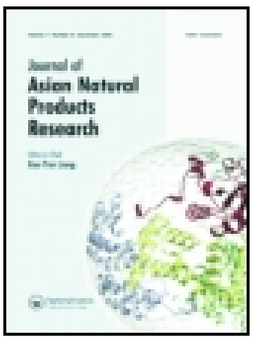

Journal of Asian Natural Products Research

ISSN: 1028-6020 (Print) 1477-2213 (Online) Journal homepage: http://www.tandfonline.com/loi/ganp20

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To cite this article: Yao Yuan, Jian-Xiang Yang, Ling-Hui Nie, Bai-Lin Li, Xu-Bing Qin, Jie-Wei Wu & Sheng-Xiang Qiu (2017): Three new kavalactone dimers from Piper methysticum (kava), Journal of Asian Natural Products Research, DOI: 10.1080/10286020.2017.1367768

To link to this article: http://dx.doi.org/10.1080/10286020.2017.1367768

JOURNAL OF ASIAN NATURAL PRODUCTS RESEARCH, 2017
https://doi.org/10.1080/10286020.2017.1367768

Three new kavalactone dimers from *Piper methysticum* (kava)

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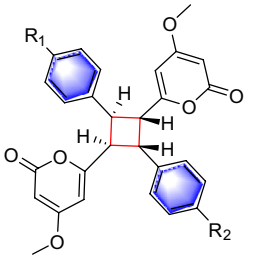
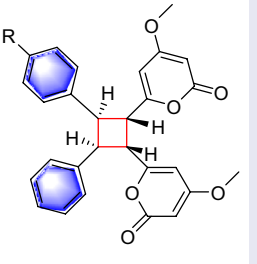
ABSTRACT

Three new dimeric kavalactones, designated as diyangonins A–C (**1**–**3**), along with two known analogs were isolated from the roots of *Piper methysticum*. Their structures were elucidated by means of extensive analysis of their 1D, 2D NMR, and mass spectroscopic data. All these dimers possess a skeleton featuring a cyclobutane ring connecting two kavalactone units in head-to-tail or head-to-head mode. Compounds **1**–**5** were evaluated for their cytotoxic activities against human tumor cell lines.



1 $R_1 = OCH_3$; $R_2 = OCH_3$
2 $R_1 = OCH_3$; $R_2 = H$
4 $R_1 = H$; $R_2 = H$



3 $R = OCH_3$
5 $R = H$

ARTICLE HISTORY
Received 17 February 2017
Accepted 11 August 2017

KEYWORDS
Piperaceae; kavalactone dimers; *Piper methysticum*; head-to-tail; head-to-head; cytotoxic activities

1. Introduction

Piper methysticum Forst. f., commonly known as kava, belongs to the family Piperaceae, and grows as a perennial shrub in the South Pacific islands [1]. The roots of this plant is traditionally used to produce a drink with sedative, anesthetic, euphoriant, and entheogenic properties for medical purposes or as an inebriant beverage that elicits physiological and psychological relaxation at social gatherings and religious and cultural ceremonies

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Supplemental data for this article can be accessed at https://doi.org/10.1080/10286020.2017.1367768.

[2]. Commercially, kava has been used as a natural remedy for insomnia, anxiety, and menopausal symptoms [3]. Due to its beneficial health effects, kava has gained popularity recently in Western countries as a dietary supplement and alternative medicine, especially for the treatment of anxiety disorders [4–6].

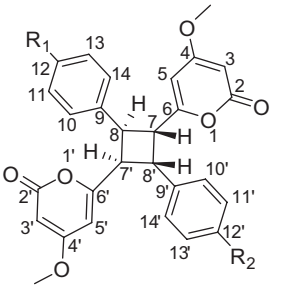
The chemistry of kava has been extensively studied in the past decades, and so far more than 40 compounds belonging to the classes of kavapyrones, alkaloids, steroids, chalcones, long-chained fatty acids, and alcohols have been isolated and identified [7]. Among these compounds, kavalactones have been recognized as the constituents responsible for the reported biological activities in kava [2].

Dimeric kavalactones were first isolated from *Polygonum nodosum* as trace constituents [8]. Our extensive phytochemical investigation on kava roots has led to the isolation of five dimeric kavalactones, three of which were characterized as new compounds, named diyangonins A–C (**1–3**), alongside two known ones, namely *rel*-, *trans*-3-bis[6-(4-methoxy-2-pyronyl)]-*cis*-2,*trans*-4-diphenyl cyclobutane (**4**) [8] and 6,6′-(3,4-diphenylcyclobutane-1,2-diyl)bis(4-methoxy-2*H*-pyran-2-one) (**5**) [9] (Figure 1). Herein, we report the details on the isolation, structural elucidation, and cytotoxic evaluation of these dimeric kavalactones.

2. Results and discussion

Compound **1** was obtained as an optically inactive yellow powder with a molecular formula of $C_{30}H_{28}O_8$ as established on the basis of HR-ESI-MS measurements wherein a protonated molecular ion was measured at *m/z* 517.1871 $[M + H]^+$, requiring 17 indices of hydrogen deficiency in the molecule.

The NMR spectra (Table 1) indicated the presence of a para-substituted aromatic ring (δ_H 7.20, d, J = 8.7 Hz, 2H; δ_H 6.83, d, J = 8.7 Hz, 2H), an ester carboxyl (δ_C 164.2), four methines (including two olefinic methines), and two methyls (δ_H 3.69, s, 3H; 3.77, s, 3H). These spectral features were similar to those of kavalactone yangonin [10], except for the absence of trans-olefinic signals, and instead, the presence of two additional methines, suggesting that **1** was a dimer of yangonin biogenetically originated via a [2 + 2] addition



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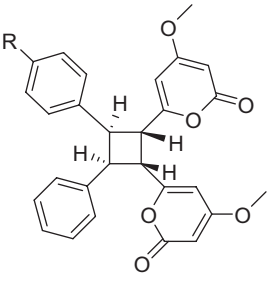

3 R = OCH_3
5 R = H

Figure 1. Structures of compounds **1–5**.

Table 1. ^{1}H and ^{13}C NMR Data for **1–3** (500 and 125 MHz, respectively) in $CDCl_3$.

	1		**2**		**3**	
No.	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1						
2		164.2		164.2		164.6
3	5.23 (d, 2.2)	87.7	5.23 (d, 2.2)	87.7	5.37 (d, 2.2)	88.3
4		170.6		170.6		171.3
5	5.73 (d, 2.2)	101.4	5.73 (d, 2.2)	101.4	5.98 (d, 2.2)	101.7
6		163.0		162.9		162.7
7	4.19 (dd, 9.8, 7.2)	45.6	4.24 (dd, 9.8, 7.2)	45.2	4.04 (dd, 9.6, 5.3)	44.8
8	4.37 (dd, 9.8, 7.2)	43.1	4.38 (dd, 9.8, 7.2)	43.2	4.44 (dd, 9.6, 5.3)	44.7
9		129.5		129.4		129.0
10	7.20 (d, 8.7)	128.6	7.21 (d, 8.7)	127.4	6.87 (d, 8.7)	130.2
11	6.83 (d, 8.7)	113.9	6.83 (d, 8.7)	114.0	6.66 (d, 8.7)	113.9
12		158.6		158.6		158.5
13	6.83 (d, 8.7)	113.9	6.83 (d, 8.7)	114.0	6.66 (d, 8.7)	113.9
14	7.20 (d, 8.7)	128.6	7.21 (d, 8.7)	127.4	6.87 (d, 8.7)	130.2
1′						
2′		164.2		164.1		164.6
3′	5.23 (d, 2.2)	87.7	5.20 (d, 2.2)	87.7	5.36 (d, 2.2)	88.3
4′		170.6		170.6		171.3
5′	5.73 (d, 2.2)	101.4	5.74 (d, 2.2)	101.4	6.00 (d, 2.2)	101.7
6′		163.0		162.8		162.7
7′	4.19 (dd, 9.8, 7.2)	45.6	4.23 (dd, 9.8, 7.2)	45.5	4.11 (dd, 9.6, 5.3)	44.1
8′	4.37 (dd, 9.8, 7.2)	43.1	4.42 (dd, 9.8, 7.2)	43.5	4.47 (dd, 9.6, 5.3)	45.2
9′		129.5		137.5		138.2
10′	7.20 (d, 8.7)	128.6	7.27–7.29 (m)	128.6	6.96 (d, 8.7)	128.0
11′	6.83 (d, 8.7)	113.9	7.27–7.29 (m)	128.6	7.13–7.16 (m)	128.5
12′		158.6	7.18–7.19 (m)	127.2	7.07–7.10 (m)	126.8
13′	6.83 (d, 8.7)	113.9	7.27–7.29 (m)	128.6	7.13–7.16 (m)	128.5
14′	7.20 (d, 8.7)	128.6	7.27–7.29 (m)	128.6	6.96 (d, 8.7)	128.0
CH_3O-4	3.69 s	56.8	3.68 s	55.8	3.76 s	56.2
CH_3O-12	3.77 s	55.2	3.76 s	55.2	3.70 s	55.4
CH_3O-4′	3.69 s	56.8	3.67 s	55.8	3.76 s	56.2
CH_3O-12′	3.77 s	55.2				

with the formation of a cyclobutane ring, which also accounts for the molecular weight of **1** as double that of yangonin.

Theoretically, two possible cyclo-adducts, either head-to-tail or head-to-head, could exist, which could be readily distinguished directly by means of EI-MS measurements rather than NMR spectroscopy [8,11]. Through this method, three typical fragment ions at *m/z* 276, 258, and 240 can be observed in head-to-head mode, while only one typical fragment ion at *m/z* 258 could be measured in head-to-tail mode (Scheme S1). The observation of the only one typical mass fragment ion at *m/z* 258 in EI-MS indicated that **1** was a dimeric kavalactone with a head-to-tail adduction mode.

The connection between the para-substituted benzene ring and C-8 postion of the cyclobutane ring was established on the basis of the HMBC correlation between protons resonating at δ_H 7.20 (H-10 and H-14) and the carbon signal of C-8 at δ_C 43.1. The proton signal at δ_H 4.19 (H-7) correlated to the carbon signals in the lactonic ring (γ-pyrone) at δ_C 101.4 (C-5) and 163.0 (C-6), indicating that the lactonic ring was located at C-7 (Figure 2). Thus, the plain structure of **1** was deduced as depicted in Figure 1.

The relative configuration of the cyclobutane ring was revealed from the coupling pattern of the methine protons on the cyclobutane ring. The coupling constants of H-7/H-8 and H-7/H-8′ were determined to be 9.8 and 7.2 Hz, respectively, which implied that **1** was a

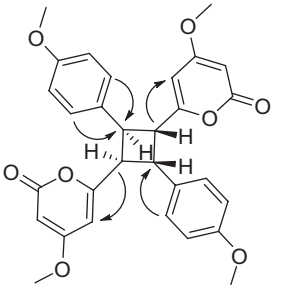

Figure 2. Key HMBC (H → C) correlations of compound **1**.

cis-cis fused dimer in head-to-tail mode [8,11]. Therefore, the structure of **1** turned to be a new dimeric kavalactone which was then given a trival name diyangonin A.

Compound **2**, also isolated as an optically inactive yellow powder, was found to possess a molecular formula of $C_{29}H_{26}O_7$ as determined by an HRESIMS ion at *m/z* 487.1695 $[M + H]^+$, indicating 17 indices of hydrogen deficiency.

The ^1H and ^{13}C NMR spectra of compound **2** (Table 1) showed close similarities to those of **1**. The main difference observed was the absence of one methoxy group signal, and the presence of one aromatic proton signal (δ_H 7.18–7.19, m, 1H; δ_C 127.2) was observed instead. Similarly, only one typical fragment ion at *m/z* 258 was measured in the EI-MS, indicated **2** was dimeric kavalactone with a head-to-tail adduction mode. In analogy to that of **1**, the coupling constants of H-7/H-8 and H-7/H-8′ in **1** were determined to be 9.8 and 7.2 Hz, respectively, implied that **2** was also a *cis-cis* fused dimeric kavalactone with a head-to-tail mode [8,11]. Thus, the structure of **2** was elucidated as depicted in Figure 1, and then named as diyangonin B.

Compound **3** shared the same molecular formula as **2**, as determined by the HRESIMS ion at *m/z* 487.1776 $[M + H]^+$. Interpretation of its NMR data (Table 1) revealed similarities between the structures of **3** and **2**, the only difference being the mode of dimerization. Three typical fragments at *m/z* 258, 228, and 210 observed in the EI-MS indicated that compound **3** was formed via a head-to-head conjunction mode, rather than the head-to-tail mode of **1** and **2**. A *cis-cis* fusion was deduced on the basis of the coupling constants of H-7/H-8 (9.6 Hz) and H-7/H-8′ (5.3 Hz) for compound **3** [8,11]. Thus, the structure of **3** was elucidated as a new dimeric kavalactone as depicted in Figure 1, and then named as diyangonin C.

Collectively, compounds **1**–**5** represented a group of dimeric kavalactones possessing a skeleton featuring a cyclobutane ring connecting two kavalactone units. While dimers **1**, **2,** and **4** formed via a head-to-tail coupling, dimers **3** and **5** were of head-to-head orientation.

The five dimeric kavalactones (**1**–**5**) were evaluated for their cytotoxic activity against three human tumor cell lines NCI-H46, SW480, and HepG2 using MTT assay. However, none showed noticeable activity (cellular proliferation inhibition rate <50% at 20 μM).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 polarimeter (Perkin-Elmer, Boston, MA, U.S.A.). UV spectra were recorded in MeOH on a Perkin-Elmer Lambda 35 UV-vis

spectrophotometer (Perkin-Elmer, Boston, MA, U.S.A.). IR spectra were acquired using a Bruker Vertex 33 infrared spectrophotometer (Bruker, Karlsruhe, Germany) with KBr disk. 1D and 2D NMR spectra were recorded on a Bruker Advance-500 spectrometer with TMS as internal standard (Bruker BioSpin AG, Fallanden, Switzerland). EIMS was carried out on a Finnigan MAT-95 mass spectrometer. HR-ESI-MS data were obtained on a Bruker Bio TOF IIIQ mass spectrometer (Bruker Daltonics, MA, U.S.A.). All solvents were analytical grade (Shanghai Chemical Plant, Shanghai, China). Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China) was used for column chromatography, and precoated silica gel GF254 plates (Qingdao Marine Chemical Inc., Qingdao, China) were used for TLC. C_{18} reversed-phase silica gel (150–200 mesh, Merck, Darmstadt, Germany), MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd., Tokyo, Japan), and Sephadex LH-20 gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd., Tokyo, Japan) were also used for column chromatography (CC). TLC spots were visualized under UV light and by dipping into 5% H_2SO_4 in alcohol followed by heating. Analytical HPLC was performed on a Waters 2690 instrument with a 996 PAD (photodiode array detector). Preparative HPLC was carried out using a P3000 HPLC pump (Beijing Tong Heng Innovation Technology Co., Ltd.) and UV3000 UV–vis detector, and the column used was 250 mm × 10 mm (Allitma C18, 10 μm).

3.2. Plant material

The roots of kava were obtained from PureWorld; Naturex, South Hackensack, NJ, U.S.A., and identified by Prof. Fu-Wu Xing of South China Botanical Garden. A voucher specimen (QH20100816) has been deposited at the Laboratory of Natural Product Chemistry Biology, South China Botanical Garden.

3.3. Extraction and isolation

Dried and powdered roots of kava (5 kg) were extracted with 95% ethanol at room temperature three times (10 × 20 L, 3 days each) and filtered, and then the filtrate was concentrated under vacuum to give a crude ethanol extract (458 g). The crude ethanol extract was suspended in H_2O (5 L) and extracted with EtOAc. The ethyl acetate fraction (410 g) was subjected to column chromatography (CC) over silica gel and eluted with n-hexane-ethyl acetate (10:1, 5:1, 2:1, 1:1, v/v) to yield fractions A–F.

Fraction E (18 g) was subjected to CC over silica gel eluted with gradient elution of chloroform-ethyl acetate (5:1, 2:1, 1:1, v/v) to yield six subfractions (Fr.E1–Fr.E6). Fr.E2 (4 g) was separated by Sephadex LH-20 with elution of chloroform-methanol (1:1, v/v) to yield two subfractions (Fr.E2-1–Fr.E2-2). Fr.E2-1 (800 mg) was further purified by preparative HPLC (CH_3CN/H_2O from 40 to 100%, flow rate: 20.0 mL/min, run time: 90 min, detection wavelength: 254 nm) to yield compound **2** (37 mg, t_R: 61.30 min). Fr.E3 (3 g) was separated by Sephadex LH-20 with elution of chloroform-methanol (1:1, v/v) to yield two subfractions (Fr.E3-1–Fr.E3-2). Fr.E3-1 (760 mg) was further purified by preparative HPLC (CH_3CN/H_2O from 40 to 100%, flow rate: 20.0 mL/min, run time: 90 min, detection wavelength: 254 nm) to yield **3** (44 mg, t_R: 60.11 min) and **5** (94 mg, t_R: 63.15 min).

Fraction F (22 g) was subjected to CC over silica gel eluted with gradient elution of chloroform-ethyl acetate (5:1, 2:1, 1:1, v/v) to yield five subfractions (Fr.F1–Fr.F5). Fr.F4 (7 g) was separated by Sephadex LH-20 with elution of chloroform-methanol (1:1, v/v) to yield

three subfractions (Fr.F4-1–Fr.F4-3). Fr.F4-1 (1200 mg) was further purified by preparative HPLC (CH_3CN/H_2O from 40 to 100%, flow rate: 20.0 mL/min, run time: 90 min, detection wavelength: 254 nm), yielding **1** (87 mg, t_R: 59.20 min) and **4** (108 mg, t_R: 63.45 min).

3.3.1. Diyangonin A (1)

Yellow power; $[\alpha]_D^{25}$ 0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 287 (0.38), 229 (0.75) nm; IR (KBr) ν_{max} 3079, 2942, 1720, 1644, 1567, 1512, 1456, 1410, 1256, 1107, 1036, 812 cm^{-1}; for ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS: *m/z* 517.1871 $[M + H]^+$ (calcd for $C_{30}H_{29}O_8$, 517.1857).

3.3.2. Diyangonin B (2)

Yellow power; $[\alpha]_D^{25}$ +2.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 287 (0.30), 229 (0.42) nm; IR (KBr) ν_{max} 2944, 1722, 1648, 1568, 1512, 1453, 1409, 1332, 1258, 1147, 1036, 973, 875, 847, 815 cm^{-1}; for ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS: *m/z* 487.1695 $[M + H]^+$ (calcd for $C_{29}H_{27}O_7$, 487.1751).

3.3.3. Diyangonin C (3)

Yellow power; $[\alpha]_D^{25}$ +1.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 283 (0.42), 228 (0.55) nm; IR (KBr) ν_{max} 2926, 1722, 1644, 1566, 1513, 1455, 1410, 1250, 1155, 1034, 825 cm^{-1}; for ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS: *m/z* 487.1776 $[M + H]^+$ (calcd for $C_{29}H_{27}O_7$, 487.1751).

3.4. Cytotoxic activity against NCI-H46, SW480 and HepG2

Compounds **1–5** were evaluated for inhibitory activity against human lung cancer cell (NCI-H46), human colorectal cancer cells (SW480), and human liver cancer cells (HepG2) using the MTT method, according to a previously described procedure [12]. Doxorubicin was used as positive control. Cells were plated in 96-well tissue plates at a density of 1×10^4 cells/well. Adherent cell lines were previously incubated for 24 h to ensure adhesion to the wells in an atmosphere of 5% CO_2. Compounds **1–5** were applied at various concentrations (0.01, 0.1, 1, 10, 100 μM) and control cells were treated with DMSO at the highest concentration used in test wells (0.5%). 1 h prior to the end of the incubation period, 20 mL of MTT (5 mg/mL in PBS, 5% MTT) were added to each well and further incubated at 37 °C for another 4 h. Supernatants were removed and 150 μL DMSO were afterward added to each well in order to dissolve the formazan crystals. The mixture was oscillatored for 10 min at room temperature and its absorbance was measured at 490 nm (Genios, Tecan, Austria). The concentration resulting in 50% of cell growth inhibition (IC_{50}) was calculated using the Probit program in SPSS 19 for windows XP (SPSS Inc., Chicago).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was financially supported by the China Postdoctoral Science Foundation [grant number 2015M582436]; the Guangdong Province Science and Technology Project [grant number

2016A010105015]; the Strategic Resources Service Network Program on Plant Genetic Resources Innovation of the Chinese Academy of Sciences [grant number ZSZC-005]; the "Twelfth Five-Year" National Science and Technology Support Project of China [grant number 2015BAD15B03] and the Dong Guan Innovative Research Team Program [grant number 2014607101005].

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