Daphnogirins A and B, Two Biflavones from *Daphne giraldii*

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Two new biflavonoids, daphnogirins A (1) and B (2), were obtained from the roots of *Daphne giraldii*. Their structures were established on the basis of the spectral data and X-ray diffraction data of the co-crystal of 1 and 2. Daphnogrins A and B have the same configuration at C-1 and opposite configurations at C-16 and C-17. Oxygen radical scavenging assay has indicated that they are of significant antioxidative activity.

Key words *Daphne giraldii*; daphnogirin A; daphnogirin B; X-ray diffraction; antioxidative activity

During the course of our investigation of the constituents of *Daphne giraldii* (Thymelaeaceae), we have reported the isolation of four known biflavonoid daphnodorins A—D.[1)] *Daphne odor*, a plant from the same genus *Daphne*, was also found to contain the same biflavonoid daphnodorins A—D.[2,3)] Apparently, *D. giraldii*, like *D. odor*, is a splendid source of some biflavonoids. Our further investigation on the EtOAc extract of the title plant led to the isolation of daphnogirins A (**1**) and B (**2**) and incidentally the co-crystal of a 1 : 1 complex of **1** and **2**. Their structures were determined by spectral analysis in combination with X-ray crystallography. Normally, flavonoids comprising of a variety of polyphenolic secondary metabolites, *e.g.* flavones, isoflavones, and flavanones, act as antioxidants against peroxy and hydroxy radicals and serve as prooxidants in the presence of Cu^{2+}. The antioxidative activity of compounds **1** and **2** was evaluated by oxygen radical scavenging assay (ORAC). Although the biological functions of biflavonoids in plants of genus *Daphne* are unclear, our research and previous reports hinted that these biflavonoids might be of chemotaxonomic significance for plants of genus *Daphne*.[1—3)]

Compound **1** was isolated as an amorphous brown solid from the co-crystal (in acetone/H_2O) of **1** and **2** (see below). FAB-MS showed only pseudomolecular ion $[M+H]^+$ at *m*/*z* 543, which was consistent with molecular formula $C_{30}H_{22}O_{10}$. The UV spectrum showed absorption at 221, 254, 291, and 311 nm. The IR spectrum exhibited absorption bands at 3433, 2930, 1640, and 1517 cm^{-1}, suggesting the presence of hydroxy and carbonyl groups and aromatic rings in the structure.

Proton and carbon signals were assigned by a combination of 1D and 2D-NMR techniques. The ^{1}H-NMR spectrum (see Table 1) of **1** showed signals of a pair of 4-oxyphenyl groups at δ 7.36 (2H, d, J=8.8 Hz) and 6.83 (2H, d, J=8.8 Hz). A 2,4,6-trioxyphenyl group, an alcoholic hydroxy group, and five phenolic hydroxy groups were also assigned on the basis of proton signals respectively at δ 6.03 (1H, d, J=2.1 Hz), 5.93 (1H, d, J=2.1 Hz), 5.32 (1H, s), and 11.64, 9.87, 8.94, 8.68, and 8.38 (each 1H, s). Meanwhile, the signals at δ 6.25 (1H, s), 4.98 (1H, br d, J=8.4 Hz), 2.26 (1H, m), 1.69 (1H, m), and 2.61 (2H, m) suggested the presence of 5,7,8-trisubstituented flavane unit. A quaternary carbon and eight benzyl carbons with attachment of oxygen atoms as well as a carbonyl carbon can be elucidated from the signals in ^{13}C-NMR spectrum (see Table 2). Careful comparison of the NMR data of **1** with those of daphnodorin E showed a small difference between them.[4)] Therefore we rationally assumed that compound **1** is the same as daphnodorin E. The relative stereochemistry of daphnodorin E between C-2″ and C-3″ was elucidated as *trans* by NOESY in a previous report.[4)] However,

Table 1. ^{1}H-NMR Data (δ) of Compounds **1** and **2** (500 MHz, in Acetone-d_6, J in Hz)

Position No.	**1**	**2**
2	4.98 (br d, 8.4)	4.87 (br d, 8.4)
3	2.13 (m)	2.26 (m)
	1.69 (m)	1.84 (m)
4	2.61 (m)	2.65 (m)
6	6.25 (s)	6.26 (s)
2′, 6′	7.13 (d, 8.8)	7.36 (d, 8.8)
3′, 5′	6.78 (d, 8.8)	6.83 (d, 8.8)
3″-OH	5.32 (br s)	5.37 (br s)
6″	6.03 (d, 2.1)	5.93 (d, 2.0)
8″	5.93 (d, 2.1)	5.92 (d, 2.0)
2‴, 6‴	7.34 (d, 8.8)	7.35 (d, 8.8)
3‴, 5‴	6.85 (d, 8.8)	6.80 (d, 8.8)
OH	11.64 (s)	11.53 (s)
	9.87 (s)	9.92 (s)
	8.94 (s)	8.94 (s)
	8.68 (s)	8.68 (s)
	8.35 (s)	8.38 (s)

Table 2. ^{13}C-NMR Data of Compounds **1** and **2** (500 MHz, in Acetone-d_6)

Position No.	**1**	**2**	Position No.	**1**	**2**
2	77.5	77.6	2″	118.3	118.2
3	29.6	30.3	3″	81.8	82.0
4	20.3	20.1	4″	193.5	193.7
4a	105.0	104.7	4″a	99.6	99.6
5	153.7	153.6	5″	160.3	160.4
6	91.7	91.6	6″	95.3	95.3
7	157.3	157.3	7″	164.6	164.6
8	108.1	107.8	8″	97.0	97.1
8a	159.1	159.1	8″a	164.6	164.6
1′	132.6	133.2	1‴	125.8	125.8
2′, 6′	127.7	127.3	2‴, 6‴	129.2	129.2
3′, 5′	115.5	115.6	3‴, 5‴	115.2	115.2
4′	158.8	158.9	4‴	167.5	167.6

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that report did not show the data of NOESY experiment. Our NOE difference experiment did not give the NOE increment in H-2‴ and H-6‴ or in 3″-OH when the proton at 3″-OH or H-2‴ and H-6‴ was alternatively irradiated. Thus compound **1** might be different from daphnodorin E in the relative configuration between C-2″ and C-3″.

Similarly, **2** had the same molecular formula as that of **1** with FAB-MS $[M+H]^+$ ion at *m*/*z* 543, indicating that **2** has the same formula. NMR data of compound **2** also showed a striking similarity with those of **1**. Careful analysis of NMR spectra revealed that **2** had the same NMR data with daphnodorin F, the isomer of daphnodorin E.

Crystalline prisms suitable for X-ray crystallographic analysis of **1** and **2** before further reverse phase HPLC separation were incidentally obtained in acetone solution. The crystal structure was a complex of two diastereoisomers at C-2″ and C-3″ (Fig. 1). That is, both **1** and **2** have the same configuration at C-2, but the opposite configuration at C-2″ and C-3″. The asymmetric unit consists of two independent molecules **1** and **2**, two acetone molecules, and three water molecules (Fig. 2). The two acetone molecules are linked to compounds **1** and **2** through intramolecular hydrogen bonds $C_{3''}$–O–H (**1**)···O=$C_{4''}$ (**2**) (2.650 Å) and $C_{3''}$–O–H′ (**2**)···O=$C_{4''}$ (**1**) (2.679 Å), respectively, while the water molecules and compounds **1** and **2** are linked through a complex intramolecular and intermolecular hydrogen bonds (see Fig. 2). The intramolecular hydrogen bonds between **1** and **2** also were observed among $C_{4'}$–O–H (**1**)···$O_{1''}$ (**2**), $O_{1''}$ (**1**)···H–O–$C_{4'}$ (**2**), $O_{1'''}$ (**1**)···H–O–$C_{5''}$ (**2**), and $C_{5''}$–O–H (**1**)···$O_{1'''}$ (**2**). Compounds **1** and **2** possess the same planar structures, which are constructed by a flavonone molecule (rings D, E, F) and a flavan molecule (rings A, B, C) through the linkage of a dihy-

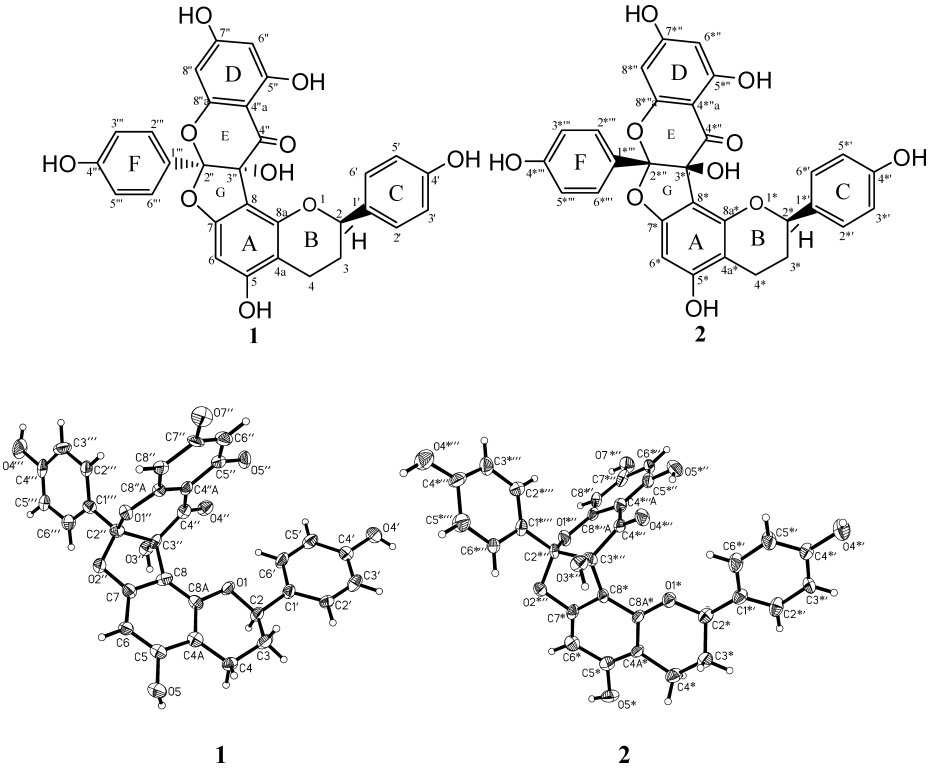

Fig. 1. Molecular Structure of **1** and **2** with Atom Labeling Scheme

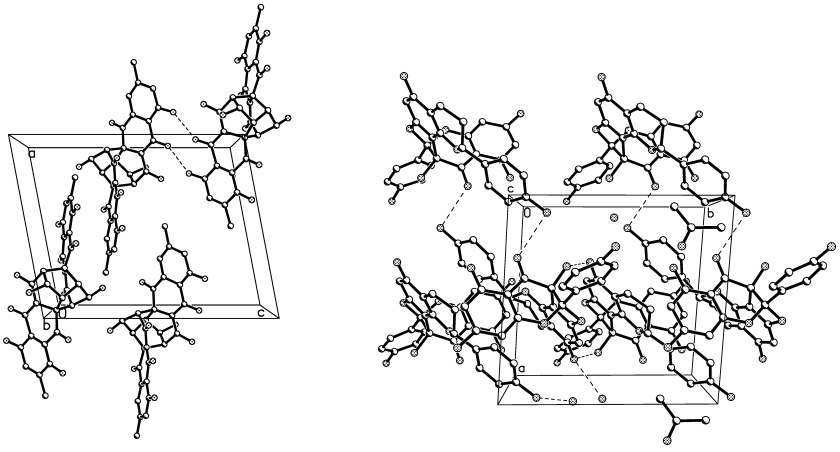

Fig. 2. Pictures of Atoms Arrangement along *b* and *c* of Crystal Lattice for the Co-crystal of **1**, **2** and Solvent Molecules

Table 3. Crystal Data and Structure Refinement for Complex of **1** and **2**

Compound	**1** and **2**
CCDC deposit no.	602798
Color/shape	Light yellow/prism
Cryst dimens (mm^3)	0.50×0.20×0.15
Chemical formula	$(C_{30}H_{22}O_{10})_2 \cdot (C_3H_6O)_2(H_2O)_3$
Formula weight	1273.18
Temperature, K	293(2)
Crystal system	Triclinic
Space group	*P*1 (No. 1)
Unit cell dimens	a=10.109(2) Å
	b=10.604(2) Å
	c=14.478(3) Å
	α=91.85(3)°
	β=99.65(3)°
	γ=93.18(3)°
Volume, $Å^3$	1526.3(5)
Z	1
Density, mg/m^3	1.385
Abs coeff, mm^{-1}	0.108
Diffractometer/scan	Mac DIP-2030K
θ range, deg	2.69—25.48
Reflections measuredd	4177
Indepnt reflns	4177
Obsd reflns [$F^2>8\sigma F^2$]	3820
Data/params	4177/833
Goodness of fit on F^2	1.75
R_1 [$I>2\sigma(I)$]	0.0728
wR_2 (all data)	0.09

$R_1=\sum||F_o|-|F_c||/\sum|F_o|$, $wR_2=[\sum[w(F_o^2-F_c^2)^2]/\sum[w(F_o^2)^2]]^{1/2}$.

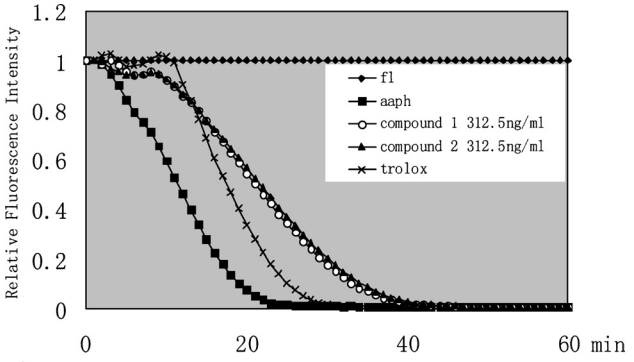

Fig. 3. Inhibition Effects of Both **1** and **2** on Fluorescence Decay Induced by AAPH

drofuran (ring G).

There are three chiral centers in both molecules and their relative configurations can be assigned as *rel*-(2*R*, 2″*S*, and 3″*R*) for **1** and *rel*-(2′*R*, 2″*R*, and 3″*S*) for **2**. The most relatively similar compounds are daphnodorin E and F.[4] However, compounds **1** and **2** are different from daphnodorin E and F with respect to the relative configurations among their chiral centers. The orientations between two groups at C-2″ and C-3″ are *trans* in daphnodorins E and F, but *cis* in compounds **1** and **2**. The CD spectra of **1** and **2** were found the same as daphnodorin E, which indicates that absolute configuration at C-2 was *S*. Thus the absolute configurations of **1** and **2** were determined as shown in Fig. 1 by considering the relative configurations revealed by X-ray analysis. Compounds **1** and **2** were here named daphnogirins A and B, respectively. Besides the configurations at C-2″ and C-3″, the differences of molecules **1** and **2** are also observed in the orientation of the phenyl ring of the flavan unit, which is indicated by some torsion angles (Fig. 1).

Table 4. Inhibition Effects of Samples on Fluorescence Decay Induced by AAPH (ORAC Value)

Samples	*AUC* (area under the curve)	*NAUC* (net area under the curve)	ORAC (trolox equivalents, μM)
AAPH	10.39		
Compound **1**	20.61	10.22	2496.77±105.33
Compound **2**	20.11	9.72	2375.55±103.65
Trolox	16.93	6.55	

The antioxidant capacities of **1** and **2** are shown in Fig. 3 and Table 4. The working curves of fluorescein oxidation were used as an index of time resistance for the oxidative reaction. Quenching curves of disodium fluorescein illustrated the ability of the sample to absorb the peroxyl radical as compared with that of the standard trolox. From the curves, it is clear that both **1** and **2** inhibited the process, and the levels remained high compared with the basal and trolox curve until 90 min. Thus, the antioxidative capacity of the sample is significant.

Experimental

General IR spectrum was recorded on a Perkin-Elmer 683 infrared spectrometer; UV spectrum was carried out by JASCO V-550 UV/Vis spectrometer; CD were measured on a Jasco J-500C; NMR spectra were recorded on a Bruker AM 500 spectrometer with TMS as internal standard; FAB mass spectra were performed on Zabspec E mass spectrometer. Preparative HPLC was performed using an ODS column (19 mm×300 mm, 10 μm, XTerra Prep. Rp18, Detector: UV at 235 nm).

Single Crystal X-Ray Diffraction The X-ray diffraction data of the complex were collected by a MAC DIP-2030K image plate diffractometer equipped with a rotating anode and Mo$K\alpha$ radiation (λ=0.71073 Å). Altogether, 36 images covering a hemisphere of reciprocal space were collected (ω scan, 5° per image). The crystal structures were solved by direct methods and refined using the SHELXTL software package.[5] The H atoms and non-H atoms were included in the calculation of structure factors and refined with isotropic and anisotropic temperature factors, respectively. In the crystal structure, some hydrogen atoms of the water molecules were not located because of the complex hydrogen bonds. A summary of crystallographic data and structural refinement parameters of the complex is listed in Table 1.

Plant Material The stems and barks of *D. giraldii* were collected at Gansu province, northwestern China in June 1999. The species of the plant was authorized by Prof. Wan-zhi Song of the Institute of Materia Medica (IMM), Chinese Academy of Medical Sciences, Beijing. A voucher specimen was deposited in the Herbarium of IMM (DG 2).

Extraction and Isolation The stems (10 kg) were chopped into small pieces and extracted with 95% of EtOH (30 l×3) under reflux. The combined EtOH extract was concentrated to dryness in vacuum and yielded 0.92 kg of ethanol extract. The ethanol extract was suspended into water and extracted successively with petroleum ether and EtOAc. 310 g of EtOAc extract was obtained after evaporation of the solvent. 50 g of the EtOAc extract was dissolved in acetone (200 ml). The acetone-soluble part (100 ml) was subjected to column chromatography on Si gel eluting in gradient $CHCl_3$/MeOH (10 : 1—1 : 2). The $CHCl_3$/MeOH (1 : 1) elute (150 mg) was separated on Sephadex LH 20 with $CHCl_3$/MeOH (1 : 1) to yield the co-crystal (35 mg) of **1** and **2**. Part (30 mg) of the co-crystal was used further to separate pure **1** and **2** by preparative reverse-phase HPLC on ODS column eluting with MeOH–H_2O (35 : 65).

Daphnogirins A (1) and B (2) Both **1** and **2** were obtained as amorphous brown solid from the preparative HPLC of a yellowish co-crystal; IR ν_{max} (KBr) cm^{-1}: 3433, 2930, 1640, 1517; UV λ_{max} MeOH (log ε) nm: 221.0 (4.53), 254.0 (3.77), 291 (4.03), 311 (3.95); CD $\Delta\varepsilon$ (c=1.21×10^{-4} μM): −1.30 (344), +8.50 (312), +1.58 (294), +3.49 (286), −3.90 (263), −1.99 (245) nm; ^{1}H- and ^{13}C-NMR data for **1** and **2**, see Tables 1 and 2; FAB-MS *m/z*: 543 $[M+H]^+$.

Oxygen Radical Absorbance Capacity (ORAC) Assay The procedure is based on a previously reported method with slight modifications.[6] Briefly,

it is described as follows: ORAC was measured using disodium fluorescein as fluorescence, and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator, which is relevant to biological systems because the peroxyl radical is the most abundant free radical. Trolox, a water-soluble analogue of vitamin E, was used as a reference standard, and the loss of fluorescence was monitored. The antioxidative effects of samples were expressed in ORAC, where one ORAC unit equals to the fluorescence decay inhibited by 1 mM trolox. Results are calculated as ORAC values using the differences of areas under the fluorescence decay curve between the blank and the sample, and are expressed as trolox equivalent. Trolox fluorescence decay curves are registered for every new solution of fluorescein.

Supporting Information Available Complete lists of refined atomic coordinates and relevant information in standard CIF format for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Center as supplementary materials. The CCDC numbers are shown in Table 3. These materials are available free of charge *via* application to CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. [Fax: (+44) 1223-336033; e-mail: deposit@ccdc.cam.ac.uk].

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