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Steroidal saponins from *Dioscorea panthaica* and their cytotoxic activity

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A new steroidal saponin, dioscoreside E (**1**), and a known compound, protodioscin (**2**), were isolated from an ethanol extract of the rhizomes of *Dioscorea panthaica*. The structure of **1** was established as 3-*O*-[*bis*-α-L-rhamnopyranosyl-(1 → 2 and 1 → 4)–β-D-glucopyranosyl]-26-*O*-β-D-glucopyranosyl-20(*R*)-methoxy-25(*R*)-furosta-5,22(23)-diene-3β,26-diol, on the basis of spectral and chemical evidence. Compounds **1** and **2** showed cytotoxic activity against a panel of tumor cell lines.

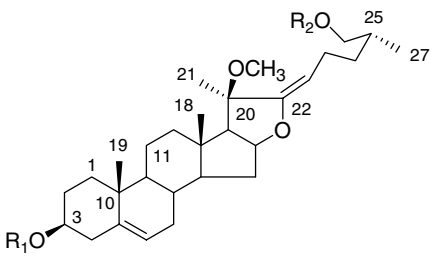
1. Introduction

Dioscorea panthaica Prain et Burkill (Dioscoreaceae), a medicinal plant native to mainland China, is widely distributed in Yunnan, Sichuan, Guizhou and Hunan Provinces of the People's Republic of China. Its rhizomes have long been known in traditional Chinese medicine as a saponin-rich herbal medicine used for the treatment of certain injection and inflammation diseases (Jiangsu New Medical College 1986). Chemical and pharmaceutical investigations have shown that saponins isolated and identified from the genus *Dioscorea* have various pharmaceutical effects, including antibacterial, antifungal, and cytotoxic activities (Li et al. 1986; Takechi et al. 1996; Nakamura et al. 1996; Mimaki et al. 1994). Our previous work on this plant resulted in the fractionation of 11 bioactive steroidal saponins that showed cytotoxicity against a panel of cancer cell lines (Dong et al. 2001a; Dong et al. 2001b). In this paper, further fractionation has led to a new steroidal saponin dioscoreside E (**1**) and the known compound protodioscin (**2**) from the same source. The cytotoxic bioassay indicated that **1** and **2** exhibited significant activity against three cultured cell lines of HeLa, L929, and A375, while were inactive against the normal human liver cell line HL-7702. The structure of **1** was elucidated on the basis of chemical evidence and spectral analysis, especially by 2D NMR techniques.

2. Investigations, results and discussion

Compound **1** was obtained as a white, amorphous powder. Its molecular weight was shown to be 1060 Daltons by negative ion mode FABMS, and the molecular formula was established as $C_{52}H_{84}O_{22}$ by HRFABMS. Acid hydrolysis of **1** afforded D-glucose and L-rhamnose, whose absolute configurations were determined by GLC of their respective trimethylsilyl derivatives (Honbu et al. 2002). The labile aglycon of **1** could not be obtained owing to its decomposition during acid hydrolysis.

Full assignments of the proton and carbon signals of **1** are listed in the Table based on an analysis of its 1D and 2D NMR spectral data. The ^{1}H NMR spectrum of **1** showed the presence of six methyl group signals at δ 0.84 (3 H, s, Me-18), 1.03 (3 H, s, Me-19), 1.08 (3 H, d, J = 6.3 Hz, Me-27), 1.40 (3 H, s, Me-21), 1.62 (3 H, d, J = 6.0 Hz, Rha Me-6‴), and 1.76 (3 H, d, J = 6.0 Hz, Rha Me-6″); four anomeric protons at δ 6.39 (1 H, br s, Rha H-1″), 5.86 (1 H, br s, Rha H-1‴), 4.95 (1 H, d, J = 7.5 Hz, Glc H-1′), and 4.86 (1 H, d, J = 7.2 Hz, Glc H-1⁗); two olefinic protons at δ 5.31 (1 H, br s, H-6) and 4.37 (1 H, br s, H-23); and a methoxyl group at δ 3.17 (3 H, s). The ^{13}C NMR spectrum of **1** showed the presence of 52 carbon atoms in the molecule, of which 24 carbon signals could be assigned to the sugar moieties, in agreement with the four anomeric carbon signals appearing at δ 100.3 (Glc C-1′), 102.2 (Rha C-1″), 103.0 (Rha C-1‴), and 105.0 (Glc C-1⁗). The broad-band decoupled and DEPT spectra displayed seven methyl, 11 methylene, 29 methine, and five quaternary carbon atoms. Four olefinic carbons at δ 140.9 (C-5), 121.8 (C-6), 157.3 (C-22) and 96.4 (C-23); and six methyl groups at δ 13.7 (C-18), 19.5 (C-19), 15.4 (C-21), 17.6 (C-27), 18.8 (Rha C-6″), and 18.6 (Rha C-6‴); and



1 R_1 = -β-D-Glc(2→α-L-Rha, 4→α-L-Rha); R_2 = -β-D-Glc

a methoxyl group at δ 48.9 were also confirmed in the ^{13}C NMR spectrum. The β-nomeric configuration for the two glucopyranosyl units were determined by their large J_{H1-H2} values (> 7.0 Hz), and the two rhamnopyranosyl units were determined to be of the α-configuration based on their C-5 (δ 69.7, 70.5) (Sang et al. 1999). The structure assignment for the aglycon was initiated from the long-range coupling networks observed between the methyl and olefinic protons with the adjacent carbons in an HMBC experiment (Fig.). Detailed analysis of the COSY, HMQC, HMBC, and NOESY NMR spectra indicated that the aglycon of **1** possesses the same partial structure in the A, B, C, and D rings as that of protodioscin (**2**) (Hu et al. 1997). However, compound **1** differed from the latter compound **2** by the presence of a methoxyl group and two sp^2 hybrid carbons (δ 96.4, d and 157.3, s). A HMBC correlation was observed between the methoxyl protons (δ 3.17, MeO−) and the C-20 carbon (δ 82.5), and HMBC correlations between H_3-21 (δ 1.40) and C-20 (δ 82.5), C-17 (δ 66.8), and C-22 (δ 157.3), and between the olefinic proton H-23 (δ 4.37) and C-22 (δ 157.3), C-24 (δ 29.7), and C-25 (δ 35.0) could also be observed (Fig.). These results confirmed that the methoxyl group is attached to C-20 and the double bond (δ 157.3, 96.4) is located at C-22, C-23. The absolute configuration of C-20 was assigned as *R* by correlation between H_3-21 (δ 1.40) and H-17 (δ 2.08) in the NOESY spectrum.

From the ^1H-^1H COSY and HMQC spectra, all proton signals belonging to each sugar moiety in **1** were identified, starting from the anomeric protons. All sugar connectivities were established using HMBC experiments. The sequence of the trisaccharide chain at C-3 of the aglycon was determined by the correlations observed in the HMBC spectrum between Glc H-1′ (δ 4.95) and C-3 (δ 78.2), Rha H-1″ (δ 6.39) and Glc C-2′ (δ 87.6), and Rha H-1‴ (δ 5.86) and Glc C-4′ (δ 78.7). The glucose attached to C-26 of the aglycon was confirmed by the HMBC correlations between Glc H-1⁗ (δ 4.86) and C-26 (δ 75.3).

Based on these data, the structure of compound **1** was established as 3-*O*-[*bis*-α-L-rhamnopyranosyl-(1 → 2 and 1 → 4)-β-D-glucopyranosyl]-26-*O*-β-D-glucopyranosyl-20(*R*)-methoxy-25(*R*)-furosta-5, 22(23)-diene-3β, 26-diol, and was assigned the initial name dioscoreside E. The cytotoxicities of compounds **1** and **2** were tested against HeLa, L929, and A375 cell lines. Compound **1** showed IC_{50} values of 8.30 ± 2.32, 10.09 ± 3.58, and 11.79 ± 3.21 μM, and compound **2** showed IC_{50} values of 4.23 ± 1.36, 5.89 ± 1.89, and 4.79 ± 2.56 μM, respectively. Cytotoxicity of compounds **1** and **2** against a normal human liver cell line HL-7702 was also tested. However, both of them were inactive (IC_{50} > 50 μM, respectively). Comparison of the data obtained from cancer cell lines and normal cells clearly showed that compounds **1** and **2** exhibited selective cytotoxic activities against cancer cell lines. More detailed analyses of the mechanisms of the cytotoxic action against target tumor cells are in progress.

Table: ^1H and ^{13}C NMR spectral data for 1 in C_5D_5N

Position	δ_H	δ_C
1	0.92, 1.67	37.6
2	1.82, 2.05	30.3
3	3.84	78.2
4	2.72, 2.78	39.0
5		140.9
6	5.31	121.8
7	1.86	32.1
8	1.50	31.1
9	0.89	50.1
10		37.1
11	1.44	20.6
12	1.09, 2.16	39.3
13		40.4
14	0.82	56.8
15	1.91, 2.04	33.6
16	4.96	84.1
17	2.08	66.8
18	0.84	13.7
19	1.03	19.5
20		82.5
21	1.40	15.4
22		157.3
23	4.37	96.4
24	1.53, 1.64	29.7
25	1.77	35.0
26	4.06	75.3
27	1.08	17.6
OCH_3	3.17	48.9
C-3 sugars		
Glc		
1′	4.95 (d, 7.5)	100.3
2′	4.42	78.6
3′	4.22	77.0
4′	4.40	78.7
5′	3.85	77.9
6′	4.10, 4.23	61.4
Rha (1 → 2)		
1″	6.39 (br s)	102.2
2″	4.81	72.6
3″	4.60	72.8
4″	4.36	74.2
5″	4.94	69.7
6″	1.76 (d, 6.0)	18.8
Rha (1 → 4)		
1‴	5.86 (br s)	103.0
2‴	4.68	72.6
3‴	4.55	72.9
4‴	4.32	74.0
5‴	4.92	70.5
6‴	1.62 (d, 6.0)	18.6
C-26 sugar		
Glc		
1⁗	4.86 (d, 7.2)	105.0
2⁗	4.03	75.3
3⁗	4.28	78.5
4⁗	4.34	71.8
5⁗	4.00	78.0
6⁗	4.35. 4.55	62.9

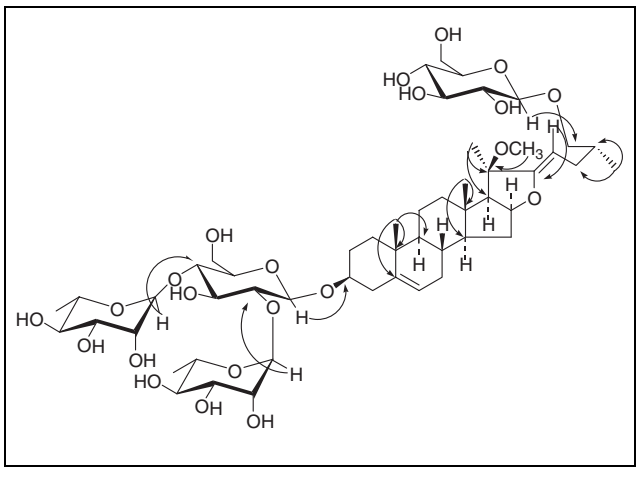


Fig.: Selected HMBC correlations for dioscoreside E (1)

3. Experimental

3.1. General procedures

Melting points were determined on a Yanaco MP-S_3 micro-melting point apparatus (uncorrected). Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter. IR spectra were measured on a Bruker IFS 55 spectrometer. ^{1}H-, ^{13}CNMR, DEPT and 2D-NMR (HMQC, HMBC, ^{1}H-^{1}H COSY, NOESY) spectra were taken on a Bruker ARX-300 MHz spectrometer in C_5D_5N solutions, tetramethylsilane (TMS) as the internal reference. HRFABMS in a glycerol matrix were recorded on a VG-Autospec-3000 instrument. Column chromatography and TLC were carried out on silica gel H (10–40 μm, Qingdao Haiyang Chemical Factory). Spots for all were detected by spraying with 10% H_2SO_4/EtOH solution or vanillin/H_2SO_4/EtOH, followed by heating. HPLC: Shimadzu LC-8A (SPD-6AV UV-VIS detector) equipped with an ODS column (Phenomenex, 10.0 mm i.d. × 250 mm). GLC: Shimadzu GC-7A, Column: silicone OV-17 on Uniport HP (80–100 mesh), 3 mm i.d. × 2.1 m; column temperature, 160 °C; carrier gas, N_2, flow rate 30 mL/min. Cells: obtained from ATCC, Rocksville, MD; RPMI 1640 medium: Gibco, Grand Island, NY; Microplate reader: Model 450, Biorad, Richmond, VA; all other chemicals used were of biochemical reagent grade.

3.2. Plant material

The rhizomes of *Dioscorea panthaica* Prain et Burkill (Dioscoreaceae) were collected from Yaan County, Sichuan Province (People's Republic of China), in August 1997, and were identified by Prof. Qishi Sun (Department of Pharmacognosy, Shenyang Pharmaceutical University, China). A voucher specimen is deposited at the herbarium of Shenyang Pharmaceutical University (No. 1068).

3.3. Extraction and isolation

The rhizomes (10 kg) of *D. panthaica* were refluxed with 90% EtOH three times and the extract was condensed to produce a residue (440 g) under reduced pressure. An aliquot (50 g) of extract was subjected to chromatographic separation on a Si gel column (1000 g) (7.5 cm × 60 cm), by elution with $CHCl_3$–MeOH (9 : 1, 6 : 1, 4 : 1, 3 : 1, 1 : 1), to afford 5 major fractions (I–V). Fr. IV was further separated by preparative HPLC with the soluent MeOH–H_2O (70 : 30) to afford compounds **1** (48 mg) and **2** (32.5 mg).

3.4. Dioscoreside E (*1*)

White amorphous powder; mp 166–168 °C; $[\alpha]_D^{25}$ – 61.2° (c 0.01, pyridine); IR (KBr) ν_{max} 3400 (OH), 2950, 1040 (C–O) cm^{-1}; ^{1}H NMR (300 MHz, C_5D_5N) and ^{13}C NMR (75 MHz, C_5D_5N), see Table 1; FABMS m/z 1059 $[M-H]^-$, 897 $[M-H-162]^-$, 751 $[M-H-162-146]^-$, 605 $[M-H-162-146\times2]^-$, 443 $[M-H-1622-1462]^-$; HRFABMS m/z 1059.5364 (calcd for $C_{52}H_{83}O_{22}$, 1059.5376).

3.5. Acid hydrolysis of 1

A solution of **1** (5 mg) in 1 M HCl (dioxane-H_2O, 1 : 1, 1 mL) was heated at 100 °C for 2 h in a sealed tube. After dioxane was removed, the solution was extracted with EtOAc (1 mL × 3). The extract was washed with H_2O and then evaporated to dryness under a vacuum. The monosaccharide portion was neutralized by passage through an exchange resin (Amberlite MB-3) column, concentrated (dried overnight), and then treated with 1-(trimethylsilyl)imidazole at room temperature for 2 h. After the excess reagent was decomposed with H_2O, the reaction products were extracted with hexane (1 mL × 2). The TMSi derivatives of the monosacchrides were identified as D-glucose and L-rhamnose in a ratio of 1 : 1 by GLC analysis with standard monosaccharides.

3.6. Cytotoxicity assays

A375 (human melanoma), L929 (murine pneumoepithelial carcinoma), HeLa (murine cervicoma), and HL-7702 (normal human liver) cells were used for the detection of cytotoxic activity. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated (56 °C for 30 min) fetal calf serum (FCS), 1% L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin sulfate. All cell lines were maintained in an incubator at 37 °C in humidified air containing 5% CO_2. The cytotoxic activity of compounds against A375, L929 and Hela cell lines was measured by an MTT assay procedure (Chen et al. 1999; Gerlier and Thomasset 1993). A dose response curve was plotted for these samples and the IC_{50} was calculated as the concentration of the test compound resulting in a 50% reduction of absorption compared to the control cells in the MTT assay.

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References

Chen JJ, Duh CY, Chen IS (1999) New tetrahydroprotoberberine *N*-oxide alkaloids and cytotoxic constituents of *Corydalis tashiroi*. Planta Med 65: 643–647.

Dong M, Feng XZ, Wang BX, Wu LJ, Ikejima, T (2001a) Two novel furostanol saponins from the rhizomes of *Dioscorea panthaica* Prain et Burkill and their cytotoxic activity. Tetrahedron 57: 501–506.

Dong M, Feng XZ, Wu LJ, Wang BX, Ikejima, T (2001b) Two novel steroidal saponins from the rhizomes of *Dioscorea panthaica* and their cytotoxic activity. Planta Med 67: 853–857.

Gerlier D, Thomasset N (1993) Use of MTT colorimetric assay to measure cell activation. J Immunol Meth 65: 55–63.

Honbu T, Ikeda T, Zhu X, Yoshihara O, Okawa M, Nafady AM, Nohara T (2002) New steroidal glycosides from the fruits of *Solanum anguivi*. J Nat Prod 65: 1918–1920.

Hu K, Dong AJ, Yao XS, Kobayashi H, Iwasaki S (1997) Antineoplastic agents; II. Four furostanol glycosides from rhizomes of *Dioscorea collettii* var. *hypoglauca*. Planta Med 63: 161–165.

Jiangsu New Medical College (1986) Dictionary of Chinese Herb Medicine. Shanghai Press of Science and Technology, Shanghai, p. 729–730.

Li BG, Tang YF, Shi Y (1986) Isolation and identification of steroidal saponins from *Dioscorea panthaica* Prain et Burkill. Zhiwu Xuebao 28: 409–414.

Mimaki K, Nakamura O, Sashida Y, Satomi Y, Nishino A, Nishino H (1994) Steroidal saponins from the bulbs of *Lilium longiflorum* and their antitumour-promoter activity. Phytochemistry 37: 227–232.

Nakamura T, Komori C, Lee YY, Hashimoto F, Yahara S, Nohara T (1996) Cytotoxic activities of solanum steroidal glycosides. Biol Pharm Bull 19: 564–566.

Sang SM, Lao A, Wang HC, Chen IL (1999) Furostanol saponins from *Allicum tuberosum*. Phytochemistry 52: 1611–1615.

Takechi M, Uno C, Tanaka K (1996) Structure-activity relationships of synthetic saponins. Phytochemistry 41: 121–123.