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•Original article•

Steroid and triterpenoid saponins from the rhizomes of *Paris polyphylla* var. *stenophylla*

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[ABSTRACT] Five new saponins, including three steroid saponins, paristenoids A−C (**1**−**3**), and two triterpenoid saponins, paristenoids D−E (**4**−**5**), along with four known ones (**6**−**9**) were isolated from the rhizomes of *Paris polyphylla* var. *stenophylla.* The structures of the isolated compounds were identified mainly by detailed spectroscopic analysis, including extensive 1D and 2D NMR, MS, as well as chemical methods. Compound **3** is a new cyclocholestanol-type steroidal saponin with a rare 6/6/6/5/5 fused-rings cholestanol skeleton, and this skeleton has been first found from the genus *Paris.* The cytotoxicities of the isolated compounds against three human three glioma cell lines (U87MG, U251MG and SHG44) were evaluated, and compound **7** displayed certain inhibitory effect with IC_{50} values of 15.22 ± 1.73, 18.87 ± 1.81 and 17.64 ± 1.69 μmol·L^{-1}, respectively.

[KEY WORDS] *Paris polyphylla* var. *stenophylla*; Steroid saponins; Triterpenoid saponins; Cyclocholestanol-type
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Introduction

The plant of the genus *Paris* (Melanthiaceae) is an erect, perennial herb and distributed in China, India, Japan, Myanmar, Vietnam, Bhutan and other countries. There are 27 species and 15 varieties mainly distributed in the southwest of China [1]. The rhizomes of *Paris* have been used as traditional Chinese medicines due to the effect of heat-cleaning, detoxification, reducing swelling-reducing and pain-relieving [2]. Two species of this genus, *Paris polyphyll*a var. *yannanensis* and *P. polyphylla* var. *chinensis*, were recorded in *Chinese Pharmacopoeia* as the source of herbal medicine Paridis Rhizoma. According to phytochemical investigations, steroidal saponins are the main chemical compositions of the genus *Paris*, with a wide range of pharmacological activities such as anti-tumor [3-5], anti-inflammatory [6], anti-fungal [7], hemostasis [8] and immunomodulatory effect [9]. *P. polyphylla* var. *stenophylla* is a variety of *P. polyphylla*, which grows at an altitude of 500−3000 meters of the undergrowth place and widely spreads from India to China. However, there are few reports concerning the phytochemical constituents of *Paris polyphylla* var. *stenophylla.* In recent years, a series of studies [10-11] have been carried out by our group on the cytotoxic steroidal saponins from the genus *Paris*. As a part of our continuous research for cytotoxic steroidal saponins from the genus *Paris*, the chemical constituents of *P. polyphylla* var. *stenophylla* were investigated. As a result, five new saponins (**1**−**5**) and four known ones (**6**−**9**) were obtained from the 70% EtOH extract of the rhizomes of *P. polyphylla* var. *stenophylla* (Fig. 1). In addition, the cytotoxicity of the isolated compounds against human cancer cell lines was evaluated. Herein, the isolation, structural elucidation and cytotoxicity evaluation were reported in this paper.

Results and Discussion

Compound **1** was obtained as white amorphous powder and positive for Liebermann-Burchard and Molish tests, which indicated that it might be a steroidal glycoside. The ion peak in the HR-ESI-MS spectrum at *m/z* 777.4044 $[M + Na]^+$ (calculated for $C_{39}H_{62}O_{14}Na$, 777.4037) was in accordance

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These authors have no conflict of interest to declare.

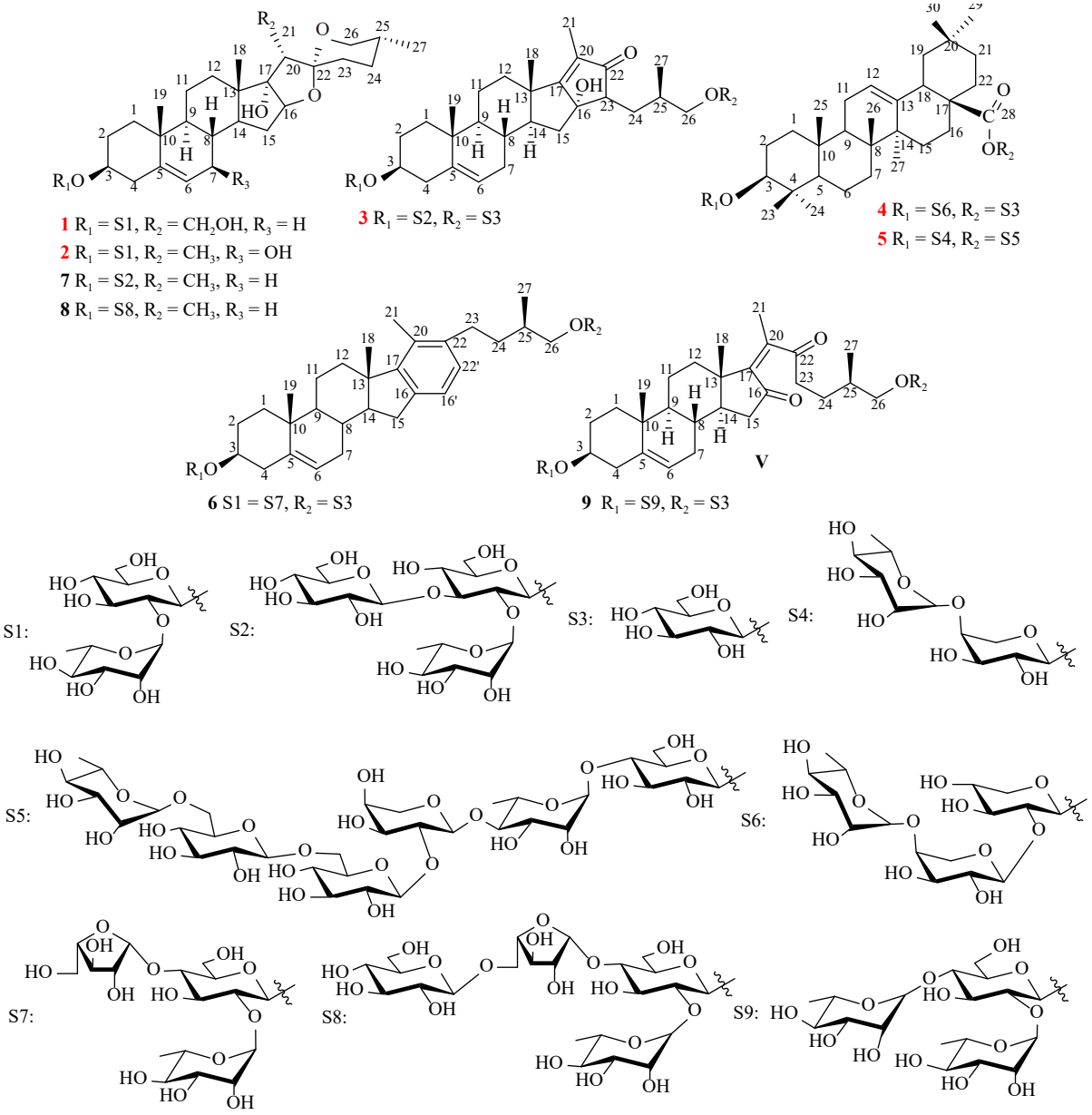

Fig. 1 Chemical structures of compounds 1−9.

with the molecular formula $C_{39}H_{62}O_{14}$. In the ^{1}H NMR spectrum, three methyl proton signals were detected at δ_H 0.79 (3H, d, J = 6.40 Hz, H-27), 0.86 (3H, *s*, H-18), 1.06 (3H, s, H-19), and one olefinic methine proton signal at δ_H 5.39 (1H, br s, H-6). Correspondingly, there were three carbon signals at δ_C 17.6 (C-27), 17.3 (C-18) and 20.0 (C-19), as well as a pair of trisubstituted double bond carbons at δ_C 142.1 (C-5) and 122.8 (C-6) in the ^{13}C NMR spectrum. A characteristic hemiacetal carbon signal of spirostanol aglycone was discovered at δ_C 111.2 (C-22) [12]. In addition, combined with the quaternary carbon signal at δ_C 91.5 (C-17) and the methylene signal at δ_C 90.7 (C-16), it was speculated that the aglycone of compound **1** was pennogenin. However, in the ^{13}C NMR, DEPT and ^{1}H NMR spectra, the characteristic methyl signal at C-21 of a pennogenin aglycone was missing, and a methylene signal was found at δ_C 59.6 with δ_{Ha} 3.61 and 3.80, which indicated that there might be a hydroxyl methyl group substitution at C-21. Moreover, it was further confirmed by the cross peaks between δ_{Ha} 3.61, δ_{Hb} 3.80 (H-21) and δ_H 2.25 (H-20) in the ^{1}H-^{1}H COSY spectrum and the correlations between δ_{Ha} 3.61, δ_{Hb} 3.80 (H-21) and δ_C 53.2 (C-20)/δ_C 110.2 (C-22), δ_H 2.25 (H-20) and δ_C 59.6 (C-21)/δ_C 91.5 (C-17)/δ_C 110.2 (C-22) in the HMBC spectrum. In addition, in the HMBC spectrum, the cross-peaks between δ_H 1.06 (H-19) and δ_C 142.1 (C-5), δ_H 5.39 (H-6) and δ_C 39.7 (C-4)/δ_C 33.8 (C-7)/δ_C 38.2 (C-10) inferred that the double bond was located at C-5/C-6 (Fig. 2). The chemical shifts of δ_C 91.5 (C-17) and δ_C 90.1 (C-16) indicated the hydroxyl group at C-17 was in *α* orientation [13-14]. In the NOESY spectrum, the correlation between δ_H 3.62 (H-3) and δ_{Ha} 1.90 (H-1) combined

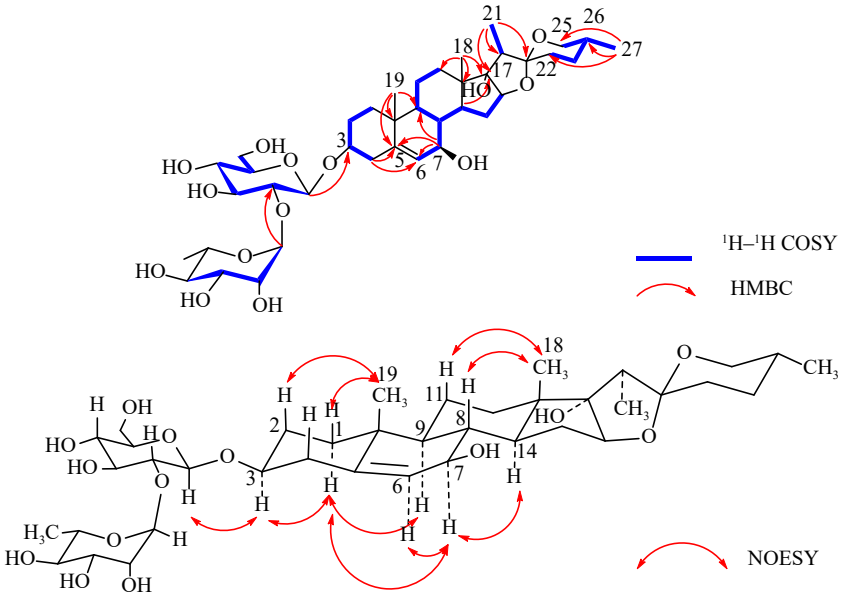

Fig. 2 Key ^{1}H–^{1}H COSY, HMBC and NOESY correlations of compound 2.

with the correlation between δ_{Hb} 1.09 (H-1) and δ_H 1.06 (H-19) suggested that H-3 was in *α*-orientation, thus the hydroxyl substituent at C-3 was in *β* configuration. The *R* configuration of C-25 was determined by the difference of geminal protons of δ_H H-26a and δ_H H-26b ($\Delta ab = 0.13 < 0.48$) [15]. All the carbon and hydrogen signals on the aglycone were assigned orderly by 1D and 2D NMR spectra (Tables 1 and 2). By combining the data and consulting the literature [16], the aglycone of compound **1** was identified as 25(*R*)-spirost-5-en-3*β*,17*α*,21-triol.

In the ^{13}C NMR spectrum, there were two anomeric carbon signals at δ_C 100.7 and δ_C 102.3. According to the HSQC spectrum, the corresponding anomeric protons were detected at δ_H 4.48 (d, 7.76) and δ_H 5.19 (br s). The above data revealed the presence of two monosaccharides in the sugar moiety of compound **1**. The monosaccharide residues were identified as D-Glc and L-Rha at a ratio of 1∶1 by acid hydrolysis and GC analysis [17]. The *β* orientation of D-Glc was deduced from the coupling constant of the anomeric proton at $J = 7.76$ Hz [18]. The *α* orientation of L-Rha was confirmed by the chemical shift of C-5 of L-Rha (δ_C 69.9) [19]. By analyzing the ^{1}H NMR, TOCSY and HSQC spectra, the protons and carbons of monosaccharide were assigned (Tables 1, 2 and 3). The linkage sequence of the sugar chain was confirmed by the cross peaks of δ_H 5.19 (Rha H-1) to δ_C 79.2 (Glc C-2) and δ_H 4.48 (Glc H-1) to δ_C 79.3 (C-3) in the HMBC spectrum (Fig. 2). Thus, the structure of compound **1** was identified as 25(*R*)-spirost-5-en-3*β*,17*α*,21-triol-3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside.

Compound **2** was obtained as white amorphous powder and positive for Liebermann-Burchard and Molish test, which indicated that it might be a steroidal glycoside. The ion peak in the HR-ESI-MS spectrum at *m/z* 777.4049 $[M + Na]^+$ (calculated for $C_{39}H_{62}O_{14}Na$, 777.4037), corresponding to the molecular formula $C_{39}H_{62}O_{14}$. Compared with compound **1**, in the ^{13}C NMR, DEPT and ^{1}H NMR spectra, the characteristic methyl signal at C-21 of a pennogenin aglycone was found at δ_C 9.3 which was missing in compound **1**. In addition, it was found that compound **2** lacked a methylene signal (C-7), and the chemical shift of (C-7 δ_C 65.9 with δ_H 3.77) moved to a lower field compared with that of compound **1** ($\Delta\delta_C$ +33.3 ppm), which indicated that a hydroxyl substitution might be attached to C-7. It was further confirmed by the cross peaks between δ_H 3.77 (H-7) and δ_H 5.58 (H-6)/δ_H 1.62 (H-8) in the ^{1}H-^{1}H COSY spectrum, and the correlations between δ_H 3.77 (H-7) and δ_C 146.5 (C-5)/δ_C 125.4 (C-6)/δ_C 43.4 (C-9), δ_H 5.58 (H-6)/δ_H 1.32 (H-9) and δ_C 65.9 (C-7) in the HMBC spectrum (Fig. 3). In the NOESY spectrum, the correlations between H-7 (δ_H 3.77) and H-1 (δ_H 3.69)/H-6 (δ_H 5.58)/H-14 (δ_H 2.22) suggested that H-7 was in *α* orientation, thus the hydroxyl substituent at C-3 was in *β* configuration.

Thus, the structure of compound **2** was identified as 25(*R*)-spirost-5-en-3*β*,7*β*,17*α*-triol-3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside.

Compound **3** was obtained as white amorphous powder and positive for Liebermann-Burchard and Molish test. The molecular formula was determined as $C_{51}H_{80}O_{23}$, based on the ion peak in the HR-ESI-MS spectrum at *m/z* 1059.4998 $[M - H]^-$ (calculated for $C_{51}H_{79}O_{23}$, 1059.5018). In the ^{1}H NMR spectrum, four methyl proton signals were detected at δ_H 1.86 (3H, s, H-21), δ_H 1.49 (3H, s, H-18), δ_H 1.15 (3H, d, $J = 6.62$ Hz, H-27) and δ_H 1.12 (3H, s, H-19), and one olefinic methine proton signal at δ_H 5.58 (1H, br s, H-6). Correspondingly, there were four methyl carbon signals at δ_C 9.0 (C-21), 16.1 (C-18), 17.6 (C-27) and 20.0 (C-19), as well as a pair of trisubstituted double bond carbons at δ_C 142.1 (C-5) and 122.8 (C-6). In addition, there were one tetra substituted double bonds at δ_C 128.7 (C-20) and 182.6 (C-17), one

Table 1 ^{1}H NMR data of the aglycones for 1−4 in CD_3OD (δ in ppm, *J* in Hz, 800 MHz)

No.	1	2	3	4
1	a 1.90 o , b 1.09 m	a 1.88 o, b 1.16 m	a 1.79 o, b 1.02 m	a 1.62 o, b 0.97 m
2	a 1.92 o, b 1.29 m	a 1.93 o, b 1.32 m	a 2.15 (t, 8.7), b 1.94 m	a 1.80 m, b 1.68 o
3	3.62 m	3.69 m	3.99 m	3.15 dd (11.7, 4.4)
4	a 2.45 m , b 2.30 t (11.2)	a 2.50 m, b 2.34 t (11.3)	a 2.83 m, b 2.80 m	–
5	–	–	–	–
6	5.39 br s	5.58 br s	5.40 br s	a 1.54 m, b 1.41 m
7	a 1.83 o, b 1.64 o	3.77 br s	a 2.01 o, b 1.63 o	a 1.72 o, b 1.60 o
8	1.65 o	1.62 m	1.72 m	–
9	0.94 m	1.32 m	1.00 m	1.58 m
10	–	–	–	–
11	a 1.62 o, b 1.51 o	a 1.61 o, b 1.52 o	a 1.58 m, b 1.53 m	a 1.90 m, b 1.82 m
12	a 1.58 o, b 1.44 m,	a 1.57 o, b 1.32m	a 2.12 m, b 1.48 br s	5.26 br s
13	–	–	–	–
14	1.71 m	2.22 o	1.14 d (7.8)	–
15	a 2.06 m, b 1.26 m	a 2.21 m, b 1.25 m	a 2.33 dd (12.7, 7.7), b 2.01 o	–
16	4.08 d (7.7)	4.05 d (7.5)	–	a 2.06 m, b 1.73 m
17	–	–	–	–
18	0.86 s	0.83 s	1.49 s	–
19	1.06 s	1.03 s	1.12 s	a 1.71 o, b 1.16 m
20	2.25 dd (8.6, 5.8)	2.10 dd (14.5, 7.20)	–	–
21	a 3.80 dd (11.2, 8.6), b 3.61 m	0.90 d (7.2)	1.86 s	a 1.40 m, b 1.23 m
22	–	–	–	–
23	a 2.02 m, b 1.56 o	a 1.98 m, b 1.56 o	2.49 dd (10.3, 3.9)	1.05 s
24	a 1.60 m, b 1.42 m	a 1.62 m, b 1.45 m	a 2.42 dd (13.6, 3.5), b 2.01 m	0.85 s
25	1.61 m	1.60 m	2.54 m	0.96 s
26	a 3.47 m, b 3.34 m	a 3.48 m, b 3.34 m	a 4.07 m, b 3.75 m	0.81 s
27	0.79 d (6.4)	0.80 d (6.4)	1.15 d (6.6)	1.16 s
28	–	–	–	–
29	–	–	–	0.90 s
30	–	–	–	0.96 s

ketone carbonyl at δ_C 212.8 (C-22) in the ^{13}C NMR spectrum. The comparison of ^{1}H and ^{13}C NMR spectra of the aglycone moiety of **3** with those of compound **9** [20] revealed the absence of one carbonyl group, one methylene and the appearance of an oxygen-bearing quaternary carbon at δ_C 83.4 (C-16) and one methine at δ_H 2.49 with δ_C 57.9 (C-23) in **3**. Therefore, it was supposed that the aglycone of compound **3** possessed a pentacyclic ring E, which was identical to that of ypsiyunnoside A [21]. The correlations of δ_H 4.07 (Ha-26) and 3.72 (Hb-26) with δ_H 2.54 (H-25), δ_H 2.54 (H-25) with δ_H 2.42 (Ha-24) and 2.01 (Hb-24), δ_H 2.54 (H-25) with δ_H 1.15 (H-27), and δ_H 2.01 (Hb-24) with δ_H 2.49 (H-23) in the ^{1}H–^{1}H COSY spectrum, combined with the correlation of δ_H 2.54 (H-25) with δ_C 57.9 (C-23) in the HMBC spectrum indicated that the methine was assigned to C-23 (Fig. 4). The observed correlations from δ_H 1.86 (H-21) to δ_C 182.6 (C-17), δ_C 128.7 (C-20), and δ_C 212.8 (C-22), from δ_H 2.49 (H-23) to δ_C 83.4 (C-16), and δ_C 212.8 (C-22), from δ_{Ha} 2.42 and δ_{Hb} 2.01 (H_2-24) to δ_C 83.4 (C-16) and δ_C 212.8 (C-22) in the HMBC spectra supported the formation of a cyclopentane with *α*, *β*-unsaturated ketone group by 23,16-aldol condensation at ring E (Fig. 4). In the HMBC spectrum, the cross-

Table 2 ^{1}H NMR data of the sugar moieties for 1−4 in CD_3OD (δ in ppm, *J* in Hz, 800 MHz)

No.	1	2	3	4
	3-*O*-*β*-D-Glc	3-*O*-*β*-D-Glc	3-*O*-*β*-D-Glc Ⅰ	3-*O*-*β*-D-Xyl
1	4.48 d (7.8)	4.49 d (7.8)	4.98 d (7.8)	4.39 d (7.8)
2	3.35 t (8.0)	3.36 t (7.9)	4.25 o	3.24 m
3	3.46 o	3.47 o	4.25 o	3.53 o
4	3.24 o	3.24 o	4.11 m	3.64 m
5	3.26 m	3.27 m	3.88 m	3.53 o, 3.59 m
6	a 3.85 m, b 3.64 m	a 3.84 m, b 3.64 m	a 4.46 m, b 4.29 m	–
	α-L-Rha	*α*-L-Rha	*α*-L-Rha	*α*-L-Ara
1	5.19 br s	5.20 br s	6.43 br s	4.42 d (7.92)
2	3.91 m	3.91 m	4.94 m	3.25 m
3	3.66 dd (9.6, 3.8)	3.66 dd (9.6, 3.7)	4.64 dd (9.5, 3.7)	3.48 m
4	3.39 o	3.39 o	4.38 m	3.55 m
5	4.14 m	4.13 m	4.99 m	a 4.10 m, b 3.80 m
6	1.24 d (6.2)	1.24 d (6.2)	1.8 d (6.1)	
			β-D-Glc Ⅲ	*α*-L-Rha
1			5.14 d (7.6)	4.85 br s
2			4.07 o	3.84 m
3			4.23 o	3.52 m
4			4.15 m	3.41 m
5			4.07 o	3.98 m
6			a 4.61 m, b 4.29 m	1.28 d (6.2)
			26-*O*-*β*-D-Glc Ⅱ	28-*O*-*β*-D-Glc
1			4.88 d (7.2)	5.35 d (8.2)
2			4.07 o	3.34 m
3			4.27 m	3.42 m
4			4.24 o	3.37 m
5			3.96 m	3.30 m
6			a 4.57 m, b 4.39 m	a 3.81 m, b 3.66 m

peaks between δ_H 1.12 (H-19) and δ_C 141.4 (C-5), δ_H 5.40 (H-6) and δ_C 39.1 (C-4)/δ_C 32.5 (C-8) inferred that the double bond was located at C-5/C-6 (Fig. 4). In the NOESY spectrum, the correlations of δ_H 1.49 (H_3-18)/δ_H 1.72 (H-8), δ_H 1.72 (H-8)/δ_H 2.01 (Hb-15), and δ_H 2.01 (Hb-15)/δ_H 2.49 (H-23) suggested that H-23 was *β*-oriented. Furthermore, the obvious correlation from δ_H 2.01 (Hb-15) to δ_H 2.49 (H-23) hinted that OH-16 was *α*-oriented [21]. The *R* configuration of C-25 was determined by the chemical shift difference between δ_H H-26a and δ_H H-26b (Δab = 0.32 < 0.48) [15]. All the carbon and hydrogen signals on the aglycone were assigned orderly by the 1D and 2D NMR spectra (Tables 1 and 2). By combining the data and consulting the literature [21], the aglycone of compound **3** was identified as 3*β*, 16*α*,26-trihydroxy-(23*R*, 25*R*)-16,23-cyclocholest-5,17(20)-dien-22-one.

The monosaccharide residues were identified as D-Glc and L-Rha in a ratio of 3∶1 by acid hydrolysis and GC analysis. In the ^{1}H NMR spectrum, four anomeric protons were observed at δ_H 4.98 (d, *J* = 7.8 Hz, H-1 of Glc Ⅰ), δ_H 4.88 (d, *J* = 7.2 Hz, H-1 of Glc Ⅱ), δ_H 5.14 (d, *J* = 7.6 Hz, H-1 of Glc Ⅲ) and δ_H 6.43 (br s, H-1 of Rha). According to the HSQC spectrum, the corresponding carbon signals were detected at δ_C 100.5, 105.8, 105.1 and 102.7. The anomeric proton coupling constants of D-glucopyranose (*J* = 7.8/7.2/7.6 Hz > 7.0 Hz) suggested the *β* orientation of three D-Glc, respectively. The *α* orientation of L-rhamnopyranosyl was confirmed by the chemical shifts of C-5 of L-Rha (δ_C 70.1). The linkage sequence of the sugar chain was confirmed by the cross peaks

Table 3 ^{13}C NMR data of **1**–**4** in CD_3OD (δ in ppm, 200 MHz)

No.	1	2	3	4	No.	1	2	3	4
1	38.8	38.3	38.0	39.9		3-*O*-*β*-D-Glc	3-*O*-*β*-D-Glc	3-*O*-*β*-D-Glc Ⅰ	3-*O*-*β*-D-Xyl
2	30.9	30.8	30.5	27.1	1	100.7	100.7	100.5	107.2
3	79.3	78.9	78.1	91.3	2	79.2	79.1	77.5	75.4
4	39.7	39.6	39.1	40.3	3	79.5	79.5	89.9	78.2
5	142.1	146.5	141.4	57.1	4	77.9	77.9	70.1	72.3
6	122.8	125.4	122.1	19.5	5	72.0	72.0	78.4	64.5
7	33.8	65.9	32.3	33.4	6	62.9	62.9	62.9	
8	*33.6*	*39.4*	*32.5*	*40.8*		*α*-L-Rha	*α*-L-Rha	*α*-L-Rha	*α*-L-Ara
9	51.6	43.4	50.9	49.2	1	102.3	102.3	102.7	104.4
10	38.2	38.9	37.7	38.0	2	72.4	72.4	73.0	75.4
11	21.7	21.5	21.1	24.7	3	72.5	72.5	73.3	76.8
12	32.4	32.7	36.2	123.9	4	74.1	74.1	74.6	79.7
13	46.3	45.7	44.5	145.0	5	69.9	69.9	70.1	69.5
14	53.9	46.7	54.2	43.1	6	18.1	18.1	19.7	
15	32.3	32.2	38.9	29.1				*β*-D-Glc Ⅲ	*α*-L-Rha
16	90.1	90.9	83.4	24.2	1			105.1	103.0
17	91.5	91.5	182.6	48.2	2			75.8	72.5
18	17.3	17.5	16.1	42.7	3			79.0	73.3
19	20.0	18.8	19.9	47.4	4			72.0	73.9
20	53.2	45.8	128.7	31.7	5			79.2	70.8
21	59.6	9.3	9.0	35.0	6			62.9	18.0
22	110.2	111.1	212.8	34.1				26-*O*-*β*-D-Glc Ⅱ	28-*O*-*β*-D-Glc
23	33.4	32.7	57.9	28.6	1			105.8	95.9
24	29.6	29.6	29.8	17.1	2			75.5	74.0
25	31.4	31.5	32.3	16.2	3			79.1	78.3
26	67.9	67.9	77.8	18.0	4			72.2	77.6
27	17.6	17.6	17.6	26.3	5			79.0	76.9
28				178.2	6			63.3	61.9
29				33.6					
30				24.3					

of δ_H 6.43 (Rha H-1) to δ_C 77.5 (Glc Ⅰ C-2), δ_H 5.14 (Glc Ⅲ H-1) to δ_C 89.9 (Glc Ⅰ C-3), δ_H 4.98 (Glc Ⅰ H-1) to δ_C 78.1 (C-3), δ_H 4.88 (Glc Ⅱ H-1) to δ_C 77.1 (C-26) in the HMBC spectrum (Fig. 4). Thus, the structure of compound **3** was characterized as 26-*O*-*β*-D-glucopyranosyl-3*β*,16*α*,26-trihydroxy-(23*R*,25*R*)-16,23-cyclocholest-5,17(20)-dien-22-one-3-*O*-*β*-D-glucuropyranosyl-(1→3)-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-glucopyranoside.

Compound **4** was obtained as white amorphous powder and positive for Liebermann-Burchard and Molish test. The ion peak in the HR-ESI-MS spectrum at *m/z* 1051.5450 $[M + Na]^+$ (calculated for $C_{52}H_{84}O_{20}Na$, 1051.5454) revealed that the molecular formula was $C_{52}H_{84}O_{20}$. In the ^{1}H NMR spectrum, seven methyl proton signals were observed at δ_H 1.05 (3H, s, H-23), δ_H 0.85 (3H, s, H-24), δ_H 0.96 (3H, s, H-25), δ_H 0.81 (3H, s, H-26), δ_H 1.16 (3H, s, H-27), δ_H 0.92 (3H, s, H-29) and δ_H 0.96 (3H, s, H-30), and one olefinic methine proton signal at δ_H 5.26 (1H, br s, H-12). Correspondingly, in the ^{13}C NMR spectrum, there were seven methyl carbon signals at δ_C 28.6 (C-23), 17.1 (C-24), 16.2 (C-25), 18.0 (C-26), 26.3 (C-27), 33.6 (C-29) and 24.3 (C-30), a pair of trisubstituted double bond carbons at δ_C 145.0 (C-13) and 123.9 (C-

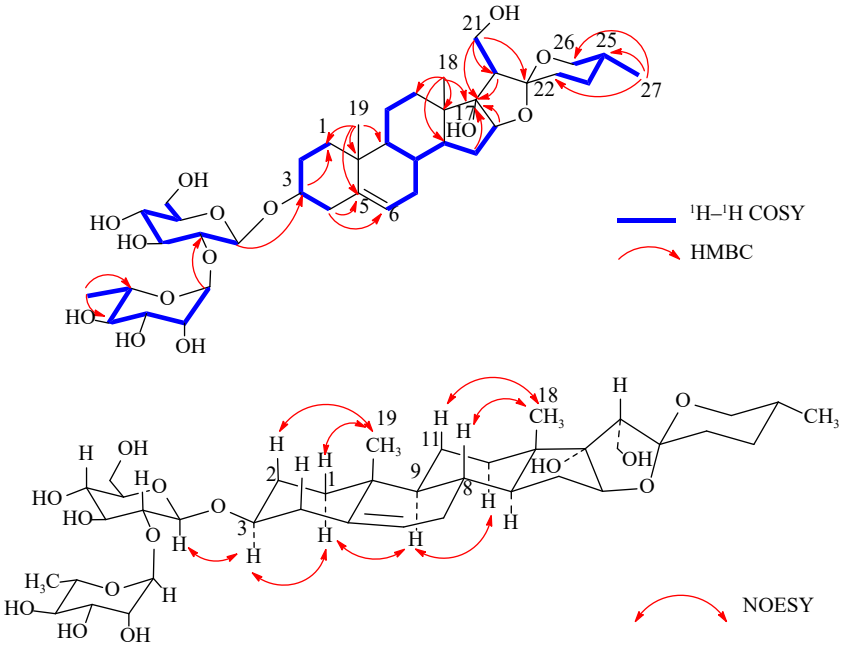

Fig. 3 Key ^{1}H–^{1}H COSY, HMBC and NOESY correlations of compound 1.

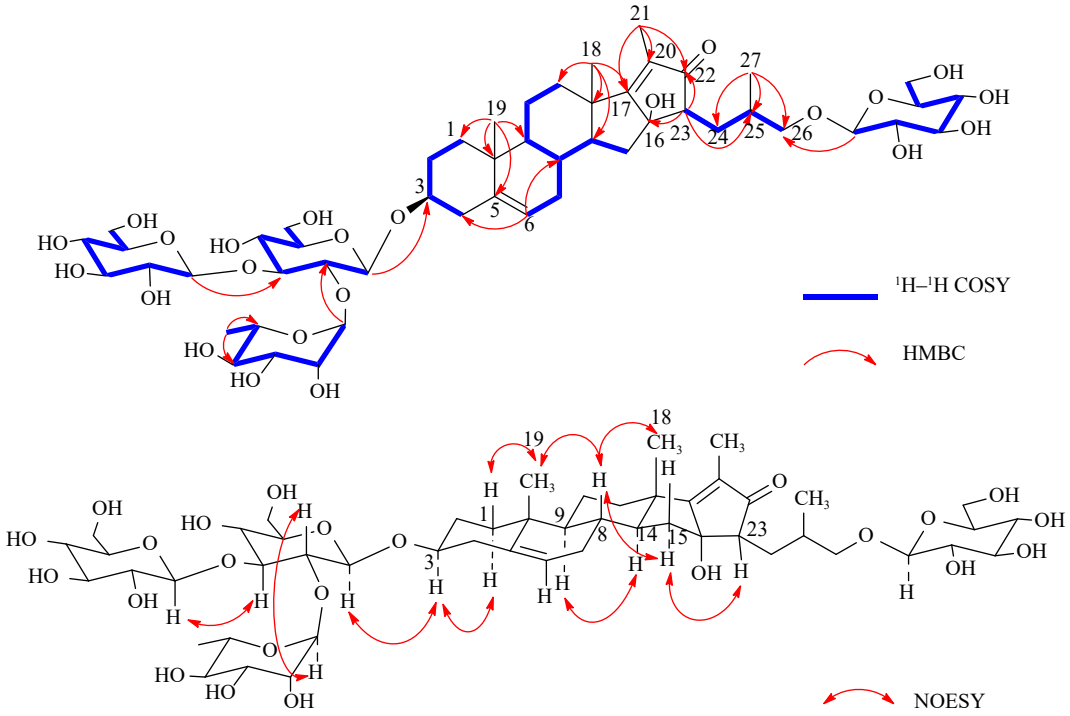

Fig. 4 Key ^{1}H–^{1}H COSY, HMBC and NOESY correlations of compound 3.

12). In addition, there were two characteristic carbon signals at δ_C 91.3 (C-3) and δ_C 178.2 (C-28). By combining the data and consulting the literature [22], the aglycone of compound **4** was identified as oleanane type structure. The correlations between δ_H 1.90 (H-11)/δ_H 5.26 (H-12)/δ_{Ha} 1.16, δ_{Hb} 1.71 (H-19)/δ_H 1.16 (H-27) and δ_C 145.0 (C-13), δ_H 2.86 (H-18) and δ_C 123.9 (C-12), δ_H 5.26 (H-12) and δ_C 49.2 (C-9)/δ_C 24.7 (C-11)/δ_C 43.1 (C-14) in the HMBC spectrum indicated that the double bond was located at C-12/C-13 (Fig. 5). In the NOESY spectrum, the correlation between δ_H 3.15 (H-3) and δ_{Ha} 1.62 (H-1) combined with the correlation between δ_{Hb} 0.97 (H-1) and δ_H 1.58 (H-9) suggested that H-3 was in α orientation, thus the hydroxyl group at C-3 was in β configuration. All the carbon and hydrogen signals on the aglycone were assigned orderly by 1D and 2D NMR (Tables 1 and 2).

In the ^{13}C NMR spectrum, there were four anomeric car-

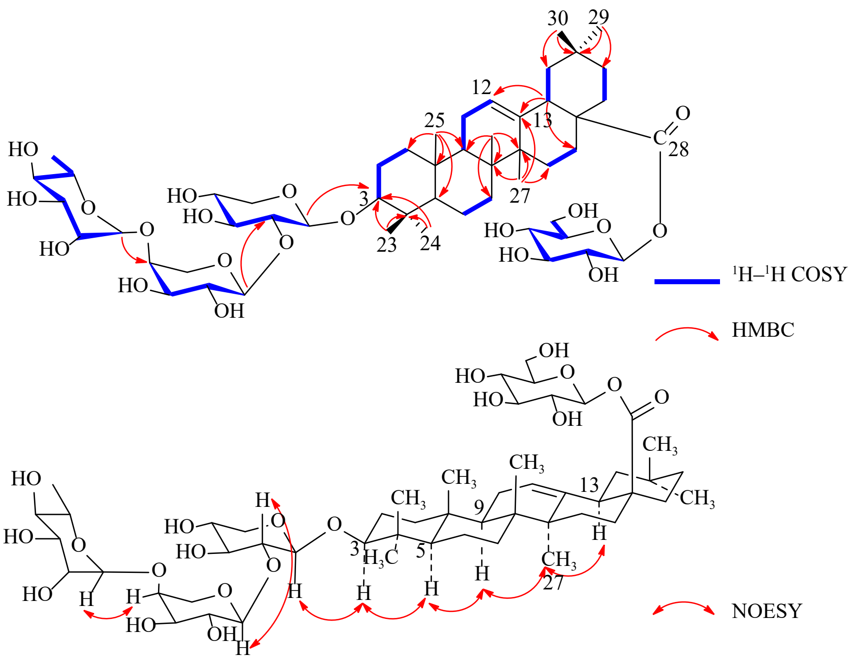

Fig. 5 Key ¹H–¹H COSY, HMBC and NOESY correlations of compound 4.

bon signals at δ_C 95.9, 107.2, 104.4 and 103.0 which indicated the presence of four monosaccharides. The monosaccharide residues were identified as D-Glc, D-Xyl, L-Ara and L-Rha in a ratio of 1 : 1 : 1 : 1 by acid hydrolysis and GC analysis. In addition, the corresponding anomeric protons were detected at δ_H 5.35 (d, J = 8.2 Hz, H-1 of Glc), δ_H 4.39 (d, J = 7.8 Hz, H-1 of Xyl), δ_H 4.42(d, J = 7.9 Hz, H-1 of Ara) and δ_H 4.85 (br s, H-1 of Rha) in the ^{1}H NMR spectrum. The coupling constants of anomeric protons of D-glucopyranose (J = 8.2 Hz > 7.0 Hz), D-xylopyranose (J = 7.8 Hz > 7.0 Hz) and L-arabopyranose (J = 7.9 Hz > 7.0 Hz) suggested that the anomeric proton of the moieites were in *β* configuration, *β* configuration and *α* configuration, respectively. The *α* configuration of L-rhamnopyranosyl was deduced from the chemical shifts of C-5 of Rha (δ_C 70.8). The linkage sequence of the sugar chain was determined by the cross peaks of δ_H 4.85 (Rha H-1) to δ_C 79.7 (Ara C-4), δ_H 4.42 (Ara H-1) to δ_C 75.4 (Xyl C-2), δ_H 4.39 (Xyl H-1) to δ_C 91.3 (C-3), δ_H 5.35 (Glc H-1) to δ_C 178.2 (C-28) in the HMBC spectrum. The above structure analysis was further verified in NOESY spectrum (Fig. 5). Thus, the structure of compound **4** was characterized as 3*β*-*O*-[*α*-L-rhamnopyranosyl-(1→4)-*α*-L-arabinopyranosyl-(1→2)-*β*-D-xylopyranosyl]-28-*O*-[*β*-D-glucopyranosyl]-oleanolate.

Compound **5** was obtained as white amorphous powder and positive for Liebermann-Burchard and Molish test. The ion peak in the HR-ESI-MS spectrum at *m/z* 1667.7680 [M + Na]$^+$ (calculated for $C_{76}H_{124}O_{38}Na$, 1667.7668) suggested that the molecular formula was $C_{76}H_{124}O_{38}$. A detailed comparison of ^{13}C NMR spectrum of **5** (Table 4) with that of **4** (Table 1) showed that these two compounds possessed the same aglycone moiety.

In the ^{1}H NMR spectrum (Table 4), there were eight anomeric protons at δ_H 4.55 (d, J = 4.6 Hz, H-1 of Ara I), δ_H 5.11 (br s, H-1 of Rha I), δ_H 5.33 (d, J = 8.2 Hz, H-1 of Glc I), δ_H 4.85 (br s, H-1 of Rha II), δ_H 4.61 (d, J = 7.8 Hz, H-1 of Ara II), δ_H 4.42 (d, J = 7.8 Hz, H-1 of Glc II), δ_H 4.41 (d, J = 7.8 Hz, H-1 of Glc III) and δ_H 4.86 (br s, H-1 of Rha III) which were accorded with eight anomeric carbon signals at δ_C 105.0, 102.2, 95.9, 102.8, 105.4, 104.8, 104.4 and 103.0 in the ^{13}C NMR spectrum. The monosaccharides were identified as D-Glc, L-Ara and L-Rha in a ratio of 3 : 2 : 3 by acid hydrolysis and GC analysis. The anomeric proton coupling constants of D-glucopyranose (J = 8.2/7.8/7.8 Hz > 7.0 Hz) and L-arabopyranose (J = 7.8 Hz > 7.0 Hz) suggested that the anomeric proton of the moieites were in *β* configuration and *α* configuration, respectively [23]. The *α* anomeric configurations of L-rhamnopyranosyl were identified by the chemical shifts of C-5 of Rha at δ_C 70.4 (Rha I), δ_C 69.3 (Rha II), δ_C 70.8 (Rha III). In HMBC spectrum (Fig. 6), the correlations from δ_H 4.86 (Rha III H-1) to δ_C 70.3 (Glc III C-6), δ_H 4.41 (Glc III H-1) to δ_C 69.6 (Glc II C-6), δ_H 4.42 (Glc II H-1) to δ_C 76.1 (Ara II C-2), δ_H 4.61 (Ara II H-1) to δ_C 83.1 (Rha II C-4), δ_H 4.85 (Rha II H-1) to δ_C 79.4 (Glc I C-), δ_H 5.33 (Glc I H-1) to δ_C 178.3 (C-28), δ_H 5.11 (Rha I H-1) to δ_C 76.8 (Ara I C-2) and δ_H 4.55 (Ara I H-1) to δ_C 90.8 (C-3) indicated the connection of two saccharide chains to aglycone. Thus, the structure of compound **5** was elucidated as 3*β*-*O*-[*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl]-28-*O*-[*α*-L-rhamnopyran-osyl-(1→6)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→2)-*α*-L-arabinopyr-anosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl]-oleanolate.

The four known steroidal saponins, were identified as 26-*O*-*β*-D-glucopyranosyl-homo-arocholest-5-ene-3-*O*-*α*-L-arabinofuranosyl-(1→4)-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-glucopyranoside (**6**) [24], pennogenin-3-*O*-*β*-D-glucuropyrano-

Table 4 ^{13}C NMR (200 MHz) and ^{1}H NMR (800 MHz) data of **5** in CD_3OD (δ in ppm)

No.	δ_C	δ_H	No.	δ_C	δ_H	No.	δ_C	δ_H
1	40.1	a 1.63 m, b 0.99 m	29	33.7	0.92 s	6	18.4	1.34 d (6.2)
2	27.2	a 1.83 m, b 1.72 m	30	24.3	0.95 s	*α*-L-Ara Ⅱ		
3	90.8	3.12 dd (11.7, 4.3)	3-*O*-*α*-L-Ara Ⅰ			1	105.4	4.61 d (7.8)
4	40.4	–	1	105.0	4.55 d (4.6)	2	76.1	3.22 m
5	57.2	–	2	76.9	3.78 m	3	78.3	3.52 m
6	19.6	a 1.55 m, b 1.41 m	3	68.6	3.79 m	4	72.3	3.70 m
7	33.5	a 1.72 m, b 1.59 m	4	77.4	3.40 m	5	64.6	a 3.58 m, b 3.52 m
8	40.9	–	5	63.9	a 3.85 m, b 3.48 m	*β*-D-Glc Ⅱ		
9	49.2	1.58 m	*α*-L-Rha Ⅰ			1	104.8	4.42 d (7.8)
10	38.1	–	1	102.2	5.11 br s	2	75.4	3.25 m
11	24.7	1.90 m	2	72.3	3.88 m	3	79.8	3.53 m
12	124.0	5.26 br s	3	73.9	3.42 m	4	71.6	3.34 m
13	145.1	–	4	74.0	3.33 m	5	77.0	3.29 m
14	43.1	–	5	70.4	3.81 m	6	69.6	a 4.08 m, b 3.80 m
15	29.1	–	6	18.2	1.23 d (6.2)	*β*-D-Glc Ⅲ		
16	24.2	a 2.05 m, b 1.72 m	28-*O*-*β*-D-Glc Ⅰ			1	104.4	4.41 d (7.8)
17	48.2	–	1	95.9	5.33 d (8.2)	2	75.5	3.23 m
18	42.7	–	2	76.9	3.33 m	3	78.3	3.77 m
19	47.4	a 1.72 m, b 1.15 m	3	78.4	3.41 m	4	71.1	3.41 m
20	31.7	–	4	79.4	3.56 m	5	77.0	3.29 m
21	35.0	a 1.48 m, b 1.32 m	5	70.8	3.97 m	6	70.3	a 4.13 m, b 3.79 m
22	34.1	–	6	62.0	a 3.82 m, b 3.67 m	*α*-L-Rha Ⅲ		
23	28.8	1.02 s	*α*-L-Rha Ⅱ			1	103.0	4.86 br s
24	17.3	0.85 s	1	102.8	4.85 br s	2	72.6	3.85 m
25	16.3	0.97 s	2	72.5	3.87 m	3	73.3	3.73 m
26	18.0	0.80 s	3	72.4	3.64 m	4	74.0	3.39 m
27	26.5	1.16 s	4	83.1	3.67 m	5	70.8	3.97 m
28	178.3	–	5	69.3	4.08 m	6	18.2	1.28 d (6.4)

syl-(1→3)-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-glucopyranoside (**7**) [25], pennogenin-3-*O*-*β*-D-glucuropyranosyl-(1→5)-*α*-L-arabinofuranosyl-(1→4)-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-glucopyranoside (**8**) [26], 26-*O*-*β*-D-glucopyranosyl-(3*β*,25*R*)-cholesta-5,17(20)-dien-16,22-dione-3-*O*-*α*-L-rhamnopyranosyl-(1→4)-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-glucopyranoside (**9**) [20] by comparing the physical and spectroscopic data with those reported in the literatures.

To determine the effective anticancer components of *P. polyphylla* var. *stenophylla*, the cytotoxic activity of saponins **1**–**9** against three glioma cell lines, U87MG, U251MG and SHG44, was evaluated using the CCK-8 method, and temozolomide was used as the positive control. The results showed that compounds **7** and **8** displayed certain inhibitory effect with IC_{50} values of 15.22 ± 1.73/31.19 ± 1.55, 18.87 ± 1.81/29.37 ± 2.04 and 17.64 ± 1.69/33.46 ± 1.72 μmol·L^{-1}, respectively, while others were inactive. From the data, we speculated that spirostanol saponins were more cytotoxic than furostanol saponins, triterpenoid saponin and cholestanol-type saponins, which meant that the opening of the F-ring would lose its antitumor activity.

Experimental

General experimental procedures

Optical rotation was measured on a Perkin-Elmer 241 MC digital polarimeter (German PerkinElmer Corporation, Boelingen, Germany). 1D and 2D-NMR spectral experiments were performed in deuterated methanol (CD_3OD) on a

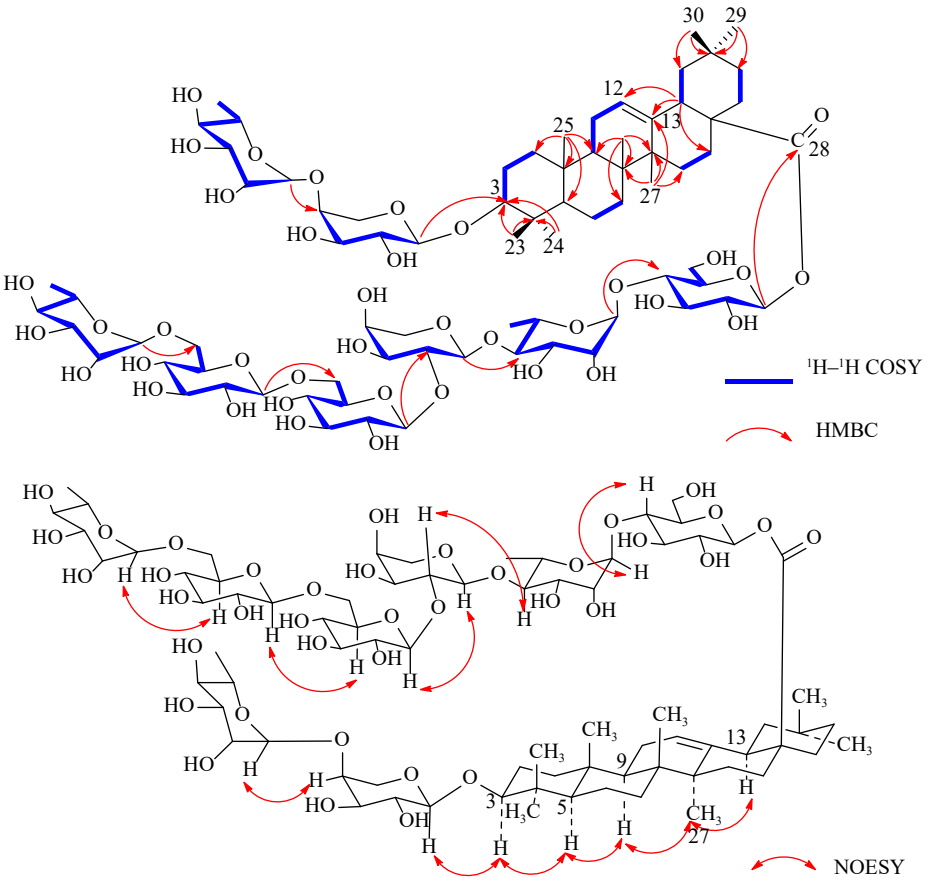

Fig. 6 Key ^{1}H–^{1}H COSY, HMBC and NOESY correlations of compound 5.

Bruker AVANCE-800^{TM} spectrometer (Burker Corporation, Karisruhe, Germany) with TMS as an internal standard on a Waters.

HR-ESI-MS spectra were obtained using an Agilent G6520 Q-TOP instrument (Agilent, Santa Clara, CA, USA). ESI-MS spectra were obtained using a Thermo Fisher FINNIGAN LTQ (Thermo Fisher Corporation, USA). Column chromatography (CC) was performed on a Sephadex LH-20 column (GE-Healthcare, Uppsala, Sweden), ODS silica gel (LiChroprep® RP-18, 40–63 μm, Merck Inc., Darmstadt, Germany), and silica gel H (10–40 μm, Qingdao Marine Chemical Inc., Qingdao, China). GC-MS analysis was performed on a Shimadzu GCMS-QP 2010 apparatus using a Rxi®-5Sil MS column (30 m × 0.25 mm, 0.25 μm) and an FID detector at an initial temperature of 120 °C for 2 min and then temperature programming to 250 °C at the rate of 10 °C·min^{-1}. The standards for D-glucose (D-Glc), D-xylose (D-Xyl), L-arabopyranose (L-Ara), L-arabinofuranose (L-Ara*f*), and L-rhamnose (L-Rha) were purchased from Sigma Chemical Co. (St, Louis, MO, USA).

Plant material

The rhizomes of *P. polyphylla* var. *stenophylla* were collected from Ankang, Shaanxi Province, China, in August 2021 and identified by the corresponding author TANG Haifeng. The voucher sample (No. 20210825) was deposited in Department of Chinese Materia Medica and Natural Medicines, School of Pharmacy, Air Force Medical University, Xi'an, China.

Extraction and isolation

The dried rhizomes of *P. polyphylla* var. *stenophylla* (1.9 kg) were chopped and refluxed with 70% ethanol (10.0 L) thrice (2 h for each). The ethanol extract was mixed and condensed with a vacuum rotary evaporator to receive a syrupy residue (484.4 g). The syrupy residue was suspended in water (3.0 L) and extracted with petroleum ether and water saturated *n*-BuOH, successively. The water saturated in the *n*-BuOH layer was evaporated and separated by silica gel column chromatography, which was eluted by CH_2Cl_2–MeOH–H_2O (50 ∶ 1 ∶ 0, 20 ∶ 1 ∶ 0, 8 ∶ 1 ∶ 0.1, 6 ∶ 1 ∶ 0.1, 4 ∶ 1 ∶ 0.1, 7 ∶ 2.5 ∶ 0.1 and 6.5 ∶ 3.5 ∶ 0.1) to offer 12 fractions (Frs. 1–12) based on the result of TLC analysis. Fr. 11 was separated by silica gel column chromatography and eluted by a gradient eluent of CH_2Cl_2–MeOH–H_2O (8 ∶ 1 ∶ 0.1, 8 ∶ 2 ∶ 0.2, 7 ∶ 2.5 ∶ 0.1 and 6 ∶ 3 ∶ 0.1) to get Frs. 11-1 (1.1 g) and 11-2 (830 mg). Fr. 11-1 was eluted by MeOH on a Sephadex LH-20 to get rid of pigmentum and separated to Frs. 11-1-1 (50 mg), 11-1-2 (45 mg) and 11-1-3 (141 mg) on ODS silica gel. Then, Frs. 11-1-1 and 11-2 were isolated by semi-preparative HPLC using MeCN–H_2O (35 ∶ 65, 40 ∶ 60) as the mobile phase at a flow rate of 10.0 mL·min^{-1} to afford compounds **1** (3.3 mg, t_R = 22.3 min) and **2** (3.9 mg, t_R = 45.8 min), respectively. Fr. 10 was eluted by

MeOH on a Sephadex LH-20 to remove pigmentum, affording Frs. 10-1 (3.9 g), 10-2 (5.1 g), 10-3 (380 mg) and 10-4 (433 mg). Frs. 10-3 and 10-4 were subjected to ODS silica gel and purified by semi-preparative HPLC using MeCN–H_2O (40 ∶ 60) as the mobile phase at a flow rate of 10.0 mL·min^{-1} to afford compounds **4** (18.3 mg, t_R = 40.2 min) and **5** (5.9 mg, t_R = 20.5 min). Fr. 6 was eluted by MeOH on a Sephadex LH-20 to remove pigmentum, affording Frs. 6-1 (2.7 g), and 6-2 (3.2 g). Fr. 6-1 was subjected to ODS silica gel and purified by semi-preparative HPLC using MeCN–H_2O (40 ∶ 60) as the mobile phase at a flow rate of 10.0 mL·min^{-1} to afford compounds **6** (11.3 mg, t_R = 15.8 min), **7** (58.6 mg, t_R = 51.2 min) and **8** (14.8 mg, t_R = 35.7 min). Fr. 8 was eluted by MeOH on a Sephadex LH-20 to remove pigmentum , affording Frs. 8-1 (1.1 g), and 8-2 (910 mg). Fr. 8-1 was subjected to ODS silica gel and purified by semi-preparative HPLC using MeCN–H_2O (20 ∶ 80) as the mobile phase at a flow rate of 10.0 mL·min^{-1} to afford compound **3** (2.5 mg, t_R = 47.6 min) and compound **9** (1.9 mg, t_R = 24.5 min). The purity of all compounds was assessed by HPLC to be more than 95%.

Compound characterization data

Compound **1**: white amorphous solid, $[\alpha]_D^{22}$ –49.2 (*c* 0.05, MeOH); positive ESI-MS *m/z* 777.47 $[M + Na]^+$, negative *m/z* 753.28 $[M - H]^-$; positive HR-ESI-MS *m/z* 777.4044 $[M + Na]^+$ (calculated for $C_{39}H_{62}O_{14}Na$, 777.4037); ^1H NMR (800 MHz, CD_3OD) and ^{13}C NMR (200 MHz, CD_3OD) data, see Tables 1−3.

Compound **2**: white amorphous solid, $[\alpha]_D^{22}$ –87.0 (*c* 0.15, MeOH); positive ESI-MS *m/z* 777.42 $[M + Na]^+$, negative *m/z* 753.25 $[M - H]^-$; positive HR-ESI-MS *m/z* 777.4049 $[M + Na]^+$ (calculated for $C_{39}H_{62}O_{14}Na$, 777.4037); ^1H NMR (800 MHz, CD_3OD) and ^{13}C NMR (200 MHz, CD_3OD) data, see Tables 1−3.

Compound **3**: white amorphous solid, $[\alpha]_D^{22}$ –80.7 (*c* 0.05, MeOH); positive ESI-MS *m/z* 1083.46 $[M + Na]^+$, negative *m/z* 1059.50 $[M - H]^-$; negative HR-ESI-MS *m/z* 1059.4998 $[M - H]^+$ (calculated for $C_{50}H_{77}O_{22}$, 1059.5018); ^1H NMR (800 MHz, CD_3OD) and ^{13}C NMR (200 MHz, CD_3OD) data, see Tables 1−3.

Compound **4**: white amorphous solid, $[\alpha]_D^{22}$ –23.0 (*c* 0.06, MeOH); positive ESI-MS *m/z* 1667.86 $[M + Na]^+$, negative *m/z* 1643.26 $[M - H]^-$; positive HR-ESI-MS *m/z* 1667.7680 $[M + Na]^+$ (calculated for $C_{76}H_{124}O_{38}Na$, 1667.7668); ^1H NMR (800 MHz, CD_3OD) and ^{13}C NMR (200 MHz, CD_3OD) data, see Tables 1−3.

Compound **5**: white amorphous solid, $[\alpha]_D^{22}$ –18.2 (*c* 0.05, MeOH); positive ESI-MS *m/z* 1051.54 $[M + Na]^+$, negative *m/z* 1027.55 $[M - H]^-$; positive HR-ESI-MS *m/z* 1051.5450 $[M + Na]^+$ (calculated for $C_{52}H_{84}O_{19}Na$, 1051.5454); ^1H NMR (800 MHz, CD_3OD) and ^{13}C NMR (200 MHz, CD_3OD) data, see Table 4.

*Acid hydrolusis and GC-MS analysis of the sugar moieties in compounds **1**–**5***

The monosaccharide compositions of compounds **1**–**5** were analyzed by GC-MS according to methods described in the literature [27]. Compounds (each 2 mg) were heated in a tube with 1 mL of 2 mol·L^{-1} trifluoroacetic acid (TFA) at 110 °C for 90 min. The reaction mixture was diluted in 2 mL of distilled water, reduced with 100 mg of NaBH4, and acetylated with acetic anhydride at 100 °C for 1 h. The acetylated products were finally analyzed by GC-MS under the following conditions: carrier gas was He (1.0 mL·min^{-1}), injector temperature was 250 °C, injection volume was 1.0 μL, and column temperature program was 120–250 °C at a rate of 3 °C·min^{-1} and maintained at 250 °C for 5 min. The absolute configurations of the sugar moieties from compounds **1**–**5** were identified as D-Glc (t_R = 26.52 min), L-Rha (t_R = 18.48 min), L-Ara (t_R = 18.20 min), and D-Xyl (t_R = 19.45 min) by comparing the retention times with standard monosaccharides.

Cytotoxic assay

U87, U251 and SHG44 (human glioblastoma cells) were purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Science. The cytotoxicity of compounds **1**–**9** against glioma cells was detected using the CCK-8 method in 96-well microplates. The DMEM or RPMI-1640 culture medium (Hyclone, USA) was supplemented with 10% FBS (GIBCO, USA), 1% penicillin, and streptomycin (Elabscience Biotechnology Co., Ltd., Wuhan, China), in which the cell lines were cultured at 37 °C with 5% CO_2. Cells in the logarithmic phase (5000 cells/well) were seeded in 96-well plates (100 μL/well) incubated for 24 h, then treated with various concentrations of saponins **1**–**11** (0.5, 1, 2, 4, 8, 16, 32, and 64 μmol·L^{-1} in medium containing < 0.1 % DMSO) for 24 h, separately. Then, 10 μL CCK-8 reagent (Elabscience Biotechnology Co., Ltd., Wuhan, China) was added to each well and incubated at 37 °C with 5% CO_2 for 2 h. The optical density of each well was measured using a microplate reader (BioTek, USA) at a wavelength of 450 nm. The IC_{50} values of saponins **1**–**9** were evaluated according to their optical densities**.** The experiments were conducted in three independent replicates, and doxorubicin (Sigma, purity ≥ 99 %) was used as the positive control.

Conclusions

This study affords 9 compounds from the rhizomes of *P. polyphylla* var. *stenophylla*, among which compounds **1**, **2**, **7** and **8** are isospirostanol-type saponins, compound **3** is a cyclocholestanol-type saponin, compounds **4** and **5** are two triterpenoid saponins, and compound **6** is a homo-aro-cholestane saponin. The discovery of the new compounds **1**–**5** extends the diversity and complexity of the saponin family of the genus *Paris*. In addition, compounds **7** and **8** exhibit certain cytotoxic activity against three glioma cell lines, and the structure-activity relationship has been discussed.

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