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Steroidal saponins with cytotoxic activity from the rhizomes of *Paris polyphylla* var. *yunnanensis*

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ABSTRACT

Two previously unreported spirostanol saponins, dianchonglouosides A and B (**1** and **2**), along with 7 known steroidal saponins (**3**–**9**) were isolated from the rhizomes of *Paris polyphylla* var. *yunnanensis*. Their structures were elucidated by extensive spectroscopic analysis (MS, 1D, and 2D NMR), and chemical methods. The cytotoxic activities of the isolated compounds **1**–**9** were also evaluated against two human cancer cell lines (HEK293 and HepG2). The results showed that compound **7** had the strongest cytotoxic activity against the two cancer cell lines with the IC_{50} values of 0.6 and 0.9 μM, respectively.

1. Introduction

The genus *Paris* (family Liliaceae) is widely distributed in many regions of the Eurasian continent from temperate zone to the tropics, which contains more than 24 species throughout the world. *Paris polyphylla* var. *yunnanensis* (“Dian Chonglou” in Chinese) is one of the most famous species of this genus, because this species has been traditionally used as anti-tumor, hemostatic, antimicrobial, and analgesic agents (Li, 1998) and is also an important ingredient of some Chinese patent medicines, such as “Gongxuening Capsules”, “Jidesheng Sheyao Tablet”, “Biyan Qingdu Keli”, *etc.* (Tian et al., 1986; Guo et al., 2006). Previous reports have revealed that the steroidal saponins were its main bioactive components and more than 70 steroidal saponins have been isolated from the medium polarity part of the extract of the titled plant (Chen and Zhou, 1981; Liu et al., 2006; Zhao et al., 2009; Wu et al., 2012; Kang et al., 2012; Qin et al., 2012). However, the chemical constituents of the water-soluble part of the crude extract are still not clear except for some furostanol saponins (Zhao et al., 2009; Matsuda et al., 2003). The objective of the present research was to elucidate the chemical constituents of the 40% EtOH eluent (water-soluble part) of a macroporous resin column from the ethanol extract of *P. polyphylla* var. *yunnanensis*. As a result, nine steroidal saponins (**1**–**9**) were obtained, two of which were previously unknown. In addition, the cytotoxicity of the isolated compounds against HEK293 and HepG2 cells was evaluated. Herein, we report the isolation, structural elucidation, and cytotoxicity of these steroidal saponins.

2. Results and discussion

The 75% EtOH extract of the rhizomes of *P. polyphylla* var. *yunnanensis* was subjected to a HPD 100 macroporous resin column and resulted in four fractions: H_2O fraction, 40% EtOH fraction, 70% EtOH fraction, and 95% EtOH fraction. The 40% EtOH fraction was repeatedly subjected to column chromatography on silica gel, Sephadex LH-20, Rp-18, and semi-preparative HPLC to yield nine compounds, including two new compounds, dianchonglouosides A (**1**) and B (**2**), together with seven known ones (**3**–**9**). The known compound were identified by comparing their spectroscopic data with published results as paris saponin XI (**3**) (Liu et al., 2006), parisyunnanoside H (**4**) (Kang et al., 2012),

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parissaponin I (**5**) (Matsuda et al., 2003), trigofoenoside A (**6**) (Gupta et al., 1985), dichotomin (**7**) (Munday et al., 1993), parisyunnanoside B (**8**) (Zhao et al., 2009), and pseudoproto-Pb (**9**) (Hirai et al., 1986).

Compound **1** was obtained as a white, amorphous powder. The molecular formula was deduced as $C_{44}H_{70}O_{18}$ based on its negative HR-ESI-MS ion peak at *m/z* 885.4466 $[M-H]^-$ (cald. 885.4484) and ^{13}C NMR spectrum (Table 1). The IR absorptions revealed the presence of hydroxyl (3440 cm^{-1}) and double bond (1632 cm^{-1}). The ^1H NMR spectrum of **1** (Table 1) showed signals for two methyl singlets at δ_H 0.95 (s, Me-18) and 1.07 (s, Me-19), two methyl doublets at δ_H 1.24 (d, *J* = 7.1 Hz, Me-21) and 1.75 (d, *J* = 6.1 Hz, Me-6″), and an olefinic proton at δ_H 5.27 (br s, H-6). The ^{13}C NMR and DEPT spectra exhibited 44 carbon signals, including 4 methyls, 13 methylenes, 22 methines, and 5 quaternary carbons, 27 of which could be contributed to the aglycone part. A characteristic quaternary carbon signal at δ_C 110.1 suggested **1** to be a spirostanol glycoside. Comparison of the ^1H and ^{13}C NMR spectroscopic data of **1** (Table 1) with those of ypslandroside C (Xie et al., 2009) allowed the identification of the aglycone of **1** as (25*S*)-spirost-5-en-3β,17α,27-triol. Furthermore, the configuration of the aglycone of **1** was confirmed by the key ROESY correlations of Me-19/H-1β, H-1α/H-3, Me-18/H-8, Me-18/H-20, H-9/H-14, and H-14/H-16. For the sugar moiety, the three anomeric protons at δ_H 4.94 (d, *J* = 7.3 Hz, H-1′), 6.28 (br s, H-1″), and 5.92 (br s, H-1‴) showed correlations with the anomeric carbon resonances at δ_C 100.0, 101.8, and 109.5 in the HSQC spectrum, respectively. Acid hydrolysis of **1** afforded D-glucose, L-rhamnose, and L-arabinose, which were determined by GC chromatographic analysis of their L-cysteine methyl ester-TMS derivates. The β-configuration for the glucopyranosyl moiety was confirmed by the *J* value ($^3J_{1,2}$ = 7.3 Hz) of the anomeric proton signal. The α-configuration of the anomeric proton of the rhamnopyranosyl was deduced from the ^{13}C NMR data of C-3″ (δ_C 72.7) and C-5″ (δ_C 69.4) with those of the corresponding carbons of methyl α- and β-rhamnopyranoside (Kasai et al., 1979). Meanwhile, the configuration of the anomeric center of arabinofuranosyl was determined as α because the anomeric proton of the arabinofuranosyl exhibited a singlet resonance at δ_H 5.92, br s (Idaka et al., 1991). The linkage points of the sugar units to each other and to C-3 of the aglycone were established from the HMBC correlations of signals at δ_H 4.94 (H-1′) with δ_C 77.9 (C-3), δ_H 6.28 (H-1″) with δ_C 77.3 (C-2′), and δ_H 5.92 (H-1‴) with δ_C 76.9 (C-4′). This was also supported by the ROESY correlations of signals at δ_H 4.94 (H-1′) with δ_H 3.83 (H-3), δ_H 6.28 (H-1″) with δ_H 4.19 (H-2′), and δ_H 5.92 (H-1‴) with δ_H 4.34 (H-4′). Based on the above information, the structure of compound **1** was elucidated as (25*S*)-spirost-5-en-3β,17α,27-triol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-arabinofuranosyl-(1 → 4)]-β-D-glucopyranoside, and given a trivial name of dianchonglouoside A (Fig. 1).

Compound **2** had the same molecular formula of $C_{44}H_{70}O_{18}$ as that of **1**, which was determined by the HR-ESI-MS (*m/z* 885.4463 $[M-H]^-$; cacl. 885.4484) and ^{13}C NMR spectroscopic data (Table 1). A comparison of the NMR and MS data of **2** with those of **1** revealed that they were similar, except for the presence of an oxymethine at C-23 (δ_C 3.96 m; δ_C 67.5) and the disappearance of an oxygenated quaternary carbon (δ_C 90.0). Thus, the aglycone of **2** was suggested as (23*S*,25*S*)-spirost-5-en-3β,23,27-triol, which was indicated by comparing its 1D NMR data with those of the aglycone of paris saponin XI (**3**). The hydroxyl group was placed at C-23 based on the ^1H–^1H COSY correlations from the proton signal at δ_H 3.96 (H-23) with H_2-24 (δ_H 2.31 and 2.04) and the HMBC correlations of H-20 (δ_H 3.06) and H-25 (δ_H 2.30) with δ_C 67.5 (C-23). The configuration of H-23 and H-25 was characterized as β-oriented by the ROESY correlations of H-23 (δ_H 3.96)/H-20 (δ_H 3.06), H-23/Me-21 (δ_H 1.20), and H-23/H-25 (δ_H 2.03) (Fig. 2). The saccharide moiety of **2** was consistent with the compounds **1**, **5**, and **8** by the NMR data and HMBC correlations. Therefore, the structure of **2** was determined as (23*S*,25*S*)-spirost-5-en-3β,23,27-triol-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-arabinofuranosyl-(1 → 4)]-β-D-glucopyranoside, and given a trivial name of dianchonglouoside B.

Considering that the cytotoxic activities of steroidal saponins from the rhizomes of this plant have been extensively examined, the *in vitro* cytotoxic activities of the steroidal saponins (**1**–**9**) were evaluated against HEK293 and HepG2 human cancer cell

Table 1
NMR data (600 MHz for ^1H and 125 MHz for ^{13}C) for **1** and **2** in pyridine-d_5 (δ in ppm and *J* in Hz).

Position	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
1	37.4 t	1.73 m 0.93 m	37.4 t	1.70 m 0.95 m
2	30.0 t	2.05 m 1.85 m	30.0 t	2.05 m 1.83 m
3	77.9 d	3.83 m	78.0 d	3.85 m
4	38.8 t	2.74 m 2.69 m	38.8 t	2.75 m 2.69 m
5	140.6 s		140.6 s	
6	121.7 d	5.27 br s	121.7 d	5.26 d (2.4)
7	32.3 t	1.89 m 1.51 m	32.0 t	2.01 m 1.47 m
8	32.2 d	1.60 m	31.4 d	1.48 m
9	50.1 d	0.95 m	50.1 d	0.87 m
10	37.0 s		37.0 s	
11	20.8 t	1.58 m 1.49 m	21.0 t	1.39 m (2H)
12	32.0 t	2.18 m 1.60 m	40.0 t	1.71 m 1.10 m
13	45.1 s		40.9 s	
14	52.9 d	2.07 m	56.5 d	1.07 m
15	31.7 t	2.19 m 1.78 m	32.2 t	2.01 m 1.79 m
16	89.9 d	4.48 pt (7.4)	81.6 d	4.65 m
17	90.0 s		62.3 d	1.88 m
18	17.0 q	0.95 s	16.4 q	1.01 s
19	19.3 q	1.07 s	19.2 q	0.98 s
20	44.8 d	2.29 q (7.0)	35.7 d	3.06 m
21	9.7 q	1.24 d (7.1)	14.6 q	1.20 d (6.9)
22	110.1 s		112.1 s	
23	31.7 t	1.78 m (2H)	67.5 d	3.96 m
24	23.5 t	1.87 m 1.79 m	33.4 t	2.31 m 2.04 m
25	38.9 d	2.05 m	40.4 d	2.30 m
26	63.8 t	4.06 m 3.89 m	63.1 t	4.08 m 3.89 m
27	64.3 t	3.73 m 3.63 br s	63.8 t	3.74 d (7.4) 3.69 dd (10.1, 7.2)
Glc-1′	100.0 d	4.94 d (7.3)	100.0 d	4.94 d (7.3)
2′	77.3 d	4.19 m	77.3 d	4.19 m
3′	77.6 d	4.23 m	77.6 d	4.23 m
4′	76.9 d	4.34 m	76.9 d	4.34 m
5′	76.6 d	3.74 m	76.6 d	3.74 m
6′	61.2 t	4.26 m 4.21 m	61.2 t	4.27 m 4.21 m
Rha-1″	101.8 d	6.28 br s	101.8 d	6.28 br s
2″	72.4 d	4.78 br s	72.4 d	4.78 m
3″	72.7 d	4.59 d (8.7)	72.7 d	4.56 m
4″	74.0 d	4.34 m	74.0 d	4.34 m
5″	69.4 d	4.92 m	69.4 d	4.93 m
6″	18.5 q	1.75 d (6.1)	18.5 q	1.75 d (6.1)
Ara-1‴	109.5 d	5.92 br s	109.5 d	5.92 br s
2‴	82.6 d	4.85 m	82.6 d	4.84 m
3‴	77.8 d	4.84 m	77.8 d	4.82 m
4‴	86.5 d	4.86 m	86.6 d	4.86 m
5‴	62.4 t	4.25 m 4.15 m	62.4 t	4.26 m 4.16 m

Assignments were based on the HMBC, HSQC, COSY and DEPT experiments.

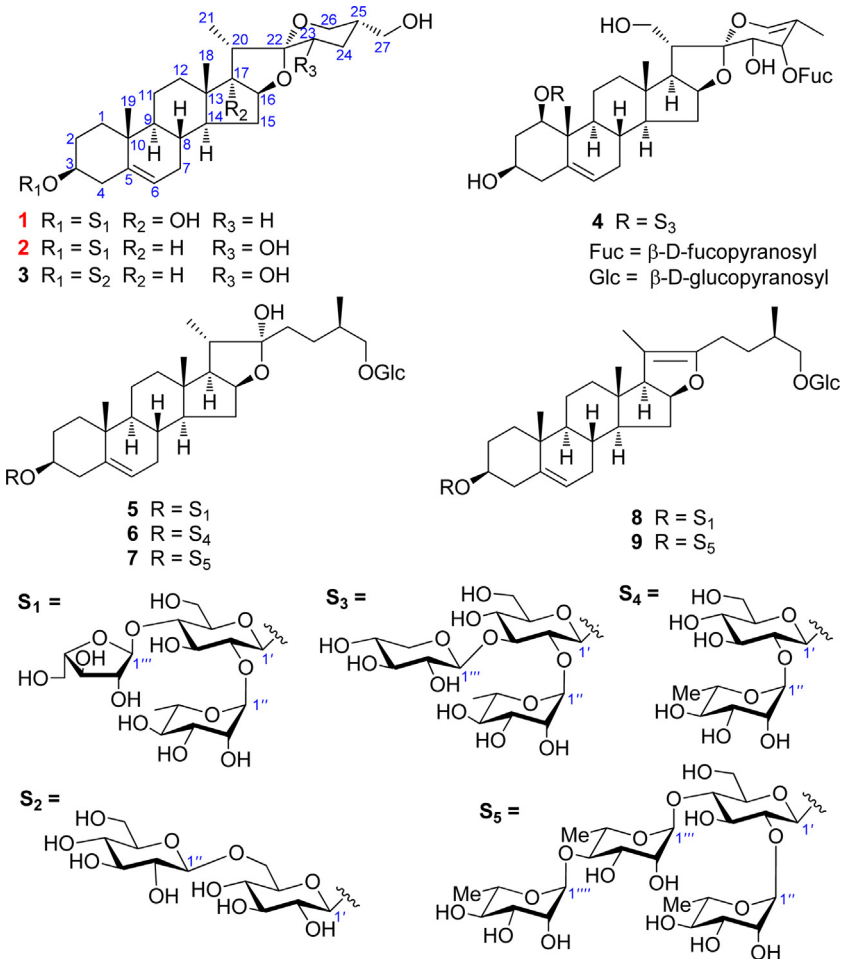

Fig. 1. The chemical structures of compounds **1**–**9**.

lines using the MTT method. (−)-OddC was used as a positive control substance with IC_{50} values of 0.30 μM and 0.17 μM against the two cell lines, respectively. The results showed that compounds **4**–**9** exhibited significant inhibitory effects against HEK293 cell lines with IC_{50} values of 0.9, 1.8, 3.4, 0.58, 2.5, and 1.8 μM, respectively, while compounds **4**–**9** exhibited significant inhibitory effects against HepG2 cell lines with IC_{50} values of 5.6, 1.8, 5.6, 0.9, 1.2, and 1.8 μM, respectively. However, the spirostanol saponins **1**–**3** were inactive. This study demonstrated that the 40% EtOH eluent of a macroporous resin column from the ethanol extract of *P. polyphylla* var. *yunnanensis* contained spirostane glycosides with one or two hydroxyl groups in F-ring except for furostanol glycosides.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a JASCO P-1020 digital polarimeter. IR spectra were recorded on a Bruker Tensor-27 infrared spectrophotometer with KBr pellets. NMR spectra were performed on Bruker AM-400 and Avance III 600 instruments with chemical shifts given in ppm (δ), using TMS (tetramethylsilane) as an internal standard. ESI-MS spectra were recorded on a Bruker HTC/Esquire spectrometer. HR-ESI-MS spectra were recorded on an API Qstar Pulsar instrument. Column Chromatography (CC) was performed on HPD100 macroporous resin column (40–60 mesh,

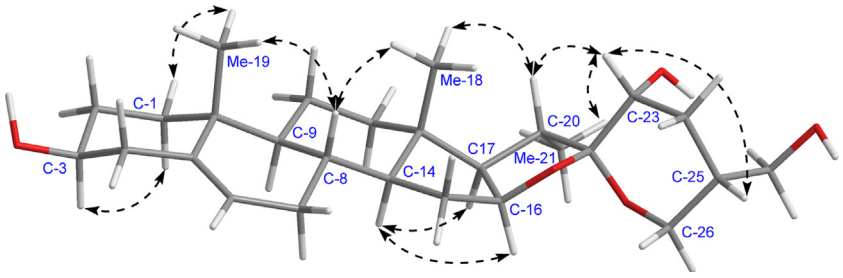

Fig. 2. Significant ROESY correlations of **2**.

Canzhou Bon Adsorber Technology Co. Ltd., China), silica gel (200–300 mesh, Qingdao Marine Chemical Co. Ltd., Qingdao, China), Rp-18 (40–63 μm, Merk). Fractions were monitored by TLC (GF254, Qingdao Marine Chemical Co. Ltd., Qingdao, China), and by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol. Semi-preparative HPLC was run on an Agilent 1100 liquid chromatograph with diode array detector (DAD), Zorbax-SB-C18 column (5 μm; 25 cm × 9.4 mm i.d.). GC analysis was performed on a HP5890 gas chromatograph equipped with an H_2 flame ionization detector.

3.2. Plant material

The dried and powdered rhizomes of *P. polyphylla* var. *yunnanensis* were purchased in November 2000 from Kunming Juhuacun traditional Chinese medicinal materials market, Yunnan Province, China. The plant material was authenticated by one of the authors, Prof. Chang-Xiang Chen. A voucher specimen (No. HY0018) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China.

3.3. Extraction and isolation

The powdered and dried rhizomes of *P. polyphylla* var. *yunnanensis* (500 g) were extracted three times (1 h per time) with 75% EtOH (2 L) by ultrasound. After evaporation, the crude extract (119 g) was chromatographed on a HPD 100 macroporous resin column, eluting with a gradient mixture of aqueous EtOH (0%, 40%, 70%, and 95%, v/v). The fraction (12.6 g) eluted by 40% EtOH was subjected to an Rp-18 silica gel column and eluted with EtOH-H_2O gradients (1:9 → 7:3). The fractions were examined by TLC and combined to give eight portions (Fr.1-8). Fr.3 (50 mg) was separated by a Sephadex LH-20 CC ($CHCl_3$-MeOH, 1:1) and then further purified by semi-prep. HPLC (MeCN-H_2O, 35:65, v/v) to afford **3** (9 mg). Fr.5 (3.8 g) were isolated by silica gel CC ($CHCl_3$-EtOH-H_2O, 8:2:0.2 → 7:3:0.5, v/v), and further purified by semi-prep. HPLC (MeCN-H_2O, 70:30, v/v) to give **1** (8 mg), **2** (13 mg), **5** (124 mg), **6** (12 mg), and **7** (14 mg). Fr.6 (100 mg) was separated by semi-prep. HPLC (MeCN-H_2O, 30:70, v/v) to afford **9** (10 mg). Compounds **4** (8 mg) and **8** (15 mg) were isolated from Fr.7 320 mg) by semi-prep. HPLC (MeCN-H_2O, 35:65 → 45:55, v/v).

3.3.1. Dianchonglouoside A

White amorphous powder; $[\alpha]_D^{22}$ −97.5 (*c* 0.8, MeOH); IR ν_{max} (KBr) cm^{-1}: 3440, 2932, 1632, 1453, 1382, 1132, 1072, 1052, 1042, 995; ^1H (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz) spectroscopic data, see Table 1; negative ESIMS *m*/*z* 921 $[M-H+Cl]^-$; HRESIMS *m*/*z* 885.4466 $[M-H]^-$ (calcd. for $C_{44}H_{69}O_{18}$, 885.4484).

3.3.2. Dianchonglouoside B

White amorphous powder; $[\alpha]_D^{22}$ −126.3 (*c* 0.1, MeOH); IR ν_{max} (KBr) cm^{-1}: 3440, 2930, 1634, 1453, 1384, 1130, 1069, 1049, 1000, 962; ^1H (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz) spectroscopic data, see Table 1; negative ESIMS *m*/*z* 885 $[M-H]^-$, 921 $[M-H+Cl]^-$; HRESIMS *m*/*z* 885.4463 $[M-H]^-$(calcd. for $C_{44}H_{69}O_{18}$, 885.4484).

3.4. Acidic hydrolysis

The hydrolysis and GC analysis of the chiral derivates of the sugars of compounds **1** and **2** were done as previously described (Qin et al., 2012). D-Glucose, L-rhamnose, and L-arabinose were detected for **1** and **2**.

3.5. Cytotoxicity assays

Cytotoxicity evaluations were performed on two human cell lines (HEK293 and HepG2) using the MTT method described in the literature elsewhere (Mosmann, 1983). (−)-OddC was used as a positive control substance. The experiments were conducted in three independent replicates, and $IC_{50} > 30$ μM was considered to be inactive.

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