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Alkamides from *Piper nigrum* L. and Their Inhibitory Activity against Human Liver Microsomal Cytochrome P450 2D6 (CYP2D6)

Subehan[a], **Tepy Usia**[a], **Shigetoshi Kadota**[a], **and Yasuhiro Tezuka**[a,b,*]

[a]*Institute of Natural Medicine and* [b]*21st Century COE Program*, *University of Toyama*, *2630 Sugitani*, *Toyama 930-0194*, *Japan*

tezuka@ms.toyama-mpu.ac.jp

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Drug−herb interaction through inhibition of cytochrome P450 alters the pharmacological response and/or toxicities of drug used concomitantly. In our screening, *Piper nigrum* L. was observed to inhibit cytochrome P450 2D6 (CYP2D6) in human liver microsomes. Thus, the MeOH extract of this plant was investigated for their chemical constituents and 19 alkamides including a new pipercyclobutanamide were isolated. Their structures were elucidated on the basis of spectroscopic analyses. The isolated compounds were tested for their inhibition on human liver microsomal dextromethorphan *O*-demethylation activity, a selective marker for CYP2D6, and pipercyclobutanamide A (**17**) showed the most potent inhibition with an IC_{50} value of 0.34 μM. The result demonstrated the potential of drug−alkamides interaction on concomitant consume of white pepper with the drugs being metabolized by CYP2D6.

Keywords: *Piper nigrum* L., cytochrome P450 2D6, CYP2D6, drug−herb interaction, pipercyclobutanamide, alkamides

Liver is the major organ responsible for the biotransformation of drugs or other xenobiotics. The smooth endoplasmic reticulum (subcellular membrane system) in hepatic tissue is rich in enzymes for metabolism, known as cytochromes P450 (CYP) [1]. This superfamily is subdivided into families and subfamilies that are defined solely on the basis of amino acid sequence homology. The CYP1, 2, and 3 families encode the enzyme involved in the majority of all drug biotransformation, while the gene products of the remaining CYP families are important in the metabolism of endogenous compounds such as steroids and fatty acids [2]. Approximately 70% of human liver CYP is accounted for by CYP1A2, CYP2A6, CYPB6, CYP2C, CYP2D6, CYP2E1, and CYP3A4 enzymes [3].

CYP2D6 is a polymorphic enzyme and the functional form is absent in 5 to 9% of Caucasians as a result of autosomal recessive inheritance of gene mutations [4]. It represents less than 5% of total CYP protein and metabolizes more than 30% of the clinically used drugs, including antidepressant, antipsychotic, and cardiovascular drugs [5]. Inhibition of CYP leads to higher plasma concentrations of drugs, which are used concomitantly and metabolized by the enzyme, to result in the increase of the toxicity or in the alteration of their efficacy levels. A variety of components in numerous species of plants have been reported to inhibit multiple forms of CYP [6,7]. Some examples include bergamotin, a component of grapefruit juice, capsaicin from chili peppers, and diallylsulfide found in garlic oil [8-10]. We also reported sesquiterpenes and flavonol glycosides of *Zingiber aromaticum*, lignans of *Piper cubeba*, and alkaloids of *Chataranthus roseus* for their CYP inhibitory activity [11-13].

In our previous study, we observed that the methanol extract of *Piper nigrum* L. (Piperaceae) strongly inhibited human liver microsomal dextromethrophan *O*-demethylation by CYP2D6 [14]. *P. nigrum* is the most popular spice in the world and has received much attention due to its many pharmacological activities [15]. In Indonesia, *P. nigrum* has been used as traditional medicine for treatment of hypertension, digestive disorder, dyspnea, and diaphoresis [16]. A

number of steroids, terpenes, lignans, flavones, and alkaloids/amides are known to occur in this plant [17] and CYP3A4 inhibitory constituents were reported [18-20]. On the CYP2D6 inhibitory constituents, however, there is no report, and thus we have carried out the phytochemical investigation of its MeOH extract and isolated 19 alkamides including a new pipercyclobutanamide (**19**). In this paper, we report the isolation and structure determination of the new compound, pipercyclobutanamide C (**19**), together with the CYP2D6 inhibitory activity of the isolated compounds.

P. nigrum fruit was extracted with MeOH and the MeOH extract was chromatographed on silica gel with a hexane−EtOAc solvent system to give nine fractions. The fractions were further separated by repeated column chromatography, followed by normal- and reversed-phase preparative TLC, to afford 19 alkamides (**1**−**19**) including a new pipercyclobutanamide (**19**) (Fig. **1**). Their structures were confirmed by comparisons of their spectral data with those in literatures to be *N*-cinnamoylpiperidine (**1**) [21], (2*E*,4*E*)-*N*-isobutyldecadienamide (**2**) [22], (2*E*)-octadec-2-enoic acid piperidide (**3**) [23], feruperine (**4**) [24], pipernonalin (**5**) [25], piperamide-A6:2(2*E*,6*E*) (**6**) [26], piperanine (**7**) [27], piperine (**8**) [28], dehydropipernonaline (**9**) [29], piperolein B (**10**) [28], piperolein A (**11**) [30], pipercide (**12**) [31], piperylin (**13**) [28], piperamide-C9:3(2*E*,4*E*,8*E*) (**14**) [28], piperamide-C9:1(8*E*) (**15**) [28], piperamide-C7:1(6*E*) (**16**) [28], pipercyclobutanamide A (**17**) [32], and nigramide R (**18**) [17].

Pipercyclobutanamide C (**19**) was isolated as colorless amorphous solid with $[\alpha]_D^{24}$ 0° (*c* 0.15, $CHCl_3$). Its IR spectrum showed absorption bands corresponding to carbonyl (1630 cm^{-1}) and methylenedioxyphenyl (930 cm^{-1}) groups. The EI-MS showed the molecular ion peak at *m*/*z* 622 and the molecular formula $C_{38}H_{42}N_2O_6$ was established by HR-EI-MS. In the NMR spectra, however, the signals ascribable to only 21 hydrogens and 19 carbons were observed. This and an intense fragment ion at *m*/*z* 311 in EI-MS suggested the symmetrical nature of **19**.

The ^1H and ^{13}C NMR data revealed the presence of a methylenedioxyphenyl group, two *trans*-olefins, a piperidine ring, and a carbonyl group. The COSY spectrum showed correlations assignable to -C(2)H−C(3)H−C(4)H=C(5)H−C(6)H=C(7)-, indicating the double bonds to be conjugated. The *E*-geometry of both double bonds were substantiated by the coupling constant (*J* = 15.3 Hz). In the HMBC spectrum, cross peaks of H-7 with C-8, C-9, and C-13 indicated the conjugated double bonds to be connected to the

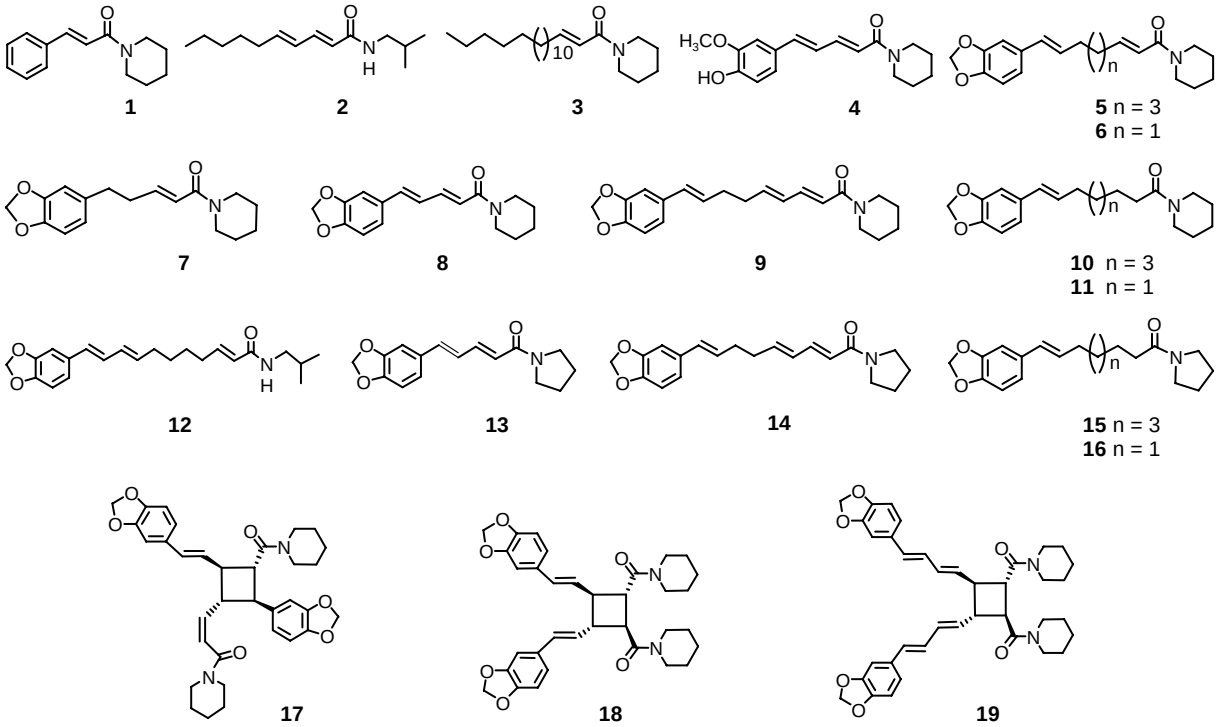

Figure 1: Chemical structures of the compounds isolated from *Piper nigrum* L.

methylenedioxyphenyl group, while the correlations of both H-5 and H-4 with C-3 and of H-2 with C-1 indicated the connection between the conjugated double bonds and the carbonyl carbon through the two methines, which led to the half unit of the molecule. The correlation of both H-2' and H-6' with C-1 also confirmed the piperidine ring to be bonded to the carbonyl carbon to form an amide linkage (Fig. **2**). Thus, **19** should be a symmetrical cyclobutanamide.

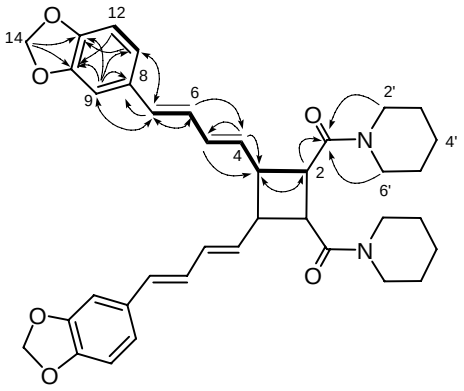

Figure 2: COSY (Bold lines) and HMBC (arrows) correlations of **19**.

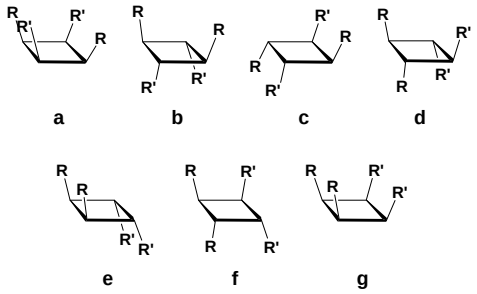

Figure 3: Possible symmetrical dimmers of cyclobutane ring.

There are seven types of possible symmetrical dimers for **19** (Fig. **3**). In the NOE difference spectrum, NOE was observed from H-2 (or H-2") to H-3 (or H-3") and vice versa, indicating the types **b** and **e** with anti configuration of H-2 (H-2") and H-3 (H-3") to be eliminated. While NOEs from H-2 (H-2") to H-4 (H-4") and H-5 (H-5"), from H-3 (H-3") to H-4 (H-4") and H-5 (H-5"), from H-4 (H-4") to H-5 (H-5"), and their vice versa should not allow the *syn* configuration of H-2 (H-2") and H-3 (H-3") (types **a**, **f**, and **g**). Thus, types **c** and **d** are the possible stereo structure of **19** (Fig. **4**). In addition, the coupling pattern of H-2 and H-3 was more similar to that of a known compound **18** with a type **d** configuration [17] than dipiperamide B with a type **c** configuration [18]. Finally, **19** was concluded to contain the cyclobutane ring with a head-to-head structure, i.e., type **d**, based on the coupling constant of H-2 (H-2") and H-3 (H-3") ($J_{2,3}$ = 6.3 Hz, $J_{2,3"}$ = −0.8 Hz, $J_{2,2"}$ = 11.3 Hz, and $J_{3,3"}$ = 10.5 Hz) obtained by a simulation (Fig. **5**) [33,34].

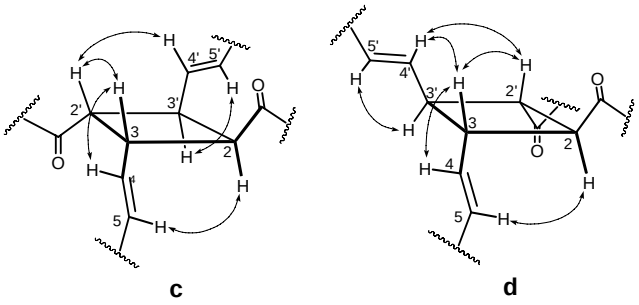

Figure 4.:NOEs detected in the difference NOE experiment of **19** implied type **c** or **d** of its stereochemistry.

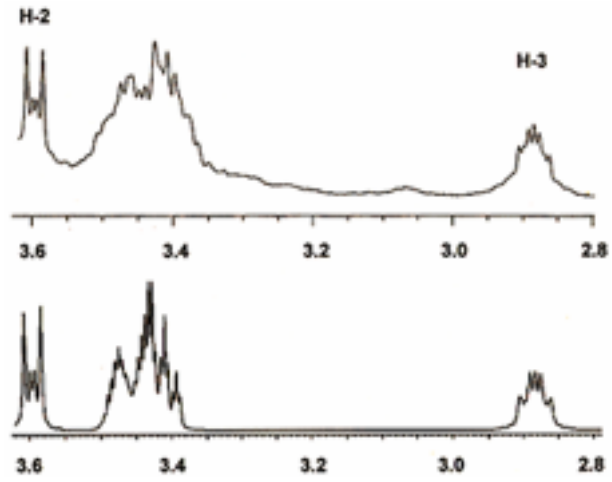

Figure 5: Experimental (above) and simulated (below) ^{1}H NMR spectra of cyclobutyl proton of **19**.

Since **17**, **18**, and **19** are optically inactive, an enzymatic cyclization may not be involved in their biosynthesis. These pipercyclobutanamide are presumably generated by [2π+2π] cycloadditions of piperine or other constituents of this plant during the process of producing white pepper [18]. This was supported by the reports that dipiperamide A isolated from *P. nigrum* can be synthesized from a solid state photodimerization of an (*E*)-cinnamic acid derivatives and piplartine-dimer A was generated by irradiation of piplartine with sunlight in *P. tuberculatum* [35,36].

The isolated compounds were tested for their CYP2D6 inhibitory activity (Table 1). Among them, **17** showed the most potent inhibitory activity with an IC_{50} value of 0.34 μM, while **2**, **6**, **9**, and **15** showed moderate in their inhibition, and compounds **1**, **3**, **4**, **11**, **12**, **18**, and **19** inhibited CYP2D6 only weakly. The presence of methylenedioxyphenyl ring and nitrogen atom is reported to be important for their inhibition on CYP2D6 [3,37]. Methylenedioxyphenyl group is metabolized to result in the generation of carbene intermediates which can form a strong covalent complex with iron center of the heme in cytochromes [37]. Many of the potent CYP inhibitors are nitrogen-containing drugs, such as imidazole, pyridines, and quinolines, which can inhibit CYP with bind to the prosthetic heme iron or to the lipophilic region of the protein [3]. Thus, inhibition of CYP2D6 by constituents of *P. nigrum* could also be due to the presence of the piperidine, pyrrolidine, or isobutylamide group and/or the methylenedioxyphenyl group.

Table 1: IC_{50} values of the isolated compounds in the metabolism mediated by CYP2D6.

Compds	IC_{50}, μM	Compds	IC_{50}, μM
1	>100	**11**	>100
2	21.8	**12**	>100
3	>100	**13**	95.2
4	>100	**14**	77.3
5	82.5	**15**	32.6
6	36.0	**16**	86.5
7	90.2	**17**	0.45
8	78.9	**18**	>100
9	20.6	**19**	>100
10	75.5	**Quinidine**	0.068

These results demonstrated the potential of drug−alkamides interaction on consumption of white pepper with drug being metabolized by CYP2D6 to result in the alteration of the toxicity and efficacy level of the drug concomitantly administrated.

Experimental

General Experimental: Optical rotations were measured on a JASCO DIP-140 digital polarimeter. IR spectra were measured with a Shimadzu IR-408 spectrophotometer in $CHCl_3$ solution. High resolution electron impact mass spectrometry (HR-EI-MS) measurements were carried out on a JEOL JMS-700T spectrometer at an ionization voltage of 40 eV. The ^1H, ^{13}C, and 2D NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard. Column chromatography was performed with silica gel 60 (Nacalai Tesque, Inc., Kyoto, Japan), and analytical and preparative TLC were conducted on precoated Merck Kieselgel $60F_{254}$ and RP-$18F_{254}$ plates (0.25 or 0.50 mm thickness).

Plant material: Fruits of *Piper nigrum* were purchased from GORO traditional market, Jakarta, Indonesia in May 2002 and identified by Drs. Sucipto (PJ. Bintang Terang Lestari, Traditional Medicine Supplier, Jakarta, Indonesia). A voucher specimen (TMPW 22278) is preserved in the Museum for Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Extraction and Isolation: The air-dried fruits (140 g) of *P. nigrum* were crushed into powder form and extracted with MeOH (24 L, 2 h, 80 °C, × 2). Evaporation of MeOH under reduced pressure, followed by lyophilization gave a MeOH extracts (10.3 g).

The MeOH extract (8.1 g) was subjected to silica gel column chromatography with a hexane–EtOAc solvent system to yield 9 fractions: fraction 1 (388 mg), 10% EtOAc–hexane elute; fraction 2 (359 mg), 20% EtOAc–hexane elute; fraction 3 (229 mg), 30% EtOAc–hexane elute; fraction 4 (1.4 g), 40% EtOAc–hexane elute; fraction 5 (3.2 g), 60% EtOAc–hexane elute; fraction 6 (650 mg), 80% EtOAc–hexane elute; fraction 7 (576 mg), EtOAc elute; fraction 8 (486 mg), 20% MeOH–EtOAc elute; and fraction 9 (572 mg), MeOH elute.

Fraction 2 was separated by reversed-phase preparative TLC (CH_3CN:MeOH:H_2O = 4:4:1) to give **1** (3.7 mg), **2** (31.3 mg), and **3** (8.7 mg), while fraction 3 gave **5** (4.2 mg) **10** (19 mg) and **11** (7.6 mg) with the same method. Fraction 4 was separated by reversed-phase preparative TLC (CH_3CN:MeOH:H_2O = 4:4:2) to yield **6** (6.4 mg), **7** (33.4 mg), **9** (6.4 mg), **15** (5.5 mg), and **12** (5 mg). Fraction 5 gave **8** (3.2 mg). Fractions 6, 7, and 8 were subjected to reversed-phase preparative TLC (CH_3CN:MeOH:H_2O = 4:4:2), and **4** (3.7 mg), **13** (31.3 mg), and **16** (8.7 mg) were isolated from fraction 6, **14** (7.4 mg) was from fraction 7, and **17** (7.1 mg), **18** (9 mg), and **19** (6 mg) were from fraction 8.

Pipercyclobutanamide C (19)

$[\alpha]_D^{24}$: 0° (*c* 0.15, $CHCl_3$).

IR ($CHCl_3$) ν_{max}:1630, 1500, 1450, 1250, 1040, 970, 930 cm^{-1}.
^1H NMR (400 MHz, $CDCl_3$): δ 6.9 (1H, s, H-9), 6.79 (1H, d, *J* = 8.0 Hz, H-12), 6.73 (1H, dd, *J* = 8.0, 1.4 Hz, H-13), 6.55 (1H, dd, *J* = 15.3, 10,4 Hz, H-6), 6.39 (1H, d, *J* = 15.3 Hz, H-7), 6.22 (1H, dd, *J* = 15.1, 10.4 Hz, H-5), 5.94 (2H, s, H-14), 5.80 (1H, dd, *J* = 15.1, 8.0 Hz, H-4), 3.65 (1H, m, H-6'), 3.59 (1H, m, H-2), 3.35−3.50 (3H, m, H-2', H-6'), 2.88 (1H, m, H-3), 1.12−1.59 (6H, m, H-3', H-4', H-5').
^{13}C NMR (100 MHz, $CDCl_3$): δ 170.4 (C-1), 148.1 (C-10), 147.2 (C-11), 133.9 (C-4), 132.1 (C-6), 131.8 (C-7,C-8), 126.8 (C-5), 121.2 (C-13), 108.4 (C-12), 105.4 (C-9), 101.1 (C-14), 46.7 (C-2'), 46.4 (C-3), 43.2 (C-6'), 42,0 (C-2), 26.8 (C-3'), 25.7 (C-5'), 24.6 (C-4').
HR-EI-MS *m/z*: 622.3010 $[M]^+$ calcd for $C_{38}H_{42}N_2O_6$: 622.3043.

CYP2D6 Inhibition Assay: Human liver microsomes (HLM; XenoTech. LLC, Kansas, USA) were stored at –80 °C prior to use. β-Nicotinamide adenine dinucleotide phosphate oxidized form ($NADP^+$), glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase (Oriental Yeast Co., LTD., Tokyo, Japan) were used as NADPH-generating system.

Inhibitory activity on the metabolism mediated by CYP2D6 *in vitro* was determined using a radiometric measurement of [^{14}C]formaldehyde formed by the reaction with [*O*-methyl-^{14}C]dextromethorphan (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) as a substrate [38]. Briefly, in disposable culture tubes (13 × 100 mm; Iwaki, Tokyo, Japan) containing phosphate buffer (pH 7.4), [*O*-methyl-^{14}C]dextromethorphan (0.1 μCi/incubation; 100 μM in 5% of MeOH), and 50 μl of HLM (4 mg/ml) were added to varying concentrations of test specimens in 500 μl of total incubation volume. After a preincubation period of 5 min in shaking water bath at 37 °C. The reaction is initiated by adding 50 μl of NADPH-generating system (4.20 mg/ml of $NADP^+$ in a solution of 100 mM G-6-P, $MgCl_2$, and 10 U/ml G-6-P dehydrogenase), and the incubation was continued for 20 min (CYP2D6) in a shaking water bath at 37 °C. The reaction was stopped by adding of 125 μl of 10% trichloroacetic acid (Nacalai Tesque, Inc., Kyoto, Japan), and the solution was centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was applied to Envi-Carb solid-phase extraction columns (Supelco, UK) and was eluted with two volumes of ultrapure water (2 × 500 μl). After adding 10 ml of Clear-sol I (Nacalai Tesque, Inc., Kyoto, Japan), the eluted radioactivity was quantified by liquid scintillation counting LS 6500 (Beckman, USA). Quinidine sulfate dihydrate (Wako Pure Chemicals Industry, Ltd., Osaka, Japan) was used as a positive control, while MeOH was used as a negative control. Correction was made for radioactivity eluted from control incubation in which HLM and NADPH-generating systems had been omitted. The assays were performed in duplicate for all test specimens, and remaining activity was analyzed using software product WinNonlin Ver.3.1 (Pharsight Corporation, Mountain View, CA. USA). IC_{50} values (concentration of test specimen causing 50% reduction in activity relative to the negative control) were calculated by linear regression analysis of the log test specimen concentration versus percentage control activity plots.

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