Limodissimin A: A New Dimeric Coumarin from *Limonia acidissima*

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Previous chemical investigations on *Limonia acidissima* L. (Rutaceae) have isolated various constituents, including coumarins, steroids, triterpenoids, benzoquinones, and tyramine derivatives from different parts of this natural source.[1-6] Particularly, plenty of interesting coumarins,[1,2,7] some of which, exhibiting anti-fungal activity have been reported in *L. acidissima*.[3,8] 'Thanaka', a root paste made from the pulp of *L. acidissima*, is a facial cosmetic to remove small spots and lesions on the skin.[9] We describe herein the isolation and structural elucidation of a new dimeric coumarin, limodissimin A (**1**), together with four known coumarins (**2-5**) from further studies on *L. acidissima*[10] and their ability to inhibit NO production in LPS-activated BV-2 cells, a microglial cell line.

Table 1. ^{1}H and ^{13}C NMR data of **1** in CD_3OD

Position	δ_H (multi, J = Hz)	δ_C	HMBC (H → C)
2		163.8	
3	6.20 (d, 9.5)	111.9	C-2, C-4, C-10
4	7.84 (d, 9.5)	146.7	C-2, C-9
5	7.33 (d, 8.5)	128.5	C-7, C-9
6	6.84 (d, 8.5)	114.8	C-8, C-10
7		161.5	
8		114.1	
9		155.4	
10		113.3	
1′	3.06 (dd, 7.5, 13.5) 3.16 (dd, 6.0, 13.5)	30.2	C-7, C-9, C-3′
2′	4.44 (dd, 6.0, 7.5)	76.3	C-8, C-4′, C-5′
3′		148.6	
4′	4.68 br s; 4.72 br s	111.4	C-2′, C-5′
5′	1.86 s	17.8	C-2′, C-4′
2″		163.9	
3″	6.18 (d, 9.5)[a]	112.5	C-2″, C-4″, C-10″
4″	7.83 (d, 9.5)[a]	146.2	C-2″, C-9″
5″	7.45 (d, 8.5)	130.8	C-7″, C-9″
6″	6.82 (dd, 2.5, 8.5)[a]	114.6	C-8″, C-10″
7″		163.2	
8″	6.72 (d, 2.5)	103.5	C-6″, C-10″
9″		157.3	
10″		113.4	

NMR data were obtained in 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively. [a]Overlapped signals.

Limodissimin A (**1**) was isolated as a yellow gum and showed a fluorescence at UV-365 nm. The molecular formula of **1** was determined to be $C_{23}H_{18}O_6$ from the $[M+H]^+$ peak at m/z 391.1189 (calcd. for $C_{23}H_{19}O_6$: 391.1182) in HRFABMS spectrum. The IR spectrum indicated that **1** possessed hydroxyl (3356 cm^{-1}), carbonyl (1660 cm^{-1}) and aromatic ring (1624, 1542 and 1510 cm^{-1}) groups. The UV spectrum absorption at 214, 258 and 325 nm showed the characteristics of a 7-oxygen-substituted coumarin for **1**.[11] The ^{1}H NMR spectrum (Table 1) of **1** displayed two vicinal pairs of typical H-3 and H-4 protons of AX system at δ_H 6.20 (1H, d, J = 9.5 Hz, H-3)/7.84 (1H, d, J = 9.5 Hz, H-4) and at δ_H 6.18 (1H, d, J = 9.5 Hz, H-3″)/7.83 (1H, d, J = 9.5 Hz, H-4″), which predicted that **1** was composed of two C-3 and C-4 unsubstituted coumarin moieties, another vicinal pair of AX system protons at δ_H 6.84 (1H, d, J = 8.5 Hz, H-6)/7.33 (1H, d, J = 8.5 Hz, H-5) and three aromatic protons of ABX system at δ_H 6.72 (1H, d, J = 2.5 Hz, H-8″), 6.82 (1H, dd, J = 2.5, 8.5 Hz, H-6″) and 7.45 (1H, d, J = 8.5 Hz, H-5″), suggesting the substitution pattern and the linkage of **1**. Further signals for two exomethylene protons at δ_H 4.68 (1H, br s, H-4′a), 4.72 (1H, br s, H-4′b), one oxygenated proton at δ_H 4.44 (1H, dd, J = 6.0, 7.5 Hz, H-2′), two methylene protons of AB system at δ_H 3.06 (1H, dd, J = 7.5, 13.5 Hz, H-1′a), 3.16 (1H, dd, J = 6.0, 13.5

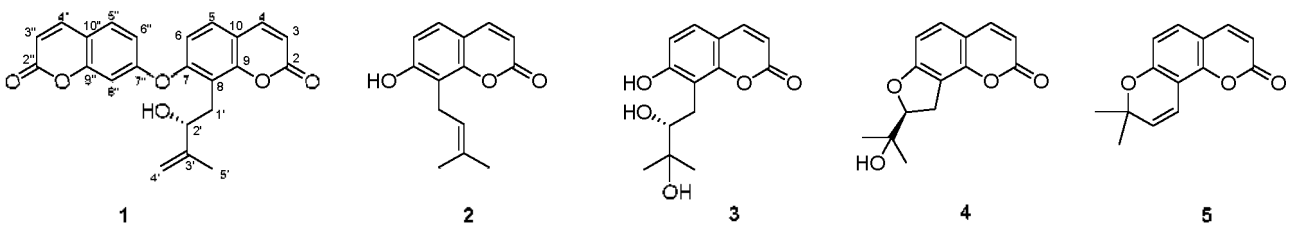

Figure 1. Structures of compounds **1-5**.

Hz, H-1'b) and one methyl proton at δ_H 1.86 (3H, s, H-5') were observed. The ^{13}C NMR spectroscopic data (Table 1) showed 23 carbon signals, which were composed of two monomeric coumarin units. The NMR resonances of **1** were similar to those of the related coumarins, umbelliferone[12] and demethylauraptenol,[13] suggesting that two coumarin moieties of **1** could be umbelliferone and demethylauraptenol.

The HMBC spectrum confirmed the above suggested structure. The signals of H-4 (δ_H 7.84) and H-4'' (δ_H 7.83) correlated with C-2 (δ_C 163.8), C-9 (δ_C 155.4) and with C-2'' (δ_C 163.9), C-9'' (δ_C 157.3), respectively and the correlations between H-2' (δ_H 4.44) with C-8 (δ_C 114.1) and between H-4' (δ_H 4.62, 4.72) with C-2' (δ_C 76.3), C-5' (δ_C 17.8) were observed in the HMBC experiment (Figure 2), allowing a hydroxyl group to be at C-2'. These assignments were further supported by NOESY experiment. On the basis of the above findings, the structure of **1** was assigned as limodissimin A (**1**) [7-[(2''-oxo-2*H*-1-benzopyran-7''-yl)oxy]-8-[2'-hydroxy-3'-methyl-3'-buten-1'-yl]-2*H*-1-benzopyran-2-one]. The absolute configuration of **1** was determined by a convenient Mosher ester procedure carried out in an NMR tube.[14] Analysis of the chemical shift differences between MTPA ester derivatives of **1** (**1r** and **1s**, Figure 2) revealed the *R*-configurated C-2'. Furthermore, the positive optical rotation value of **1** ($[\alpha]_D^{25}$ +3.8°) was in good agreement with that of related (2'*R*)-hydroxy-coumarin derivatives.[15,16]

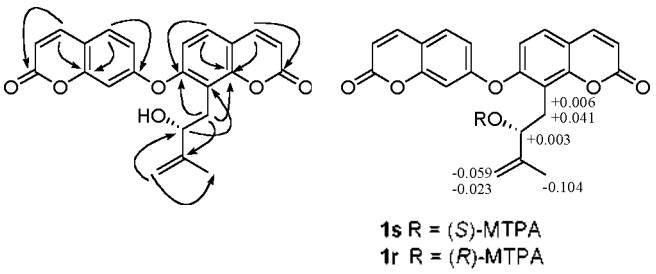

Figure 2. Key HMBC correlations and values of $\delta_S - \delta_R$ (data obtained in pyridine-d_5) of the MTPA esters of **1**.

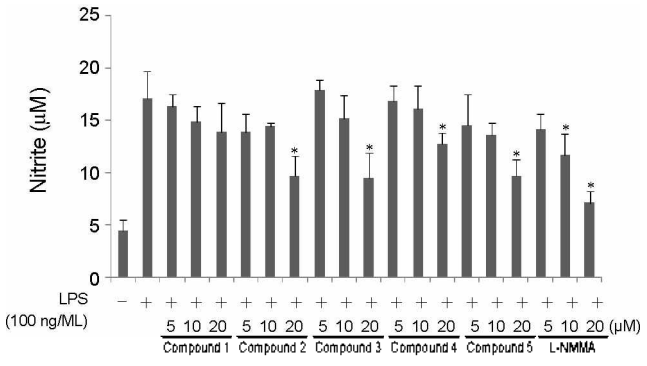

Figure 3. Effects of compounds **1-5** and NMMA on LPS-induced NO production in BV-2 microglia cells. Cells were pretreated with compounds **1-5** and NMMA (5, 10, and 20 μM) for 30 min and then stimulated with LPS (100 ng/mL) for another 24 h. The culture medium was then collected for a nitrite assay. Nitrate was measured using a Griess reaction. All data were presented as the mean ± S.E.M of three independent experiments. *$p < 0.05$ indicates statistically significant differences compared to treatment with LPS alone.

The known compounds, osthenol (**2**),[17] (2'*R*)-7-hydroxy-8-(2',3'-dihydroxy-3'-methylbutyl)-2*H*-1-benzopyran-2-one (**3**),[18] columbianetin (**4**),[19] and seselin (**5**)[20] were also isolated. Their structures were identified by comparing their spectroscopic data with those in the literature. The isolated known compounds **3-5** were reported from this source for the first time.

We evaluated the inhibitory effects of isolated compounds **1-5** on NO production in LPS-activated BV-2 cells, a microglial cell line. As shown in Figure 3, compounds **2**, **3**, **4** and **5** significantly inhibited the LPS-induced NO production, with IC_{50} values of 22.3, 21.6, 33.5 and 23.1 μM, respectively. Compound **1** did not show the significant inhibitory effect on NO production in ranges from 5 to 20 μM. In summary, the present study demonstrated that coumarins (compounds **2**, **3**, **4** and **5**) isolated from *L. acidissima* exert anti-inflammatory effects in LPS- stimulated microglia cells. These results suggest that coumarins **2**, **3**, **4** and **5** might be good lead compounds to modulate neurological diseases associated with inflammatory processes.

Experimental Section

General Procedures. Optical rotations were measured on a Jasco P-1020 polarimeter in MeOH. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. UV spectra were recorded using a Schimadzu UV-1601 UV-Visible spectrophotometer. FAB and HRFAB mass spectra were obtained on a JEOL JMS700 mass spectrometer. NMR spectra, including ^1H-^1H COSY, HMQC, HMBC and NOESY experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^1H) and 125 MHz (^{13}C) with chemical shifts given in ppm (δ). Preparative HPLC was conducted using a Gilson 306 pump with Shodex refractive index detector and Apollo Silica 5 μ column (250 × 22 mm). Silica gel 60 (Merck, 70 - 230 mesh and 230 - 400 mesh) was used for column chromatography. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co.). Spots were detected on TLC under UV light or by heating after spraying with 10 % H_2SO_4 in C_2H_5OH (v/v).

Plant Materials. The dried bark of *L. acidissima* was imported from Yangon, Union of Myanmar, in October 2006 and identified by Dr. W. Bae. A voucher specimen (SKKU 2006-10) was deposited at the R & D Institute, Miwon Commercial Co., LTD, Ansan, Korea.

Extraction and Isolation. The dried bark of *L. acidissima* (3 kg) was extracted with 85% EtOH three times at 85 °C. The resulting ethanol extract (250 g) was suspended in distilled water (7.2 L) and then partitioned with EtOAc, yielding an EtOAc soluble extract (50 g). The EtOAc soluble fraction (50 g) was separated over a silica gel column, eluted gradiently with *n*-hexane-EtOAc (10:1, 5:1, 1:1, and 0:1, v/v) to afford 12 fractions (Fr. A to Fr. L). Fr. H (810 mg) was separated further over a silica gel column ($CHCl_3$-MeOH, 17:1, v/v) to give three subfractions (Fr. H1 to Fr. H3). Fr. H2 (450 mg) was separated over a Sephadex LH-20 (Pharmacia Co.), using a solvent system of CH_2Cl_2-MeOH (1:1, v/v) to yield three subfractions (Fr. H21 to Fr. H23). Fr. H23 (120 mg) was separated by preparative HPLC, over 30 min at a flow rate of

2.0 mL/min (Apollo Silica 5 μ column; 250 × 22 mm; 5 μ particle size; Shodex refractive index detector) to obtain **1** (8 mg, *n*-hexane-EtOAc, 1:1, v/v), **2** (7 mg, *n*-hexane-EtOAc, 1:1, v/v), **3** (11 mg, *n*-hexane-EtOAc, 1:1, v/v), **4** (6 mg, $CHCl_3$-MeOH, 12:1, v/v) and **5** (7 mg, $CHCl_3$-MeOH, 10:1, v/v).

Limodissimin A (1). yellow gum. $[\alpha]_D^{25}$ +3.8° (*c* 0.08, MeOH); IR (KBr) ν_{max} cm^{-1}: 3356, 2948, 1660, 1624, 1542, 1510, 1032, 670; UV λ_{max} (MeOH) nm (log ε): 325 (4.27), 258 (w), 214 (4.38); FABMS *m/z* 391 $[M + H]^-$; HRFABMS *m/z* 391.1189 $[M + H]^+$ (calcd. for $C_{23}H_{19}O_6$: 391.1182); ^1H and ^{13}C NMR: see Table 1.

Preparation of the (*R*)- and (*S*)-MTPA Ester Derivatives of 1 by a Convenient Mosher Ester Procedure. Compound **1** (0.7 mg) in deuterated pyridine (0.75 mL) was transferred into a clean NMR tube. (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride, (*S*)-MTPA-Cl (5 μL) was added into the NMR tube immediately under a N_2 gas stream, and then the NMR tube was shaken carefully to mix the sample and (*S*)-MTPA-Cl evenly. The reaction NMR tube was permitted to stand at room temperature overnight. The reaction was then completed to afford the (*R*)-MTPA ester derivative (**1r**) of **1**. In the manner described for **1r**, (*S*)-MTPA ester derivative of **1** (**1s**) was obtained. The ^1H-NMR spectra of **1r** and **1s** were measured with the reaction NMR tubes directly. **1s:** ^1H-NMR (500 MHz, pyridine-d_5): δ 6.251 (1H, dd, *J* = 6.0, 7.5 Hz, H-2′a), 5.047 (1H, br s, H-4′a), 4.802 (1H, br s, H-4′b), 3.569 (1H, m, H-1′a), 3.514 (1H, m, H-1′b), 1.958 (3H, s, H-5′); FAB-MS *m/z* 606 $[M]^-$. **1r:** ^1H-NMR (500 MHz, pyridine-d_5): δ 6.248 (1H, dd, *J* = 6.0, 7.5 Hz, H-2′a), 5.106 (1H, br s, H-4′a), 4.825 (1H, br s, H-4′b), 3.528 (1H, m, H-1′a), 3.508 (1H, m, H-1′b), 2.062 (3H, s, H-5′); FAB-MS *m/z* 606 $[M]^-$.

Measurement of NO Production and Cell Viability. A murine microglia cell line, BV-2 cells, was stimulated with 100 ng/mL of LPS in the presence or absence of samples for 24 h. Nitrite in the culture media, a soluble oxidation product of NO, was determined using the Griess reaction. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.[21] N^G-monomethyl-L-arginine (L-NMMA, Sigma), a well-known NOS inhibitor, was tested as a positive control.

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