Two New Triterpenoid Saponins Isolated from *Polygala japonica*

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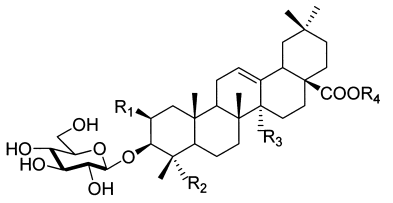
Bioassay guided investigation of whole parts of *Polygala japonica* afforded two new triterpenoid saponins, characterized as 3-*O*-β-D-glucopyranosyl medicagenic acid 28-*O*-{β-D-xylopyranosyl(1→4)-[β-D-apiofuranosyl (1→3)]-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl} ester (1), 3-*O*-β-D-glucopyranosyl 2-oxo-olean-12-en-23, 28-dioic acid 28-*O*-{β-D-xylopyranosyl(1→4)-[β-D-apiofuranosyl(1→3)]-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl} ester (2), together with four known triterpenoid saponins (3—6). Their structures were elucidated by spectroscopic and chemical methods. Saponins 3, 4 and 5 showed significant anti-inflammation effects on carrageenan-induced acute paw edema in mouse.

Key words *Polygala japonica*; Polygalaceae; triterpenoid saponin; anti-inflammatory activity

Polygala japonica HOUTT. (Polygalaceae) widely distributes in Asian, primarily in eastern China. It has long history of using *P. japonica* in traditional Chinese medicines for the treatment of various inflammatory disorders, such as acute tonsillitis, pharyngitis, myelitis and nephritis.[1] Our bioassay guided investigation of *P. japonica* has showed that the methanol (MeOH) extract have significant anti-inflammation effect against carrageenan-induced mouse paw edema, and the ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) fractions from it are the main active fractions. Our efforts further extended to isolate and identify the bioactive compounds in these fractions, which will support the wide utility of *P. japonica* in traditional Chinese medicines. We have isolated and elucidated two new triterpenoid saponins (**1**, **2**), along with four known triterpenoid saponins, 3-*O*-β-D-glucopyranoside bayogenin 28-*O*-β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl ester (**3**),[2] polygalasaponin V (**4**),[3] bayogenin-3-*O*-β-D-glucopyranoside (**5**)[4] and tenuifolin (**6**).[5] The structures of the known compounds were elucidated by comparison of their physical and spectral data with literature values. We report herein the isolation and structure elucidation of two new triterpenoid glycosides as well as the anti-inflammatory activity using the carrageenan-induced mouse paw edema for all the isolated saponins (Fig. 1).

Saponin **1** was obtained as a white amorphous powder, and assigned a molecular formula of $C_{58}H_{92}O_{28}$, as deduced from HR-ESI-MS and ^{13}C-NMR (Table 1) data analysis. The NMR data (Tables 1, 2) were characteristic of triterpenoid saponin with five sugar units. Detailed analysis of the ^{13}C-NMR spectrum revealed that 28 signals assignable to the sugar moiety and 30 to the aglycone. After acidic hydrolysis, D-apiose, D-xylose, D-glucose and L-rhamnose were identified by HPLC and GC analysis with authentic samples. The ^{1}H-NMR spectrum showed the presence of six tertiary methyl groups (δ_H 1.99, 1.56, 1.22, 1.14, 0.89, 0.84), five anomeric protons (δ_H 5.11, d, J=7.8 Hz; δ_H 5.35, d, J=7.7 Hz; δ_H 6.07, d, J=4.5 Hz; δ_H 6.18, br s; δ_H 6.24, d, J=7.1 Hz) and one trisubstituted olefinic proton (δ_H 5.43, t-like). Additionally, the ^{13}C-NMR spectra displayed six methyl groups, five anomeric carbons, and two olefinic carbons. A detailed comparison between **1** and the reference data of medicagenic acid,[6] a major aglycone present in this plant, implied that **1** possessed of the aglycone of medicagenic acid.

Complete assignments of each glycosidic proton system were achieved by analysis of 2D NMR experiments (TOCSY, HSQC and HMBC). Evaluation of spin–spin couplings and chemical shifts allowed the identification of two β-D-glucopyranosyl units, one α-L-rhamnopyranosyl unit, one β-D-xylopyranosyl unit and one β-D-apiofuranosyl unit. Anomeric configurations of the glucose units and xylose unit were all β configurations determined from $J_{H\text{-}1,H\text{-}2}$, whereas that of rhamnose was deduced as α- from the $J_{C1–H1}$ value (169) and the comparable ^{13}C-NMR chemical shifts of the rhamnose moiety in α-L-rhamnopyranosides as opposed to β-L-rhamnopyranosides.[7,8] A β-configuration of the anomeric center of the apiose was determined by the comparison of the ^{13}C-NMR data for **1** with those for α- and β-D-apiofuranosides,[9] and the $J_{H\text{-}1,H\text{-}2}$ coupling constant of the apiose was similar to the reported data of β-D-apiofuranoside.[10] The sugar sequences of the oligosaccharide chains as well as the glycosidic sites were subsequently determined by a combination of HSQC and HMBC experiments (Fig. 2), a β-D-glu-



Saponin	R_1	R_2	R_3	R_4
1	OH	COOH	CH_3	Glc^2--$^1Rha^4$--^1Xyl ^{3}| ^1Api
2	=O	COOH	CH_3	Glc^2--$^1Rha^4$--^1Xyl ^{3}| ^1Api
3	OH	CH_2OH	CH_3	Glc^2--$^1Rha^4$--^1Xyl
4	OH	CH_2OH	CH_3	Glc^2--$^1Rha^4$--^1Xyl ^{3}| ^1Api
5	OH	CH_2OH	CH_3	H
6	OH	COOH	CH_2OH	H

Fig. 1. Structures of Isolated Triterpenoid Glycosides **1—6**

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Table 1. ^{13}C-NMR Spectroscopic Assignment for Saponins **1** and **2** in Pyridine-d_5

Aglycone	**1**	**2**	Sugar	**1**	**2**
			C-3		
1	44.2	54.6	Glc-1	105.3	103.8
2	70.2	207.3	2	75.2	75.0
3	86.0	85.3	3	78.4	78.7
4	52.8	58.2	4	71.6	71.5
5	52.5	52.4	5	78.3	78.6
6	21.2	20.8	6	62.7	62.7
7	32.3	32.3	C-28		
8	40.4	39.9	Glc-1	94.7	94.8
9	48.7	47.9	2	78.4	78.1
10	36.8	43.2	3	78.6	78.8
11	23.9	23.8	4	71.3	71.3
12	122.8	122.1	5	78.6	78.8
13	144.0	144.2	6	62.4	62.4
14	42.2	42.2	Rha-1	101.7	101.8
15	28.0	28.2	2	71.2	71.4
16	23.5	23.4	3	82.2	82.8
17	46.9	47.0	4	78.7	78.7
18	42.0	42.0	5	68.6	68.6
19	46.3	46.2	6	19.0	19.0
20	30.7	30.8	Xyl-1	105.3	105.3
21	33.9	33.9	2	75.7	75.7
22	33.0	32.8	3	78.6	78.6
23	180.7	178.8	4	71.5	71.3
24	14.2	13.6	5	67.2	67.2
25	16.9	16.6	Api-1	111.6	111.6
26	17.5	17.1	2	77.6	77.6
27	26.0	26.0	3	79.5	79.5
28	176.4	176.4	4	74.6	74.6
29	33.0	33.1	5	64.6	64.6
30	23.8	23.7			

Table 2. ^{1}H-NMR Assignment of Sugar Moieties of Saponins **1** and **2** in Pyridine-d_5

H	**1**	**2**
C-3 sugar		
Glc-1	5.11 (1H, d, J=7.8 Hz)	5.16 (1H, d, J=7.6 Hz)
2	4.00 (1H, t, J=7.8 Hz)	4.01 (1H, t, J=7.6 Hz)
3	4.15	4.15
4	4.21	4.19
5	3.92 (1H, m)	3.88 (1H, m)
6	4.31	4.28
	4.46 (1H, dd, J=2, 12 Hz)	4.41 (1H, dd, J=2, 12 Hz)
C-28 sugar		
Glc-1	6.24 (1H, d, J=7.1 Hz)	6.22 (1H, d, J=7.1 Hz)
2	4.26 (1H, dd, J=2.5, 7.1 Hz)	4.26 (1H, dd, J=2.5, 7.1 Hz)
3	4.29	4.29
4	4.27	4.27
5	3.97 (1H, m)	3.96 (1H, m)
6	4.32	4.32
	4.38 (1H, dd, J=2, 12 Hz)	4.38
Rha-1	6.18 (1H, br s)	6.23 (1H, br s)
2	5.01 (1H, br s)	5.02 (1H, br s)
3	4.67 (1H, dd, J=3, 9 Hz)	4.65 (1H, dd, J=3, 9 Hz)
4	4.52 (1H, t, J=9 Hz)	4.52 (1H, t, J=9 Hz)
5	4.49 (1H, m)	4.48 (1H, m)
6	1.78 (3H, d, J=6 Hz)	1.78 (3H, d, J=6 Hz)
Xyl-1	5.35 (1H, d, J=7.7 Hz)	5.33 (1H, d, J=7.6 Hz)
2	3.99	3.98
3	4.09 (1H, t, J=7.7 Hz)	4.09 (1H, t, J=7.6 Hz)
4	4.16	4.17
5	3.50 (1H, t, J=10 Hz)	3.50 (1H, t, J=10 Hz)
	4.23	4.21
Api-1	6.07 (1H, d, J=4.5 Hz)	6.06 (1H, d, J=4.5 Hz)
2	4.78 (1H, d, J=4.5 Hz)	4.77 (1H, d, J=4.5 Hz)
3		
4	4.19 (1H, d, J=9 Hz)	4.19 (1H, d, J=9 Hz)
	4.58 (1H, d, J=9 Hz)	4.57 (1H, d, J=9 Hz)
5	4.04 (1H, d, J=11 Hz)	4.05 (1H, d, J=11 Hz)
	4.02 (1H, d, J=11 Hz)	4.02 (1H, d, J=11 Hz)

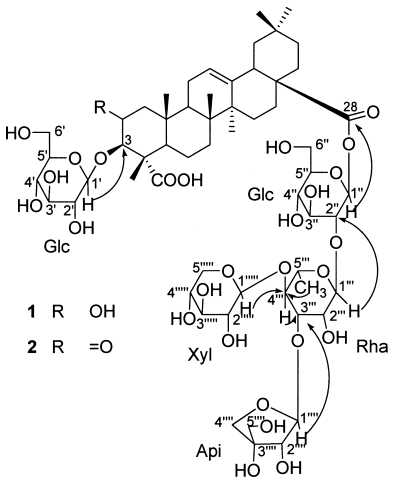

Fig. 2. Selected HMBC Correlations of Saponins **1** and **2**

copyranosyl (δ_H 5.11, δ 105.3) attached to C-3 of medicagenic acid, a β-D-glucopyranosyl ester (δ_H 6.24, δ 94.7) attached to C-28 of medicagenic acid, an α-L-rhamnopyranosyl (δ_H 6.18, δ 101.7) attached to C-2″ of β-D-glucopyranosyl ester and two terminal sugars: β-D-xylopyranosyl and β-D-apiofuranosyl. Comparing spectroscopic data with that of polygalasaponin XXII,[6] the ^{1}H- and ^{13}C-NMR data of **1** were similar to those of polygalasaponin XXII, except data of C-3″ of 28-glucose, C-3‴, C-4‴ of rhamnose. Due to the attachment of a terminal apiose to C-3″ of 28-glucose, a significant downfield shift was observed in polygalasaponin XXII at C-3″ (δ 86.5), while in saponin **1** the chemical shifts is at δ 78.6. The deshielding of C-3‴ (δ 82.2) and C-4‴ (δ 78.7) of rhamnose in **1** indicated two terminal sugars attached to it, instead of a terminal sugar attached to C-4‴ of rhamnose in polygalasaponin XXII [C-3‴ (δ 72.4), C-4″ (δ 84.4)]. In the HMBC spectrum of **1** (Fig. 2), the correlations could be achieved between the anomeric proton of apiose at δ_H 6.07 (d, J=4.5 Hz) and C-3‴ of rhamnose at δ 82.2, as well as between H-3‴ (δ_H 4.67) of rhamnose and the anomeric carbon of apiose at δ 111.6, which supported the terminal apiose attached to C-3‴ of rhamnose. All the data assigned that saponin **1** is as 3-*O*-β-D-glucopyranosyl medicagenic acid 28-*O*-{β-D-xylopyranosyl(1→4)–[β-D-apiofuranosyl(1→3)]-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl} ester.

Saponin **2** was obtained as a white amorphous powder. Its molecular formula was deduced as $C_{58}H_{90}O_{28}$ from the HR-ESI-MS and ^{13}C-NMR (Table 1). A comparison between the ^{13}C-NMR data of **2** and **1** revealed that the carbon signals of the aglycon of the two saponins were almost identical, except that a carbon signal corresponding to a carbonyl group in **2** (δ 207.3, C-2) was observed instead of a secondary alcohol methine carbon (δ 70.2, C-2) in **1** (Table 1). In addition, the ^{13}C-NMR data for the sugar moiety of **2** were observed as a close resemblance to those of **1**, suggesting that **2** has a silimiar sugar substitution pattern to **1**, that is, D-xylopyranosyl(1→4)-[β-D-apiofuranosyl(1→3)]-α-L-rhamnopyra-

Table 3. Effects of Saponins **1**—**6** on Carrageenan-Induced Mouse Paw Edema

Group (n=10)	Dose (μmol/kg)	Edema rate percentage (mean±S.E.M.)			
		1 h	2 h	3 h	4 h
Control		31.69±6.69	52.63±10.84	38.01±9.22	31.81±9.43
Indomethacin	55	15.93±6.05**	20.51±7.87**	17.49±7.62**	13.23±7.33**
Saponin **1**	0.1	39.85±10.93	55.74±7.40	34.65±8.82	39.85±10.93
Saponin **2**	0.1	36.59±9.98	53.86±11.14	39.90±9.90	34.22±8.28
Saponin **3**	0.1	8.07±1.92**	15.95±4.64**	11.21±2.34**	7.66±3.46**
Saponin **4**	0.1	23.12±7.24*	26.95±9.36**	21.57±6.91**	23.57±9.82
Saponin **5**	0.1	14.82±6.33**	16.00±8.52**	16.15±8.79**	13.39±5.31**
Saponin **6**	0.1	26.75±4.91	61.34±11.64	40.20±9.62	33.44±6.09

Values are expressed as mean±standard error (S.E.M.). Data were analyzed by a one-way ANOVA, followed by Dunnett's test. * $p<0.05$, ** $p<0.01$ significantly different from the control group.

nosyl(1→2)-β-D-glucopyranosyl at C-28 and a β-D-glucopyranosyl at C-3 (Table 1). These assignments were further confirmed by acidic hydrolysis, with the same method depicted for **1** (D-apiose, D-xylose, D-glucose and L-rhamnose detected by HPLC and GC) and the combination analysis of TOCSY, HMBC and HSQC experiments. Thus, from the above evidences saponin **2** was characterized as 3-*O*-β-D-glucopyranosyl 2-oxo-olean-12-en-23,28-dioic acid 28-*O*-{β-D-xylopyranosyl(1→4)-[β-D-apiofuranosyl(1→3)]-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl} ester.

In this study, carrageenan-induced mouse paw edema was selected as an *in vivo* model to evaluate the anti-inflammation activity. In the dose of 0.1 μmol/kg, saponins **3**, **4** and **5** significantly inhibited mouse paw edema induced by carrageenan, while saponins **1**, **2** and **6** did not exhibit anti-inflammatory effects although the similar structure were observed between them (Table 3). By comparing the chemical structure of the saponins, CH_2OH group at C-4 and oxygenated function (carboxyl or glycosyl ester) at C-17 of bayogenin seems crucial to maintain the acute anti-inflammatory effect. Further investigations are needed to develop the structure–activity relationship, which will facilitate the more rational modification of the compounds.

Experimental

General Experimental Procedures All melting points were measured on a X-4 digital micromelting point apparatus and were uncorrected. Optical rotations were determined on a JASCO P-1020 Polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded at 303 K, 500 MHz using an AV-500 spectrometer in C_5D_5N with TMS as internal standard. HR-ESI-MS was performed on an IonSpec 4.7 Tesla mass spectrometer. ESI-MS and MS/MS experiments were determined on a LC-MSD-Trap mass spectrometer. Silica gel (silica gel 60, 0.040—0.063 mm, Merck) and RP-18 (40—75 μm, Merck) were used for MPLC. HPLC analysis was performed on a Shimadzu apparatus equipped with a LC-10AT pump, an Alltech evaporative light scattering detector (ELSD) 500 detector and a Class-VP software using carbohydrate analysis column (Cosmosil, 4.6×250 mm, 5 μm). GC analysis was performed on a VARIAN Saturn 2100T GC-MS [column, CP SIL-5CB (0.25 mm×30 m); column temperature, 230 °C; carrier gas, N_2].

Plant Material The whole herb of *P. japonica* was purchased from Jiangsu Medical Material Company (Jiangsu, China), and was identified by Dr. Zenglai Xu (Jiangsu Zhongshan Arboretum, Nanjing, China). The voucher specimen (ZDN020518) was deposited at the Herbarium of China Pharmaceutical University.

Extraction and Isolation The dried whole plant of *P. japonica* (9 kg) was ground to a coarse powder and extracted with methanol for 12 h. The methanol extract was evaporated *in vacco* to yield a dark residue (1651 g, yield 18.34%). Except 5% of the residue left for bio-guided test, all the remaining was suspended in water, and then partitioned successively with petroleum ether, ethyl acetate and *n*-butanol, yielding three fractions, PEF (148.0 g, yield 1.73%), EAF (1103.6 g, yield 12.9%) and *n*-BF (108.28 g, yield 1.27%). Part of the fraction EAF was first subjected to chromatography column (CC, 8 cm×120 cm) over silica gel (100—200 mesh) using $CHCl_3$/MeOH as a solvent system. A total of 102 fractions were collected and then combined on the basis of similar TLC profiles to give ten sub-fractions denoted as I—X. Then, the sub-fractions IV (14.2 g) and V (6.3 g) eluted with $CHCl_3$/MeOH (1000 ml, 8/1) and (1000 ml, 5/1) by MPLC over silica gel 60 (0.040—0.063 mm) (150, 80 g), respectively, were further chromatographied on a reversed-phase RP18 MPLC (30 g, 2 cm×40 cm) using a gradient of MeOH/H_2O (500 ml each, from 7/3 to 9/1) to afford saponin **5** (25 mg) and **6** (16 mg), respectively. Part of the *n*-BF fraction was applied to CC (6 cm×100 cm) over silica gel (200—300 mesh) eluting with a gradient of $CHCl_3$/MeOH to give nine sub-fractions A—I according to their TLC behavior. Sub-fraction F (5.6 g) eluted with $CHCl_3$/MeOH (3/1) was further separated by reversed-phase RP18 MPLC (30 g, 2 cm×40 cm) using a gradient of MeOH/H_2O (800 ml each, from 6/4 to 8/2) to give saponin **3** (25 mg) and saponin **4** (36 mg), while sub-fraction H (5.1 g) eluted with $CHCl_3$/MeOH (2/1) was separated by reversed-phase RP18 MPLC (30 g, 2 cm×40 cm) using a gradient of MeOH/H_2O (800 ml each, from 5/5 to 7/3) to give saponin **1** (27 mg) and saponin **2** (30 mg).

Saponin (**1**): A white powder, mp 246—248 °C, $[\alpha]_D^{23}$ −22.5° (c=0.15, MeOH), HR-ESI-MS *m/z*: 1259.5663 $[M+Na]^+$ (Calcd for $C_{58}H_{92}O_{28}Na$: 1259.5673), ESI-MS (negative-ion mode) *m/z*: 1236 $[M-H]^-$. For ^{1}H- and ^{13}C-NMR (C_5D_5N) data, see Tables 1 and 2.

Saponin (**2**): A white powder, mp 241—243 °C, $[\alpha]_D^{23}$ −21.6° (c=0.22, MeOH), HR-ESI-MS *m/z*: 1257.5532 $[M+Na]^+$ (Calcd for $C_{58}H_{90}O_{28}Na$: 1257.5516), ESI-MS (negative-ion mode) *m/z*: 1234 $[M-H]^-$. For ^{1}H- and ^{13}C-NMR (C_5D_5N) data, see Tables 1 and 2.

Acidic Hydrolysis of Saponins 1, 2 Each saponin of **1**, **2** (5 mg) was refluxed with 2 ml of 2 M HCl (dioxane–H_2O, 1 : 1) at 100 °C for 4 h. After dioxane was removed, the solution was diluted with H_2O and extracted with EtOAc (1 ml×3) to remove the aglycone. The water layer was neutralized by passing through an Amberlite IRA 400 column, eluted with water and concentrated. Portion of the residue was examined for nature of sugars by HPLC analysis against standard samples [conditions: column, Cosmosil carbohydrate analysis column (4.6×250 mm, 5 μm); solvent, CH_3CN–H_2O (85 : 15); flow rate, 1 ml/min; detector, Alltech ELSD 500 detector; drift tube temperature, 90 °C; retention time, D-glucose (15.6 min), L-rhamnose (9.2 min), D-xylose (6.9 min), D-apiose (6.3 min)]. The absolute configurations of the sugars were determined according to the reported method using GC after being converted to their thiazolidine derivatives.[11] Another portion of water layer residue was dissolved in pyridine (100 μl) and stirred with D-cysteine methyl ester (5 mg) for 1.5 h at 60 °C. To the reaction mixture, hexamethyldisilazane (100 μl) and trimethylsilylchloride (100 μl) were added and the mixtures were stirred for 30 min at 60 °C. The supernatant was then analyzed by GC experiment with standard samples [conditions: column, CP SIL-5CB (0.25 mm×30 m); column temperature, 230 °C; carrier gas, N_2; retention time, D-glucose (10.4 min), L-rhamnose (8.6 min), D-xylose (7.8 min), D-apiose (7.5 min)]. From the new saponins **1** and **2**, D-glucose, L-rhamnose, D-xylose and D-apiose were detected.

Carrageenan-Induced Paw Edema The initial hind paw volume of the Institute of Cancer Research (ICR) mice was determined volumetrically. A 1% solution of carrageenan in saline (0.05 ml/mouse) was injected subcutaneously into the right hind paw 1 h after the test samples had been administered orally. The control group received the vehicle. Paw volumes were

measured at 0, 1, 2, 3 and 4 h after injection, and the volume of the edema was measured with a plethysmometer. Indomethacin was used as a standard drug.[12] This study complied with current ethical regulations on animal research (National Research Council of U.S.A., 1996) and all animals used in the experiment received humane care.

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