New Flavan and Benzil Isolated from *Fissistigma latifolium*

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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 formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)/cytochalasin B (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 oral epidermoid carcinoma cell *in vitro*.^{6,13} Our previous investigation of *Fissistigma* species resulted in the isolation of alkaloids, furano-fissoshamione, cyclopentenones and flavonoids.^{2,4,8,13–18} Among these compounds, bracteolatum, piperactam A, and fissilandione showed inhibitory effects on formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-induced superoxide anion (O₂^{•−}) 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 (O₂^{•−}) generation in human neutrophils.

Compound 1 was obtained as a white amorphous solid. The molecular formula of compound 1 was determined to be C₁₉H₂₂O₅ by high resolution-electron ionization-mass spectra (HR-EI-MS) (*m/z* 330.1479, Calcd 330.1467). IR spectrum indicated the presence of phenyl (1612, 1490 cm^{−1}) group. The UV spectrum exhibited maximal absorptions at 208 and 280 nm. The ¹H-NMR 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 correlation spectroscopy (COSY) and heteronuclear multiple quantum coherence (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 ¹³C-NMR 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. Heteronuclear multiple bond connectivity (HMBC) correlations between 5-OCH₃ (δ_{H} 3.88)/C-5 (δ_{C} 151.3), 6-OCH₃ (δ_{H} 3.80)/C-6 (δ_{C} 136.7), and 7-OCH₃ (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_{C} 108.8/136.7/148.8/152.8 and the nuclear Overhauser effect spectroscopy (NOESY) cross peak between δ_{H} 6.49 and δ_{H} 3.85 (7-OCH₃). The upfield methoxy group (δ_{H} 3.02) connected to C-2 (δ_{C} 100.4) was assigned by observing the HMBC correlation at δ_{H} 3.02 (2-OCH₃), δ_{H} 2.27 and 1.79 (H-3), and 2.67 (H-4b) with δ_{C} 100.4 (C-2). The NOESY cross peaks between δ_{H} 3.02 (2-OCH₃) 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, C₁₇H₁₆O₆, was confirmed by HR-EI-MS (*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 ¹³C-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

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Fig. 1. Chemical Structures of Compounds **1** and **2** Fig. 2. Selected HMBC Correlations for **1** and **2** Fig. 3. Assignment of EI-MS Fragments for **1** and **2**

groups (C-1, 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'-OCH₃)

further confirmed this assignment. Furthermore, the structure of compound **2** was deduced on the basis of the MS spectrum. Prominent fragment ions in the EI-MS of **2** were observed at m/z 105 and 211 which arose from rings A (C₁₀H₁₁O₅) and B (C₇H₅O), 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 IC₅₀ values of 6.0±1.9 μM .

Experimental

General Experimental Procedures IR spectra were measured on a IRPrestige-21 spectrophotometer. ¹H-NMR (400 MHz, using CDCl₃ as solvent for measurement), ¹³C-NMR (100 MHz), HMQC, HMBC, ¹H-¹H COSY, distortionless enhancement by polarization transfer (DEPT), and NOESY spectra were recorded with Bruker Avance-400 NMR spectrometers. FAB-MS were collected on a JEOL JMS-SX/SX 102A mass spectrometer. EI-MS 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 car-

Table 1. ¹H- and ¹³C-NMR Spectroscopic Data of Compounds **1** and **2**

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

ried out on Si gel GF₂₅₄ pre-coated plates with detection using 50% H₂SO₄ 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 H₂O (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 CHCl₃ (5 L), EtOAc (5 L) and MeOH (10 L) as elution solvents. This yielded 4 fractions (*n*-hexane fraction (EH, 70.8 g), CHCl₃ fraction (EC, 39.7 g), EtOAc fraction (EE, 2.5 g) and a MeOH fraction (EM, 8.9 g)). The CHCl₃ fraction (EC, 39.8 g) was separated on a silica gel column (8×16 cm, CHCl₃-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 (O₂^{•−}) Generation The measurement of the generation of O₂^{•−} was based on the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c*.²³ In brief, after supplementing with ferricytochrome *c* (0.5 mg/mL), neutrophils (6×10⁵/mL) were equilibrated at 37°C for 2 min and incubated with either control or different concentrations of tested compounds for 5 min. Cells were activated 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 (CH₃OH) 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}; ¹H-NMR (CH₃OH, 200 Hz): see Table 1; ¹³C-NMR (CH₃OH, 50 Hz): see Table 1; FAB-MS *m/z* (rel. int. %): 330 ([M]⁺, 7), 196 (8), 154 (100), 136 (73), 77 (22); HR-FAB-MS: *m/z*=330.1479 [M]⁺, Calcd for C₁₉H₂₂O₅.

2'-Hydroxy-4',5',6'-trimethoxybenzil (2**):** Yellow amorphous solid. UV (CH₃OH) 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}; ¹H-NMR (CH₃OH, 200 Hz): see Table 1; ¹³C-NMR (CH₃OH, 50 Hz): see Table 1; EI-MS (70 eV) *m/z* 316 [M]⁺ (4), 211 (100), 105 (8), 77 (7); HR-EI-MS: *m/z*=316.0955 [M]⁺, Calcd for 316.0947.

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