Two New Bis-coumarin Glycosides from *Daphne giraldii* NITSCHE

Juan SU,[a] Zhi-Jun WU,[c] Wei-Dong ZHANG,[a,b] Chuan ZHANG,*[,a] Hui-Liang LI,[a] Run-Hui LIU,[a] and Yun-Heng SHEN*[,a]

[a] *Department of Phytochemistry, School of Pharmacy, Second Military Medical University; Shanghai 200433, P. R. China:* [b] *School of Pharmacy, Shanghai Jiao Tong University; Shanghai 200030, P. R. China: and* [c] *Department of Research & Development, Shanghai Center of Research & Development of New Drugs; Shanghai 201203, P. R. China.*

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Two new bis-coumarin glycosides were isolated from the stem barks of *Daphne giraldii* NITSCHE. Their structures were elucidated as 6-*O*-α-L-rhamnopyranosyl daphnogirin (1), and 6-*O*-β-D-apiofuranosyl daphnogirin (2), on the basis of detailed spectroscopic analysis. Compounds 1 and 2 were tested inhibitory effects against production of nitric oxide (NO) in RAW264.7 cells, and cytotoxicity of human tumor cell lines A549, LOVO, QGY-7703, 6T-CEM. The results showed that compounds 1 and 2 neither reduced production of NO, nor inhibited human tumor cell lines.

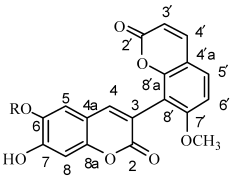
Key words *Daphne giraldii*; bis-coumarin glycoside; daphnogirin; nitric oxide; cytotoxic activity

Daphne giraldii NITSCHE (Thymelaeaceae) is mainly distributed in Shanxi, Gansu, and Qinghai provinces in China. The stems and roots of this plant (Chinese name 'Zu Shima') have been used in Chinese folk medicine to treat ache and rheumatism, especially for toothache, waist ache, rheumatoid arthritis, quadriplegia.[1)] Earlier phytochemical investigations on this plant were mainly focused on diterpenoids.[2—6)] In our search for natural products with biological activities, two new bis-coumarin glycosides were isolated from the alcoholic extract of the stem barks of this plant. Herein, we report the isolation and structural elucidation of two new bis-coumarin glycosides, along with their inhibitory effects against production of NO in RAW264.7 cells and growth of four human tumor cell lines.

The EtOH extract of *D. giraldii* was partitioned with petroleum ether, $CHCl_3$, EtOAc and *n*-BuOH, respectivey. The EtOAc fraction was submitted to column chromatography over silica gel and Rp-18 to afford compounds **1** and **2**.

Compound **1** (Fig. 1) was obtained as white powder, showing a molecular formula of $C_{25}H_{22}O_{11}$ as deduced by HR-ESI-MS. The UV spectrum exhibited the absorption maximal signals at 210.0, 325.5 nm. In the ^{1}H-NMR spectrum (Table 1) of **1**, an anomeric proton signal at δ_H 5.52 (1H, br s), together with characteristic methyl resonance at δ_H 1.13 (3H, d, J=6.0 Hz), suggested an α-rhamnnopyranosyl in **1**. The above deduction was confirmed by the carbon resonances (Table 1) for a rhamnose at δ_C 99.3, 71.7, 70.1, 69.8, 69.7, and 17.8. Acid hydrolysis of **1** and GC analysis of chiral derivatives of sugars in the acid hydrolysate afforded an L-rhamnnopyranosyl. In addition to the sugar unit, the aglycone moiety of **1** exhibited 18 sp^2 carbons, including two ester carbonyls at δ_C 159.1 and 159.8, together with a methoxyl. The ^{1}H-NMR spectrum displayed three singlet aromatic protons at δ_H 8.00, 7.22, and 7.13, and four doublet proton resonances at δ_H 8.09 (1H, d, J=10.0 Hz), 7.74 (1H, d, J=9.0 Hz), 7.21 (1H, d, J=9.0 Hz), and 6.30 (1H, d, J=10.0 Hz). Considering chemical constituents previously reported from the genus *Daphne*, it was hinted that **1** should be a bis-coumarin glycoside. By comparison of the ^{1}H- and ^{13}C-NMR data of **1** with those of known daphnogirin isolated from *D. giraldii* by Liao S. G. *et al.*,[4)] **1** was determined as a rhamnnopyranoside of daphnogirin analog. The aglycone of **1** was obtained by acid hydrolysis, and identified to be 6-hydroxyl daphnogirin (**1a**) based on inspection of its NMR data. The rhamnnopyranosyl was attached to C-6 of the aglycose based on the hetero multiple bonding connectivity (HMBC) correlation between the anomeric proton (δ_H 5.52) of rhamnose and C-6 (δ_C 148.1). The above assignment was evidenced through the observation of the nuclear Overhouser enhanced and exchange spectroscopy (NOESY) correlations between 7-OH and H-8, and between the anomeric proton (δ_H 5.52) of rhamnnose and H-5, and between H-5 and H-4. Therefore, the structure of compound **1** was finally elucidated as 6-*O*-α-L-rhamnnopyranosyl-7-hydroxy-3-(7-methoxy-2-oxo-2*H*-1-benzopyran-8-yl)-2*H*-1-benzopyran-2-one, and named 6-*O*-α-L-rhamnnopyranosyl daphnogirin.

Compound **2** was isolated as white powder. The HR-ESI-MS showed a $[M+Na]^+$ ion peak, corresponding to a molecular formula $C_{24}H_{20}O_{11}$. The ^{1}H- and ^{13}C-NMR data (Table 1) of **2** was quite similar to those of **1**, with the exception of one additional apiofuranosyl instead of α-L-rhamnnopyra-



1 R = α-L-rhamnopyranosyl
1a R= H
2 R = β-D-apiofuranosyl

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

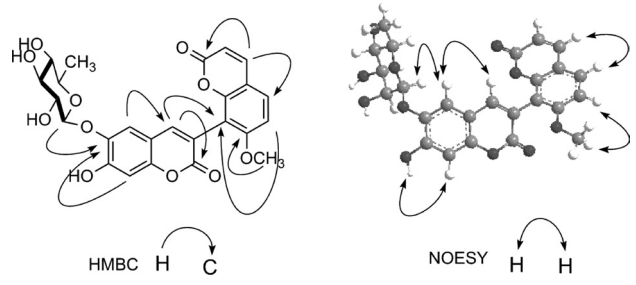


Fig. 2. Key HMBC and NOESY Correlations of **1**

* To whom correspondence should be addressed. e-mail: shenyunheng9217018@yahoo.com.cn

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for **1** and **2** (δ in ppm, J in Hz, in DMSO-d_6)

No.	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
2	159.1 s		160.0	
3	116.5 s		116.4	
4	144.5 d	8.00 s	144.5	8.00 s
4a	112.9 s		112.7	
5	103.9 d	7.22 s	103.5	7.09 s
6	148.1 s		148.9	
7	144.5 s		144.4	
8	112.8 d	7.13 s	112.6	7.13 s
8a	147.4 s		147.5	
2′	159.8 s		159.8	
3′	112.5 d	6.30 d (10.0)	112.5	6.31 d (10.0)
4′	144.4 d	8.09 d (10.0)	144.5	8.07 d (10.0)
4′a	112.6 s		112.7	
5′	129.7 d	7.74 d (9.0)	129.7	7.76 d (9.0)
6′	108.3 d	7.21 d (9.0)	108.3	7.20 d (9.0)
7′	160.0 s		160.0	
8′	111.5 s		111.6	
8′a	152.3 s		152.3	
1″	99.3 d	5.52 br s	107.8	5.67 br s
2″	69.8 d	4.00 br s	76.2	4.27 m
3″	70.1 d	3.84 m	78.8	
4″	71.7 d	3.36 m	74.7	3.84 m
				4.27 m
5″	69.7 d	3.53 m	62.5	3.44 m
6″	17.8 t	1.13 d (6.0)		
OCH_3	56.5 t	3.87 s	56.5	3.87 s
OH		9.51 s		9.50 s

nosyl in **1**. Acid hydrolysis of **2** and GC analysis of chiral derivatives of sugars in the acid hydrolysate further confirmed the presence of D-apiofuranosyl. The long-range correlation of the anomeric proton (δ_H 5.67) of apiofuranose with C-6 (δ_C 148.9) suggested the presence of 6-*O*-β-D-apiofuranosyl. All proton and carbon signals were assigned by heteronuclear single quantum coherence (HSQC), HMBC and NOESY experiments. Thus, **2** was determined as 6-*O*-β-D-apiofuranosyl-7-hydroxy-3-(7-methoxy-2-oxo-2*H*-1-benzopyran-8-yl)-2*H*-1-benzopyran-2-one, and named 6-*O*-β-D-apiofuranosyl daphnogirin.

Nitric oxide (NO) plays an important role in the regulation of many physiological functions, such as host defence, neurotoxicity, and vasodilation.[7] However, the excess production of NO has been implicated for immunological and inflammatory diseases including septic shock, rheumatoid arthritis, graft rejection, and diabetes.[8] Therefore, inhibition of NO production is apparently an important therapeutic consideration in the development of anti-inflammatory agents.

The stems and roots of *D. giraldii* have been used in Chinese folk medicine to treat rheumatism, and coumarins of this plant are commonly considered as the major bioactive constituents. In our test, compounds **1** and **2** were found not to reduce production of NO in RAW264.7 cells, and showed no cytotoxicity against RAW264.7 cells at the concentration of 100 μg/ml. Mono-coumarin, such as daphnetin isolated from *D. giraldii*, has been reported to possess unambiguous anti-inflammatory activity, while bis-coumarin glycosides **1** and **2** showed no inhibitory effects. It maybe result from structural difference or other action mechanism.

Compounds **1** and **2** were also screened cytotoxicity against four human tumor cell lines A549, LOVO, QGY-7703, 6T-CEM by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, but showed no inhibitory effects with IC_{50} values of more than 30 μg/ml.

Experimental

Optical rotations were measured on a Perkin-Elmer-341 polarimeter. UV spectra were performed on a Shimadzu UV-210A, λ_{max} (log ε) in nm. IR spectra were obtained on a Perkin-Elmer-577 spectrometer, in cm^{-1}. Gas chromatography analysis was operated on an HP-5892 II with an FID detector, and an HP-20M (Carbowx 20M) capillary column (25 m×0.32 mm×0.3 μm) was used. ^{1}H- and ^{13}C-NMR, HSQC, HMBC and NOESY spectra were recorded on a Bruker AVANCE^{II} 600 NMR. HR-ESI-MS were performed on a JMS-HX-110 instrument.

Plant Material The stem barks of *D. giraldii* were collected in Shanxi province, P. R. China in May 2005, and authenticated by Prof. CeMing Tan. The voucher specimen (No. 2005051509) is deposited in Department of Phytochemistry, Second Military Medical University.

Extraction and Isolation The stem barks of *D. giraldii* (11 kg) were extracted with 95% EtOH at room temperature for three times. After removal of EtOH, the water suspension was partitioned with petroleum ether, $CHCl_3$, EtOAc and *n*-BuOH. The EtOAc fraction (200 g) was submitted over Si-gel CC (100—200 mesh) eluting with gradient $CHCl_3$–MeOH (15 : 1 to 5 : 1) to give fractions 1—5. Fraction 3 (40 g) was purified by Si-gel CC (100—200 mesh) eluting with gradient $CHCl_3$–MeOH (10 : 1 to 7 : 1) and Rp-18 chromatography eluting with gradient MeOH–H_2O (5 : 5 to 7 : 3) to provide **1** (51 mg), and **2** (32 mg).

Compound **1**: White powder, $[\alpha]_D^{20}$ −146° (c=0.22, MeOH), UV (MeOH): 210, 325.5 nm; IR (KBr) cm^{-1}: 3397, 1733, 1628, 1497, 1297, 1116, 972, 580; ^{1}H-NMR (600 MHz, in DMSO-d_6) and ^{13}C-NMR (150 MHz, in DMSO-d_6): see Table 1; HR-ESI-MS m/z: 521.1061 [M+Na]$^+$, Calcd for $C_{25}H_{22}O_{11}Na$ 521.1060.

Compound **1a**: White powder, obtained by acid hydrolysis of **1** and **2**, and identified as 6-hydroxyl daphnogirin based on inspection of the ^{1}H- and ^{13}C-NMR spectra. ^{1}H-NMR (600 MHz, in CD_3OD) δ_H: 7.80 (1H, s, H-4), 7.00 (1H, s, H-5), 6.83 (1H, s, H-8), 6.26 (1H, d, J=9.6 Hz, H-3′), 7.94 (1H, d, J=9.6 Hz, H-4′), 7.68 (1H, d, J=9.0 Hz, H-5′), 7.15 (1H, d, J=9.0 Hz, H-6′); ^{13}C-NMR (150 MHz, in CD_3OD) δ_C: 162.2 (s, C-2), 116.4 (s, C-3), 146.9 (d, C-4), 114.5 (s, C-4a), 103.5 (d, C-5), 152.2 (s, C-6), 144.7 (s, C-7), 113.1 (d, C-8), 150.4 (s, C-8a), 162.9 (s, C-2′), 113.5 (d, C-3′), 146.0 (d, C-4′), 113.5 (s, C-4′a), 130.9 (d, C-5′), 109.5 (d, C-6′), 163.0 (s, C-7′), 113.1 (s, C-8′).

Compound **2**: White powder, $[\alpha]_D^{20}$ −133° (c=0.29, MeOH), UV (MeOH): 210, 325.5 nm; IR (KBr) cm^{-1}: 3397, 1733, 1628, 1502, 1297, 1144, 1049, 973, 589; ^{1}H-NMR (600 MHz, in DMSO-d_6) and ^{13}C-NMR (150 MHz, in DMSO-d_6): see Table 1; HR-ESI-MS m/z: 507.0906 [M+Na]$^+$, Calcd for $C_{24}H_{20}O_{11}Na$ 507.0903.

Acid Hydrolysis and GC Analysis of 1 and 2 Each compound (5 mg) was heated in 2.0 mol/l HCl–MeOH (1 : 1, v/v, 10 ml) at 90 °C for 4 h. The reaction mixture was evaporated to dryness, and then partitioned between EtOAc and H_2O. The EtOAc fraction was purified by Sephadex LH-20 (MeOH) to yield aglycone **1a**. The H_2O layer was neutralized by passing through an ion-exchange resin (Amberlite MB-3) column and concentrated to yield a sugar residue. The residue was treated with dry pyridine and L-cysteine methyl ester hydrochloride at 60 °C for 2 h with stirring, and then concentrated to dryness. Trimethylsilylimidazole was added to the residue, and the mixture was heated for 1 h at 60 °C, followed by partition between *n*-hexane and water. The organic layer was analyzed by GC [HP-5892 II with an FID detector, and an HP-20M (Carbowx 20M) capillary column (25 m×0.32 mm×0.3 μm)] and their retention times were compared with those of authentic sugars.[9] L-Rhamnnopyranose was detected from **1** (t_R: 5.012 min), and D-apiofuranose was detected from **2** (t_R: 4.680 min).

Determination of Nitrite Production The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess method as described previously.[10] An amount of 100 μl of each culture supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid, and 0.1% naphthylethylenediamine dihydrocholoride in water) and the absorbance of the mixture at 550 nm was measured using a microplate reader. Nitrite concentrations were calculated from a standard curve of sodium nitrite prepared in the culture medium.

MTT Assay Compounds were tested for cytotoxicity using the microtitre MTT tetrazolium dye assay under conditions of continuous drug exposure reported,[11] but with some modifications. Cells culture was diluted

with fresh medium to 4×10^4 cell ml^{-1} and plated in 96-well microplates at 100 μl $well^{-1}$. After 24-h incubation at 37 °C in a 5% CO_2 atmosphere, the tested compounds of variety concentrations (10^{-2}—10^2 μg/ml) were added to the microplates in 10 μl amounts. The RAW264.7 cells and four tumour cell lines A549, LOVO, QGY-7703, 6T-CEM, were exposed to the drugs for another 72 h. The absorbance was read on a Wellscan reader (MK-2, Labsystems, Finland) at 570 nm. Topotecan (purchased from Nanjing Tianzun Zezhong Chemical Co., Ltd., P. R. China) was used as positive reference substance with concentrations of 10^{-3}—10^2 μg/ml.

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