Note

Chemical and Pharmaceutical Bulletin

New Flavan and Benzil isolated from *Fissistigma latifolum*

Yu-Hsuan Lan,[a*] Yi-Ting Peng,[a] Tran-Dinh Thang,[b] Tsong-Long Hwang,[c] Do-Ngoc Dai,[d] Yann-Lii Leu,[c] Wan-Chun Lai[e] and Yang-Chang Wu[f*]

[a]*School of Pharmacy, China Medical University, Taichung 404, Taiwan;* [b]*Department of Chemistry, Vinh University, Vinh City, Vietnam;* [c]*Graduate Institute of Natural Products, Chang Gung University, Tao-Yuan 333, Taiwan;* [d]*Institute of Ecology and Biologycal Resources, Vietnam Academy of Science and Technology, Vietnam;* [e]*Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan; and* [f]*Graduate Institute of Integrated Medicine, China Medical University, Taichung 404, Taiwan*

[*] To whom correspondence should be addressed. Tel: +886-4-22053366 EXT. 5138; FAX: +886-4-22060248; E-MAIL: lanyh@mail.cmu.edu.tw

Abstract—Further investigation of the methanolic extract of *Fissistigma latifolium* resulted in two new compounds whose structures were assigned as 2,5,6,7-tetramethoxyflavan (**1**) and 2'-hydroxy-4',5',6'-trimethoxybenzil (**2**). These two compounds were determined on the basis of chemical and spectroscopic evidences. Compound **2** is the first report of benzil from *Fissistigma* species. 2,5,6,7-tetramethoxyflavan (**1**) showed a potent inhibitory effect on superoxide anion production in fMLP/CB-activated human neutrophils.

Key words: *Fissistigma latifolium*; Annonaceae; flavan; benzil.

Fissistigma genus (Annonaceae), consisting of about 90 species, has been shown to be a rich source of bioactive compounds. Until now, only about 16 species have been investigated for their constituents.[1-12] In literature, these species were reported to contain alkaloids, flavonoids, triterpenoids, benzenoids, cyclohexenones and cyclopentenones etc. [1-12] Bioactive components such as aristolactams from *F. balance* and *F. oldhamii* display antiplatelet aggregation activity;[2] kuafumine, fissistin and isofissistin from *F. glaucescens* and *F. lanuginosum* show potent cytotoxicity against KB cell *in vitro*.[6, 13] Our previous investigation of *Fissistigma* species resulted in the isolation of alkaloids, furano-fissohamione, cyclopentenones and flavonoids.[2, 4, 8, 13-18] Among these compounds, bracteolatum, piperoactam A, and fissislandione showed inhibitory effects on formyl-L-methionyl-L-leucyl-L-phenylalanine(fMLP)-induced superoxide anion ($O_2^{\cdot-}$) generation in human neutrophils and nitric oxide (NO) generation by RAW 264.7 macrophages in response to lipopolysaccharide (LPS). In the continuation of our search for constituents of *F. latifolium*, the isolation of two new compounds, 2,5,6,7-tetramethoxyflavan (**1**) and 2'-hydroxy-4',5',6'-trimethoxybenzil (**2**), is reported herein. The chemical structures of these two compounds were established by means of mass and related spectral experiments. These two compounds were also investigated for their effects on fMLP-induced superoxide anion ($O_2^{\cdot-}$) generation in human neutrophils.

Compound **1** was obtained as a white amorphous solid. The molecular formula of compound **1** was determined to be $C_{19}H_{22}O_5$ by HREIMS (*m/z* 330.1479, calcd. 330.1467). IR spectrum indicated the presence of phenyl (1612 and 1490 cm^{-1}) group. The UV spectrum exhibited maximal absorptions at 208 and 280 nm. The ^{1}HNMR spectrum showed the presence of five aromatic protons at δ_H 7.58 (2H, d, *J*=7.2 Hz), 7.43 (2H, t, *J*=7.2 Hz), and 7.37 (1H, d, *J*=7.2 Hz) of a mono-substituted benzene ring. Four protons of two adjacent methylene groups at δ_H 1.79, 2,27, 2,67 and 2.82 were evidenced by the COSY and HMQC spectrum. One singlet peak at δ_H 6.49 and three singlet peaks at δ_H 3.80, 3.85, and 3.88 indicated the presence of one aromatic proton and three methoxy groups, respectively. The ^{13}CNMR spectrum showed nineteen carbon signals, including four methoxy, five methine, two methylene and seven quaternary carbons (*Table 1*). By comparison with literature data,[19,20] the above-mentioned

information suggested a flavan skeleton. HMBC correlations between 5-OCH_3 (δ_H 3.88)/C-5 (δ_C 151.3), 6-OCH_3 (δ_H 3.80)/C-6 (δ_C 136.7), and 7-OCH_3 (δ_H 3.85)/C-7 (δ_C 152.8) permitted the location of three methoxy groups at C-5, C-6, and C-7. The proton signal at δ_H 6.49 assigned to C-8 was due to its HMBC correlations between δ_H 6.49 and δ_C108.8/136.7/148.8/152.8 and the NOESY cross peak between δ_H 6.49 and δ_H 3.85 (7-OCH_3). The upfield methoxy group (δ_H 3.02) connected to C-2 (δ_C100.4) was assigned by observing the HMBC correlation at δ_H 3.02 (2-OCH_3), δ_H 2.27 and 1.79 (H-3), and 2.67 (H-4b) with δ_C100.4 (C-2). The NOESY cross peaks between δ_H 3.02 (2-OCH_3) and δ_H 7.58 (H-2', H-6') further confirmed this assignment. The structure of **1** was further confirmed by the mass spectrum. A prominent ion at *m/z* 196, consistent with a retro-Diels-Alder fragmentation, indicated that three methoxyl units were on ring A. Consequently, compound **1** was deduced to be 2,5,6,7-tetramethoxyflavan and is a new compound, reported here for the first time. Compound **1** is a racemate which was confirmed by the lack of Cotton effects in the circular dichroism (CD) spectrum.

Compound **2** was isolated as a yellow amorphous solid. The molecular formula, $C_{17}H_{16}O_6$, was confirmed by HREIMS (*m/z* 316.0955, calcd. 316.0947). The IR spectrum showed absorptions at 3404, 1712, and 1681 cm^{-1}. The ^1H NMR protons at δ_H 7.91 (2H, d, *J*=7.2 Hz), 7.63 (1H, t, *J*=7.2 Hz), and 7.52 (2H, t, *J*=7.2 Hz) showed the presence of a mono-substituted benzene ring. Three singlets at δ_H 3.94, 3.71, and 3.60 indicated the presence of three methoxy groups. One downfield singlet at δ_H 12.14 revealed the presence of one hydrogen-bonded hydroxyl group. A singlet at δ_H 6.29 was due to an aromatic proton. The ^{13}C NMR spectrum showed seventeen carbon signals, including three methoxy, six methane and eight quaternary carbons. Among the eight quaternary carbons, two signals at δ_C 190.9 and 198.2 were identified as carbonyl groups (C-1 and C-2). HMBC correlations at δ_H 6.29 with C-1' (δ_C 106.9), C-2'(δ_C 162.9), C-4'(δ_C 163.3) and C-5'(δ_C 134.3) revealed that the proton was located at position 3' and the NOESY cross peak between δ_H 6.29 and δ_H 3.94 (4'-OCH_3) further confirmed this assignment. Furthermore, the structure of compound 2 was deduced on the basis of the MS spectrum. Prominent fragment ions in the EIMS of **2** were observed at *m/z* 105 and 211 which arose from rings A ($C_{10}H_{11}O_5$) and B (C_7H_5O), respectively. Based on the NMR and MS spectra, the structure of **2** was established as

2'-hydroxy-4',5',6'-trimethoxybenzil. The natural occurrence of benzils is rare. For instance, 2,4,2-trihydroxy-4'-methoxybenzil was isolated from *Zollernia paraensis* and vijayosin from *Pterocarpus marsupium*.[21, 22] Until now, no benzil has ever been reported from *Fissistigma* species. Compound **2** was the first benzil isolated from this species.

Two compounds were evaluated for their anti-inflammatory activities. The inhibitory activity of 2,5,6,7-tetramethoxyflavan (**1**) and 2'-hydroxy-4',5',6'-trimethoxybenzil (**2**) against the inflammatory response in human neutrophils was investigated. Compound **1** showed significant anti-inflammatory activity and inhibited the superoxide anion generation by fMLP/CB-induced human neutrophils, with IC_{50} values of 6.0 ± 1.9 μM.

Experimental

General experimental procedures. IR spectra were measured on a IRPrestige-21 spectrophotometer. ^{1}H NMR (400 MHz, using $CDCl_3$ as solvent for measurement), ^{13}C NMR (100 MHz), HMQC, HMBC, ^{1}H-^{1}H COSY, DEPT, and NOESY spectra were recorded with Bruker Avance-400 NMR spectrometers. FABMS were collected on a JEOL JMS-SX/SX 102A mass spectrometer. EIMS were collected on a MAT-95XL mass spectrometer. Silica gel 60 (Merck; 70-230, 230-400 mesh), sephadex LH-20 (GE healthcare) was used for column chromatography. TLC analysis was carried out on Si gel GF_{254} pre-coated plates with detection using 50% H_2SO_4 followed by heating on a hot plate.

Plant material. The plants of *F. latifolium* (Annonaceae) were collected from Phong Nha-Ke Bang National Park, Vietnam, in May, 2008, and the origin identified and authenticated by Dr. Tran Huy Thai (Institute of Ecology and Biological Resources, Vietnamese Academy of Science and Technology). A voucher specimen (20080515VN-TW) was deposited in the Herbarium of Vinh University, Vietnam.

Extraction and isolation. *F. latifolium* (8.5 kg) was extracted repeatedly with MeOH (15 L × 3) at room temperature. The combined MeOH extracts were evaporated under reduced pressure to afford a syrup. The MeOH extracts (186.6 g) of *F. latifolium* were partitioned between EtOAc and H_2O (1:1, v/v) to give an EtOAc fraction (121.9 g), which was then

purified by column chromatography (10×25 cm, Celite 545) using *n*-hexane (5 L), followed by $CHCl_3$ (5 L), EtOAc (5 L) and MeOH (10 L) as elution solvents. This yielded 4 fractions (*n*-hexane fraction (EH, 70.8 g), $CHCl_3$ fraction (EC, 39.7 g), EtOAc fraction (EE, 2.5 g) and a MeOH fraction (EM, 8.9 g)). The $CHCl_3$ fraction (EC, 39.8 g) was separated on a silica gel column (8×16 cm, $CHCl_3$-MeOH, gradient) to give 12 fractions. Fraction 5 (8.1 g) was purified on a silica gel column to afford 10 fractions. Subfraction 5-4 (2.7 g) was separated by silica gel column chromatography to provide 6 fractions. Subfraction 5-4-2 (1.3 g) was purified by repeated silica chromatography and then further purified twice by preparative thin layer chromatography to yield 2,5,6,7-tetramethoxyflavan (**1**) (7.5 mg) and 2'-hydroxy-4',5',6'-trimethoxybenzil (**2**) (3.2 mg).

Measurement of Superoxide Anion ($O_2^{\cdot-}$) Generation. The measurement of the generation of $O_2^{\cdot-}$ was based on the superoxide dismutase(SOD)-inhibitable reduction of erricytochrome *c*.[23] In brief, after supplementing withferricytochrome *c* (0.5 mg/mL), neutrophils (6× 105/mL) were equilibrated at 37 °C for 2 min andincubated with either control or different concentrations of tested compounds for 5 min. Cells wereactivated by fMLP (0.1 μM) or PMA (5 nM) for 10 min. When fMLP was used as stimulant, cytochalasin B (CB, 1 μg/mL) was incubated for 3 min before peptide activation. The changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring. Calculation is based on the difference of the reactions with and without SOD (100 U/mL) divided by the extinction coefficient for the reduction of ferricytochrome *c* (ε = 21.1/mM/10 mm).

2,5,6,7-tetramethoxyflavan (**1**): white amorphous solid. UV (CH_3OH) λ_{max} nm (log ε): 208 (4.20), 226 (3.64), 280 (3.17); IR (KBr) ν_{max} 2937, 2833, 1612, 1593, 1490, 1465, 1450, 1413, 1261, 1192, 1130, 1101 cm^{-1};^{1}H-NMR (CH_3OH, 200 Hz): see **Table 1**; ^{13}C-NMR (CH_3OH, 50 Hz): see **Table 1**; FAB-MS *m/z* (*rel. int.%*): 330 ($[M]^+$, 7), 196 (8), 154 (100), 136 (73), 77 (22) ;HRFABMS: *m*/z = 330.1479 $[M]^+$, calc. for $C_{19}H_{22}O_5$.

2'-hydroxy-4',5',6'-trimethoxybenzil (**2**): yellow amorphous solid. UV (CH_3OH) λ_{max} nm (log ε): 285 (4.80), 340 (4.30); IR (KBr) ν_{max} 3404, 3016, 2927, 2852, 1712, 1681, 1610, 1487,

1450, 1350, 1298, 1259, 1240, 1205, 1109 cm^{-1}; ^{1}H-NMR (CH_3OH, 200 Hz): see **Table 1**; ^{13}C-NMR (CH_3OH, 50 Hz): see **Table 1**; EI-MS (70 eV) *m/z* 316 $[M]^+$ (4), 211 (100), 105 (8), 77 (7); HREIMS: *m*/z = 316.0955$[M]^+$, calc. for 316.0947.

Acknowledgement The investigation was supported by a research grant from the National Science Council of the Republic of China (NSC 97-2320-B-039-020-MY3) and a grant from China Medical University, Taichung, Taiwan (CMU100-TS-15).

References and notes

1) Tran V. S., Trinh P. L., Trinh T. T., Nguyen T. H. A., Nguyen H. V., Tran D. Q., Nguyen T. A., *Adv. Nat. Sci.*, **9**, 55-67 (2008).

2) Chia Y. C., Chang F. R., Teng C. M., Wu Y. C., *J. Nat. Prod.*, **63**, 1160-1163 (2000).

3) Yang Z., Niu Y., Le Y., Ma X., Qiao C., *Phytomedicine*, **17**, 139-141 (2010).

4) Lo W. L., Chang F. R., Wu Y. C., *J. Chin. Chem. Soc.*, **47**, 1251-1256 (2000).

5) Hao X., Lu Y., Shang L., Zheng Q., *Chinese J. Med. Chem.*, **5**, 143-145 (1995).

6) Alias Y., Awang K., Hadi A. H., Thoison O., Sevenet T., Pais, M., *J. Nat. Prod.*, **58**, 1160-1166 (1995).

7) Alias A., Hazni H., Jaafar F.M., Awang K., Ismail N.H., *Molecules*, **15**, 4583-4588 (2010)

8) Chia Y. C., Wu J. B., Wu Y. C., *Tetrahedron Lett.*, **41**, 2199-2201 (2000).

9) Trinh T. T., Tran V. S., Nguyen T. H., *Tạp chí Hóa học*, **44**, 412-417 (2006).

10) Vichien J., Rapepol B., Porntip T., Rungruedee R., Kittisak L., *ScienceAsia*, **25**, 31-33 (1999).

11) Liao Y. H., Guo J., Xu L. Z., Yang S. L., *Acta Pharm. Sinica*, **34,** 207-209 (1999).

12) Uddin M. J., Khan S. H., Mamun M. I. R., Nahar N., Aziz M. A., Mosihuzzaman M., *J. Bangladesh Chem. Soc.*, **13,** 175-179 (2000).

13) Wu Y. C., Lu S. T., Wu T. S., Lee K. H., *Heterocycles*, **26**, 9-12 (1997).

14) Wu Y. C., Kao S. C., Huang J. F., Duh C. Y., Lu S. T., *Phytochemistry*, **29**, 2387-2388 (1990).

15) Chia Y. C., Chang F. R., Li C. M., Wu Y. C., *Phytochemistry*, **48**, 367-369 (1998).

16) Chia Y. C., Chang F. R., Wu Y. C., *J. Nat. Prod.*, **61**, 1430-1432 (1998).

17) Chia Y. C., Chang F. R., Wu Y. C., *Tetrahedron Lett.*, **40**, 7513-7514 (1999).

18) Lan Y. H., Chia Y. C., Chang F. R., Hwang T. L., Liaw C. C., Wu Y. C., *Helv. Chim. Acta.*, **88**, 905-909 (2005).

19) Lee D. Y., Kim D. H., Lee H. J., Lee Y., Ryu K. H., Jung B. I., Song Y. S., Ryu J. H. *Bioorg. Med. Chem. Lett.*, **20**, 3764-3767 (2010).

20) Ho J. C., Chen C. M., *Phytochemistry*, **61**, 405-408 (2002).

21) Maurya R., Singh R., Deepak M., Handa S. S., Yadav P. P., Mishra P. K., *Phytochemistry*, **65**, 915-920 (2004).

22) Ferrari F., Lima R. A., Bettolo G. B. M., *Phytochemistry*, **23**, 2691-2692 (1984).

23) Hwang T. L., Su Y. C., Chang H. L., Leu Y. L.. Chung P. J., Kuo L. M., Chang Y. J., *J. Lipid Res.*, **50**, 1395-1408 (2009).

Legends

Tables

Tables 1. ^{1}H and ^{13}C NMR spectroscopic data of compounds **1** and **2**.

Figures

Figure 1. Chemical structures of compounds **1** and **2**.

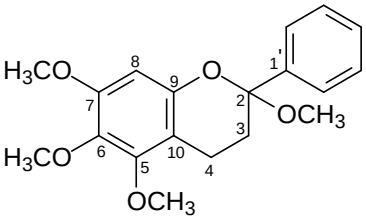
Figure 2. Selected HMBC correlations for **1** and **2**.

Figure 3. Assignment of EIMS fragments for **1** and **2**.

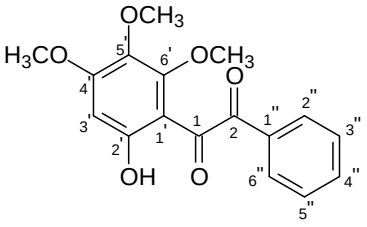
Table 1. ^{1}H and ^{13}C NMR spectroscopic data of compounds **1** and **2**.

1			2		
position	δ (H)	δ (C)	position	δ (H)	δ (C)
			1'		106.9
2		100.4	2'		162.9
3a	2.27 (1H, dd, *J*=13.6, 6.4 Hz)	34.0	3'	6.29	96.3
3b	1.79 (1H, ddd, *J*=13.6, 6.4, 2.4 Hz)		4'		163.3
4a	2.82 (1H, ddd, *J*=16.4, 11.6, 6.4Hz)	16.6	5'		134.3
4b	2.67 (1H, ddd, *J*=16.4, 6.2, 2.4Hz)		6'		154.7
5		151.3	1"		133.3
6		136.7	2", 6"	7.91, d, *J*=7.2 Hz	129.7
7		152.8	3", 5"	7.52, t, *J*=7.2 Hz	129.3
8	6.49	97.2	4"	7.63, t, *J*=7.2 Hz	134.4
9		148.8	1		198.3
10		108.8	2		190.1
1'		137.0	4'-OCH_3	3.94	56.8
2', 6'	7.58, d, *J*=7.2 Hz	126.3	5'-OCH_3	3.71	61.4
3', 5'	7.43, t, *J*=7.2 Hz	128.4	6'-OCH_3	3.60	60.9
4'	7.37, t, *J*=7.2 Hz	128.3			
2-OCH_3	3.02	49.4			
5-OCH_3	3.88	60.1			
6-OCH_3	3.80	60.5			
7-OCH_3	3.85	55.5			

Figure 1. Chemical structures of compounds **1** and **2**.

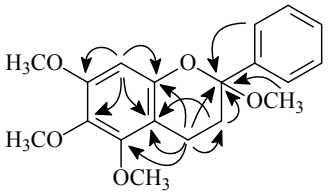


1

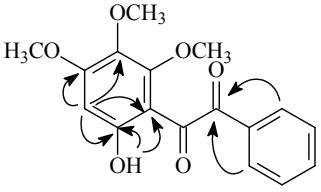


2

Figure 2. Selected HMBC correlations for **1** and **2**.

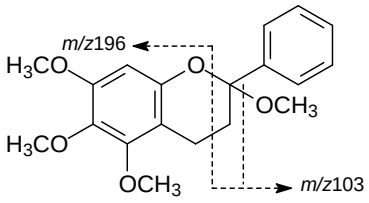


1

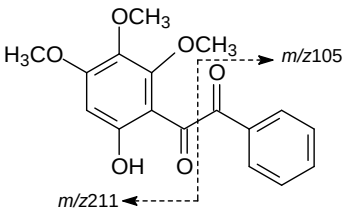


2

Figure 3. Assignment of EIMS fragments for **1** and **2**.



1



2