Biflavonoids from *Daphne feddei* and Their Inhibitory Activities against Nitric Oxide Production

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Received July 29, 2008; accepted September 23, 2008; published online September 30, 2008

Bioassay-directed fractionation led to the isolation of two new biflavonoids and 13 known biflavonoids from a sample of the dried stem barks of *Daphne feddei*. The structures of compounds 1 and 2 were elucidated as 2″-methoxy-daphnodorin C (1) and 2″-methoxy-2-epi-daphnodorin C (2) on the basis of detailed spectroscopic analysis and X-ray crystallography. All 15 biflavonoids were tested for inhibitory activities against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages. Compounds 1, 2, 8, 10, 14 and 15 showed varying degrees of inhibitory activities against the production of NO in tested concentration of 25, 50, 75 and 100 μg/ml.

Key words *Daphne feddei*; biflavonoid; RAW 264.7 macrophages; nitric oxide

Daphne feddei LEVL. is a common evergreen shrub native to Yunnan, Sichuan and Guizhou provinces in China. Its stem barks are used for the treatment of injuries from falls and bruises as folk medicine.[1)] In a previous chemical investigation of *D. feddei*, the occurrence of four diterpenes had been reported.[2)] In course of our studies on the constituents of thymelaeaceous plants,[3—5)] two new biflavonoids along with 13 known biflavonoids were isolated from the titled plant. This paper concerned with the structural elucidation of compounds **1** and **2** and inhibitory activities of all 15 compounds against nitric oxide (NO) increase by lipopolysaccharide (LPS)-induced in macrophages.

The EtOAc-soluble fraction from the methanolic extract of the stem barks of *D. feddei* was subjected to silica gel column chromatography, Rp-18, and Sephadex LH-20 in various solvent systems to afford two new biflavonoids, compounds **1** and **2**, together with 13 known biflavonoids. By comparing the physical and spectroscopic data with those in literatures, 13 known biflavonoids were identified as: daphnodorin A (**3**),[6,7)] daphnodorin B (**4**),[6,7)] daphnodorin C (**5**),[7,8)] daphnodorin I (**6**),[9)] daphnodorin J (**7**),[10)] a mixture of daphnodorins M and N (**8**),[11)] dihydrodaphnodorin B (**9**),[10)] wikstrol A (**10**),[12)] wikstrol B (**11**),[12)] genkwanol B (**12**),[13)] genkwanol C (**13**),[14)] neochamaejasmin B (**14**),[15)] isoneochamaejasminin A (**15**).[16)]

Compound **1** (Fig. 1) was obtained as white optically active needle (MeOH), showing a molecular formula of $C_{31}H_{24}O_{10}$ by HR-ESI-MS ([M+Na]$^+$ at *m/z* 579.1267). In the ^{1}H-NMR spectrum, two pairs of A_2X_2 aromatic protons at δ_H 6.98 (2H, d, J=8.4 Hz), 6.66 (2H, d, J=8.4 Hz), 7.16 (2H, d, J=8.4 Hz) and 6.70 (2H, d, J=8.4 Hz) indicated the existence of two 4-oxyphenyl groups, and two aromatic protons situated in meta positions (δ_H 5.79, d, J=1.8 Hz and δ_H 5.35, d, J=1.8 Hz) indicated the existence of a 2,4,6-trioxyphenyl group. The ^{1}H-NMR spectrum also showed the presence of a 2,5,6-trisubstituted dihydropyran ring [δ_H 4.78

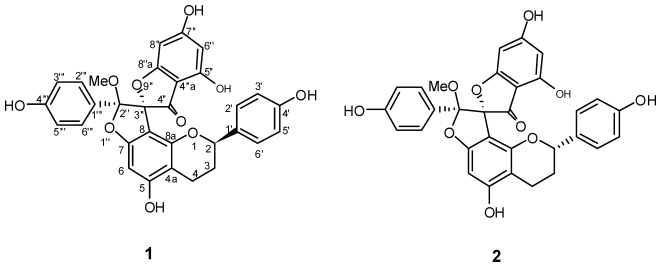

Fig. 1. The Structures of Compounds **1** and **2**

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for **1** and **2** (δ in ppm, J in Hz, in CD_3OD)

Carbon	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
2	78.3	4.78 d (10.8)	77.8	4.70 d (10.8)
3	20.6	2.59 m, 2.69 m	20.4	2.59 m, 2.69 m
4	30.7	2.20 m, 1.70 m	30.2	2.22 m, 1.68 m
4a	104.7		104.7	
5	160.6		160.6	
6	91.2	6.16 s	91.3	6.14 s
7	173.4		173.4	
8	103.5		103.8	
8a	154.5		154.4	
1′	133.8		133.6	
2′	127.4	6.98 d (8.4)	127.2	6.81 d (8.4)
3′	115.8	6.66 d (8.4)	115.7	6.53 d (8.4)
4′	157.3		157.3	
5′	115.8	6.66 d (8.4)	115.7	6.53 d (8.4)
6′	127.4	6.98 d (8.4)	127.2	6.81 d (8.4)
2″	117.0		117.1	
3″	105.4		105.6	
4″	194.3		194.3	
4″a	104.7		104.7	
5″	162.2		162.2	
6″	97.2	5.79 d (1.8)	97.3	5.81 d (1.8)
7″	159.4		159.3	
8″	90.8	5.35 d (1.8)	90.9	5.50 d (1.8)
8″a	170.8		170.8	
1‴	125.6		126.7	
2‴	129.5	7.16 d (8.4)	129.5	7.15 d (8.4)
3‴	115.9	6.70 d (8.4)	115.9	6.70 d (8.4)
4‴	159.2		159.3	
5‴	115.9	6.70 d (8.4)	115.8	6.70 d (8.4)
6‴	129.5	7.16 d (8.4)	129.5	7.15 d (8.4)
OCH_3	51.9	3.20 s	51.9	3.20 s

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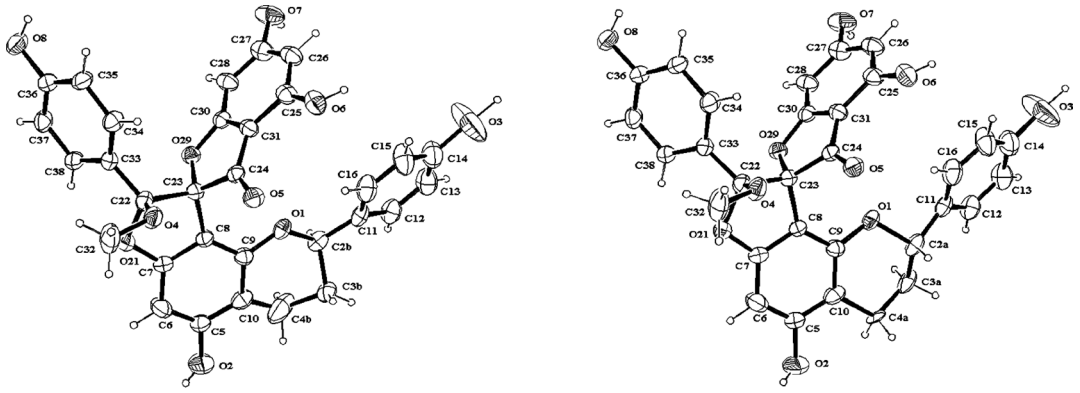

1 **2**

Fig. 2. X-Ray Crystal Structures of **1** and **2**

Table 2. Effect of 15 Compounds on LPS-Induced NO Production in RAW264.7 Macrophages (n=4, Means±S.D.)

Groups	Dose (μg/ml)	Concentration (NO)	Inhibiton rate (%)	Groups	Dose (μg/ml)	Concentration (NO)	Inhibiton rate (%)
LPS[a)]	1		100	AG[b)]	25 μM		50.4
1	100	4.5±0.1**	32	**2**	100	2.8±0.1**	58
	75	5.5±0.4**	18		75	5.5±0.1*	18
	50	8.0±0.5**	0		50	13.4±1.3	0
	25	10.3±0.4	0		25	13.4±0.5	0
8	100	3.7±0.1**	44	**10**	100	5.7±0.1	39
	75	4.8±0.2**	28		75	6.6±0.1	29
	50	5.1±0.3	23		50	9.4±0.4	0
	25	6.8±0.9	0		25	11.2±0.6	0
14	100	4.4±0.2**	42	**15**	100	5.1±0.3**	17
	75	4.5±0.1**	41		75	5.8±0.1	6
	50	6.3±0.9	17		50	6.4±0.3	0
	25	7.2±0.1	2		25	7.6±1.3	0
Ocs[c)]	100		0				
	75		0				
	50		0				

a) Negative control; *b*) aminoguanidine, positive control; *c*) other compounds, including compounds **3**, **4**, **5**, **6**, **7**, **9**, **11**, **12**, **13**. $*p<0.05$ *vs.* LPS group; $**p<0.011$ *vs.* LPS group.

(d, J=10.8 Hz), 2.59 (m), 2.69 (m), 2.20 (m), and 1.70 (m)]. Further, the ^{1}H- and ^{13}C-NMR (DEPT) spectra showed signals assignable to a methoxy group [δ_H 3.20 (3H, s), δ_C 51.9]. These NMR data of **1** were very similar to those of known daphnodorin C,[7,8)] expect for an additional methoxy group. The fact that the signal at δ_H 3.20 (3H, s, OCH_3) had long-range correlation with the resonances at δ_C 117.0 (C-2″) suggested that the methoxy group was attached to C-2″. Thus, compound **1** was deduced and named 2″-methoxy-daphnodorin C (Fig. 1).

Compound **2** was isolated as white optically active powder (MeOH). The HR-ESI-MS showed a $[M+Na]^+$ ion peak, corresponding to a molecular formula $C_{31}H_{24}O_{10}$. The NMR data of **2** were very similar to those of **1**, expect for signals due to C(2)–H (δ_C 77.8 and δ_H 4.70, d, J=10.8 Hz) instead of the signals due to C(2)–H (δ_C 78.3 and δ_H 4.78, d, J=10.8 Hz) in **1**. The same planar structure as that of **1** was deduced from the HSQC, HMBC, and ^{1}H, ^{1}H-COSY data of **2**, which suggested that **2** would be a diastereoisomer of **1**. Therefore, the structure of **2** was deduced as 2″-methoxy-2-epi-daphnodorin C (Fig. 1) on the basis of the above evidence and the biogenetic evidence provided by the cooccurrence of daphnodorin C.

A white needle crystal mixture of compounds **1** and **2** was obtained in the course of isolation. An X-ray crystal-structure analysis[17)] confirmed further the planar constitution and the relative configurations of **1** and **2** (Fig. 2).

NO plays an important role in the regulation of many physiological functions, such as host defence, neurotoxicity, and vasodilation.[18)] However, the excess production of NO has been implicated for immunological and inflammatory diseases including septic shock, rheumatoid arthritis, graft rejection, and diabetes.[19)] Therefore, inhibition of NO increase is apparently an important therapeutic consideration in the development of anti-inflammatory agents.

All 15 isolates were tested for inhibitory activities against LPS-induced NO increase in RAW 264.7 macrophages. Compounds **1**, **2**, **8**, **10**, **14** and **15** inhibited the increase of NO to different extent in tested concentration of 25, 50, 75 and 100 μg/ml (Table 2), which implied that biflavonoids may be responsible for the traditional usages of *D. feddei* to treat injuries from falls and bruises.

Experimental

Column chromatography (CC): silica gel H (10—40 μm) from Zhifu Huangwu Silica Gel D & R Plant, Yantai, China; Sephadex LH-20 and ODS from Pharmacia and Merck, resp. TLC: plates precoated with silica gel HF_{254} (5—7 μm) from Zhifu Huangwu Silica Gel D & R Plant, Yantai, China. Optical rotations: Perkin-Elmer 343 polarimeter. IR Spectra: Bruker

Vector-22 spectrometer; KBr pellets; in cm^{-1}. NMR Spectra: Bruker AVANCE 600 NMR spectrometer; ^1H- and ^{13}C-NMR, HSQC, HMBC and NOESY spectra; CD_3OD solns. with $SiMe_4$ as internal standard; δ in ppm, J in Hz. HR-TOF-MS: ESI mode; Q-Tof-Micro-Mass spectrometer in *m/z*.

Plant Material The plant material was collected in July 2006 in Kunming City, Yunnan province, China, and identified as *Daphne feddei* LEVL. by Prof. Li-shan Xie of Kunming Institute of Botany. A voucher specimen has been deposited in the Herbarium of the School of Pharmacy, Second Military Medical University, Shanghai (No. 200607-12).

Extraction and Isolation The air-dried and powdered stem barks of *D. feddei* (6.5 kg) were extracted with methanol for 3×2 h. The solvent was evaporated under vacuum. Then the extract was suspended in water and partitioned with petroleum ether, EtOAc and *n*-butanol successively. EtOAc extract (400 g) was subjected to silica gel CC (200—300 mesh, 1000 g), eluting successively with gradient $CHCl_3$–MeOH mixtures of increasing polarity. The 4% MeOH eluates were purified on silica gel CC with $CHCl_3$–MeOH followed by Sephadex LH-20 eluting with MeOH to give **1** (80 mg), **2** (40 mg), **3** (800 mg), **5** (600 mg), **7** (900 mg), **8** (400 mg), **12** (70 mg), **13** (40 mg), **14** (200 mg) and **15** (140 mg). The 10% MeOH eluates were rechromatographed on ODS (CH_3OH–H_2O, 10 : 90—60 : 40) followed by Sephadex LH-20 to give **4** (700 mg), **6** (15 mg), **9** (170 mg), **10** (20 mg), and **11** (20 mg).

Compound **1**: White needle (MeOH), $[\alpha]_D^{17}$ −42 (c=0.87, MeOH), IR (KBr) cm^{-1}: 3397, 1733, 1628, 1497, 1297, 1116, 972, 580; ^1H-NMR (600 MHz, in CD_3OD) and ^{13}C-NMR (150 MHz, in CD_3OD): see Table 1; HR-ESI-MS *m/z*: 579.1269 $[M+Na]^+$. Calcd for $C_{25}H_{22}O_{11}Na$ 579.1267.

Compound **2**: White powder (MeOH), $[\alpha]_D^{17}$ −25 (c=1.0, MeOH), IR (KBr) cm^{-1}: 3394, 1730, 1635, 1482, 1115, 975, 581; ^1H-NMR (600 MHz, in CD_3OD) and ^{13}C-NMR (150 MHz, in CD_3OD): see Table 1; HR-ESI-MS *m/z*: 579.1269 $[M+Na]^+$. Calcd for $C_{25}H_{22}O_{11}Na$ 579.1267.

Assay for Inhibitory Ability against LPS-Induced NO Increase in RAW 264.7 Macrophages RAW 264.7 macrophages were seeded in 24-well plates (10^5 cells/well). The cells were co-incubated with drugs and LPS (1 μg/ml) for 24 h. The amount of NO was assessed by determining the nitrite concentration in the cultured RAW 264.7 macrophage supernatants with Griess reagent. Aliquots of supernatants (100 μl) were incubated, in sequence, with 50 μl 1% sulphanilamide and 50 μl 0.1% naphthyl ethylene diamine in 2.5% phosphoric acid solution. The absorbance at 570 nm was read using a microtiter plate reader. Results are expressed as means±S.D. Statistical analysis was performed using Student's *t*-test, and $p<0.05$ was considered significant.[20,21]

Acknowledgment The work was supported by program for Changjiang Scholars and Innovative Research Team in University (PCSIRT), NCET Foundation, NSFC (30725045), National 863 Program (2006AA02Z338), China Postdoctoral Science Foundation (20070410711), "973" program of China (2007CB507400), Shanghai Leading Academic Discipline Project (B906) and in part by the Scientific Foundation of Shanghai China (07DZ19728, 06DZ19717, 06DZ19005).

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17) Crystal data: $C_{31.50}H_{35}O_{15.50}$, M=661.59, monoclinic system, space group C2, a=28.735(8), b=15.081(4), c=16.185(4) Å, V=6958(3) Å^3, Z=8, d=1.263 mg/m^3. Crystal size; 0.20×0.15×0.10 mm. CCDC 694965 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge *via* www.ccdc.cam.ac.uk/data_request/cif.
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