}H_{18}O_6$ as determined by HREIMS. Its UV spectrum showed absorption maxima at 217, 240, and 282 nm, suggesting its furofuranic-type lignan nature [11]. Assignment of the ^1H (Table 1) and ^{13}C NMR spectroscopic resonances (Table 2) was achieved by the analysis of COSY, HMBC (Figure 1) and NOESY (Figure 2) data. The ring junction of the bis-tetrahydrofuran ring system was established as *cis* as its ^1H NMR spectrum exhibited signals at δ 3.21 (2H, m) and at δ 4.95 (2H, d, J = 5.2 Hz), corresponding to H-1 and H-5, and H-2 and H-6, respectively [11]. NOESY data supports axial orientations of both aromatic substituents, thus allowing for two types of stereoisomers (the *R*,*R*,*R*,*R* or *S*,*S*,*S*,*S* configuration at C-1, C-2, C-5, and C-6.) [11]. According to the literature data, all (+)-sesamin-type lignans acquire *R* absolute configuration at the bridge carbons C-1 and C-5 [11], therefore absolute configuration at C-1, C-2, C-5, and C-6 was assigned to be *R*. Thus, on the basis of these data, structure **1** was assigned to (+)-diasesamin, which was isolated for the first time from nature, though it has been synthesized previously [12].

The other isolated lignans, based upon the analysis of their NMR spectral data (Tables 1 and 2), were identified as (+)-sesamin (**2**) [4] and (+)-episesamin (**3**) [5], respectively.

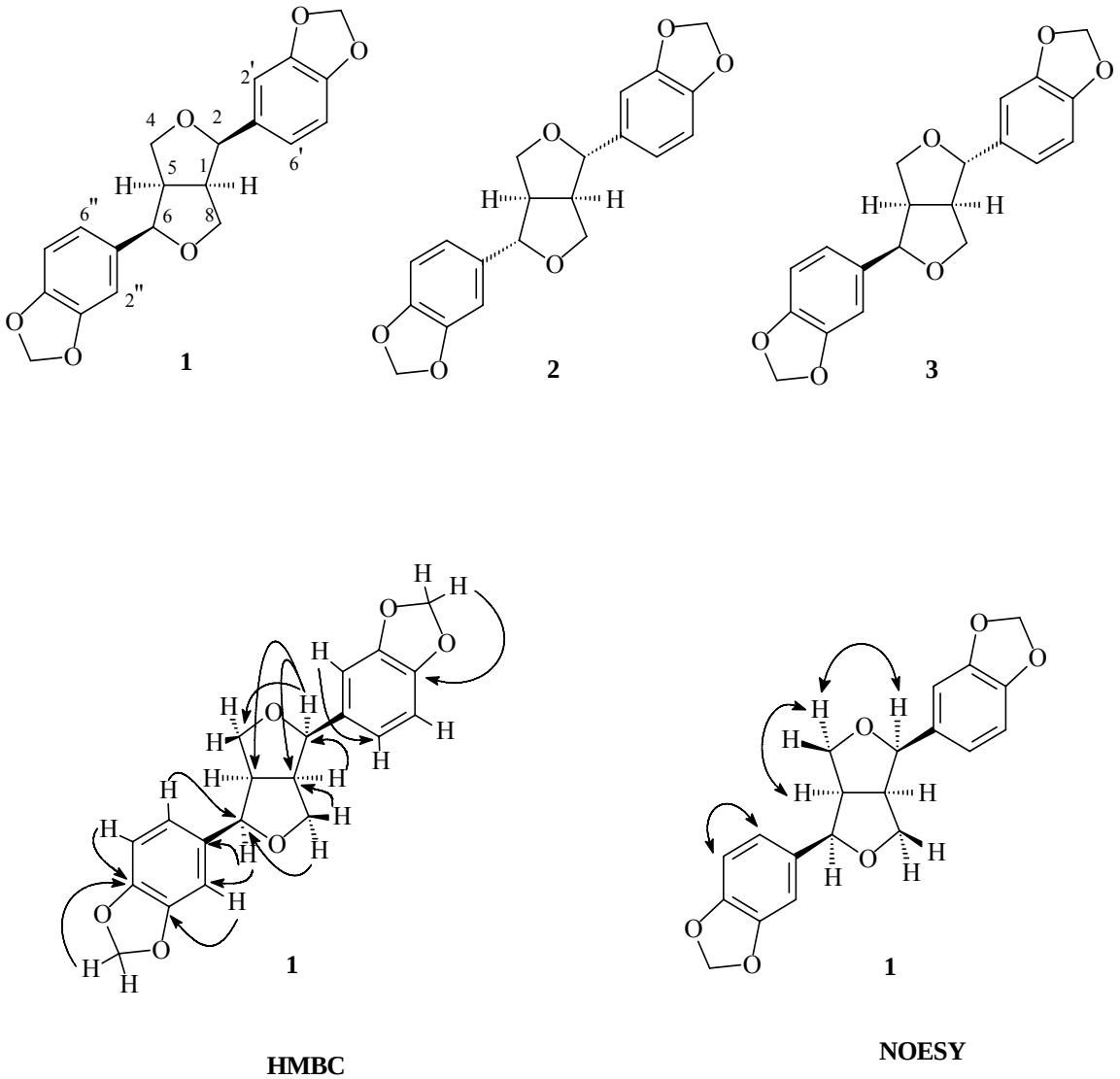

Figure 1: Chemical structure of lignans [(+)-diasesamin (**1**), (+)-sesamin (**2**), and (+)-episesamin (**1**)], HMBC and NOESY correlations of **1**.

Compound **2** was examined for its cytotoxic effects on the cell cycle of HeLa and Hep G2 cells. Both HeLa and Hep G2 cells were incubated with **2** for 24 hours and the PI fluorescence was measured by flow cytometry after fixation with methanol. The different phases of the cell cycle were evaluated based on PI fluorescence and the results are shown in Figure 2. As shown in Figure 2A, the percentage of the S and G2M phase of HeLa cells slightly increased after 24 hours of incubation with **2**. In Figure 2B, the decrease of the cell percentage in the S phase of Hep G2 cells demonstrated a dose response relationship with increasing concentrations of **2** incubated for 24 hours. The percentage of cells in the S phase decreased to 30% after incubation with 200 μM **2** for 24 hours.

Experimental

General: UV spectra were obtained in CH_3CN. IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^1H NMR (400 MHz), HETCOR, HMBC, NOESY, and DEPT spectra were obtained on a Varian (Unity Plus) NMR spectrometer. Low resolution FABMS and low-resolution EIMS spectra were collected on a Jeol JMS-SX/SX 102A mass spectrometer or Quattro GC/MS spectrometer having a direct inlet system. High-resolution EIMS spectra were measured on a Jeol JMS-HX 110 mass spectrometer. silica gel 60 (Merck, 70~230 mesh, 230~400 mesh) was used for column chromatography. Precoated silica gel plates (Merck,

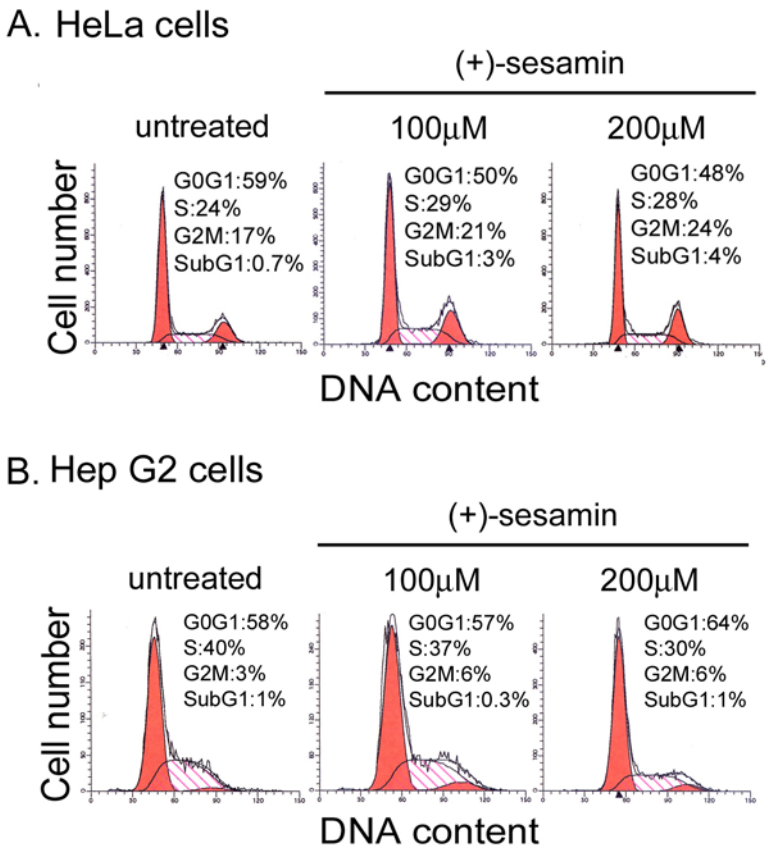

Figure 2: Effects of (+)-sesamin (**2**) on the cell cycle of HeLa and Hep G2 cells. The cells were treated with indicated doses of **2** for 24 hours. Cells were then harvested, stained with propidium iodide, and analyzed by flow cytometry. The data are presented as % of cell cycle of total cells in (A) HeLa cells and (B) Hep G2 cells. The data represent the mean for 3 separate experiments performed in one experiment.

Kieselgel 60 F-254), 0.20 mm and 0.50 mm, were used for analytical TLC and preparative TLC, respectively, visualized with 50% H_2SO_4.

Plant material: The stems of *C. camphora* were collected from Fooyin University, Kaohsiung County, Taiwan, July 2003. Plant material was identified by Dr. Horng-Liang Lay (Graduate Institute of Biotechnology, National Pingtung University of Science and Technology). A voucher specimen was deposited in the Basic Medical Science Education Center, Fooyin University, Kaohsiung County,Taiwan.

Extraction and separation: The air-dried stems of *C. camphora* (12.0 kg) were extracted with MeOH (80 L x 6) at room temperature and the extract (304.2 g) concentrated under reduced pressure. The MeOH extract, suspended in H_2O (1 L), was partitioned with $CHCl_3$ (2 L x 5) to give fractions soluble in $CHCl_3$ (212.4 g) and H_2O (56.8 g). The $CHCl_3$-soluble fraction (212.4g) was chromatographed over silica gel (800 g, 70~230 mesh) using *n*-hexane/EtOAc /acetone as eluent to produce five fractions. Fraction 1 [5.31 g, *n*-hexane-EtOAc (20:1)] was further purified using another silica gel column (150 g, 230-400 mesh) and eluting with *n*-hexane:acetone to obtain stearic acid (**4**) (1.72 g) and palmitic acid (**5**) (2.34 g). Fraction 2 [10.05 g, *n*-hexane-EtOAc (1:1)] was further purified on another silica gel column (170 g, 230-400 mesh) using the $CHCl_3$-MeOH system to obtain a mixture of β-sitosterol (**6**) and stigmasterol (**7**) (7.89 g). Fraction 3 [3.53 g, EtOAc-acetone (20:1)] was further separated and purified on a silica gel column (150 g, 230-400 mesh), and by preparative TLC [$CHCl_3$-MeOH (40:1)] in sequence to give (+)-diasesamin (**1**) (21 mg), (+)-sesamin (**2**) (2.67 g) and (+)-episesamin (**3**) (45 mg), respectively. Fraction 4 [6.54 g, EtOAc-acetone (10:1)] was separated on a silica gel column (200 g, 230-400. mesh) using EtOAc-acetone to give a mixture of β-sitosterol-D-glucoside (**8**) and stigmasterol-D-glucoside (**9**) (3.84 g).

Table 1: ^{1}H NMR ($CDCl_3$, 400 MHz).spectral data for compounds **1-3**.

Proton	**1**	**2**	**3**
1	3.21 (1H), m	3.04 (1H), m	2.87 (1H), m
2	4.95 (1H), d, *J* = 5.2 Hz	4.72 (1H), d, *J* = 4.4 Hz	4.84 (1H), d, *J* = 5.2 Hz
4 eq.	4.21 (1H),dd, *J* = 9.2, 6.8 Hz	4.23 (1H), dd, *J* = 9.2, 6.6 Hz	3.85 (1H), m
4 ax.	4.02 (1H), dd, *J* = 9.2, 3.6 Hz	3.87 (1H), dd, *J* = 9.2, 3.6 Hz	3.38 (1H), m
5	3.21 (1H), m	3.04 (1H), m	3.26 (1H), m
6	4.95 (1H), d, *J* = 5.2 Hz	4.72 (1H), d, *J* = 4.4 Hz	4.40 (1H), d, *J* = 6.8 Hz
8 eq.	4.21 (1H), dd, *J* = 9.2, 6.8 Hz	4.23 (1H), dd, *J* = 9.2, 6.6 Hz	4.13 (1H), m
8 ax.	4.02 (1H), dd, *J* = 9.2, 3.6 Hz	3.87 (1H), dd, *J* = 9.2, 3.6	3.85 (1H), m
2' 2''	6.92 (2H), d, *J* = 1.2 Hz	6.85 (2H), d, *J* = 1.2 Hz	6.85 (1H), d, *J* = 1.2 Hz 6.87 (1H), d, *J* = 1.2 Hz
5' 5''	6.77 (2H), d, *J* = 8.4 Hz	6.78 (2H), d, *J* = 8.4 Hz	6.78 (1H), d, *J* = 8.4 Hz 6.76 (1H), d, *J* = 8.4 Hz
6' 6''	6.80 (2H),dd, *J* = 8.4, 1.2 Hz	6.79 (2H), dd, *J* = 8.4, 1.2 Hz	6.79 (1H), dd, *J* = 8.4, 1.2 Hz 6.80 (1H), dd, *J* = 8.4, 1.2 Hz
O*CH*$_2$O	5.94 (4H), s	5.94 (4H), s	5.94 (2H), s 5.92 (2H), s

Table 2: ^{13}C NMR ($CDCl_3$, 100 MHz) spectral data for compounds **1-3**.

Carbon No.	**1**	**2**	**3**
C-1	50.1(d)	54.2(d)	54.4(d)
C-2	84.2(d)	85.6(d)	88.0(d)
C-4	68.8(t)	71.5(t)	71.6(t)
C-5	50.1(d)	54.2(d)	54.4(d)
C-6	84.2(d)	85.6(d)	88.0(d)
C-8	68.8(t)	71.5(t)	69.8(t)
C-1'	133.1(s)	135.0(s)	135.1(s)
C-1''	133.1(s)	135.0(s)	133.2(s)
C-2'	106.6(d)	106.4(d)	106.7(d)
C-2''	106.6(d)	106.4(d)	106.4(d)
C-3'	147.0(s)	146.9(s)	147.1(s)
C-3''	147.0(s)	146.9(s)	147.0(s)
C-4'	147.2(s)	147.8(s)	147.9(s)
C-4''	147.2(s)	147.8(s)	147.2(s)
C-5'	108.1(d)	108.2(d)	108.2(d)
C-5''	108.1(d)	108.2(d)	108.0(d)
C-6'	119.1(d)	119.2(d)	119.3(d)
C-6''	119.1(d)	119.2(d)	119.0(d)
OCH_2O	101.0(t)	101.1(t)	100.9(t)

(+)-Diasesamin (1)

White powder ($CHCl_3$).
MP:165-168°C.
$[\alpha]_D^{21}$: +214° (*c* 0.3, $CHCl_3$).
IR (KBr) ν_{max}: 1040, 950 cm^{-1}.
UV/Vis λ_{max} (CH_3CN) nm (log ε): 217 (4.10), 240 (4.14), 282 (4.06).
^{1}H NMR: Table 1.
^{13}C NMR: Table 2.
MS (EI, 70 eV): *m/z* (%): 354 $[M]^+$ (39), 203 (20), 178 (15), 161 (36), 150 (32), 149 (100), 135 (45), 131 (40), 122 (13), 121 (20), 117 (6), 115 (8), 103 (5), 91 (10), 77 (12), 65 (12), 63 (4).
HRMS-EI: *m/z* $[M]^+$ calcd for $C_{20}H_{18}O_6$: 354.1103; found: 354.1106.

(+)-Sesamin (2)

Colourless needles (EtOH).
MP: 119-121°C.
$[\alpha]_D^{25}$ +50° (*c* 0.5, $CHCl_3$).
IR (KBr) ν_{max}: 1040, 950 cm^{-1}.
UV/Vis λ_{max} (CH_3CN) nm (log ε): 207 (4.13), 235 (4.19), 286 (4.10).
^{1}H NMR: Table 1.
^{13}C NMR: Table 2.
MS (EI, 70 eV): *m/z* (%): 354 $[M]^+$ (30), 203 (22), 178 (11), 161 (34), 150 (35), 149 (100), 135 (50), 131 (35), 122 (28), 121 (21), 117 (10), 115 (18), 103 (16), 91 (10), 77 (18), 65 (15), 63 (10).

(+)-Episesamin (3)

Colourless needles (EtOH).
MP: 123-125°C.
$[\alpha]_D^{25}$ +130° (*c* 0.5, $CHCl_3$).
IR (KBr) ν_{max}: 1040, 950 cm^{-1}.
UV/Vis λ_{max} (CH_3CN) nm (log ε): 211 (4.13), 235 (4.17), 285 (4.09) nm.
^{1}H NMR: Table 1.
^{13}C NMR: Table 2.
MS (EI, 70 eV): *m/z* (%): 354 $[M]^+$ (35), 203 (20), 178 (18), 161 (36), 150 (30), 149 (100), 135 (47), 131 (45), 122 (21), 121 (20), 117 (6), 115 (8), 103 (8), 91 (10), 77 (12), 65 (5), 63 (5).

Preparation of culture medium with (+)-sesamin (2) [10]***:*** The basal medium for Hep G2 or HeLa cell culture was DMEM supplemented with 10% FCS (10%), penicillin G (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (250 μg/ml). The stock

solution of (+)-sesamin (2) (200 mM) was dissolved in DMSO and the experimental concentrations of (+)-sesamin (2) were prepared in the aforementioned basal medium with a final DMSO concentration of 0.1%.

(+)-Sesamin (2) treatment and cell cycle assay [10]***:*** Propidium iodide analysis was used to determine cell cycle distributions of baicalein treated and untreated samples. Hep G2 or HeLa (5 x 10^5 cells/dish) cells in 60-mm dishes were cultured overnight and then exposed to (+)-sesamin (**2**) (0, 100, and 200 μM), as described above. After 48 hours, cells were harvested by trypsinization and fixed at 4°C with PBS-methanol (1:2, volume/volume) solution. Aliquots of 1-2 x 10^6 fixed cells were resuspended in a solution containing DNase-free RNase A (50 μg/ml) and PI (50 μg/ml) for 30 minutes at room temperature in the dark. DNA fluorescence of PI-stained cells was evaluated by excitation at 488 nm and monitored through a 630/22-nm band pass filter using a Becton-Dickinson FACS-Calibur flow cytometer. A minimum of 10,000 cells were analyzed per sample, and the DNA histograms were gated and analyzed further using ModFit software to estimate the percentage of cells in various phases of the cell cycle.

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