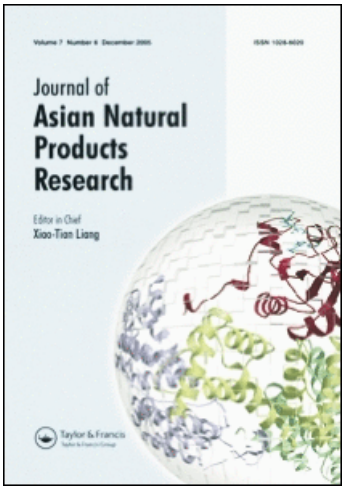

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Two new triterpenoid saponins from *Pulsatilla cernua* (Thunb.) Bercht. et Opiz

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Two new triterpenoid saponins, named cernuaside C and D, have been isolated from *Pulsatilla cernua* (Thunb.) Bercht. et Opiz. The structures of the two new triterpenoid saponins were elucidated as 3-O-β-D-xylopyranosyl(1 → 2)-[α-L-rhamnopyranosyl(1 → 3)]-α-L-arabinopyranosyl oleanolic acid 28-α-L–rhamnopyranosyl(1 → 4)-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranoside. **(1)** and 3-O-α-L–arabinopyranosyl (1 → 3)-α-L-rhamnopyranosyl(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28–O–β-D-glucopyranoside **(2)** by 1D, 2D-NMR techniques, ESI-MS analysis as well as chemical methods.

Keywords: Ranunculaceae; *Pulsatilla cernua* (Thunb.) Bercht. et Opiz; Triterpenoid saponin; Cernuaside C and D

1. Introduction

Pulsatilla cernua (Thunb.) Bercht. et Opiz is distributed in northeast China. Its root has been used as a Chinese traditional medicine for treatment of amoebic dysentery and bacterial dysentery in northeast China. Phytochemical studies on this plant were reported previously [1]. In the search for new and bioactive components from traditional Chinese medicines, we investigated the roots of *Pulsatilla cernua*. In the present paper, we report the isolation and structure elucidation of two new triterpenoid saponins, namely cernuaside C (**1**) and cernuaside D (**2**), by using 1D, 2D-NMR techniques, ESIMS analysis as well as chemical methods.

2. Results and discussion

Cernuaside C **(1)** obtained as a white amorphous powder, turned red upon colouration with Liebermann–Burchard reaction. Acidic hydrolysis of compound **1** with 1M HCl gave

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oleanolic acid as aglycon and arabinose, rhamnose, glucose and xylose as the sugar components identified on TLC by comparison with authentic samples. Compound **1** exhibited the molecular formula $C_{64}H_{104}O_{29}$ by its HRMS spectrum. Its ESIMS spectrum showed quasimolecular ion at *m/z* 1335[M-H]$^-$, indicating a molecular weight of 1336. An ion at *m/z* 865[M-H-470(162 + 162 + 146)]$^-$, taken as evidence for the direct elimination of two hexoses and one deoxyhexose, indicated the presence of a trisaccharide unit at C-28 of the aglycone [2,3]. In addition, significant ions at *m/z* 733[M-H-470-132]$^-$, 587[M-H-470-132-146]$^-$, 455[M-H-470-132-146-132]$^-$, 1203[M-H-132]$^-$, 1057[M-H-132-146]$^-$, 587[M-H-132-146-470]$^-$, 445[M-H-132-146-470-132]$^-$, suggested the presence of arabinose, rhamnose, glucose and xylose. It appeared from this fragmentation that the trisaccharide was branched with rhamnose and a pentose individually linked to another pentose which was attached to the aglycon. Comparison of the ^{13}C NMR data of the aglycone of **1** (table 1) with those of oleanolic acid [4] showed that C-3(δ88.80) was shifted 10.8 ppm downfield, while C-2(δ26.24) and C-28(δ176.67) were respectively shifted 1.96 ppm and 3.53 ppm upfield, indicating that **1** must be a bisdesmosidic saponin in which the trisaccharide chain of arabinose, rhamnose, xylose was bound to the aglycon by a glycosidic linkage at C-3, while glucose was bound by a glycosidic ester linkage at C-28. The ^{1}H NMR spectrum of **1** showed characteristic resonances for oleanolic acid saponin, with seven

Table 1. ^{13}C-NMR spectral data of compound **1** and **2** (δ_C, 125 MHz, C_5D_5N).

		1				*2*	
1	39.17	C-3 Ara-1	106.28	1	39.13	C-3 Ara-1	106.26
2	26.24	2	75.30	2	26.22	2	81.41
3	88.80	3	81.10	3	88.74	3	72.18
4	39.76	4	68.50	4	39.72	4	68.99
5	56.31	5	65.07	5	56.26	5	65.44
6	18.65	Rha-1	106.65	6	18.63	Rha-1	101.62
7	33.28	2	72.13	7	33.24	2	71.26
8	40.05	3	72.72	8	40.04	3	83.49
9	48.22	4	72.92	9	48.13	4	73.03
10	37.20	5	70.09	10	37.16	5	69.11
11	23.93	6	18.65	11	23.91	6	18.74
12	123.02	Xyl-1	104.60	12	123.07	Ara-1′	104.85
13	144.26	2	74.08	13	144.23	2′	71.95
14	42.27	3	78.21	14	42.25	3′	73.32
15	28.37	4	70.41	15	28.34	4′	69.92
16	23.86	5	67.17	16	23.77	5′	67.18
17	47.19	C-28 Glc-1	95.81	17	47.11	C-28 Glc-1	95.90
18	41.92	2	74.02	18	41.84	2	74.29
19	46.38	3	79.03	19	46.32	3	79.64
20	30.90	4	71.04	20	30.88	4	71.67
21	34.15	5	78.80	21	34.13	5	79.15
22	32.70	6	69.37	22	32.65	6	62.35
23	28.37	Glc-1′	105.00	23	28.34		
24	17.38	2′	75.50	24	17.38		
25	15.84	3′	76.64	25	15.78		
26	17.66	4′	78.37	26	17.56		
27	27.03	5′	77.30	27	27.01		
28	176.67	6′	61.42	28	176.53		
29	33.28	Rha-1′	102.79	29	33.24		
30	23.52	2′	71.78	30	23.55		
		3′	72.39				
		4′	72.98				
		5′	69.93				
		6′	18.75				

tertiary methyl signals at δ0.74(s), 0.76(s), 0.78(s), 0.95(s), 1.08(s), 1.12(s) and 1.29(s), one trisubstituted olefinic proton at δ5.26 (1H, br, s), two doublets for H-3 at δ3.54 (1H, dd, $J = 4.0$ Hz, 12 Hz) and for H-18 atδ3.17 (1H, dd, $J = 4.0$ Hz, 14.0 Hz). The anomeric proton signals were observed at δ4.72 (1H, d, $J = 6.7$ Hz), 4.86 (1H, d, $J = 7.7$ Hz), 5.76(1H, br, s), 5.88(1H, d, $J = 7.6$ Hz), 6.40(1H, br, s), 6.11(1H, d, $J = 8.0$ Hz). These coupling constants indicated that the glycosidic linkage of arabinose, rhamnose were α configuration, and those of xylose glucose were β configuration [5,6]. The ^{13}C NMR spectrum of **1** (table 1) showed signals corresponding to two olefinic carbons (δ123.03, 144.26), one carboxylic carbon (δ176.67) and six anomeric carbons (δ95.81, 102.79, 104.60, 105.00, 105.28, 106.65).

Table 2. ^{1}H NMR spectral data of compounds **1** and **2** (δ_H, 500 MHz, C_5D_5N).

	1		***2***
3	3.54 (dd, 4.0, 11.5 Hz)	3	3.58 (dd, 4.0, 11.5 Hz)
12	5.26 (br. s)	12	5.25 (br. s)
18	3.17 (dd, 4.0, 14.0 Hz)	18	3.16 (dd, 4.0, 14.0 Hz)
23	1.12 (s)	23	1.16 (s)
24	1.08 (s)	24	1.06 (s)
25	0.95 (s)	25	0.83 (s)
26	0.76 (s)	26	0.73 (s)
27	1.29 (s)	27	0.95 (s)
29	0.74 (s)	29	0.70 (s)
30	0.78 (s)	30	0.83 (s)
C-3 Ara-1	4.72 (d, 6.7 Hz)	C-3 Ara-1	4.64 (d, 6.6 Hz)
2	4.63	2	4.52
3	4.26	3	4.23
4	4.75	4	4.20
5	3.78, 4.29	5	3.75, 4.25
Rha-1	6.40 (br, s)	Rha-1	5.29 (br, s)
2	4.90	2	4.83
3	4.56	3	4.65
4	4.35	4	4.48
5	4.40	5	4.61
6	1.75 (d, 7.6 Hz)	6	1.57 (6.1)
Xyl-1	5.88 (d, 7.6 Hz)	Ara-1′	4.77 (d, 6.6 Hz)
2	4.08	2′	4.48
3	4.18	3′	4.20
4	4.26	4′	4.29
5	3.57, 4.46	5′	3.79, 4.29
C-28 Glc-1	6.11 (d, 8.0 Hz)	C-28 Glc-1	5.29 (d, 8.0 Hz)
2	4.12	2	4.28
3	4.23	3	4.26
4	4.25	4	4.27
5	4.06	5	4.17
6	4.28, 4.54	6	4.35, 4.61
Glc-1′	4.86 (d, 7.7 Hz)		
2′	3.89		
3′	4.31		
4′	3.85		
5′	3.94		
6′	4.06, 4.28		
Rha-1′	5.76 (br, s)		
2′	4.65		
3′	4.5		
4′	4.35		
5′	4.81		
6′	1.71 (d,6.0 Hz)		

The assignments of the ^{1}H NMR and ^{13}C NMR signals of **1** and **2** were made by comparison with those of oleanolic acid and oleanolic acid saponins [4], and were confirmed by ^{1}H-^{1}H COSY, DEPT, HMQC and HMBC spectral analysis (tables 1 and 2).

The positions of the sugar residues in **1** were defined unambiguously by HMBC experiment (figure 1). A cross-peak due to long-range correlations between H-1(δ 4.72) of arabinose and C-3 (δ88.80) of the aglycon indicated that arabinose was linked to C-3 of the oleanolic acid; a cross-peak between H-1(δ5.88) of xylose and C-2 (δ75.30) of arabinose indicated that xylose was linked to C-2 of the arabinose; a cross-peak between H-1(δ6.40) of rhamnose and C-3 (δ81.10) of arabinose indicated that rhamnose was linked to C-3 of the arabinose. These assignments showed that a trisaccharide moiety, 3-O-[β-D−xylopyranosyl-(1 → 2)]-[α−L−rhamnopyranosyl-(1 → 3)]-arabinopyranoside, was linked to the oleanolic acid at C-3. Additionally, correlations between H-1′(δ5.76) of a terminal rhamnose and C-4′(δ78.37) of glucose; H-1′(δ4.86) of glucose and C-6(δ69.37) of inner glucose; and H-1(δ6.11) of inner glucose and C-28 carbonyl carbon resonance at δ176.67, definitively proved that an ester linkage of the trisaccharide unit was linked to C-28 of the oleanolic acid. These assignments showed that a trisaccharide moiety, 28-O-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside, was linked to C-28 of the oleanolic acid. On the basis of all of these evidences, **1** was identified as 3-O-β-D-xylopyranosyl(1 → 2)-[α-L-rhamnopyranosyl(1 → 3)]-α-L-arabinopyranosyl oleanolic acid 28-α-L-rhamnopyranosyl(1 → 4)-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranoside.

Cernuaside D **(2)** obtained as a white powder, turned red upon reaction with Liebermann–Burchard reaction. The IR spectrum showed absorption for hydroxyl groups (3425 cm^{-1}), ester carbonyl (1729 cm^{-1}) and double bond (1639 cm^{-1}). An acidic hydrolysis of **2** with mineral acid afforded oleanolic acid as aglycon and arabinose, rhamnose and glucose as the sugar components identified on TLC by comparison with authentic samples. Compound **2** exhibited the molecular formula $C_{52}H_{83}O_{20}$ by its HRMS spectrum.The ESIMS of **2** showed a quasimolecular ion peak at 1027[M-H]$^{-}$, indicating a molecular weight of 1028, and significant ion peak at *m/z:*865[M-H-162]$^{-}$, corresponding to the loss of one hexosyl moiety,

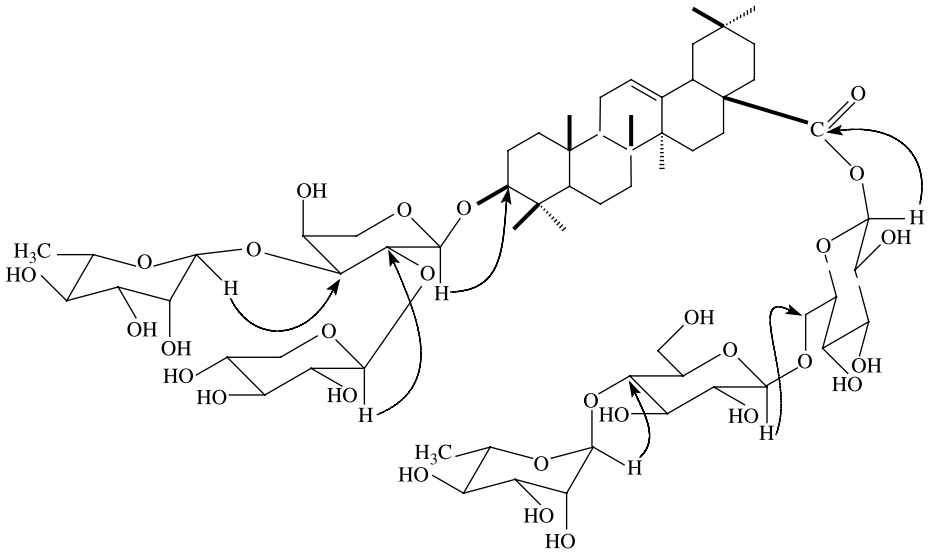

Figure 1. Structure and Key HMBC correlations for **1**.

suggested that a hexose was linked to oleanolic acid by an ester function. Additionally, significant ions at *m/z* 733[M-H-162-132]$^-$, 587[M-H-162-132-146]$^-$, 455[M-H-162-132-146-132]$^-$, showed the presence of a linear sugar chain of arabinose-rhamnose-arabinose. In the ^{13}C NMR spectrum of **2** (table 1), the downfield signals atδ88.74 and 176.53 suggested glycosidation at C-3 and C-28 of the aglycon. In oleanolic acid, the signals for C-3 and C-28 are observed at δ78.0 and 180.2, respectively [4]. These data indicated that **2** must be a bisdesmosidic saponin in which the trisaccharide chain with two arabinose and a rhamnose was bound to the oleanolic acid by a glycosidic linkage at C-3, while a glucose was bound by a glycosidic ester linkage at C-28.

The positions of the sugar residues in **2** were defined unambiguously by the HMBC experiment (figure 2). A cross-peak due to the long-range correlations between H-1(δ4.64) of inner arabinose and C-3 (δ88.74) of the aglycon indicated that the arabinose was linked to C-3 of the oleanolic acid; a cross-peak between H-1(δ5.29) of rhamnose and C-2 (δ81.41) of inner arabinose indicated that the rhamnose was linked to C-2 of the inner arabinose, and a cross-peak between H-1′(δ4.77) of terminal arabinose and C-3(δ83.49) of rhamnose indicated that terminal arabinose was linked to C-3 of the rhamnose. These assignments showed that a trisaccharide moiety, 3-O-α-L-arabinopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside, was linked to the oleanolic acid at C-3. Additionally, a cross-peaks between H-1(δ5.29) of glucose and C-28 carbonyl carbon (δ176.53) definitively proved that the glucose was linked to C-28 of the oleanolic acid. On the basis of all of these evidences, **2** was identified as 3-O-α-L−arabinopyranosyl (1 → 3)-α-L-rhamnopyranosyl(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28−O−β-D-glucopyranoside.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a Kofler-microscope apparatus and are uncorrected. The IR spectra were measured on a Y-Zoom scroll Fourier transform infrared spectrometer with a KBr disc. The ESIMS was recorded on LCQ-1700 ESIMS instrument. The NMR

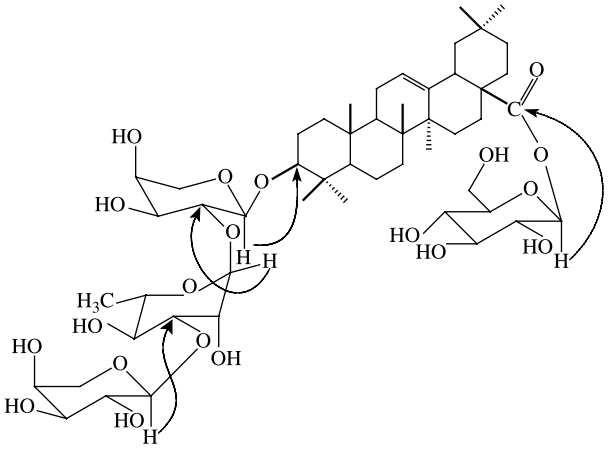

Figure 2. Structure and Key HMBC correlations for **2**.

spectra were obtained on an Bruker AM-500 instrument, using TMS as internal standard. Column chromatography was performed on silica gel (200–300 mesh, Qingdao Oceanic Chemical Industry) and ODS reversed silica gel (25 × 2.5 cm, Nacalai Tesque, Kyoto, Japan). Macroporous resin D_{101} made in Tianjin gel. TCL was conducted on silica gel 60 F_{254} (Merck). Spots were detected after spraying with 10% H_2SO_4.

3.2 Plant material

The roots of *Pulsatilla cernua* were purchased from the company of Chinese Medicinal Materials in Yanbian, Jilin Province, China and identified by Professor Minglu Deng, Changchun College of traditional Chinese medicine. A voucher specimen (990926) has been deposited in the Herbarium of Academy of Traditional Chinese Medicine and Material Medica of Jilin Province.

3.3 Extraction and isolation

The dried and powdered roots (7.5 kg) of the plant were exhaustively extracted with 70% EtOH, and the mixed extract was concentrated under reduced pressure, to obtain a crude residue (670*g*), which was chromatographed over a D_{101} macroporous resin column (10 × 80 cm), eluted successively with H_2O, 30%EtOH, 40%EtOH, 70%EtOH. The 70% EtOH eluate was concentrated to dryness (218*g* crude saponin mixture) and chromatographed over a silica gel column (200–300 mesh, 500*g*) eluted with $CHCl_3$ in increasing amounts of MeOH[65:10–40:10]. The fractions were combined by TLC monitors. The fractions were submitted to chromatography on silica gel column to afford compounds **1** (108 mg), **2** (79.5 mg).

3.4 Acid hydrolysis

Acid hydrolysis of **1** and **2**: Each saponin (each 10 mg) was heated with 2M HCl-MeOH (10 ml) under reflux for 3 h. The reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The sapogenin was detected in the $CHCl_3$ layer by TLC. The water layer was neutralized with Na_2CO_3, concentrated, and subjected to co-TLC analysis with authentic samples of D-glucose, L-arabinose, D-xylose and L-rhamnose,and developed with H_2O-MeOH-AcOH-EtOAc(15:15:20:65). Detection was carried out with aniline phthalate spray.

3.5 Structure and identification

Cernuaside C (1). White amorphous powder, mp 213–215°C, $[\alpha]_D^{18}$–16 (c 0.28,MeOH), IR (KBr) (νcm^{-1}): 3413, 2937, 1736, 1639, 1460, 1387, 1460, 1259, 1066, 1043, 921, 813. HRMS *m/z*: 1335.65618 [M-1]$^-$, (calcd for $C_{64}H_{104}O_{29}$,1336.6663). ESI MS *m/z* 1335[M-H]$^-$, 865[M-H-470]$^-$, 733[M-H-470-132]$^-$, 587[M-H-470-132-146]$^-$, 455[M-H-470-132-146-132]$^-$, 1203[M-H-132]$^-$, 1057[M-H-132-146]$^-$, 587[M-H-132-146-470]$^-$, 445[M-H-132-146-470-132]$^-$. The ^{1}H and ^{13}C NMR (500 MHz, pyridine-d_5) data are given in table 1 and table 2.

Cernuaside D (2). White powder, mp 203–206°C, $[\alpha]_D^{18}$–17 (c 0.31,MeOH), IR (KBr) (νcm^{-1}): 3425, 2943, 1729, 1639, 1461, 1388, 1365, 1256, 1230, 1075, 1043, 922, 894, 814, 732. HRMS *m/z*: 1027.54648[M-1]$^-$, (calcd for $C_{52}H_{83}O_{20}$,1027.5478). ESIMS *m/z* 1027[M-H]$^-$,

865[M-H-162]$^-$, 733[M-H-162-132]$^-$, 749[M- H-162-132]$^-$, 587[M-H-162-132-146]$^-$, 455[M-H-162-132-146-132]$^-$. The ^{1}H and ^{13}C NMR (500 MHz, pyridine-d_5) data are given in table 1 and table 2.

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