Triterpenoid Saponins from *Lysimachia davurica*

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Three new saponins were isolated from the whole plants of *Lysimachia davurica*. On the basis of 1D and 2D NMR (^{1}H–^{1}HCOSY, HMBC, HMQC, and TOCSY) techniques, MS, and hydrolysis, their structures were found to be 3-*O*-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl-13,28-epoxy-3β-hydroxy-16-oleanaone (1), 3-*O*-β-D-glucopyranosyloxyuronic acid-(1→2)-β-D-xylopyranosyl-cyclamiretin A (2), and 3-*O*-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl-cyclamiretin A (3), respectively. Compounds 2 and 3 showed significant cytotoxicities against human A-2780 cells.

Key words *Lysimachia davurica*; triterpene; saponin; 3-*O*-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl-13,28-epoxy-3β-hydroxy-16-oleanaone; 3-*O*-β-D-glucopyranosyloxyuronic acid-(1→2)-β-D-xylopyranosyl-cyclamiretin A; 3-*O*-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl-cyclamiretin A

Lysimachia davurica belongs to the Primulaceae family and grows in northeast China. As a folk medicinal plant, its whole plants have been used for treating hypertension.[1)] In the course of our ongoing screening for active antitumor constituents, it was found that the 70% ethanol extracts displayed cytotoxicity. We isolated eight saponins from this plant, together with some organic acids and flavones,[2—10)] and one of the saponins was found to have better antitumor activity.[10)] Here we report the isolation and structural elucidation of three new saponins, 3-*O*-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl-13,28-epoxy-3β-hydroxy-16-oleanaone (**1**), 3-*O*-β-D-glucopyranosyloxyuronic acid-(1→2)-β-D-xylopyranosyl cyclamiretin A (**2**), and 3-*O*-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl cyclamiretin A (**3**). Saponins **2** and **3** showed significant cytotoxic activities against human A-2780 cells.

Compound **1** was obtained as an amorphous white powder, giving a positive result in the Liebermann–Burchard test. Its positive and negative ESI-MS showed signals of quasi-molecular ion peaks at *m/z* 935 $[M+Na]^+$ and 911 $[M-H]^-$, respectively. Thus the molecular formula was determined to be $C_{47}H_{76}O_{17}$ from HR-FAB-MS. The seven tertiary methyl groups (δ 1.30, 1.17, 1.07, 1.02, 0.89, 0.86, 0.80) observed in the ^{1}H-NMR spectrum as well as the information from the ^{13}C-NMR spectrum (seven sp^3 carbons at δ 33.4, 28.1, 23.5, 21.8, 18.8, 16.5, 16.2 analyzed in the DEPT and HMQC spectra) indicated that the compound was a triterpenoid saponin. D-Glucose and L-arabinose were detected in GC analysis after acid hydrolysis and preparation of their thiazolidine derivatives.[11)] Assignments of all carbon signals (see Table 1) were achieved based on its HMQC and HMBC spectra. A quaternary carbon signal at δ 86.2 ppm due to C-13 showed that there should be a 13,28-epoxy moiety in this molecule. Correlations between the carbonyl of a ketone (δ 213.0) and the protons of H_2-28 [δ 3.90 (1H, d, J=8.5 Hz), 3.50 (1H, d, J=8.5 Hz)] and H_2-15 [δ 2.82 (1H, d, J=16.0 Hz), 1.99 (1H, d, J=16.0 Hz)], respectively, were made in its HMBC spectrum, which confirmed that the ketone carbonyl was located at C-16. The α conformation of H-3 was deduced by its spin–spin coupling constants (J=11.5, 4.5 Hz, respectively). The above analysis revealed the aglycone of **1** was 13,28-epoxy-3β-hydroxy-16-oleanaone.[12)]

Three sugar units were obtained from the HMQC spectrum of **1**, in which their anomeric protons at δ 5.17 (1H, d, J=7.5 Hz), 5.13 (1H, d, J=7.5 Hz), and 4.93 (1H, d, J=5.0 Hz) were correlated with carbon signals at δ 105.8, 105.5, and 104.4, respectively. The spin systems associated with monosaccharides were identified by a HMQC-TOCSY experiment with the aid of the ^{1}H–^{1}HCOSY spectrum. All carbon signals of the sugar moieties were assigned based on the HMQC experiment as shown in Table 1. Combining with spin–spin couplings, the three sugar units were identified as two β-glucopyranoses (glc) and one α-arabinopyranose (ara).

The sugar sequences of the oligosaccharide chain as well as the glycoside sites were subsequently determined based on the HMBC spectrum. In the HMBC spectrum of **1**, the correlations could be achieved between the anomeric proton of arabinose at δ 4.93 (1H, d, J=5.0 Hz) and C-3 of aglycone at δ 88.9, the anomeric proton of glucose-I at δ 5.17 (1H, d, J=7.5 Hz) and the C-2 of arabinose at δ 80.8, the anomeric

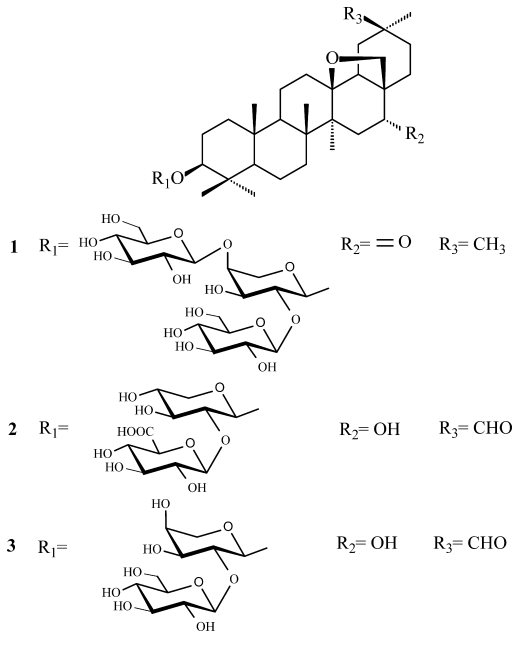

Fig. 1

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Table 1. ^{13}C-NMR (125 MHz) Spectral Data of **1**, **2**, and **3** in Pyridine-d_5

No.	**1**	**2**	**3**	No.	**1**	**2**	**3**
1	39.1	39.6	39.3	Sugar	Ara	Xyl	Ara
2	26.5	26.8	26.6	1	104.4	107.3	104.8
3	88.9	88.7	88.7	2	80.8	79.8	81.0
4	39.6	39.0	39.2	3	72.4	78.4	73.5
5	55.6	55.6	55.2	4	77.0	71.4	68.2
6	18.9	18.5	18.9	5	63.5	66.2	64.9
7	33.9	33.3	33.8	Sugar	Glc I at Ara C-2	Glc A at Xyl C-2	Glc I at Ara C-2
8	49.9	42.5	42.6	1	105.8	106.8	106.0
9	50.3	50.0	50.4	2	76.1	77.1	76.4
10	35.7	36.8	36.8	3	78.6	78.5	78.1
11	17.8	19.5	19.7	4	71.8	73.3	73.3
12	31.9	36.8	36.1	5	78.2	77.9	78.3
13	86.2	86.3	86.5	6	62.8	170.0	62.5
14	43.0	44.6	44.1	Sugar	Glc II at Ara C-4		
15	45.8	42.5	42.7	1	105.5		
16	213.0	75.8	74.5	2	75.7		
17	56.2	44.0	43.8	3	78.4		
18	54.7	53.3	53.1	4	71.5		
19	36.8	30.4	30.0	5	78.0		
20	40.1	48.3	48.5	6	62.7		
21	31.8	34.3	34.9				
22	25.1	32.6	32.3				
23	28.1	28.6	28.1				
24	16.5	16.4	16.3				
25	16.2	16.5	16.7				
26	18.8	17.9	18.0				
27	21.8	19.1	19.3				
28	75.1	77.6	76.8				
29	33.4	24.1	24.7				
30	23.5	207.5	207.2				

Table 2. ^{1}H-NMR (500 MHz) Spectral Data of **1**, **2**, and **3** in Pyridine-d_5

No.	**1**	**2**	**3**	No.	**1**	**2**	**3**
3	3.15 (dd, 11.5, 4.5)	3.22 (dd, 12.0, 4.5)	3.20 (dd, 12.0, 4.5)	Sugar	Ara	Xyl	Ara
5	0.84 (t, 7.5)	0.87 (t, 7.0)	0.84 (t, 7.0)	1	4.95 (d, 5.0)	4.70 (d, 7.0)	4.78 (d, 6.0)
12	1.97 (t, 7.0)	2.03 (t, 7.0)	2.06 (t, 7.0)	2	4.52	3.66	4.46
15	2.82 (d, 16.0)	2.16 (d, 7.0)	2.23 (d, 7.5)	3	4.43	3.59	4.14
	1.99 (d, 16.0)			4	4.47	3.52	4.05
16		4.23 (m)	4.19 (m)	5	3.82	4.01	3.79
18	2.26 (dd, 13.5, 4.0)	2.23 (dd, 14.0, 4.0)	2.20 (dd, 14.0, 4.5)	Sugar	Glc I at Ara C-2	Glc A at Xyl C-2	Glc I at Ara C-2
23	1.17	1.04	1.07	1	5.17 (d, 7.5)	5.28 (d, 8.0)	5.25 (d, 7.5)
24	1.02	1.00	1.02	2	4.03	4.07	4.04
25	0.80	0.83	0.85	3	3.83	3.76	3.82
26	1.30	1.30	1.33	4	4.27	4.19	4.21
27	1.07	1.54	1.56	5	3.96	3.92	3.99
28	3.90 (d, 8.5)	3.54 (d, 9.0)	3.42 (d, 9.0)	6	4.54		4.50
	3.50 (d, 8.5)	3.15 (d, 9.0)	3.17 (d, 9.0)	Sugar	Glc II at Ara C-4		
29	0.89	1.20	1.22	1	5.13 (d, 7.5)		
30	0.86	9.60	9.62	2	4.01		
				3	3.95		
				4	4.20		
				5	3.87		
				6	4.56		

proton of glucose-II at δ 5.13 (1H, d, J=7.5 Hz) and the C-4 of arabinose at δ 77.0, respectively, which suggested that the sugar sequences of the oligosaccharide chain were as shown in Fig. 1.

Thus the structure of **1** was established to be 3-*O*-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl-13,28-epoxy-3β-hydroxy-16-oleanaone.

Compound **2** was obtained as an amorphous white powder and gave a positive result in the Liebermann–Burchard test. Signals of quasi-molecular ion peaks at m/z 803 $[M+Na]^+$ and 779 $[M-H]^-$, respectively, were seen in the FAB-MS. The molecular formula was determined to be $C_{41}H_{64}O_{14}$ from HR-FAB-MS. The six tertiary methyl groups (δ 1.54, 1.30, 1.20, 1.04, 1.00, 0.83) observed in the ^{1}H-NMR spectrum as well as the carbon signals in the ^{13}C-NMR spectrum (six sp^3 carbons at δ 28.6, 24.1, 19.1, 17.9, 16.5, 16.4 analyzed in the

DEPT and HMQC spectra) revealed that the compound is a triterpenoid saponin. D-Glucopyranosyloxyuronic acid and L-arabinose were also detected in GC analysis in the same procedure as used for **1**. The ^{13}C-NMR spectrum of **2** showed broadly similar signals of the aglycone to those of a known compound, cyclamiretin A (3β,16α-dihydroxy-13,28-epoxy-oleanane-30-aldehyde),[13] except for that attributable to C-3, which downshifted for 10.9 ppm, and thus the glycoside linkage was suggested to be at C-3 of the aglycone. All the above analyses revealed that the aglycone of **2** was cyclamiretin A.

From the HMQC spectrum of **2**, we confirmed that two sugar units should exist in this molecule, and their anomeric protons at δ 5.28 (1H, d, J=8.0 Hz) and 4.70 (1H, d, J=7.0 Hz) were correlated with carbons signals at δ 106.8 and 107.3, respectively. The spin systems associated with monosaccharides were identified in its ^{1}H–^{1}HCOSY spectrum. All carbon signals of the sugar moieties were assigned by the HMQC experiment as shown in Table 1. Combining with spin–spin couplings, the two sugar units were identified as one β-xylopyranose (xyl) and one β-glucopyranosyloxyuronic acid (glc A).

The sugar sequences of the oligosaccharide chain as well as the glycoside sites were subsequently determined based on the HMBC spectrum. In the HMBC spectrum of **2** (see Fig. 1), correlations could be observed between the anomeric proton of xylose at δ 4.70 (1H, d, J=7.0 Hz) and C-3 of aglycone at δ 88.7, the anomeric proton of glucopyranosyloxyuronic acid at δ 5.28 (1H, d, J=8.0 Hz) and the C-2 of xylose at δ 79.8, respectively, suggesting the sugar sequences of the oligosaccharide chain shown in Fig. 1.

Thus the structure of **2** was established to be 3-*O*-β-D-glucopyranosyloxyuronic acid-(1→2)-β-D-xylopyranosyl cyclamiretin A.

Compound **3** was also obtained as an amorphous white powder and its molecular formula was determined to be $C_{41}H_{66}O_{13}$ based on its HR-FAB-MS. By Comparing the NMR data of **3** with those of **2**, we found that **3** showed signals identical to those of **2** in the aglycone portion but different in the sugar portion. D-Glucose and L-arabinose were also identified using the same procedure as for **1**.

The sugar sequences of the oligosaccharide chain as well as the glycoside sites were subsequently determined using the same procedures mentioned above. Therefore the structure of **3** was established to be 3-*O*-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl cyclamiretin A.

Experimental

General Melting points were measured on a Fisher–Johns apparatus and are uncorrected. Optical rotations were obtained on a Perkin-Elmer 341 polarimeter. IR spectra were recorded on a Perkin-Elmer 983G spectrometer. NMR spectra were recorded on a Bruker AM-500 (500 MHz) instrument. FAB-MS were obtained on a Zabspec E spectrometer and ESI-MS were obtained on an Esquire-LC00054 spectrometer. For column chromatography, AB-8 resin (Tianjin Nankai), silica gel (200—300 mesh, Qingdao Haiyang), and ODS C_{18} (35—50 μm, Alltech) were used. TLC and HPTLC (silica gel GF_{254} precoated plates, Qingdao Haiyang) detections were obtained by spraying with 10% H_2SO_4 following heating. GC analysis was carried out on a Shimadzu QP5050A gas chromatograph.

Extraction and Isolation The whole plants of *L. davurica* were collected in Jilin province, China, and identified by Dr. Bao-Lin Guo, Institute of Medicinal Plants Development, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher specimen is deposited in the Institute of Medicinal Plants Development, Chinese Academy of Medical Sciences and Peking Union Medical College. The dried plant materials (10 kg) were ground and extracted twice with 95% EtOH and then twice with 50% EtOH under reflux. The 95% EtOH extract and 50% EtOH extract were combined. After removal of the solvents by evaporation, the combined extracts (2.8 kg) were partitioned successively between H_2O and petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH. The *n*-BuOH extract (1.5 kg) was chromatographed over an AB-8 resin column eluted with H_2O and 30%, 50%, 70%, and 95% EtOH, respectively. The 50% EtOH eluate (27 g) was then chromatographed on a silica gel column eluted with $CHCl_3$/MeOH (MeOH contained 5% H_2O) in a gradient from 90 : 10 to 20 : 80. Fraction 7 ($CHCl_3$/MeOH 75 : 35) (0.7 g) was finally separated on an ODS C_{18} (35—50 μm) column, using MeOH/H_2O (63 : 37) as eluents to afford **2** (23 mg) and **3** (19 mg); fractions 11—14 ($CHCl_3$/MeOH 65 : 35) (1.5 g) were also separated on an ODS C_{18} (35—50 μm) column (2.0×40.0 cm) using MeOH/H_2O (54 : 46) as eluents to afford **1** (22 mg).

Compound **1**: White amorphous powder, $[\alpha]_D^{20}$ −18.75° (c=0.10, MeOH); IR (KBr) ν_{max} cm^{-1}: 3420 (OH), 2960, 2870, 1740, 1475, 1360, 1207, 1030, 940; positive ESI-MS *m/z*: 935 [M+Na]$^+$; negative ESI-MS *m/z*: 911 [M−H]$^-$; HR-FAB-MS *m/z*: 935.4829 [M+Na]$^+$ (Calcd for $C_{47}H_{76}O_{17}Na$, 935.4980); ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

Compound **2**: White amorphous powder, $[\alpha]_D^{20}$ −5.00° (c=0.10, MeOH); IR (KBr) ν_{max} cm^{-1}: 3400 (OH), 2965, 2860, 1720, 1470, 1350, 1200, 1010; positive ESI-MS *m/z*: 803 [M+Na]$^+$; negative ESI-MS *m/z*: 779 [M−H]$^-$; HR-FAB-MS *m/z*: 803.4017 [M+Na]$^+$ (Calcd for $C_{41}H_{64}O_{14}Na$, 803.4194); ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

Compound **3**: White amorphous powder, $[\alpha]_D^{20}$ −7.30° (c=0.25, MeOH); IR (KBr) ν_{max} cm^{-1}: 3340 (OH), 2965, 2870, 1720, 1470, 1310, 1240, 1020; positive ESI-MS *m/z*: 789 [M+Na]$^+$; negative ESI-MS *m/z*: 765 [M−H]$^-$; HR-FAB-MS *m/z*: 789.4325 [M+Na]$^+$ (Calcd for $C_{41}H_{66}O_{13}Na$, 789.4401); ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

Acid Hydrolysis of 1—3 Each saponin (5 mg) dissolved in water (100 ml) and 2 M HCl (100 ml) was heated at 100 °C for 1 h. The mixture was then passed through an Amberlite IRA-60E column (6×50 mm) and the eluate was concentrated. The residue was dissolved in pyridine (25 ml) and stirred with D-cysteine methyl ester (4.0 mg) for 1.5 h at 60 °C. To the reaction mixture, hexamethyldisilazan (10 ml) and trimethylsilyl chloride (10 ml) were added and the mixture was stirred for 30 min at 60 °C. The supernatant was then analyzed with GC [column, DB-50, 25 mm×30 m; column temperature, 235 °C; carrier gas, N_2; retention time, D-Glc A (18.7 min), L-Glc A (18.4 min), D-Glc (16.5 min), L-Glc (16.1 min), D-Xyl (10.0 min), L-Xyl (9.7 min), D-Ara (9.5 min), L-Ara (10.1 min)]. From the new saponins D-Glc A (18.7 min), D-Glc, D-Xyl, and L-Ara were detected.

Cytotoxic Activity The three isolated saponins (**1**—**3**) were evaluated for their cytotoxicities against the human A-2780 cell line using the methylene blue dye assay and the anticancer drug hydroxycamptothecin (HCPT), as positive controls.[14] Among them, saponins **2** and **3** exhibited cytotoxicity against human A-2780 cells, with IC_{50} values of 1.92×10^{-3} μM/ml and 2.48×10^{-3} μM/ml, respectively while the positive control HCPT had an IC_{50} value of 1.05×10^{-3} μM/ml. However, saponin **1** displayed no cytotoxic effects against human A-2780 cells (>10 μg/ml). Considering the relationships between their structures and activities, triterpenes with an α-OH at the C-16 position, such as saponins **2** and **3**, showed more potent cytotoxic efficacies than other tested triterpenes without 16α-OH.

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