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Chemical constituents investigation of *Daphne tangutica*

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ABSTRACT

A phytochemical study of an ethanol-soluble extract from the root barks of *Daphne tangutica* Maxim., a traditional Tibetan herb medicine, led to the isolation of 30 compounds, including eight daphnane diterpenes, nine coumarines, six lignans, five phenylpropanoid derivatives, β-sitosterol and p-hydroxy benzonate. Two compounds out of these isolates are new daphne diterpene analogs, and their structures were established as 1,2α-dihydro-5β-hydroxy-6α,7α-epoxy-resiniferonol-14-benzonate, and 1,2β-dihydro-5β-hydroxy-6α,7α-epoxy-resiniferonol-14-benzonate, respectively, on the basis of spectroscopic methods. Additionally, this is the first time that 13 known compounds have been isolated and identified from this traditional Tibetan medicinal plant.

1. Introduction

Daphne tangutica Maxim. (Thymelaeaceae), an evergreen shrub mainly distributed in west China, has been used as a traditional Tibetan medicine named "Shenxingnama" for the treatment of rheumatoid arthritis and apoplexia [1,2]. Previous phytochemical research on *D. tangutica* has revealed that daphnane diterpenes, coumarines as well as lignans are major principles isolated from this plant [3–5]. Among those reported secondary metabolites, daphnane diterpenes are structurally unique compounds only found in the families of Euphorbiaceae and Thymelaeaceae, and have been documented as the irritant and toxic principles of these plants with a wide spectrum of activities including abortifacient, neurotrophic, insecticidal, tumor promoting, antileukaemia and anticancer [6–8].

As part of our efforts to search for new pharmaceutical agents from plants, *D. tangutica*, a locally medicinal plant collected from Qinghai province of China, was selected for the phytochemical investigation. In our present study, two new daphnane derivatives (**1** and **2**), together with 28 known compounds, including six daphnane diterpenes, vesiculosin (**3**) [9], isovesiculosin (**4**) [9], gniditrin (**5**) [10], gnidicin (**6**) [10], daphnetoxin (**7**) [11] and excoecariatoxin (**8**) [12], nine coumarines, umbelliferone [13] (**9**), daphnoretin (**10**) [14], daphneticin (**11**) [5b], isodaphneticin (**12**) [15], daphnetin (**13**) [5c], daphnorin (**14**) [5c], daphnin (**15**) [16], daphnetin 8-*O*-β-D-glucopyranoside (**16**) [16] and daphneside (**17**) [16], six lignans, (−)-piperitol (**18**) [17], (−)-pinoresinol (**19**) [18], (−)-syringaresinol (**20**) [5c], syringaresinol 4′-*O*-β-D-glucopyranoside (**21**) [19], (−)-pinoresinol glucoside (**22**) [18] and syringaresinol 4′,4″-di-*O*-β-D-glucopyranoside (**23**) [19], five phenylpropanoid derivatives, caffeic acid octadecyl ester (**24**) [20], *trans*-ferulic acid (**25**) [21], isoferulic acid (**26**) [22], icariside H_1 (**27**) [23] and syringin (**28**) [24], a steroid, β-sitosterol (**29**) and a small molecular compound, methyl p-hydroxy benzonate (**30**) [20] were purified and structurally identified. This is the first time that 13 known compounds, including **3**, **4**, **6**, **12**, **14**, **17**, **22**, **24**–**28**, and **30**, were reported as secondary metabolites isolated from this plant. Herein, the structure elucidation of new compounds **1** and **2** by using spectroscopic methods including HRESIMS and 2D NMR will be present.

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2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 automatic polarimeter at 589 nm. IR spectra were recorded on a Perkin-Elmer FTIR spectrometer. 1D and 2D NMR spectra were recorded on a Bruker Advance 600 spectrometer with TMS as internal standard. HRESIMS were obtained on a BioTOF Q mass spectrometer. Column chromatography was performed with silica gel (160–200 and 200–300 mesh, Qingdao Marine Chemical Co. Ltd., People's Republic of China) and RP-C18 (40–63 μm, Merck KGaA, Darmstadt, Germany). Thin-layer chromatography (TLC) plates were prepared with silica gel GF_{254} (Qingdao Marine Chemical Co. Ltd., People's Republic of China). A 150 mm × 19 mm i.d., 5 μm Sunfire $PrepC_{18}$ column (Waters, Milford, MA) was used for preparative HPLC, along with a Waters system including a 600 controller, a 717 Plus autosampler, and a 2487 dual wavelength absorbance detector.

2.2. Plant material

The fresh root barks (15 kg) of *D. tangutica* were collected from Huzhubei mountain at altitude of 2500–3000 m in Qinghai of China, in July 2004. A voucher specimen has been deposited at the Herbarium of the Northwest Plateau Institute of Biology, CAS.

2.3. Extraction and isolation

The fresh root barks of *E. wallichii* (15 kg) was sliced into small pieces and was extracted using 80% EtOH (3 × 15 L, each for 7 days) at rt. The pooled solvents were removed under reduced pressure to give a residue (1100 g), which was then suspended in water and partitioned successively with EtOAc (3 × 3 L) and *n*-butanol (3 × 3 L) to yield 160 g of EtOAc-soluble extract and 110 g of *n*-butanol-soluble extract, respectively. An aliquot (156 g) of EtOAc-soluble extract was subjected to separation over a silica gel column (12 × 100 cm, 65–250 mesh, 2000 g), and eluted with a gradient solvent system of $CHCl_3$/acetone to yield 12 subfractions, denoted as F1–F12. Compound **29** (500 mg) was precipitated from fraction F3 as a white crystal. The residue of fraction F3 (5 g) was chromatographied on a silica gel column and eluted in a gradient manner with hexanes/acetone (15:1 to 1:1), to give three subfractions (F301–F303). Compound **30** (10 mg) was purified from subfraction F302 by recrystallization in ethyl acetate. Compound **18** was separated from subfraction F303 on a silica gel column eluted with petroleum ether/acetone (5:1–1:1), then purified by recrystallization in acetone. Aliquot of fraction F4 (7 g) was subjected to separation over a silica gel column with a solvent system of hexanes/acetone (20:1 to 1:1) to yield two subfractions (F401and F402). Fraction F401 was further chromatographied on silica gel column using hexanes/acetone (8:1 to 1:1) as eluent to afford **24** (70 mg). Fraction F402 was separated on silica gel column eluted with hexanes/acetone (5:1–1:1) to give **9** (110 mg) and **10** (50 mg). Fraction F5 was chromatographied on a silica gel column and with hexanes/acetone (15:1 to pure acetone) as the eluent, to give five subfractions (F501–F505). Compound **25** (50 mg) was recrystallized from acetone solution of subfraction F501. Subfraction F502 was separated on silica gel column with $CHCl_3$/acetone (30:1 to 5:1) as solvent system to yield **5** (40 mg). A silica gel column chromatography (hexane/acetone, 4:1 to pure acetone) of subfraction F504 led to the isolation of compounds **26** (5 mg) and **19** (30 mg). Compound **12** (30 mg) was obtained from subfraction F505 by using a silica gel column eluted with $CHCl_3$/acetone (15:1 to 2:1) Fraction F6 (7 g) was subjected to separation over a silica gel column and eluted with a solvent system of hexane/acetone (10:1 to pure acetone) to yield four subfractions (F601–F604). Compound **6** (60 mg) was purified from subfraction F603 by using preparative TLC ($CHCl_3$/acetone 5:1, $R_f = 0.6$). Fraction F7 (11 g) was chromatographied a silica gel column and eluted with a solvent system of $CHCl_3$/acetone (20:1 to 2:1) to yield four subfractions (F701–F704). Compound **11** was purified from subfraction F701 with recrystallization in methanol. Compound **8** (10 mg) was purified from subfraction F702 by using preparative TLC (hexane/acetone 1:1, $R_f = 0.5$). Compound **13** (5 mg) was purified from subfraction F703 by using preparative TLC ($CHCl_3$/acetone 5:1, $R_f = 0.3$). A part of fraction F8 (4 g) was separated on a silica gel using a solvent system of $CHCl_3$/acetone (20:1 to 2:1) as eluent to yield four subfractions (F801–F804). F804 was further subjected to separation on a silica gel column (hexane/acetone 4:1 to 1:1) to give two subfraction, compound **7** (10 mg) was purified from the more polar subfraction by using preparative TLC ($CHCl_3$/acetone 5:1, $R_f = 0.5$). An aliquot of fraction F9 (4.5 g) was separated on a silica gel column using a solvent system of $CHCl_3$/acetone (15:1 to pure acetone) as eluent to yield three subfractions (F901–F903), and F901 was further subjected to separation on a silica gel column ($CHCl_3$/acetone 15:1 to pure acetone) to give two subfraction (F901A and F901B). A further separation of the subfraction F901A on a silica gel column ($CHCl_3$/acetone 15:1 to 2:1) yielded a mixture of compounds **1** (3 mg) and **2** (2 mg), which was purified by preparative HPLC using a solvent system of MeOH/water (60:40) as eluent. Subfraction F901B was subjected to a preparative TLC to yield compound **3** ($CHCl_3$/acetone 2.5:1, $R_f = 0.2$) and **4** ($CHCl_3$/acetone 2.5:1, $R_f = 0.4$). Fraction F10 was chromatographied on a silica gel column and with $CHCl_3$/methanol (20:1 to 2:1) as the eluent, to give three subfractions (F1001–F1003). Compound **27** (45 mg) was precipitated as a white powder

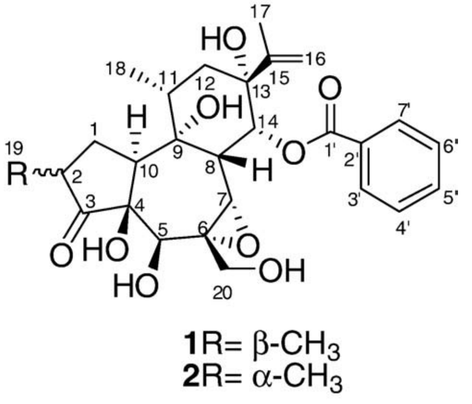

Fig. 1. Structures of compounds **1** and **2**.

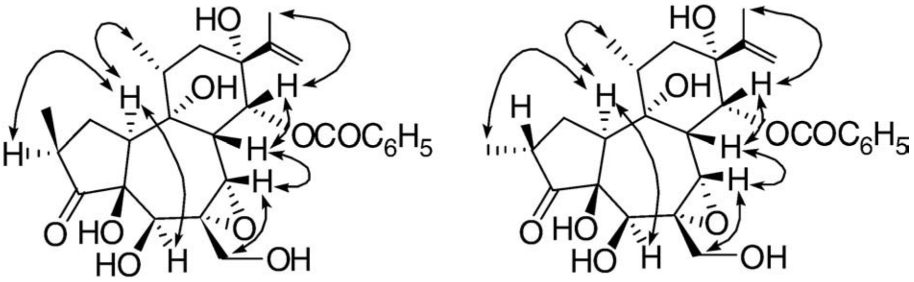

Fig. 2. Selected NOE correlations of compounds **1** and **2**.

from an ethanol solution of subfraction F1001. The chromatography of subfraction F1002 on a silica gel column using a mixture of hexane/EtOAc (3:1 to pure EtOAc) yielded three subfractions (F1002A and F1002B). Compound **14** (60 mg) was purified from subfraction F1002A by recrystallized from methanol, and compound **15** (8 mg) was purified from subfraction F1002B by using preparative TLC ($CHCl_3$/MeOH 5:1, $R_f = 0.3$). An aliquot (96 g) of *n*-butanol-soluble extract was subjected to separation over a silica gel column (12 × 100 cm, 65–250 mesh, 1000 g), and eluted with a solvent system of $CHCl_3$/MeOH with a gradient polarity to yield six subfractions, denoted as F1–F6. Fraction F1 was chromatographied on a silica gel column ($CHCl_3$/acetone, 10:1 to pure acetone) to yield four subfractions (F101–F104). Subfraction F102 was separated on a silica gel column using a mixture of hexane/acetone (3:1 to pure acetone) as eluent to give compound **28** (45 mg). Compound **21** (60 mg) was recrystallized from one polar subfraction of fraction F103 after with a silica gel column chromatography (hexane/acetone 3:1 to pure acetone). A yellow powder was deposited from fraction F2 to give compound **22** (50 mg) after recrystallization from methanol. Fraction F3 was chromatographied on a silica gel column ($CHCl_3$/MeOH 10:1 to 1:1) to yield three subfractions, and compound **16** (30 mg) was recrystallized from the most polar subfraction. Compound **23** (75 mg) precipitated as a white powder from fraction F4, and was further purified by recrystallization from acetone. An aliquot of fraction F4 (8 g) was subjected to separation on a silica gel column using a mixture of $CHCl_3$/MeOH (8:1 to pure methanol) to yield compound **17** (300 mg).

*2.4. 1,2α-dihydro-5β-hydroxy-6α,7α-epoxy-resiniferonol-14-benzoate (**1**)*

Colorless resin; $[\alpha]_D^{23} + 42$ (*c* 0.05, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 231 (4.75) nm; IR ν_{max} (film) 3588, 2965, 2920, 1728 (br), 1695, 1647, 1458, 1380, 1125 cm^{-1}; HRESIMS obsd *m/z* 525.2082 $[M+Na]^+$, calcd for $C_{27}H_{34}O_9Na$ 525.2095; the spectroscopic data (^1H and ^{13}C NMR) see Table 1.

*2.4. 1,2β-dihydro-5β-hydroxy-6α,7α-epoxy-resiniferonol-14-benzoate (**2**)*

Colorless resin; $[\alpha]_D^{23} + 23$ (*c* 0.05, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 231 (4.62) nm; IR ν_{max} (film) 3585, 2963, 2918, 1730 (br), 1690, 1644, 1440, 1365, 1125 cm^{-1}; HRESIMS obsd *m/z* 525.2082 $[M+Na]^+$, calcd for $C_{27}H_{34}O_9Na$ 525.2095; the spectroscopic data (^1H and ^{13}C NMR) see Table 1.

All the known compounds were identified by comparison the spectroscopic data (^1H and ^{13}C NMR) with those published values.

3. Results and discussion

Compound **1** (Fig. 1) was obtained as a colorless resin. The molecular formula was determined as $C_{27}H_{34}O_9$ based on the sodiated molecular ion peak at *m/z* 525.2082 $[M+Na]^+$ (calcd 525.2095) in the HRESIMS. In the ^1H NMR spectrum, proton signals at δ_H 7.46–7.58 (3H, m) and 8.11 (2H, br d, $J = 8.2$ Hz) suggested the existence of a benzoyl group, two olefinic protons at δ_H 5.19 (br s, 2H) were ascribed to a terminal double bond, and methyl groups at δ_H 1.90 (s, 3H), 1.06 (d, $J = 7.0$ Hz, 3H) and δ_H 1.26 (d, $J = 7.0$ Hz, 3H), were consistent with the presence of

Table 1
^1H NMR and ^{13}C NMR chemical shifts of compounds **1** and **2**.[a]

No	1		2	
	δ_C [a]	δ_H [a]	δ_C [a]	δ_H [a]
1	30.2 t	2.13 m 1.69 m	29.8 t	2.28 m 1.54 m
2	42.2 d	2.26 m	38.7 d	2.76 m
3	218.7 s		218.6 s	
4	75.9 s		75.9 s	
5	73.5 s	4.83 br s	73.3 s	4.88 br s
6	61.9 s		61.8 s	
7	67.3 d	3.29 s	67.0 d	3.30 s
8	39.6 d	3.20 d (2.9)	39.4 d	3.14 d (2.9)
9	73.6 s		73.7 s	
10	53.8 d	2.04 m	54.9 d	2.06 m
11	35.4 d	1.50 m	35.8 d	1.57 m
12	34.3 d	2.15 m 1.63 m	33.8 d	2.14 m 1.63 m
13	73.5 s		73.5 s	
14	76.9 d	5.96 br s	76.9 d	5.99 br s
15	145.1 s		145.0 s	
16	114.0 t	5.19 br s	114.0 t	5.17 br s
17	19.0 q	1.90 s	18.9 q	1.89 s
18	15.8 q	1.06 d (7.0)	15.4 q	1.03 d (7.0)
19	16.7 q	1.26 d (7.0)	16.0 q	1.16 d (7.0)
20	66.0 t	4.02 d (12.0) 3.35 d (12.0)	66.0 t	4.08 d (12.0) 3.28 d (12.0)
1'	167.2 s		167.1 s	
2'	130.0 s		130.0 s	
3', 7'	129.8 d	8.11 br d (8.2)	129.8 d	8.11 br d (8.2)
4', 6'	128.6 d	7.46 m	128.6 d	7.46 m
5'	133.3 d	7.58 m	133.3 d	7.58 m

[a] ^1H NMR measured at 600 MHz; ^{13}C NMR measured at 150 MHz; obtained in $CDCl_3$ with TMS as internal standard; *J* values (Hz) are given in parentheses. Assignments are based on ^1H-^1H COSY, HSQC, and HMBC spectroscopic data.

a tertiary methyl group and two secondary methyl groups, respectively. After subtraction of the benzoyl group mentioned above, there were 20 carbon signals remaining in the ^{13}C NMR spectrum, which were sorted by ^{13}C DEPT NMR experiment into one keto group, eight oxygenated carbons (including one primary, three secondary and four tertiary), a terminal double bond, two methylenes, four methines, and three methyls. These NMR data were very similar to those known daphne diterpene analogs isolated from plants Euphorbiaceae and Thymelaeaceae. When the ^{13}C NMR data of compound **1** were compared with those of daphne diterpenes previously isolated from this plant, the quaternary oxygenated carbon signal around δ_C 117, characteristic of the presence of the *ortho*-ester group was absent, and a carboxy signal at δ_C 167.2 appeared. This observation suggested that compound **1** does not possess the *ortho*-ester group on ring C, which was found in most known daphnane diterpene derivatives. By thus far, the reports on the naturally occurring daphnane diterpenes without this unusual feature are very limited [8,9]. Another notable difference between compound **1** and those known analogs found in the ring A [10–12]. In the ^{1}H NMR spectrum, instead of the signals of an olefinic proton and a vinylic methyl belonging to the α, β unsaturated carboxy group, protons of a methene group (δ_H 2.26, m, 2H) and a secondary methyl group (δ_H 1.26, d, $J = 7.0$, 3H) were observed. Furthermore, a downfield shift around 10 ppm of the ketone carbon signal (δ_C 218.7, C-3) on ring A suggested the absence of the conjugate effect. All these analysis implied that the endocyclic 1(2)-double bond of compound **1** is saturated. In the HMBC spectrum, key correlations of H_3-19/C-3, H-5/C-4, H-20/C-6, H_3-18/C-11, H_3-17/C-3, H-16/C-17 confirmed the presumed daphne diterpene skeleton of compound **1**, and the cross peaks of oxymethine proton signal at δ_H 5.96 (1H, br s, H-14) with C-13 and C-8, suggested the esterification of the benzoyl group on C-14. The stereochemistry of compound **1** was identical with those known daphne diterpene derivatives [10–12] based on the analysis of the NOESY spectrum. The β orientation of C-19 (the methyl group at C-2) was determined by the NOE correlations of H_3-19/H-1β, and H-2/H-10, the α position of the benzoyl group was deduced from the key NOE correlations between H-14 and H-8 (Fig. 2). Thus, the chemical structure of compound **1** was determined as 1,2α-dihydro-5β-hydroxy-6α,7α-epoxy-resiniferonol-14-benzoate (Fig. 1).

The HRESIMS of compound **2** gave a sodiated molecular ion peak at m/z 525.2082 $[M+Na]^+$ (calcd 525.2095), consistent with a molecular formula of $C_{27}H_{34}O_9$, as same as that of compound **1**. The NMR spectra of compound **2** were very similar with those of compound **1**, only with slight difference on ring A. A downfield shift of the proton signal of H-2 from δ 2.28 to 2.76, an upfield shift of H_3-19 from δ 1.26 to 1.16 in ^{1}H NMR, as well as an upfield shift of 4.5 ppm for the carbon signal of C-2 in ^{13}C NMR spectrum were observed. These subtle differences of NMR shifts implied that the methyl group (C-18) on C-2 might adopt the α position rather than the β position in compound **1**. This presumption was further confirmed by the key NOE effect between H_3-19 and H-10 (Fig. 2). Thus, compound **2** was deduced as an isomer of compound **1** at C-2, with the structure determined as 1,2β-dihydro-5β-hydroxy-6α,7α-epoxy-resiniferonol-14-benzoate (Fig. 1).

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