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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