

Potential Anti-Inflammatory Activities of Bractelactone and other Compounds Isolated from *Fissistigma bracteolatum*

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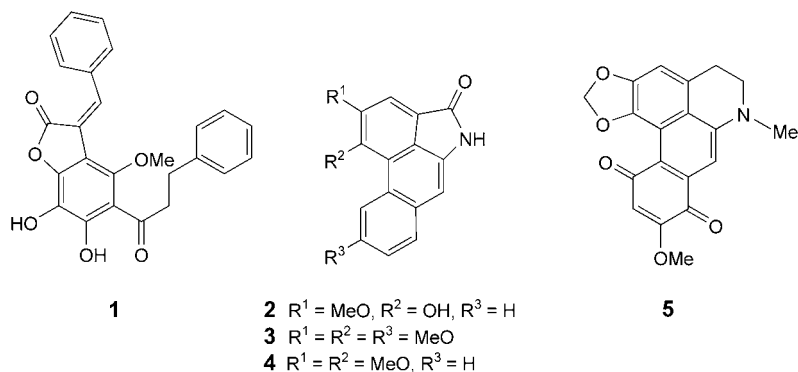
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From the stems of *Fissistigma bracteolatum*, a novel natural product with an unprecedented skeleton, bractelactone (**1**), was isolated, together with four known compounds: piperolactam A (**2**), aristololactam BIII (**3**), aristololactam BII (**4**), and fissilandione (**5**). The structure of **1** was established on the basis of spectroscopic data as (3*Z*)-6,7-dihydroxy-4-methoxy-3-(phenylmethylidene)-5-(3-phenylpropanoyl)-1-benzofuran-2(3*H*)-one. This compound may be derived from a hybrid of a chalcone and a cinnamic acid, or from a degradation product of a dichalcone. Compounds **1**, **2**, and **5** showed inhibitory effects on NO generation by RAW264.7 macrophages in response to lipopolysaccharide. Compounds **2** and **5** showed inhibitory effects on formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-induced superoxide anion (O_2^-) generation in human neutrophils.

Introduction. – In our previous studies, alkaloids, furano-fissohamione, cyclopentenones, and flavonoids have been reported from *Fissistigma* plants [1–8]. *Fissistigma bracteolatum* CHATT. (Annonaceae), a climbing shrub, grows mainly in the southern part of China and Vietnam. In traditional medicine, this plant is used for curing broken bones and to stop bleeding [9]. This paper describes the structural elucidation and biological activity of a novel compound named bractelactone (**1**), isolated from the stems of *F. bracteolatum*, together with four known compounds: piperolactam A (**2**) [10], aristololactam BIII (**3**) [11], aristololactam BII (**4**) [11], and fissilandione (**5**) [6]. Compounds **1**–**5** were investigated for their effects of formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-induced superoxide anion (O_2^-) generation in human neutrophils, as well as the nitric oxide (NO) generation by RAW264.7 macrophages in response to lipopolysaccharide (LPS).

Results and Discussion. – Compound **1**, obtained as yellow needles, displayed UV absorptions at 294 and 228 nm. The molecular formula, $C_{25}H_{20}O_6$, was confirmed by HR-FAB-MS (m/z 416.1252; calc. 416.1260). The presence of OH and C=O groups was indicated from IR absorptions at 3492, and 1776 and 1640 cm^{-1} , respectively. From the ¹H-NMR spectrum of **1** (Table 1), a dihydrochalcone moiety comprising a 2'-OH group¹⁾ on ring A was identified, with signals for a mono-substituted aromatic ring at $\delta(H)$ 7.30–7.43 (3 H) and 7.22 (2 H), α - and β -CH₂ signals at $\delta(H)$ 3.41 and 3.05 (2t,

¹⁾ Arbitrary atom numbering, see Fig. 1.



$J = 7.6$ Hz each), and a H-bonded phenolic signal at $\delta(\text{H})$ 13.50, which was oriented towards a C=O group. As shown in *Fig. 1, a*, these assignments were confirmed by HMBC correlations from $\delta(\text{H})$ 13.50 to $\delta(\text{C})$ 152.3 (C(2')), 101.8 (C(1')), and 132.1 (C(3')); from $\delta(\text{H})$ 3.05 to $\delta(\text{C})$ 203.6 (C(β')), 140.6 (C(1)), and 128.5 (C(2)); and from $\delta(\text{H})$ 3.41 to $\delta(\text{C})$ 203.6 (C(β')) and 140.6 (C(1)). The NOESY correlation between $\delta(\text{H})$ 5.50 (OH) and 13.50 (2'-OH) indicated an additional 3'-OH group. A MeO signal at $\delta(\text{H})$ 4.26 showed correlations with the aromatic signals of ring B and the β -CH₂ moiety, but no correlation with the 3'-OH group. Thus, the MeO group had to be attached at C(6').

Table 1. ¹H- and ¹³C-NMR Data of Compound **1**. At 400/100 MHz, in CDCl₃; δ in ppm, J in Hz. Assignments based on ¹H,¹H-COSY, NOESY, DEPT, HMQC, and HMBC experiments.

Position ¹⁾	¹ H	¹³ C	Position ¹⁾	¹ H	¹³ C
C(1)	–	140.6	6'-MeO	4.27 (s)	60.6
H-C(2,6)	7.30–7.43 (<i>m</i>)	128.5	2'-OH	13.51 (s)	–
H-C(3,5)	7.30–7.43 (<i>m</i>)	128.5	3'-OH	5.50 (s)	–
H-C(4)	7.22 (<i>m</i>)	126.2	C(1'')	–	164.8
CH ₂ (α)	3.41 (<i>t</i> , $J = 7.2$)	44.4	C(2'')	–	119.1
CH ₂ (β)	3.05 (<i>t</i> , $J = 7.2$)	30.0	H-C(3'')	8.13 (s)	142.7
C(β')	–	203.6	C(4'')	–	133.7
C(1')	–	101.8	H-C(5'',9'')	8.00–8.02 (<i>m</i>)	131.5
C(2')	–	152.3	H-C(6'',8'')	7.43–7.45 (<i>m</i>)	130.7
C(3')	–	132.1	H-C(7'')	7.43–7.45 (<i>m</i>)	128.3
C(4')	–	146.5			
C(5')	–	107.6			
C(6')	–	147.3			

The remaining ¹H- and ¹³C-NMR signals of **1** ($\delta(\text{H})$ 8.00–8.02 (2 H), 7.43–7.45 (3 H), and 8.13 (1 H); $\delta(\text{C})$ 119.1, 128.3, 130.7 (2C), 131.5 (2C), 133.7, 142.7, and 164) indicated a mono-substituted aromatic ring, a trisubstituted C=C bond, and a conjugated C=O system. HMBC correlations from $\delta(\text{H})$ 8.13 (H-C(3'')) to $\delta(\text{C})$ 133.7 (C(4'')), 164.8 (C(1'')), and 107.6 (C(5')) confirmed the linkage between C(2'') and C(5'). The resonance at $\delta(\text{C})$ 164.8 (C(1'')) indicated a lactone ring with an

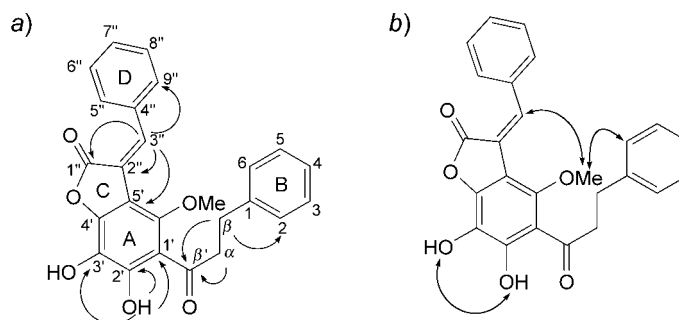


Fig. 1. Key HMBC (a) and NOESY (b) correlations for **1**

O–C(4') linkage. These assignments were further confirmed by analysis of the MS fragmentation pattern (Fig. 2).

NOESY Correlations observed between the 6'-MeO group and H–C(3'') indicated the C=C bond in **1** to be (*Z*)-configured (Fig. 1, b). The same conclusion was drawn from the ^{13}C -NMR chemical shift of C(3''). On the basis of the previous reports [12][13], the chemical shift of the exocyclic methine C-atom of (*E*)-isoaurone is *ca.* 152 ppm, whereas that of (*Z*)-isoaurone is *ca.* 141 ppm. Hence, the observed $\delta(\text{C})$ value of 142.7 for C(3'') of **1** was consistent with a (*Z*)-configured olefin. From these data, the structure of compound **1** was determined as (3*Z*)-6,7-dihydroxy-4-methoxy-3-(phenylmethylidene)-5-(3-phenylpropanoyl)-1-benzofuran-2(3*H*)-one, and named *bractelactone*.

Bractelactone (**1**) represents an unprecedented natural product, with a hexasubstituted aromatic ring. Probably, **1** is formed as a hybrid of a chalcone and a cinnamic acid, or by degradation of a dichalcone. In general, 3,8-linked diflavonoids represent a common skeleton in the classes of diflavonoids [14]. It suggests that a 5, α -linked dichalcone may also be a reasonable precursor of **1**.

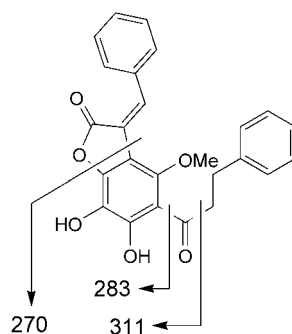


Fig. 2. Assignment of EI-MS fragments for **1**. In m/z.

According to our previous studies, piperolactam A (**2**) shows significant inhibitory effects against arachidonic acid and collagen [10]. To investigate the biological activities of compounds **1**–**5** from *F. bracteolatum*, and to rationalize the folk-medicinal

use of this plant, all compounds were screened for inhibition of O_2^- release from human neutrophils. The effects on NO generation by RAW264.7 macrophages in response to LPS were also examined.

Our screening showed that compounds **2** and **5** significantly inhibit the fMLP-induced O_2^- generation (Table 2). Compounds **1**, **2**, and **5** showed inhibitory effects on NO generation by RAW264.7 macrophages in response to LPS. However, compound **2** showed a cytotoxic effect towards RAW264.7 macrophages. These results, thus, support the beneficial folk-therapeutic use of this interesting plant.

Table 2. Inhibitory Effects of Compounds **1–5** on a) Superoxide Anion (O_2^-) Generation by Human Neutrophils in Response to formyl-MetLeuPhe (fMLP) and b) Nitric Oxide Generation by RAW264.7 Macrophages in Response to Lipopolysaccharide (LPS). For details, see *Exper. Part*. ‘Diphenylene-iodonium’ (DPI), an NADPH oxidase inhibitor, and *N*^ω-nitro-L-arginine methyl ester (L-NAME) were used as positive controls, resp. All values were averaged ($n = 3–4$).

Compound	IC_{50} [$\mu\text{g/ml}$] ^{a)}		Cytotoxic ^{b)}
	fMLP/CB	LPS	
1	n.t. ^{c)}	1.55 ± 0.42	no
2	4.68 ± 0.75	2.47 ± 0.76	yes
3	> 10	> 10	no
4	> 10	> 10	no
5	2.34 ± 0.09	0.85 ± 0.30	no
DPI	0.13 ± 0.06	–	–
L-NAME	–	47.31 ± 0.99	no

^{a)} Concentration required for 50% inhibition. ^{b)} Examined by LDH release at a level of 10 $\mu\text{g/ml}$. ^{c)} Not tested. Compound **1** caused reduction of cytochrome *c* in a cell-free system.

Experimental Part

General. Column chromatography (CC): silica gel 60 (230–400 mesh; Merck). Thin-layer chromatography (TLC): silica gel GF₂₅₄ precoated plates; detection by spraying with 50% H₂SO₄, followed by heating on a hot plate. Melting points (m.p.): Yanagimoto micro-melting-point apparatus; uncorrected. IR Spectra: Mattson Genesis II spectrophotometer; in cm^{-1} . ¹H- and ¹³C-NMR Spectra (including HMQC, HMBC, ¹H,¹H-COSY, DEPT, and NOESY): Varian Unity Plus spectrometer; at 400/100 MHz, resp., in CDCl₃, δ in ppm, *J* in Hz. EI-MS: JEOL JMS-SX/SX 102A mass spectrometer; in *m/z*. High-resolution (HR) FAB-MS: MAT-95XL mass spectrometer; in *m/z*.

Plant Material. Fresh stems of *F. bracteolatum* were collected in Kunming, Yunnan, P. R. China. A voucher specimen (FB 1) was deposited at the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Isolation. The MeOH extract (48 g) of *F. bracteolatum* stem was subjected to CC (SiO₂; hexane/CHCl₃/AcOEt/MeOH gradient of increasing polarity): 15 fractions (*Fr.*) on the basis of TLC. *Fr.* 6, eluted with hexane/AcOEt 5:1, afforded aristolactam BII (**4**; 1.8 mg) and fissilandione (**5**; 3.5 mg). *Fr.* 7, eluted with CHCl₃, gave bractelactone (**1**; 42 mg). *Fr.* 8, eluted with hexane/AcOEt 3:1, provided aristolactam BIII (**3**; 4.2 mg); and *Fr.* 10, eluted with CHCl₃, gave piperlactam A (**2**; 2.4 mg).

Neutrophil Superoxide Anion Generation. Human neutrophils from venous blood of healthy, adult volunteers (18–32 years old) were isolated by means of standard dextran sedimentation prior to centrifugation in Ficoll–Hypaque gradient and hypotonic lysis of erythrocytes [15]. The generation of O_2^- was determined with superoxide dismutase (SOD), which inhibits cytochrome *c* reduction. Cells were activated by formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) for 10 min. When fMLP was used as stimulant, cytochalasin B (CB; 1 $\mu\text{g/ml}$) was incubated for 3 min before peptide activation.

Macrophage Nitric Oxide Generation. RAW264.7 Cells were obtained from *American Type Culture Collection* (Manassas, VA). Cells were seeded at a density of 10^6 cells/ml in 24-well *Costar* plates, and cultured in *Dulbecco's* modified *Eagle* medium containing 2% heat-inactivated fetal calf serum (FCS) [16]. The cells were stimulated with lipopolysaccharide (LPS; 1 μ g/ml) for 24 h. Measurement of NO was performed via an assay based on the accumulation of nitrite in the medium, as determined by colorimetry with *Griess* reagent [17], *N^ω*-nitro-L-arginine methyl ester (L-NAME) being used as positive control.

Lactate Dehydrogenase (LDH) Release. Cytotoxicity was expressed as the percent LDH activity obtained in cell-free medium compared to the total LDH activity. The latter was determined by lysing cells with 0.1% *Triton X-100* for 30 min at 37°.

Bractelactone (= (3*Z*)-6,7-Dihydroxy-4-methoxy-3-(phenylmethylidene)-5-(3-phenylpropanoyl)-1-benzofuran-2(3*H*)-one; **1**). Yellow needles. M.p. 132–134°. IR (KBr): 3492, 3026, 2938, 2217, 1776, 1640, 1600, 1424, 1348, 1268, 1174, 1008, 957. ¹H- and ¹³C-NMR: see *Table 1*. EI-MS (70 eV): 416 (97, *M*⁺), 398 (10, [*M* – H₂O]⁺), 325 (5), 311 (10), 283 (23), 105 (37), 91 (100). HR-FAB-MS: 416.1252 (*M*⁺, C₂₅H₂₀O₆⁺; calc. 416.1260).

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