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A New Triterpenoid Saponin from *Pulsatilla cernua*†

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Abstract – A new oleanane-type triterpenoid saponin together with six known saponins were isolated from the roots of *Pulsatilla cernua*. Their structures were elucidated on the basis of spectroscopic data, including 2D NMR spectra and chemical evidence. Compounds **1** and **6** are reported from this genus for the first time.
Keywords – *Pulsatilla cernua*, Ranunculaceae, Triterpenoid saponin

Introduction

The root of *Pulsatilla cernua* (Thunb.) Bercht et Opiz. (Ranunculaceae) is a well known herbal medicine for treatment of amoebic dysentery, malaria, and chills in Northeast China and Korea. Previous phytochemical investigations on this plant have reported a number of triterpenoid saponins (Kang, 1989; Zhang, *et al.*, 2000a; Zhang, *et al.*, 2000b; Bang, *et al.*, 2005a; Xu, *et al.*, 2006; Xu, *et al.*, 2007; Fu, *et al.*, 2008; Xu, *et al.*, 2010; Yang, *et al.*, 2010; Liu, *et al.*, 2012), and on our previous study, some neuroprotective components were isolated from this plant (Liu, *et al.*, 2012). In continuation to our study, a new saponin (**7**) together with six known compounds (**1**-**6**) were obtained from this plant, This paper deals with the isolation and identification of these compounds on the basis of spectroscopic analysis.

Experimental

General experimental procedures – The NMR spectra were measured in pyridine-d_5, on a Bruker AV600 instrument. ESI-MS spectra were recorded on Waters Quattro micro API LC/MS/MS spectrometer (Waters, USA). HR-TOF-MS spectra were performed on Agilent LC/MS spectrometer (Agilent, USA). HPLC was performed on JAI LC9103 Recycling preparative HPLC (Japan Analytical Industries, Japan) equipped with JAIGEL-ODS-AP-P column and JAIGEL-GS310 column using a JAI refractive index detector and a JAI UV-3702 detector with MultiChro 2000 workstation. TLC was performed on pre-coated GF_{254} plates (Merck, Germany) and detected by spraying with 10% H_2SO_4 followed heating. GC analyses were performed using an Agilent GC 6890 instrument on an HP-5 column (320 μm × 30 m, 0.25 μm).

Plant material – The roots of *P. cernua* were collected in May 2011 at Fushun, Liaoning Province, China, and authenticated by Professor Jincai Lu (The School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University). A voucher specimen has been deposited in our laboratory (voucher No. pc-2011-001).

Extraction and Isolation – The dry roots of *P. cernua* (40 kg) were extracted with 70% EtOH (70 - 80 ºC, 3 × 80 L, 2 h, each). The 70% EtOH solution was heated on steam bath to remove EtOH. Aqueous solution with petroleum ether, ethyl acetate, n-butyl alcohol extract three times, condensed into concrete. The n-butyl alcohol extract part was chromatographed over a D101 macroporous resin column (15 × 150 cm), eluted successively with water, 30% EtOH, 50% EtOH, 70% EtOH, 95% EtOH. The 50% EtOH eluate and 70% EtOH eluate were evaporated to dryness to give 50% EtOH extract (150 g), and 70% EtOH extract (264 g), respectively. The 70% EtOH crude extract (100 g) was chromatographed on silica gel (1.2 kg, 200 mesh) with $CHCl_3$-MeOH-H_2O [(90 : 10 : 1, lower layer → 7 : 3 : 0.5, lower layer), to afford five fractions fr.1 (2.6 g), fr.2 (3.5 g), fr.3 (45 g),

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fr.4 (12.4 g), fr.5 (18.5 g). Fr.1and fr.4 separated with recycling preparative HPLC (different ratio of methanol-water as eluent, at room temperature, 50 - 70 minutes a cycle) to give compounds **1** (20 mg), **2** (14 mg), **3** (130 mg), **4** (180 mg), **5** (21 mg), **6** (11 mg), and **7** (10 mg), respectively.

Acid hydrolysis and GC analysis – Compound **7** (4 mg) was treated with 1 M HCl (4 mL) at 90 ºC for 2 h. The reaction mixture was then extracted with $CHCl_3$ (3 × 5 mL). Acid hydrolysis of **2 - 4** was performed likewise. Each remaining aqueous layer was concentrated to dryness to give a residue and was dissolved in pyridine (2 mL), and then L-cysteine methyl ester hydrochloride (2 mg) was added to the solution. Then the mixture was heated at 60 ºC for 1 h, and trimethylchlorosilane (0.5 mL) was added, followed by heating at 60 ºC for 30 min. Then, the solution was concentrated to dryness and taken up in water (1 mL × 3), followed by extraction with n-hexane (1 mL × 3). The supernatant was subjected to GC analysis under the following conditions: Agilent GC 6890 instrument equipped with FID (detection temperature 280 ºC). Column: HP-5 column (320 μm × 30 m, 0.25 μm). Column temperature: 160 - 200 ºC with the rate of 4 ºC/min, then kept for 5 min, and then 200 - 240 ºC with the rate of 10 ºC/min and kept for 10 min. The carrier gas was N_2 (1.0 mL/min), split ratio 1/10, injection temperature: 270 ºC. Injection volume: 10 μL. The absolute configurations of the monosaccharides were confirmed to be L-arabinose, L-rhamnose, and D-glucose by comparison of the retention times of monosaccharide derivatives with those of standard samples: L-arabinose (12.67 min), L-rhamnose (12.85 min), and D-glucose (14.41 min), re- spectively.

Hederagenin 3-*O*-*α*-L-arabinopyranosyl-28-*O*-*β*-D-glucopyranosyl ester (1) – White amorphous powder. ESI-MS: *m/z* 789.2 $[M + Na]^+$, 805.2 $[M + K]^+$. ^{1}H-NMR (600 MHz, C_5D_5N) *δ*: 0.84 (6H, s, 2 × CH_3), 0.88, 0.92, 1.09, 1.14 (each 3H, s, CH_3), 4.98 (1H, d, *J* = 6.6 Hz, H-1 of Ara), 5.39 (1H, brs, H-12), 6.28 (1H, d, *J* = 8.4 Hz, H-1 of Glc); ^{13}C-NMR (150 MHz, C_5D_5N) data see Table 1.

Hederagenin 3-*O*-*β*-D-glucopyranosyl(1 → 4)-*α*-L-arabinopyranoside (2) – White amorphous powder. ESI-MS: *m/z* 782.9 $[M + Na]^+$, 805.1 $[M + K]^+$. ^{1}H-NMR (600 MHz, C_5D_5N) *δ*: 0.88, 0.89, 0.91, 0.96, 0.98, 1.20 (each 3H, s, CH_3), 4.86 (1H, d, *J* = 7.2 Hz, H-1 of Ara), 5.20 (1H, d, *J* = 7.8 Hz, H-1 of Glc), 5.44 (1H, brs, H-12); ^{13}C-NMR (150 MHz, C_5D_5N) data see Table 1.

Hederagenin 3-*O*-*α*-L-rhamnopyranosyl(1 → 2)-[*β*-D-glucopyranosyl(1 → 4)]-*α*-L-arabinopyranoside (3) – White amorphous powder. ESI-MS: *m/z* 935.3 $[M + Na]^+$, 911.2 $[M - H]^-$. ^{1}H-NMR (600 MHz, C_5D_5N) *δ*: 0.89, 0.90, 0.96, 0.97, 1.04, 1.20 (each 3H, s, CH_3), 1.61 (3H, d, *J* = 6.6 Hz, Rha-6), 4.94 (1H, d, *J* = 6.6 Hz, H-1 of Ara), 5.05 (1H, d, *J* = 7.8 Hz, H-1 of Glc), 5.42 (1H, brs, H-12), 6.19 (1H, s, H-1 of Rha); ^{13}C-NMR (150 MHz, C_5D_5N) data see Table 1.

Hederagenin 3-*O*-*β*-D-glucopyranosyl(1 → 4)-*β*-D-glucopyranosyl(1→3)-*α*-L- rhamnopyranosyl(1→2)-*α*-L-arabinopyranoside (4) – White amorphous powder. ESI-MS: *m/z* 1073.2 $[M - H]^-$, 1109.1 $[M + Cl]^-$. ^{1}H-NMR (600 MHz, C_5D_5N) *δ*: 0.89, 0.90, 0.96, 0.97, 1.06, 1.20 (each 3H, s, CH_3), 1.51 (3H, d, *J* = 6.0 Hz, Rha-6), 4.97 (1H, d, *J* = 6.6 Hz, H-1 of Ara), 5.10 (1H, d, *J* = 7.8 Hz, H-1 of Glc'), 5.37 (1H, d, *J* = 7.8 Hz, H-1 of Glc), 5.42 (1H, brs, H-12), 6.17 (1H, s, H-1 of Rha); ^{13}C-NMR (150 MHz, C_5D_5N) data see Table 1.

Hederagenin 3-*O*-*β*-D-glucopyranosyl(1 → 3)-*α*-L-rhamnopyranosyl(1 → 2)-[*β*-D-glucopyranosyl(1 → 4)]-*α*-L-arabinopyranoside (5) – White amorphous powder. ESI-MS: *m/z* 1073.3 $[M - H]^-$, 1097.3 $[M + Na]^+$. ^{1}H-NMR (600 MHz, C_5D_5N) *δ*: 0.89 (6H, s, 2 × CH_3), 0.96, 0.97, 1.07, 1.21 (each 3H, s, CH_3), 1.53 (3H, d, *J* = 6.6 Hz, Rha-6), 4.86 (1H, d, *J* = 7.2 Hz, H-1 of Ara), 5.04 (1H, d, *J* = 7.8 Hz, H-1 of Glc'), 5.43 (1H, d, *J* = 7.8 Hz, H-1 of Glc), 5.43 (1H, brs, H-12), 6.14 (1H, s, H-1 of Rha); ^{13}C-NMR (150 MHz, C_5D_5N) data see Table 1.

Oleanolic acid 3-*O*-*α*-L-rhamnopyranosyl(1 → 2)-*α*-L-arabinopyranosyl-28-*O*-*β*-D-glucopyranosyl ester (6) – White amorphous powder. ESI-MS: *m/z* 919.3 $[M + Na]^+$, 935.3 $[M + K]^+$. ^{1}H-NMR (600 MHz, C_5D_5N) *δ*: 0.81, 0.84, 0.87, 1.03, 1.06, 1.12, 1.21 (each 3H, s, CH_3), 1.59 (3H, d, *J* = 6.0 Hz, Rha-6), 4.85 (1H, d, *J* = 6.0 Hz, H-1 of Ara), 5.38 (1H, brs, H-12), 6.12 (1H, s, H-1 of Rha), 6.29 (1H, d, *J* = 7.8 Hz, H-1 of Glc); ^{13}C-NMR (150 MHz, C_5D_5N) data see Table 1.

Oleanolic acid 3-*O*-*β*-D-glucopyranosyl(1 → 3)-*α*-L-rhamnopyranosyl(1 → 2) [*α*-L-rhamnopyranosyl(1 → 6)-*β*-D-glucopyranosyl(1 → 4)]-*α*-L-arabinopyranoside (7) – White amorphous powder. ESI-MS: *m/z* 1203.1 $[M - H]^-$. ^{1}H-NMR (600 MHz, C_5D_5N) *δ*: 0.79 (3H, s, CH_3), 0.91 (6H, s, 2 × CH_3), 0.96 (3H, s, CH_3), 1.10 (3H, s, CH_3), 1.26 (6H, s, 2 × CH_3), 1.54 (3H, d, *J* = 6.0 Hz, Rha-6), 1.52 (3H, d, *J* = 6.0 Hz, Rha"-6), 4.78 (1H, d, *J* = 6.0 Hz, H-1 of Ara), 5.10 (1H, d, *J* = 7.8 Hz, H-1 of Glc), 5.39 (1H, s, H-1 of Rha"), 5.41 (1H, d, *J* = 7.8 Hz, H-1 of Glc'), 5.42 (1H, brs, 12-H), 6.18 (1H, s, H-1 of Rha); ^{13}C-NMR (150 MHz, C_5D_5N) data see Table 1.

Result and Discussion

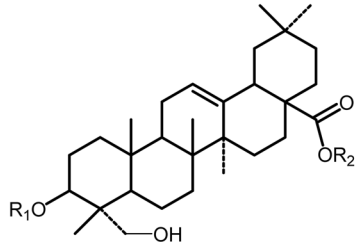
The EtOH exract of the roots of *P. cernua* was isolated

Table 1. ^{13}C-NMR data of compounds **1** - **7** (pyridine-d_5, δ).

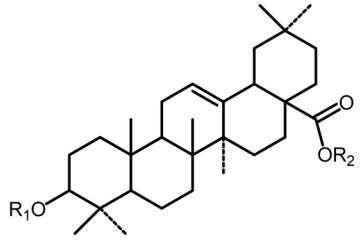
No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	39.0	38.3	38.6	38.7	38.7	38.5	38.5	C-3							
2	26.3	25.7	26	26.1	26.2	26.1	26.3	ara	106.9	106.1	104.1	104.7	104.7	104.4	105.0
3	82.1	81.6	80.7	80.9	81.0	88.3	88.3		73.3	73.4	75.8	75.1	75.7	75.5	75.3
4	43.7	43.1	43.1	43.3	43.3	39.2	39.2		75.0	74.3	74.8	74.5	74.9	73.7	75.0
5	47.8	47.2	47.4	47.3	47.3	55.6	55.6		69.9	79.5	80.2	69.5	80.6	68.3	81.5
6	18.4	17.8	17.8	17.9	17.8	18.2	18.3		67.2	66.0	65.2	66.2	65.8	64.3	65.4
7	33.0	32.5	32.5	32.6	33.0	32.7	32.8	glc		106.5	106.4	104.6	106.7		104.7
8	40.2	39.4	39.4	39.5	39.5	39.5	39.3			75.5	75.1	74.9	75.6		71.6
9	48.4	47.8	47.8	47.9	47.9	47.6	47.7			78.5	78.4	78.2	78.2		76.4
10	37.2	36.6	36.5	36.6	36.6	36.6	36.7			70.9	70.8	71.2	70.9		72.7
11	24.1	23.3	23.4	23.5	23.5	23.2	23.4			78.1	78.2	77.9	78.6		76.1
12	123.2	122.2	122.2	122.3	122.3	122.4	122.2			62.2	62.2	62.1	62.3		68.2
13	144.4	144.4	144.5	144.6	144.5	143.7	144.1	rha"							102.4
14	42.4	41.8	41.8	41.9	41.9	41.7	41.8								71.4
15	28.5	28.0	28	28.1	28.1	27.6	27.9								72.3
16	23.6	23.5	23.3	23.4	23.4	23	23.4								73.7
17	47.2	46.0	46.3	46.4	46.4	46.6	46.4								69.3
18	42.0	41.6	41.6	41.7	41.7	41.3	41.6								18.1
19	46.4	46.3	46.1	46.1	46.2	45.7	45.4	rha			101.3	101.2	101.2	101.3	101.3
20	31.0	30.6	30.6	30.7	30.7	30.3	30.6				71.9	71.5	71.4	72.0	71.3
21	34.2	33.8	33.9	33.9	34.0	33.5	33.8				72.1	83.1	82.6	72.2	83.2
22	32.8	32.9	32.9	33.0	32.6	32.1	32.8				73.7	72.8	72.7	73.6	69.5
23	64.7	64.1	63.6	63.7	63.6	27.8	27.8				69.3	69.4	69.6	69.5	69.0
24	13.8	13.2	13.7	13.9	13.9	16.5	16.8				18.3	18.2	18.3	18.1	18.1
25	16.4	15.7	15.7	15.8	15.8	15.2	15.2	glc'				106.3	106.7		106.2
26	17.8	17.1	17.1	17.2	17.2	17	17					75.2	75.2		74.2
27	26.1	25.8	25.8	25.9	25.9	25.6	25.8					76.4	78.2		77.8
28	176.7	179.8	179.7	180.0	179.9	176	180.1					80.8	71.4		74.4
29	33.3	32.9	32.9	33.0	33.0	32.7	32.9					76.4	78.4		76.2
30	23.9	23.4	23.4	23.5	23.5	23.4	23.4					61.5	62.2		61.4
								C-28							
								glc	96.0					95.3	
									74.4					73.4	
									79.6					78.5	
									71.3					70.6	
									79.1					78.9	
									62.4					61.7	

and further purified to give seven saponins. Compounds **1** and **6** are reported from this genus for the first time, and the structures were identified as hederagenin 3-*O*-α-L-arabinopyranosyl-28-*O*-β-D-glucopyranosyl ester (**1**) (Kizu, *et al.*, 1985), hederagenin 3-*O*-β-D-glucopyranosyl(1 → 4)-α-L-arabinopyranoside (**2**) (Li, *et al.*,1990), hederagenin 3-*O*-α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 4)]-α -L-arabinopyranoside (**3**) (Zhang, *et al*., 2000a), hederagenin 3-*O*-β-D-glucopyranosyl(1 → 4)-β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside (**4**) (Bang, *et al.*, 2005b), hederagenin 3-*O*-β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1 → 4)]-α-L-arabinopyranoside (**5**) (Mimaki, *et al.*, 2009), oleanolic acid 3-*O*-α-L-rhamnopyranosyl(1 → 2)-α-L-arabinopyranosyl-28-*O*-β-D-glucopyranosyl ester (**6**) (Saito, *et al.*, 1993).

Compound **7** was obtained as white amorphous powder, ESI-MS: *m/z* 1203.1 $[M-H]^-$. The molecular



1 R_1=ara- R_2=glc-
2 R_1=glc(1→4)ara- R_2=H
3 R_1=rha(1→2)[glc(1→4)]ara- R_2=H
4 R_1=glc(1→4)glc(1→3)rha(1→2)ara- R_2=H
5 R_1=glc(1→3)rha(1→2)[glc(1→4)]ara- R_2=H



6 R_1=rha(1→2)ara- R_2=glc-
7 R_1=rha(1→6)glc(1→4)[glc(1→3)rha(1→2)]ara- R_2=H

Fig. 1. Structures of compounds **1** - **7**.

formula of **7** was determined as $C_{59}H_{96}O_{25}$ from HR-TOF-MS (*m/z* 1227.6181 $[M + Na]^+$, calc. 1227.6133). The compound displayed 59 carbon signals in the ^{13}C-NMR spectrum, of which 30 were assigned to the aglycone and the remaining 29 to the sugar moieties. The seven methyl carbon signals at δ_C 15.2, 16.8, 17.0, 23.4, 25.8, 27.8, and 32.9, and the two olefinic carbon signals at δ_C 122.2 and 144.1, coupled with the ^{1}H-NMR data, seven tertiary methyl proton singlets at δ_H 0.79, 0.91, 0.91, 0.96, 1.10, 1.26, and 1.26, and a broad triplet-like olefinic proton signal at δ_H 5.42 (brs), indicated that the aglycone possessed an olean-12-ene skeleton. The ^{1}H-NMR spectrum showed five anomeric proton signals at δ_H 4.78 (d, J = 6.0 Hz, H-1 of Ara), 5.10 (d, J = 7.8 Hz, H-1 of Glc), 5.39 (s, H-1 of Rha"), 5.41 (d, J = 7.8 Hz, H-1 of Glc'), 5.42 (brs, 12-H), 6.18 (1H, s, H-1 of Rha), as well as two methyl doublets of rhamnose at δ_H 1.54 (3H, d, J = 6.0 Hz, rha-6), 1.52 (3H, d, J = 6.0 Hz, rha"-6), and the corresponding anomeric carbon signals at δ_C 101.3, 102.4, 104.7, 105.0 and 106.2, respectively (Table 1). The chemical shifts of δ_C 88.3 (C-3) revealed that **7** was a monodesmosides saponin with a glycosidic linkage at C-3 through an *O*-heterosidic bond. The linkage of the sugar moiety at C-3 of the aglycone was established from the HMBC correlations between δ 105.0 (ara-1), δ 4.78 (d, J = 7.8 Hz, H-1 of Ara) and δ 88.3 (C-3), δ 5.10 (d, J = 7.8 Hz, H-1 of Glc) and δ 81.5 (ara-4), δ 6.18 (1H, s, H-1 of Rha) and δ 75.3 (ara-2), δ 5.41 (d, J = 7.8 Hz, H-1 of Glc') and δ 83.2 (rha-3), δ 5.39 (s, H-1 of Rha") and δ 68.2 (glc-6), Based on the above evidence, the structure of **7** was determined to be oleanolic acid 3-*O*-*β*-D-glucopyranosyl(1→3)-*α*-L-rhamnopyranosyl (1→2)[*α*-L-rhamnopyranosyl(1→6)-*β*-D-glucopyranosyl(1→4)]-*α*-L-arabinopyranoside.

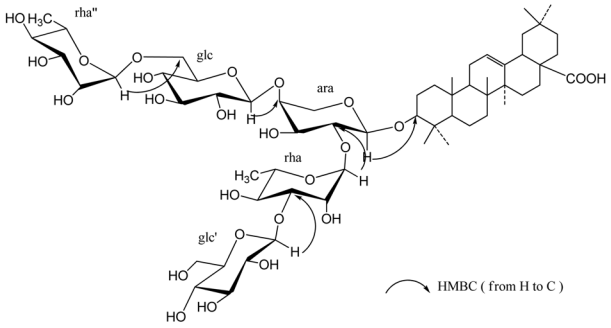


Fig. 2. Key HMBC correlations of compound **7**.

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