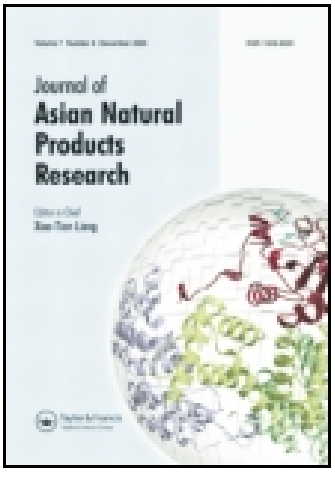

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Two new triterpenoid saponins from rhizome of *Anemone amurensis*

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Two new triterpenoid saponins were isolated from the 70% ethanol extract of the rhizome of *Anemone amurensis*, they are oleanolic acid 28-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester (**1**) and 23,27-dihydroxy oleanolic acid 3-*O*-α-L-arabinopyranoside (**2**). The structures of **1** and **2** were elucidated on the basis of chemical and spectral analysis, including 1D and 2D NMR data and HR-ESI-MS. Compounds **1** and **2** were tested for cytotoxicities against three human cancer cell lines (A549, Hep-G2, and MCF-7). Compound **1** showed potent cytotoxicity with IC_{50} values of 34.76, 41.17, and 28.92 μM, respectively, while compound **2** with $IC_{50} > 100$ μM.

Keywords: *Anemone amurensis*; triterpenoid saponins; NMR; cytotoxic activity

1. Introduction

Anemone amurensis (Korsh.) Kom, a member of the genus *Anemone*, is mainly distributed in Russia, Korea, and northeast of China, including Liaoning, Jilin, and Heilongjiang Provinces [1]. Its rhizome is used as a folk medicine in China which has been used for colds [2]. However, there was very few reports about the chemical composition. In this paper, we describe the isolation and the structural elucidation of two new triterpenoid saponins obtained from the 70% EtOH extract of *A. amurensis*. Their structures (Figure 1) were established by extensive spectroscopic data analysis and comparison with those of literature values. Meanwhile, they were evaluated for its cytotoxicities against A549, Hep-G2 and MCF-7 cancer cell lines. Compound **1** showed potent cytotoxicity with IC_{50} values of 34.76, 41.17, and 28.92 μM, respectively, while compound **2** with $IC_{50} > 100$ μM.

2. Results and discussion

Compound **1** was obtained as white needles and showed positive Molish and Liebermann–Burchard reaction tests. The molecular formula was established as $C_{54}H_{88}O_{22}$ according to HR-ESI-MS at *m/z* 1089.5851 $[M + H]^+$. The ^1H and ^{13}C NMR data of compound **1** (Table 1) indicated a pentacyclic triterpenoid saponin containing one triterpene aglycone and four monosaccharides. The NMR data of compound **1** displayed the characteristic signals for seven angular methyl groups (δ_H 0.82, 0.88, 0.92, 0.96, 1.08, 1.26, and 1.27, each 3H, s). In addition, the spectrum also revealed an olefinic group (δ_H 5.42 and δ_C 122.9, 144.1). The ^{13}C NMR spectrum of compound **1** showed 54 carbon signals, of which 30 were assigned to the aglycon. Compared with the data published in the literature, the aglycon was identified to be oleanolic acid [3].

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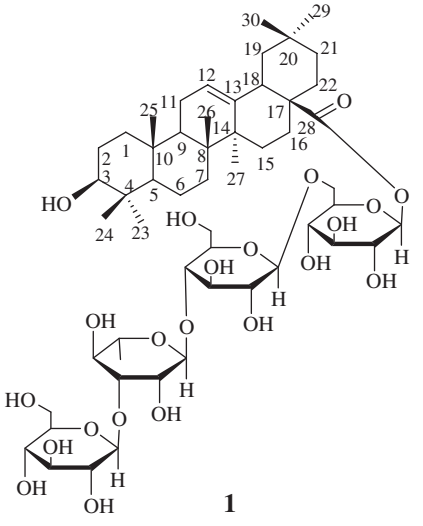
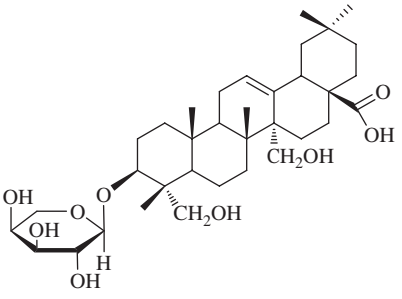


1



2

Figure 1. Structures of compounds **1** and **2**.

On acid hydrolysis of compound **1**, D-glucose and L-rhamnose were isolated from the water layer by preparative thin-layer chromatography (PTLC). The absolute configuration of each sugar was affirmed by measuring optical rotation. Meanwhile, according to the 2D NMR (^{1}H–^{1}H COSY, HSQC, and HMBC) spectra, three glucose units (δ_C 95.6, 73.8, 78.7, 70.7, 77.9, and 69.3; δ_C 105.1, 75.3, 76.4, 77.3, 77.0, and 61.2; δ_C 106.6, 75.9, 78.3, 71.3, 78.3, and 62.3) and a rhamnose unit (δ_C 102.4, 71.9, 83.8, 72.9, 70.0, and 18.4) were identified. Four anomeric proton signals at δ_H 4.92 (1H, d, $J = 7.0$ Hz), 5.36 (1H, d, $J = 7.6$ Hz), 5.86 (1H, br s), and 6.23 (1H, d, $J = 7.0$ Hz) were assigned to four anomeric carbon signals at δ_C 105.1, 106.6, 102.4, and 95.6 in the HSQC experiment. From the coupling constants of the anomeric signals at δ_H 4.92 (1H, d, $J = 7.0$ Hz), 5.36 (1H, d, $J = 7.6$ Hz), and 6.23 (1H, d, $J = 7.0$ Hz), the glucoses were deduced to be β configuration. The α configuration for the L-rhamnose was confirmed by comparison of the ^{13}C NMR data [4].

In the HMBC experiment, the long-range correlation between the anomeric proton at δ_H 5.36 (Glc$^{////}$) and the carbon at δ_C 83.8 (Rha$^{///}$-C-3) indicated that the glucose (Glc$^{////}$) was attached to position 3 of the rhamnose. The long-range correlation between the anomeric proton at δ_H 5.86 (Rha$^{///}$) and the carbon at δ_C 77.3 (Glc$^{//}$-C-4) hinted that the rhamnose was attached to position 4 of the glucose (Glc$^{//}$) (Figure 2). Similarly, the correlation between the proton at δ_H 4.92 (Glc$^{//}$) and the carbon at δ_C 69.3 (Glc$^{/}$-C-6) hinted that the glucose (Glc$^{//}$) was attached to position 6 of the glucose (Glc$^{/}$). The long-range correlation between the anomeric proton signal at δ_H 6.23 (Glc$^{/}$) and C-28 at δ_C 176.5 suggested that the glucose (Glc$^{/}$) was linked with the aglycone via C-28. The ^{1}H and ^{13}C NMR data of compound **1** were assigned on the basis of the ^{1}H–^{1}H COSY, HSQC, and HMBC (see Table 1).

Thus, the structure of **1** was determined as oleanolic acid 28-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester.

Compound **2** was obtained as white needles and showed positive Molish and Liebermann–Burchard reaction tests. The molecular formula was established as C_{35}

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compounds **1** and **2** in C_5D_5N.

	1			**2**	
No.	δ_H	δ_C	No.	δ_H	δ_C
1	0.76–0.78, 1.35–1.37 m	38.7	1	1.52–1.55, 1.09–1.11 m	38.7
2	1.78–1.80, 2.21–2.23 m	28.3	2	1.98–2.00, 2.18–2.20 m	26.1
3	3.53 dd (11.2, 4.0)	77.2	3	4.22 dd (12.0, 4.8)	82.0
4		39.4	4		43.5
5	0.73–0.75 m	55.8	5	1.79–1.81 m	47.7
6	1.16–1.18, 1.44–1.46 m	18.4	6	1.37–1.39, 1.72–1.74 m	18.2
7	1.30–1.32, 1.45–1.47 m	33.2	7	1.34–1.36, 2.00–2.04 m	33.4
8		39.9	8		40.5
9	1.60 overlapped	48.0	9	2.36 q (3.6, 6.6)	48.8
10		36.9	10		37.1
11	1.84–1.86 m	23.7	11	1.96–1.98 m	24.5
12	5.42 (br s)	122.9	12	5.84–5.85 m	127.7
13		144.1	13		139.8
14		42.1	14		47.9
15	1.23–1.25, 2.29–2.31 m	28.3	15	1.38–1.41, 2.10–2.14 m	24.1
16	1.96–1.98 m	23.4	16	1.92–1.94 m	23.7
17		47.0	17		46.5
18	3.17 dd (3.4, 13.2)	41.7	18	3.38 dd (4.2, 13.8)	41.8
19	1.24–1.28, 1.75–1.77 m	46.3	19	1.73–1.76, 1.35–1.36 m	45.5
20		30.8	20		31.0
21	1.07–1.11, 1.34–1.36 m	34.0	21	1.37–1.39, 1.17–1.19 m	34.1
22	1.73–1.75, 1.82–1.86 m	32.5	22	1.77–1.79, 2.05–2.07 m	33.2
23	1.27 s	28.3	23	4.28, 3.69 d (10.8)	64.6
24	0.96 s	17.0	24	0.92 s	13.5
25	0.82 s	15.6	25	0.94 s	16.6
26	1.08 s	17.5	26	1.04 s	18.9
27	1.26 s	26.1	27	3.76, 4.02 d (12.0)	64.5
28		176.5	28		180.3
29	0.92 s	33.2	29	0.88 s	33.2
30	0.88 s	23.7	30	1.02 s	23.9
Glc′-1	6.23 d (7.0)	95.6	Ara-1	4.97 d (7.2)	106.7
2	4.13–4.15 m	73.8	2	4.42 dd (7.8, 1.2)	73.2
3	4.20–4.22 m	78.7	3	4.08 dd (3.6, 9.0)	74.8
4	4.35 d (7.6)	70.7	4	4.25–4.27 m	69.7
5	4.05 overlapped	77.9	5	4.28–4.30, 3.76–3.78 m	67.1
6	4.29–4.31, 4.62–4.64 m	69.3			
Glc″-1	4.92 d (7.0)	105.1			
2	3.85–3.87 m	75.3			
3	4.10 overlapped	76.4			
4	4.41 overlapped	77.3			
5	3.53 d (8.7)	77.0			
6	4.01–4.03, 4.14-4.16 m	61.2			
Rha‴-1	5.86 (br s)	102.4			
2	4.89 overlapped	71.9			
3	4.62–4.64 m	83.8			
4	4.49–4.51 m	72.9			
5	5.01–5.03 m	70.0			
6	1.65 d (6.0)	18.4			
Glc⁗-1	5.36 d (7.6)	106.6			
2	4.10 overlapped	75.9			

(*Continued*)

Table 1 – *continued*

	1			2	
No.	δ_H	δ_C	No.	δ_H	δ_C
3	4.20–4.23 m	78.3			
4	4.26–4.28 m	71.3			
5	3.82–3.83 m	78.3			
6	4.34–4.36, 4.41–4.43 m	62.3			

Note: Coupling constants (*J*) in Hz are given in parentheses; chemical shift values are expressed in parts per million.

$H_{56}O_9$ according to HR-ESI-MS at *m/z* 643.3821 $[M + Na]^+$. The ^1H NMR spectrum of compound **2** (Table 1) displayed only five angular methyl groups (δ_H 0.88, 0.92, 0.94, 1.02, and 1.04, each 3H, s). The ^{13}C NMR spectrum (Table 1) of compound **2** showed 35 carbon signals, of which 30 were the aglycone. Compared with the hederagenin, the angular methyl signal C-27 (δ_C 26.2) disappeared, meanwhile a signal at δ_C 64.5 appeared. The results hinted that C-27 may be a hydroxymethyl group [5]. Therefore, based on the literature, the aglycone of compound **2** was identified to be 23,27-dihydroxy oleanolic acid [6,7].

With the above methods, the glycosyl unit of compound **2** was identified as α-L-arabinose. In the HMBC experiment, the long-range correlation between the anomeric proton at δ_H 4.97 (Ara-H-1) and the carbon at δ_C 82.0 (C-3) indicated that the arabinose linked the aglycone via C-3. Accordingly, compound **2** was established as 23,27-dihydroxy oleanolic acid 3-*O*-α-L-arabinopyranoside.

The cytotoxic activities of compounds **1** and **2** were evaluated against lung cancer (A549), human hepatocarcinoma (HepG2), and human breast adenocarcinoma (MCF-7) cell lines by the MTT method [8], using 5-fluorouracil as the positive control. Compound **1** showed cytotoxicity against the three human cancer cell lines with IC_{50} values of 34.76, 41.17, and 28.92 μM, respectively, while compound **2** with $IC_{50} > 100$ μM.

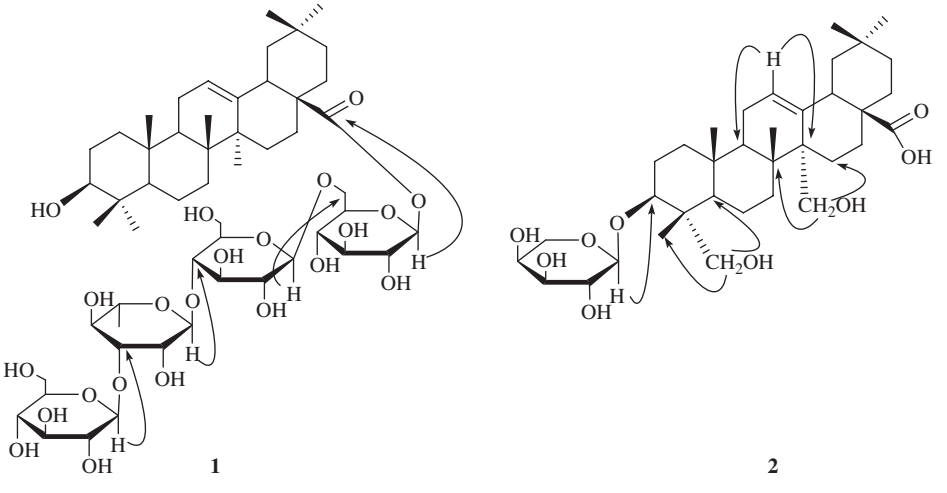


Figure 2. Key HMBC correlations of **1** and **2**.

3. Experimental

3.1 General experimental procedures

Melting points were measured on a Yanaco-53 micromelting point apparatus (Yanaco Co., Tokyo, Japan) and uncorrected. IR spectra were obtained on a Shimadzu ftir-8400s spectrophotometer (Shimadzu Corporation, Kyoto, Japan). UV spectra were recorded on a Shimadzu Pharma-Spec UV-1700 UV-Visable Spectrophotometer (Shimadzu Corporation). Optical rotations were determined on a POLAX-2L automatic digital polarimeter (Atago Co., Tokyo, Japan). NMR spectra were recorded on Bruker ARX-600 instruments (Bruker Co., Billerica, MA, USA). HR-ESI-TOF-MS experiments were performed on a Micro TOF spectrometer (Bruker Co., Karlsruhe, Germany). Preparative HPLC was conducted using a Shimadzu LC-10A instrument with an SPD-10A detector (Shimadzu Corporation) and a YMC-Pack ODS-A column (250 mm × 10 mm, 5 mm). Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), silica gel (200–300 mesh, Qingdao Marine Chemistry Ltd, Qingdao, China), and Cosmosil octadecyl silane (ODS) (20–45 μm, Merck, Darmstadt, Germany) were used for column chromatography (CC). TLC was conducted on silica gel GF254 (Qingdao Marine Chemistry Ltd).

3.2 Plant material

The rhizomes (5.7 kg) of *A. amurensis* were collected in Kuandian Country, Liaoning Province of China, in June 2012, and authenticated by Prof. Jin-Cai Lu (Department of Pharmacognosy, Shenyang Pharmaceutical University). The voucher specimen (No. 20120626001) is kept in the Pharmacognosy Laboratory of Shenyang Pharmaceutical University, Shenyang, China.

3.3 Extraction and isolation

Air-dried and powered rhizome of *A. amurensis* (5.7 kg) was refluxed with 70% ethanol for three times. The combined alcohol extracts were evaporated under reduced pressure to afford a residue (1250 g). The residue was suspended in H_2O and then successively extracted with $CHCl_3$, EtOAc, and *n*-butanol. The *n*-butanol extract was subjected to silica gel CC (10 cm × 60 cm) with a gradient mixture of CH_2Cl_2–MeOH–H_2O (100:1:0–50:50:25) to afford nine fractions (1–9). Fraction 3 (4.6 g) was further purified over an ODS CC (1.8 cm × 30 cm) using MeOH and H_2O as the mobile phase with a gradient from 45% to 95% to afford fractions $F_{3\text{-}1}$–$F_{3\text{-}8}$. $F_{3\text{-}3}$ (1.4 g) was subjected to another silica gel CC (2.2 cm × 15 cm) and eluted with CH_2Cl_2:MeOH:H_2O (8.5:2.5:0.25) to afford fractions $F_{3\text{-}3\text{-}1}$–$F_{3\text{-}3\text{-}7}$ based on TLC analysis. $F_{3\text{-}3\text{-}4}$ (360 mg) was subjected to semi-preparative HPLC eluted with CH_3OH–H_2O (15:85) at 1.3 ml/min (t_R 22 and 38 min) to yield compounds **1** (36 mg) and **2** (25 mg).

3.3.1 Compound *1*

White needles (MeOH); m.p. 222–225°C; $[\alpha]_D^{25}$ + 9.2 (c = 0.50, MeOH); UV (MeOH) λ_{max}: 204 nm. IR (KBr) ν_{max} (cm^{-1}): 3439, 2935, 1735, 1640, 1062. For ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS: *m/z* 1089.5851 $[M + H]^+$ (calcd for $C_{54}H_{89}O_{22}$, 1089.5845).

3.3.2 Compound *2*

White needles (MeOH); m.p. 208–211°C; $[\alpha]_D^{25}$ + 22.3 (c 0.12, MeOH); UV (MeOH) λ_{max}: 204 nm. IR (KBr) ν_{max} (cm^{-1}): 3450, 2950, 1700, 1636, 1071. For ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS: *m/z* 643.3821 $[M + Na]^+$ (calcd for $C_{35}H_{56}O_9Na$, 643.3810), 613.3619 $[M + Na–OCH_2]^+$, 599.3861 $[M–2OCH_2 + K]^+$, 173.0388 $[Ara + Na]^+$.

3.4 Acid hydrolysis of compounds 1 and 2

An aqueous solution (6 ml) of each sample (15 mg) was refluxed with 2 M HCl (6 ml) for 5 h. After neutralization with $NaHCO_3$ solution, the reaction mixture was extracted with $CHCl_3$ (3×10 ml). The water layer was concentrated and submitted to silica gel PTLC (EtOAc/CH_3OH/H_2O; 7:3:0.4) to yield the sugars [4]. Then, they were identified by TLC ($CHCl_3$/CH_3OH/H_2O; 16:9:2) in comparison with authentic samples and optical rotation as the following: D-glucose, $[\alpha]_D^{25} + 45.1$ ($c = 0.12$, H_2O); L-arabinose, $[\alpha]_D^{25} + 80.2$ ($c = 0.10$, H_2O); L-rhamnose, $[\alpha]_D^{25} + 9.2$ ($c = 0.10$, H_2O). Spots were visualized by spraying with EtOH/H_2SO_4/anisaldehyde (17:2:1) followed by heating.

3.5 Cytotoxicity assay

Lung cancer (A549), human hepatocarcinoma (Hep-G2) and human breast adenocarcinoma (MCF-7) cell lines were routinely cultured in DMEM supplemented with 10% fetal bovine serum and maintained at 37°C with 5% CO_2. The *in vitro* cell viability effects of compounds were determined by the MTT assay [7]. The cells (1×10^5 cells/ml) were seeded into 96-well culture plates. After overnight incubation, the cells were treated with various concentrations of agents for 72 h. Then, 10 μl of MTT solution was added to each well and incubated for an additional 4 h at 37°C. After centrifugation (200*g*, 10 min), the medium with MTT was aspirated, followed by the addition of 100 μl DMSO. The absorbances at 492 nm were measured for the cells using a Thermo microplate reader. IC_{50} measurements for each compound were done three times.

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