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Steroidal saponins with induced platelet aggregation activity from the aerial parts of *Paris verticillata*

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ABSTRACT

In order to utilize and protect the resources of Rhizoma Paridis rationally, we carried out a phytochemical investigation on the non-medicinal (aerial) parts of *Paris verticillata* that led to the isolation of fifteen steroidal saponins. Among them, three are new spirostanol saponins, named parisverticosides A–C (**1**–**3**), as well as one new cholestane glycoside, named parisverticoside D (**4**). Their structures were elucidated by extensive spectroscopic analysis and acid hydrolysis. The aglycone of compound **1** is a new spirostane and identified as (23*S*,24*S*,25*S*)-spirost-5-en-1β,3β,23, 24-tetraol. The selected isolates were evaluated for induced platelet aggregation activity and compound **5** showed 62% maximal platelet aggregation rate at the concentration of 300 μg/mL.

1. Introduction

The genus *Paris* (family Liliaceae) consists of more than 24 species of perennial herbs distributed from Europe to eastern Asia. *Paris* is notable in China for its medicinal value. The species with thick rhizomes are traditional medicinal herbs and the major source of raw material for some patent medicines, e.g. 'Gongxuening Capsule', well-known for its use as a hemostatic [1]. Recently, Cong's group reported that pennogenin glycosides with a spirostanol structure isolated from *Paris polyphylla* var. *yunnanensis* were the active ingredients in promoting hemostasis *in vivo* and directly induced platelet activation by dense granule secretion of ADP, which in turn activated the P2Y1 and P2Y12 receptor signaling pathways [2,3]. Due to long growth cycle and the expansion of industrial demand, the wild populations of this genus are dramatically decreased, which reminds us to find alternative resources urgently. However, the non-medicinal parts (including leaves, stems, fibril, etc.) of this herb, which can regenerate every year, were thrown away. In order to utilize the discarded resources rationally, our group is carrying out systematic phytochemical investigations of the constituents and bioactivity of the non-medicinal parts of the genus *Paris* [4,5].

Paris verticillata is widely distributed in the north of China and has been used to treat febrile convulsion, snake bites, and sore throat in Chinese folk medicine [6]. Previous chemical investigations resulted in the isolation of steroids, steroidal saponins, phenols, pyrrolizidine alkaloids, and cyclopropanoic fatty acid glycosides, some of which showed cytotoxicity and anti-neuroinflammatory effect [7–11]. We found that the 75% EtOH eluant of the 70% ethanolic extract of the aerial parts of *P. verticillata* on a macroporous resin column showed 53% maximal platelet aggregation rate at the concentration of 1.5 mg/mL. With the aim of searching for the active constituents, we have examined the 70% EtOH eluant and obtained four new steroidal saponins, named parisverticosides A–D (**1**–**4**), and 11 known steroidal saponins. This paper presents the isolation, structural elucidation, and bioactivities of these steroidal saponins.

2. Experimental

2.1. General methods

Optical rotations were measured on a Jasco P-1020 digital polarimeter. IR spectra were obtained on Bruker Tensor-27 infrared spectrophotometer with KBr pellets. ESI-MS spectra were recorded on a Bruker HTC/Esquire spectrometer, HREIMS spectra were recorded on a Waters AutoSpec Premier P776 instrument.

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HRESIMS spectra were recorded on a Shimadzu LCMS-IT-TOF mass spectrometeter instrument. NMR experiments were performed on Bruker DRX-500 instrument with TMS as the internal standard. Chemical shifts (δ) were expressed in ppm with reference to the solvent signals. Column chromatography (CC) was performed on YWD-3F macroporous resin, MCI-gel CHP20P (75–150 μm; Mitsubishi Chemical Co.), silica gel (200–300 mesh, Qingdao Marine Chemical Co., China), RP-18 (40–63 μm, Merck), and Sephadex LH-20 (GE Healthcare, Sweden). TLC was performed on $HSGF_{254}$ (0.2 mm, Qingdao Marine Chemical Co., China) or RP-18 F_{254} (0.25 mm, Merck). Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. GC analysis was performed on a HP5890 gas chromatograph equipped with an H_2 flame ionization detector. Semi-preparative HPLC was run on Agilent 1100 liquid chromatograph with diode array detector (DAD), Zorbax-SB-C18 column (5 μm; 25 cm × 9.4 mm i.d).

2.2. Plant material

The aerial parts of *P. verticillata* were collected in July 2012 from Panshi, Jilin Province, China, and identified by Prof. Heng Li of the Kunming Institute of Botany. A voucher specimen (No. HY0014) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China.

2.3. Extraction and isolation

The dried aerial parts of *P. verticillata* (5 kg) were extracted three times with 70% EtOH (20 L × 3) under reflux for a total of 6 h and the combined extract was concentrated under reduced pressure. Then the concentrated extract was dissolved in H_2O and passed through an YWD-3F macroporous resin column, eluting with H_2O and 75% EtOH. The 75% EtOH fraction was subjected to a MCI gel column chromatography (MeOH–H_2O, 0:1 → 1:0, v/v) to give six fractions. Fr.2 (110 g) was separated by a silica gel column chromatography ($CHCl_3$–MeOH, 3:1 → 0:1) to give five fractions. Fr.2-2 (120 mg) was then separated by a silica gel column chromatography ($CHCl_3$–MeOH, 3:1) and sem-prep. HPLC (MeCN–H_2O, 25:75) to yield **8** (8 mg). Fr.2-3 (2.6 g) was passed through an RP-18 column (MeOH–H_2O, 15:85 → 30:70) to give three fractions. Fr.2-3-2 (230 mg) and Fr.2-3-3 (150 mg) were separated by semi-prep. HPLC (MeCN–H_2O, 22:78) respectively to give **11** (30 mg) and **12** (62 mg). Fr.2-4 (45 g) was chromatographed by an RP-18 column (MeOH–H_2O, 10:90 → 30:70) to give Fr.2-3-1, Fr.2-3-2, Fr.2-3-3, and **14** (1.9 g). Fr.2-3-2 (50 mg) and Fr.2-3-3(46 mg) were purified by semi-prep. HPLC (MeCN–H_2O, 20:80) to afford **13** (18 mg) and **15** (15 mg), respectively. Fr.2-5 (200 mg) was separated by an RP-18 column chromatography (MeOH–H_2O, 15:85 → 30:70) then semi-prep. HPLC (MeCN–H_2O, 15:85) to yield **4** (11 mg). Fr.3 (9 g) was isolated with a silica gel column chromatography ($CHCl_3$–MeOH, 4:1 → 1:1) to give four fractions. Fr.3-1 (120 mg) was purified by semi-prep. HPLC (MeCN–H_2O, 25:75) to afford **3** (51 mg). Fr.3-2 (1.1 g) was subjected to an RP-18 column chromatography (MeOH–H_2O, 30:70 → 40:60) to give four fractions. Fr.3-2-2 (35 mg) and Fr.3-2-3 (41 mg) were separated by semi-prep. HPLC (MeCN–H_2O, 30:70) to yield **1** (11 mg) and **2** (10 mg), respectively. Fr.3-3 (2.3 g) was subjected to an RP-18 column chromatography (MeOH–H_2O, 30:70 → 40:60) to give three fractions. Fr.3-3-2 (100 mg) was then given to semi-prep. HPLC (MeCN–H_2O, 23:77) to afford **9** (43 mg). Fr.3-3-3 (80 mg) was purified by semi-prep. HPLC (MeCN–H_2O, 25:75) to yield **10** (31 mg). Fr.4 (19 g) was isolated by an RP-18 column chromatography (MeOH–H_2O, 30:70 → 50:50) to give Fr.4-1, Fr.4-2, and **5** (1.5 g). Fr.4-1 was then given to semi-prep. HPLC (MeCN–H_2O, 30: 70) – yield **6** (11 mg) and **7** (62 mg).

2.3.1. Parisverticoside A (**1**)

White, amorphous powder; $[\alpha]_D^{23}$ −55.1 (*c* 0.24, MeOH); ESI: *m/z* 925 $[M+Na]^+$; HRESI-MS: *m/z* 902.4540 $[M]^+$ (Calc. for $C_{44}H_{70}O_{19}$, 902.4511); IR (KBr) ν_{max} (cm^{-1}): 3439, 3424, 2955, 2927, 2855, 1708, 1687,1656, 1640, 1631, 1452, 1415, 1380, 1273, 1251, 1216, 1202, 1156, 1044, 982; ^1H and ^{13}C NMR data see Table **1**.

2.3.2. Parisverticoside B (**2**)

White, amorphous powder; $[\alpha]_D^{24}$ −51.1 (*c* 0.32, MeOH); ESI: *m/z* 909 $[M+Na]^+$; HRESI-MS: *m/z* 886.4535 $[M]^+$ (Calc. for $C_{44}H_{70}O_{18}$, 886.4562); IR (KBr) ν_{max} (cm^{-1}): 3426, 2953, 2930, 2909, 1620, 1460, 1380, 1362, 1311, 1275, 1253, 1201, 1157, 1044, 1001, 985, 964, 945, 900, 610; ^1H and ^{13}C NMR data see Table **1**.

2.3.3. Parisverticoside C (**3**)

White, amorphous powder; $[\alpha]_D^{23}$ −74.5 (*c* 0.22, MeOH); ESI: *m/z* 1231 $[M+Na]^+$; HREI-MS: *m/z* 1231.5629 $[M+Na]^+$ (Calc. for $C_{57}H_{92}O_{27}Na$, 1231.5718); IR (KBr) ν_{max} (cm^{-1}):3441, 3420, 2933, 1640, 1455, 1434, 1380, 1284, 1268, 1247, 1132, 1043, 978, 910, 837, 803, 630, 594, 579, 520; ^1H and ^{13}C NMR data see Table 1.

2.3.4. Parisverticoside D (**4**)

White, amorphous powder; $[\alpha]_D^{24}$ −47.4 (*c* 0.32, MeOH); ESI: *m/z* 1233 $[M+Na]^+$; HRESI-MS: *m/z* 1209.5768 $[M-H]^-$ (Calc. for $C_{57}H_{93}O_{27}$, 1209.5910); IR (KBr) ν_{max} (cm^{-1}): 3439, 3428, 3028, 2934, 1657, 1639, 1631, 1460, 1452, 1422, 1407, 1384, 1307, 1273, 1253, 1233, 1197, 1130, 1041, 985; ^1H and ^{13}C NMR data see Table 1.

2.3.5. Acid hydrolysis of compounds **1**–**4** and GC analysis

Compounds **1**–**4** (2 mg) were refluxed with 2 M HCl (1,4 dioxane/H_2O 1:1, 2 ml) on a water bath for 2 h. After cooling, the reaction mixture was neutralized with 1 M NaOH and filtered. The filtrate was extracted with $CHCl_3$ (3 × 5 ml). The aqueous layer was evaporated to dryness. The dried residue was dissolved in 1 mL of anhydrous pyridine and treated with L-cysteine methyl ester hydrochloride (1.5 mg) stirred at 60 °C for 30 min. Trimethylsilylimidazole (1.0 ml) was added to the reaction mixtures, and they were kept at 60 °C for 30 min. The supernatants (4 μL) were analyzed by GC, respectively, under the following conditions: H_2 flame ionization detector. Column: 30 QC2/AC-5 quartz capillary column (30 m × 0.32 mm). Column temperature: 180–280 °C with the rate of 3 °C/min, and the carrier gas was N_2 (1 ml/min); injector temperature: 250 °C; split ratio: 1/50. The configurations of D-glucose, L-rhamnose, and D-xylose for compounds **1**–**4** were determined by comparison of the retentions times of the corresponding derivatives with those of standard D-glucose, D-xylose, and L-rhamnose giving a single peak at 19.01, 18.34, and 15.43 min, respectively. These assignments of absolute configurations are based on the assumption that the corresponding enantiomeric sugar derivatives of D-cysteinyl methyl ester would in fact be separable from the L-cysteinyl derivatives under our GC conditions.

2.4. Platelet aggregation assays

Turbidometric measurements of platelet aggregation of the samples were performed in a Chronolog Model 700 Aggregometer (Chronolog Corporation, Havertown, PA, USA) according to Born's method [12,13]. The blood from the rabbits by cardiac puncture, were anticoagulated with 3.8% sodium citrate (9:1, v/v). Platelet-rich plasma (PRP) was prepared shortly after blood collection by spinning the sample at 180 g for 10 min at 22 °C. The PRP was carefully removed and the remaining blood centrifuged at 2400 g for 10 min to obtain platelet-poor plasma (PPP). The centrifuge temperature was maintained at 22 °C. Platelet counts were adjusted by the addition of PPP to the PRP to achieve a count of

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data for compounds **1** and **2** (δ in ppm, J in Hz, C_5D_5N).

Pos.	**1**		**2**		Pos.	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H		δ_C	δ_H	δ_C	δ_H
1	84.5 d	3.77 o	84.3 d	3.84 o	24	73.2 d	4.05 o	38.8 t	2.07 m
2	37.9 t	2.60 o	37.9 t	2.65 o					1.76 o
		2.36 m		2.39 dd (11.8, 11.6)	25	36.2 d	1.83 o	31.8 d	1.79 o
3	68.2 d	3.70 o	68.1 d	3.74 o	26	60.8 t	3.75 o	65.9 t	3.52 m
4	43.8 t	2.59 o	43.8 t	2.63 o			3.38 dd (11.4, 4.7)		3.44 t (10.3)
		2.47 m		2.51 m	27	13.1 q	0.91 d (6.7)	16.9 q	0.70 d (5.8)
5	139.4 s		139.3 s		1-Glc				
6	124.9 d	5.46 br s	124.8 d	5.51 d (5.6)	1′	100.2 d	4.72 d (8.3)	100.1 d	4.78 d (7.4)
7	32.0 t	1.76 o	31.9 t	1.79 m	2′	76.5 d	4.12 m	76.4 d	4.16 o
		1.42 o		1.48 o	3′	88.7 d	3.99 m	88.5 d	4.04 o
8	33.1 d	1.45 o	33.0 d	1.50 o	4′	70.4 d	3.79 o	70.2 d	3.84 o
9	50.5 d	1.58 o	50.3 d	1.62 o	5′	77.8 d	3.73 o	77.8 d	3.77 m
10	42.9 s		42.7 s		6′	63.4 t	4.42 o	63.3 t	4.47 m
11	24.3 t	2.79 m	24.3 t	2.83 m			4.14 m		4.19 o
		1.57 o		1.62 o	2′-Rha				
12	40.8 t	1.64 m	40.7 t	1.70 o	1″	101.8 d	6.37 br s	101.8 d	6.42 br s
		1.43 o		1.49 o	2″	72.6 d	4.73 o	72.5 d	4.77 o
13	40.8 s		40.8 s		3″	72.6 d	4.53 m	72.5 d	4.56 m
14	57.2 d	1.14 m	57.1 d	1.20 m	4″	74.3 d	4.26 t (9.6)	74.2 d	4.29 dd (9.9, 9.0)
15	32.3 t	1.85 o	32.3 t	1.99 m	5″	69.6 d	4.77 m	69.6 d	4.80 m
		1.33 o		1.48 o	6″	19.3 q	1.69 d (5.9)	19.3 q	1.72 d (6.1)
16	83.1 d	4.44 m	81.6 d	4.51 m	3′-Xyl				
17	61.5 d	1.75 o	62.6 d	1.88 m	1‴	105.4 d	4.88 d (7.5)	105.3 d	4.91 d (7.6)
18	17.1 q	1.00 s	17.1 q	1.07 s	2‴	74.9 d	3.91 m	74.8 d	3.94 dd (8.0, 7.8)
19	15.1 q	1.31 o	15.0 q	1.34 s	3‴	78.5 d	4.03 o	78.4 d	4.06 o
20	36.9 d	2.94 t (6.8)	35.8 d	3.03 t (6.9)	4‴	70.7 d	4.04 o	70.6 d	4.08 o
21	14.7 q	1.09 d (6.8)	14.7 q	1.16 d (6.9)	5‴	67.3 t	4.19 m	67.3 t	4.23 o
22	112.8 s		111.7 s				3.63 t (10.1)		3.66 t (10.4)
23	68.8 d	3.76 o	67.4 d	3.83 m					

(m: multiplet; o: overlapped).

$500 \times 10^9\ L^{-1}$. Platelet aggregation studies were completed within 3 h of preparation of PRP. Immediately after preparation of PRP, 250 μL was incubated in each of the test tubes at 37 °C for 5 min and then 2.5 μL of inducers (or compounds) was added. The change of optical density as a result of platelet aggregation was recorded. The extent of aggregation was estimated by the percentage of maximum increase in light transmission, with the buffer representing 100% transmittance. AA (Arachidonic acid) as the positive control and showed 48% maximal platelet aggregation rate at the concentration of 20 μM.

3. Results and discussion

The EtOH extract of the aerial parts of *P. verticillata* was subjected repeatedly to YWD-3F macroporous resin, silica gel, RP-18, and semi-preparative HPLC column chromatography to yield three new spirostanol saponins and one new cholestane saponin, named parisverticosides A–D (**1**–**4**, Fig. 1). In addition, 11 known steroidal saponins were obtained and identified by direct comparison with the MS and NMR spectra data reported in the literature, paris saponin VII (**5**) [14], pennogenin 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranoside (**6**) [15], diosgenin 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranoside (**7**) [16], parispseudoside A (**8**) [17], pregnane 5,16-dien-3β-ol-20-oxo 3-*O*-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (**9**) [14], smilaxchinoside B (**10**) [18], parispseudoside C (**11**) [17], pseudoproto Pb (**12**) [19], saponin Th (**13**) [20], dichotomin (proto-Pb) (**14**) [21], and 26-*O*-β-D-glucopyranosyl-22-methoxy-3β, 26-dihydroxy-25(*R*)-furost-5-en-3-*O*-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**15**) [22].

Compound **1** was obtained as amorphous powder with molecular formula of $C_{44}H_{70}O_{19}$ determined by its HRESI-MS at *m/z* 902.4540 $[M]^+$ (calc. for 902.4511) and ^{13}C NMR data (Table 1). The ^{1}H NMR spectrum of **1** showed signals for two tertial methyl groups at δ_H 1.00 and 1.31 (each 3H, s); three secondary methyl groups at δ_H 0.91 (3H, d, J = 6.7 Hz), 1.09 (3H, d, J = 6.8 Hz), and 1.69 (1H, d, J = 5.9 Hz); three anomeric proton signals at δ_H 4.72 (1H, d, J = 8.3 Hz), 4.88 (1H, d, J = 7.5 Hz), 6.37 (1H, br s); and an olefinic proton signal at δ_H 5.46 (1H, s). The signal at δ_H 1.69 was due to the methyl group of rhamnose. These ^{1}H NMR signals and two olefinic carbon signals at δ_C 139.4 (s, C-5) and 124.9 (d, C-6) together with a quaternary carbon signal at δ_C 112.8 (s) in its ^{13}C NMR spectrum suggested **1** to be a $\Delta^{5,6}$-spirostanol triglycoside [23]. Acid hydrolysis of **1** with 1 M HCl in dioxane-H_2O (1:1) yielded D-glucose, D-xylose, and L-rhamnose as carbohydrate moieties, which were determined by GC analysis of their corresponding trimethylsilated L-cysteine adducts. The ^{13}C NMR spectrum of **1** showed a total of 44 resonance lines, 17 of which were attributed to three monosaccharide units. This implied a $C_{27}H_{42}O_7$ molecular formula for the aglycone moiety, which suggested the aglycone to be a spirostanol with five oxygen atoms on the skeleton. Detailed comparison of NMR data of **1** with those of padelaoside A [24] (isolated from *Paris delavayi*, (23*S*,24*S*,25*S*)-spirost-5-ene-1β,3β,21,23,24-pentol 1-*O*-[α-L-rhamnopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl]-24-*O*-β-L-fucopyranoside) indicated the presence of a methyl (δ_H 1.09; δ_C 14.7) and the disappearance of a hydroxymethyl and a fucopyranosyl unit attached to C-24. The methyl group (δ_C 14.7) was fixed at C-20 based on the ^{1}H–^{1}H COSY from δ_H 1.09 (3H, d, J = 6.8 Hz) to 2.94 (t, H-20) and the HMBC correlations of δ_H 1.09 (3H, d, J = 6.8 Hz) with δ_C 36.9 (C-20), δ_C 61.5 (C-17), and δ_C 112.8 (C-22). The β-orientations of 1-OH and 3-OH were deduced from ROESY correlations of H-1/H-9, H-1/H_α-2, and H-3/H_α-2. The ROESY correlations of H-23 (δ_H 3.76)/H-20 (δ_H 2.94), H-23 (δ_H 3.76)/H-25 (δ_H 1.83), H-24 (δ_H 4.05)/H-23 (δ_H 3.76), H-24 (δ_H 4.05)/H-25 (δ_H 1.83) allowed us to assign the 23*S*, 24*S*, and

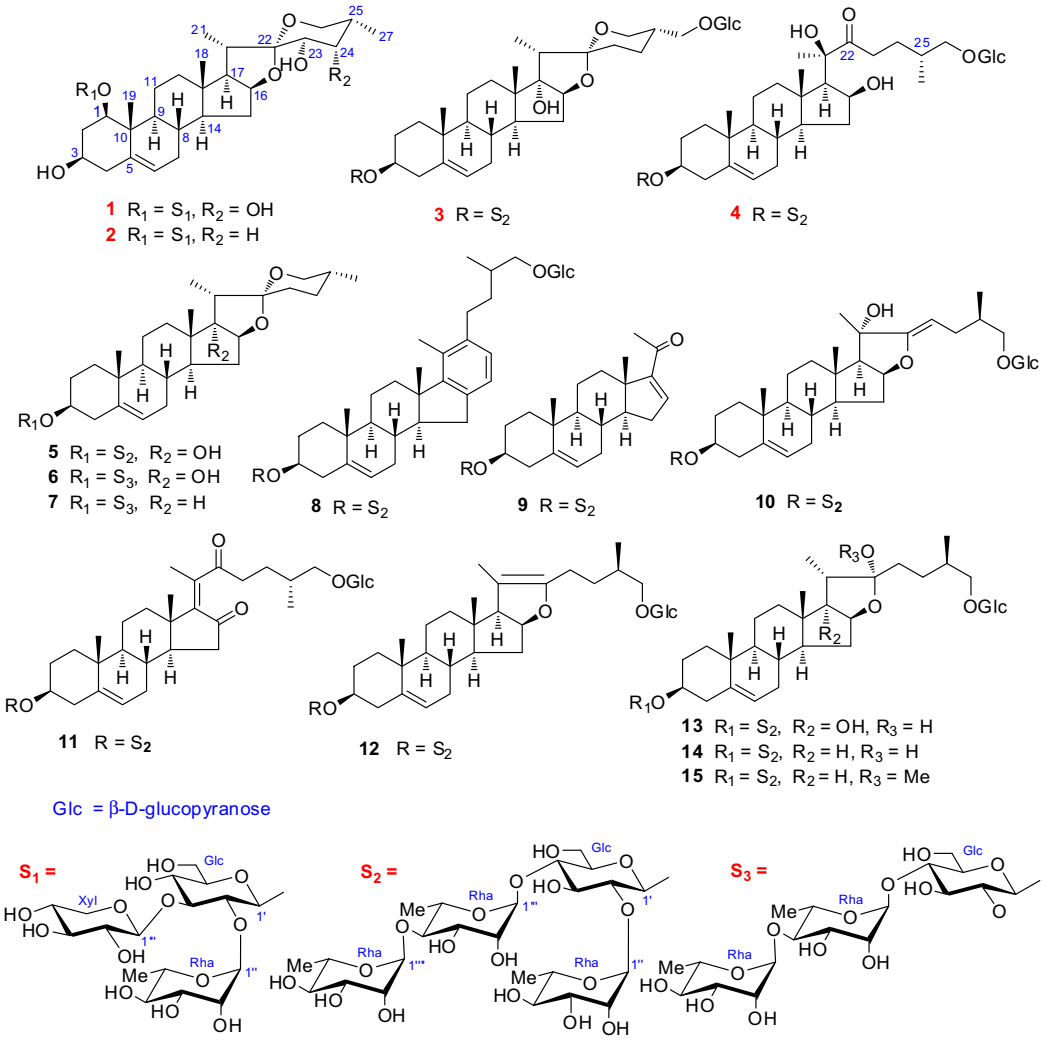

Fig. 1. The structures of compounds **1**–**15**.

25*S* configurations. Thus, the aglycone of **1** was elucidated as (23*S*,24*S*,25*S*)-spirost-5-ene-1β, 3β,23,24-tetraol, a new spirostanol sapogenin.

The glucopyranoside and xylopyranoside were β-configuration on the ground of the coupling constants ($^3J_{1,2}$ > 7 Hz) of the anomeric protons. The α-configuration for the rhamnopyranoside was deduced by comparing the ^{13}C NMR spectroscopic data for C-3″ (δ_C 72.6) and C-5″ (δ_C 69.6) of rhamnopyranoside with those reported in the literature [25]. The sequence and linkage positions of the sugars were verified by detailed 2D NMR spectroscopic analysis. In the HMBC spectrum, the correlations of H-1′ (δ_H 4.72) with C-1 (δ_C 84.5), H-1″ (δ_H 6.37) with C-2′ (δ_C 76.5), and H-1‴ (δ_H 4.88) with C-3′ (δ_C 88.7), were observed, which hinted that a β-xylopyranosyl and an α-rhamnopyranosyl attached at C-3′ and C-2′ of β-glucopyranosyl, respectively, and the β-glucopyranosyl was attached at C-1 of the aglycone. Therefore, the structure of **1** was determined as (23*S*,24*S*,25*S*)-spirost-5- en-1β,3β,23,24-tetraol 1-O-α-L-rhamnopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranoside and named as parisverticoside A.

Compound **2**, isolated as white amorphous powder, had a molecular formula of $C_{44}H_{70}O_{18}$ as deduced by HREI-MS with a *m/z* 886.4535 $[M]^+$ (calc. 886.4562) and ^{13}C NMR data (Table 1), which lacks that of compound **1** by an oxygen atom. The ^{1}H and ^{13}C NMR spectroscopic data of **2** (Table 1) was similar to those of compound **1** except for the presence of a methylene (δ_C 38.8) and the absence of an oxymethine. The methylene was inferred to be located at C-24 on the ground of ^{1}H-^{1}H COSY correlation of δ_H 3.83 (1H, m, H-23) with δ_H 2.07(1H, m, H-24a) and δ_H 1.76 (1H, m, H-24b), of δ_H1.79 (1H, m, H-25) with δ_H 2.07 (1H, m, H-24a) and δ_H 1.76 (1H, m, H-24b) and the HMBC correlations from δ_H 0.70 (3H, d, *J* = 5.8 Hz, Me-27) to δ_C 38.8 (t, C-24), 31.8 (d, C-25), and 65.9 (t, C-26). Based on the ROESY correlations of H-23 (δ_H 3.83) with H-20 (δ_H 3.03) and H-25 (δ_H 1.79), the absolute configurations of C-23 and C-25 were deduced as *S* and *R*, respectively. Thus, the structure of **2** was elucidated as (23*S*,25*R*)-spirost-5-en-1β,3β,23-triol 1-*O*-α-L-rhamnopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranoside and named as parisverticoside B.

The molecular formula of compound **3** was identified as $C_{57}H_{92}O_{27}$ on the basis of its quasimolecular ion at *m/z* 1231.5629 $[M+Na]^+$ (calc. 1231.5718) in the HRESI-MS and ^{13}C NMR data. In the ^{1}H NMR spectrum (Table 2), signals for three methyls of aglycone at δ_H 0.93 (3H, s, Me-18), 1.07 (3H, s, Me-19), 1.18 (3H, d, *J* = 7.0 Hz, Me-21) and five anomeric proton signals of sugar moieties at δ_H 4.92 (1H, d, *J* = 7.2 Hz, H-1′), 6.39 (1H, br s, H-1″), 5.83 (1H, br s, H-1‴), 6.28 (1H, br s, H-1⁗), 4.74 (1H, d, *J* = 7.7 Hz, H-1⁗′) were observed. A characteristic quaternary carbon resonance assignable to C-22 of the spirostanol skeleton and five anomeric carbon signals could be showed at δ_C 110.2 (s, C-22), 100.4 (d, C-1′), 102.2 (d, C-1″), 102.3 (d, C-1‴), 103.2

Table 2
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data for compounds **3** and **4** (δ in ppm, J in Hz, C_5D_5N).

Pos.	**3**		**4**		Pos.	**3**		**4**	
	δ_C	δ_H	δ_C	δ_H		δ_C	δ_H	δ_C	δ_H
1	37.6 t	1.74 o	37.5 t	1.53 o	3-Glc				
		0.94 o		0.91 o	1′	100.4 d	4.92 d (7.2)	100.4 d	4.90 d (8.3)
2	30.2 t	2.04 o	30.2 t	2.00 o	2′	78.2 d	4.20 o	78.0 d	4.16 o
		1.84 m		1.79 o	3′	77.8 d	4.19 o	77.7 d	4.17 o
3	78.1 d	3.83 m	78.2 d	3.82 o	4′	77.8 d	4.38 o	77.8 d	4.36 o
4	39.0 t	2.76 o	39.1 t	2.74 o	5′	77.0 d	3.58 m	76.9 d	3.55 o
		2.71 o		2.67 o	6′	61.3 d	4.16 o	61.2 t	4.14 o
5	140.9 s		140.9 s				4.03 o		4.00 o
6	121.9 s	5.29 br s	121.9 d	5.27 br s	2′-Rha				
7	32.5 t	1.90 m	32.1 t	1.84 o	1″	102.2 d	6.39 br s	102.2 d	6.37 br s
		1.61 o		1.46 o	2″	72.5 d	4.83 o	72.6 d	4.81 o
8	32.4 d	1.50 o	31.3 d	1.45 o	3″	72.9 d	4.61 m	72.7 d	4.59 m
9	50.3 d	0.95 o	50.5 d	0.81 m	4″	74.2 d	4.34 o	74.2 d	4.32 o
10	37.2 s		37.1 s		5″	69.6 d	4.93 o	69.6 d	4.90 o
11	21.0 t	1.58 o	20.9 t	1.37 o (2H)	6″	18.7 q	1.57 d (5.7)	18.7 q	1.54 d (5.7)
		1.49 m			4′-Rha				
12	32.1 t	2.14 m	39.8 t	1.59 o	1‴	102.3 d	5.83 br s	102.3 d	5.81 br s
		1.50 o		1.08 m	2‴	72.9 d	4.48 o	72.9 d	4.47 o
13	45.2 s		42.5 s		3‴	73.2 d	4.53 o	73.4 d	4.51 o
14	53.1 d	2.06 o	54.8 d	0.74 o	4‴	80.4 d	4.47 o	80.5 d	4.39 o
15	31.8 t	2.21 o	37.6 t	2.20 m	5‴	68.4 d	4.91 o	68.4 d	4.88 o
		1.52 o		1.66 o	6‴	18.9 q	1.57 d (5.7)	19.0 q	1.54 d (5.7)
16	90.2 d	4.40 o	72.5 d	4.82 o	4″-Rha				
17	90.2 s		59.9 d	1.84 d (6.9)	1⁗	103.2 d	6.28 br s	103.4 d	6.26 br s
18	17.2 q	0.93 s	15.8 q	1.34 s	2⁗	72.6 d	4.87 o	72.7 d	4.86 o
19	19.5 q	1.07 s	19.5 q	1.00 s	3⁗	72.9 d	4.48 m	73.0 d	4.46 o
20	44.9 d	2.24 o	82.6 s		4⁗	74.0 d	4.28 o	74.1 d	4.26 o
21	9.6 q	1.18 d (7.0)	28.7 q	1.70 s	5⁗	70.4 d	4.33 o	70.5 d	4.31 o
22	110.2 s		215.9 s		6⁗	18.4 q	1.74 d (6.1)	18.5 q	1.71 d (6.3)
23	31.6 t	1.72 o	36.1 t	3.24 m	27-Glc			26-Glc	
		1.65 o		3.04 m	1⁗′	105.0 d	4.74 d (7.7)	104.9 d	4.78 d (7.8)
24	23.5 t	1.68 o (2H)	27.9 t	1.94 o	2⁗′	75.2 d	3.99 o	75.3 d	3.97 m
				1.74 o	3⁗′	78.5 d	4.20 o	78.6 d	4.20 o
25	36.6 d	2.03 o	33.5 d	1.98 o	4⁗′	71.8 d	4.21 o	71.7 d	4.19 o
26	63.6 t	3.96 o	75.2 t	3.97 o	5⁗′	78.6 d	3.93 o	78.6 d	3.89 o
		3.71 t (11.3)		3.56 o	6⁗′	62.9 t	4.54 o	62.9 t	4.50 o
27	72.0 t	3.91 o	17.5 q	0.96 d (6.4)			4.37 o		4.34 o
		3.43 t (8.5)							

m: multiplet; o: overlapped.

(d, C-1⁗′), 105.0 (d, C-1⁗′) in the ^{13}C NMR and DEPT spectrum of **3**. Comparison of the ^{13}C NMR signals of **3** (Table 2) with those of paris saponin VII (**5**) [14] showed their structural similarity except for the presence of an oxymethylene (δ_C 72.0) and an additional glucopyranosyl and the absence of Me-27. The ^{1}H–^{1}H COSY correlations of H-25/H_2-27 (δ_H 3.91 and 3.43) and H-25/H_2-26, along with the HMBC correlations from H_2-27 (δ_H 3.91 and 3.43) to δ_C 23.5 (C-24), 36.6 (C-25), 63.6 (C-26), and 105.0 (C-1⁗′) confirmed that the oxymethylene was unambiguously placed at C-27 and linked with the glucose. Acid hydrolysis of 3 afforded D-glucose and L-rhamnose as sugar residues. The sequence of the tetrasaccharide attached to C-3, which was the same as the known compounds **5**, **8**–**15**, was demonstrated by the further HMBC correlations: H-1′ (δ_H 4.92) of 3-Glc with C-3 (δ_C 78.1) of the aglycone, H-1″ (δ_H 6.39) of 2′-Rha with C-2′ (δ_C 78.2) of 3-Glc, H-1‴(δ_H 5.83) of 4″-Rha with C-4′ (δ_C 77.8) of 3-Glc, and H-1⁗′ (δ_H 6.28) of 4‴-Rha with C-4‴ (δ_C 80.4) of 4″-Rha. Accordingly, the structural of **3** was elucidated as 27-*O*-β-D-glucopyranosyl-(25*R*)-spirost-5-en-3β,17α,27-triol-3-*O*-α-L- rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside and named as parisverticoside C.

Compound **4** gave a pseudo-molecular ion peak $[M-H]^-$ at *m/z* 1209.5768 (calc 1209.5910) in its HRESI-MS. Combined with ^{13}C NMR data, its molecular formula was defined as $C_{57}H_{94}O_{27}$. The ^{1}H NMR spectrum of **4** (Table 2) showed four steroid methyl groups at δ_H 1.34 (s, Me-18), 1.00 (s, Me-19), 1.70 (s, Me-21), 0.96 (d, J = 6.4 Hz, Me-27), an olefinic proton at δ_H 5.27 (br s, H-6), as well as five anomeric proton signals at δ_H 4.90 (1H, d, J = 8.3 Hz, H-1′), 6.37 (1H, br s, H-1″), 5.81 (1H, br s, H-1‴), 6.26 (1H, br s, H-1⁗′), and 4.78 (d, J = 7.8 Hz, H-1⁗′). The above ^{1}H NMR spectrum of **4** was similar to those of parispseudoside C (**11**) [17]. In the ^{13}C NMR spectrum of **4**, the signals due to the aglycone moiety except for C-15, C-16, C-17, C-20, and C-21, and the signals of the sugar moiety were in good agreement with those of **11** [17]. The significant differences were the presence of a methine (δ_C 59.9), an oxymetine (δ_C 72.5) and an oxygenated quaternary carbon (δ_C 82.6) instead of the carbonyl group at C-16 and a double bond between C-17 and C-20 in the latter, respectively. This was confirmed by the ^{1}H-^{1}H COSY correlation from δ_H 4.82 (H-16) to δ_H 1.84 (H-17) and the HMBC correlations of Me-21 (δ_H 1.70) with δ_C 59.9 (C-17), δ_C 82.6 (C-20), and 215.9 (C-22) and of δ_H 4.82 (H-16) with δ_C 42.5 (s, C-13), 54.8 (d, C-14), 37.6 (t, C-15), 59.9 (d, C-17) and δ_C 82.6 (s, C-20). The OH-16 was assigned as β-oriented on the ground the ROESY correlations of H-17 (δ_H 1.84)/H-16 (δ_H 4.82), H_β-15 (δ_H 1.66)/Me-18 (δ_H 1.34), and H-16/H_α-15 (δ_H 2.20). The configuration of the C-20 (δ_C82.6) was indicated to be *R* on the comparison with the chemical shift values of goniopectenoside B [δ_C 82.1 (C-20*R*)] [26], which was confirmed by the ROESY cross-peaks of Me-18 (δ_H 1.34) with H_β-12 (δ_H 1.59) and Me-21 (δ_H 1.70), of H_α-12 (δ_H 1.08) with H-9 (δ_H 0.81) and Me-21 (δ_H 1.70). The HMBC experiment showed that the tetrasaccharide and monosaccharide moieties were linked to C-3

and C-26 of the aglycone, respectively. Moreover, the anomeric protons signals at δ_H 4.90 (H-1′), 6.37 (H-1″), 5.81 (H-1‴), 6.26 (H-1⁗), and 4.78 (H-1⁗′) with δ_C 78.2 (C-3 of aglycone), 78.0 (C-2′ of 3-Glc), 77.7 (C-3′ of 3-Glc), 80.5 (C-4‴ of 4′-Rha), and 75.2 (C-26 of aglycone), respectively. On the basis of the foregoing data, the structure of **4** was elucidated as 26-*O*-β-D-glucopyranosyl-(20*R*,25*R*)-cholestan-5-en-3β,16β,20,26-tetraol-22-one-3-*O*-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 4)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside and named as parisverticoside D.

The platelet aggregation activities of the 75% EtOH eluant from the macroporous resin column and saponins **1**–**8** were evaluated. The results indicated that only compound **5** showed 62% maximal platelet aggregation rate at the concentration of 300 μg/mL, while the EtOH eluant showed 53% maximal platelet aggregation rate at the test concentration (1.5 mg/mL). This study demonstrated that the aerial parts of *P. verticillata* were rich in steroidal saponins and paris saponin VII (**5**) was the main hemostatic ingredient of the titled plant. The results remind us that the non-medicinal parts of *P. verticillata* may be potential candidates to exploit and utilize.

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