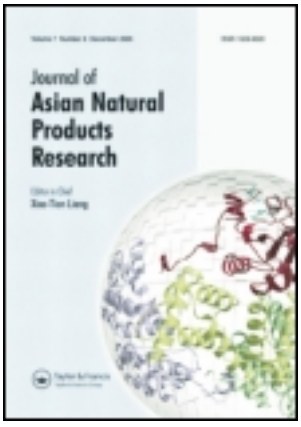

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Clematomandshurica saponin E, a new triterpenoid saponin from *Clematis mandshurica*

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A new triterpenoid saponin, clematomandshurica saponin E, together with four known saponins were isolated and characterized from the roots and rhizomes of *Clematis mandshurica* (Ranunculaceae), a commonly used traditional Chinese medicine with anti-inflammatory and antirheumatoid activities. On the basis of spectroscopic analysis, including HR-ESI-MS, IR, 1D, and 2D NMR spectral data and hydrolysis followed by chromatographic analysis, the structure of the new triterpenoid saponin was elucidated as 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside.

Keywords: *Clematis mandshurica*; Ranunculaceae; triterpenoid saponins; clematomandshurica saponin E

1. Introduction

The genus *Clematis* belongs to the family Ranunculaceae and comprises about 350 species which grow worldwide. About 150 species of this genus are distributed in China [1]. Pharmacological investigations demonstrated that many plants from this genus possess antitumor [2,3], antimicrobial [4,5], anti-inflammatory [6,7], and diuretic activities [8]. *Clematis mandshurica* Rupr., a plant widely distributed in the northeast of China, is one of the three sources of a commonly used traditional Chinese medicine called 'Wei-LingXian' with anti-inflammatory and antirheumatoid activities. The phytochemical researches carried out in the past years on the roots and rhizomes of *C. mandshurica* revealed the occurrence of triterpenoid saponins [9], lignans [10], alkaloids [11], macrocyclic glycosides [12], and phenolic glycosides [13]. In extensive investigations aimed at the discovery of new bioactive saponins from *C. mandshurica* growing in the Changbai Mountain, Jilin Province of China, five triterpenoid saponins were isolated and characterized, including a new triterpenoid saponin, 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside (**1**, clematomandshurica saponin E), as well as four known triterpenoid saponins (**2**–**5**) (Figure 1). In this paper, we report the isolation and structural characterization of these compounds.

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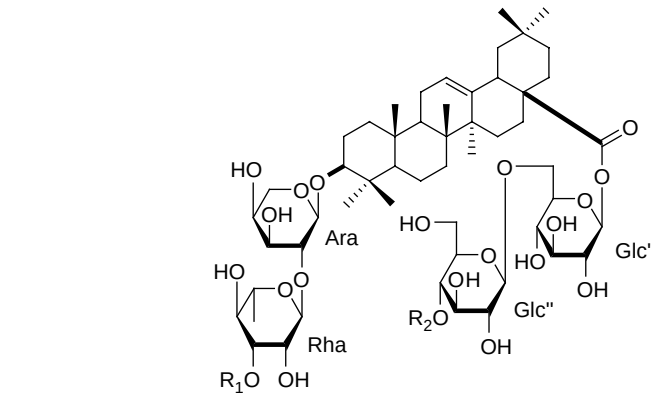

	R_1	R_2
1	S-1	H
2	S-1	S-5
3	S-2	S-5
4	S-3	S-5
5	S-4	S-5

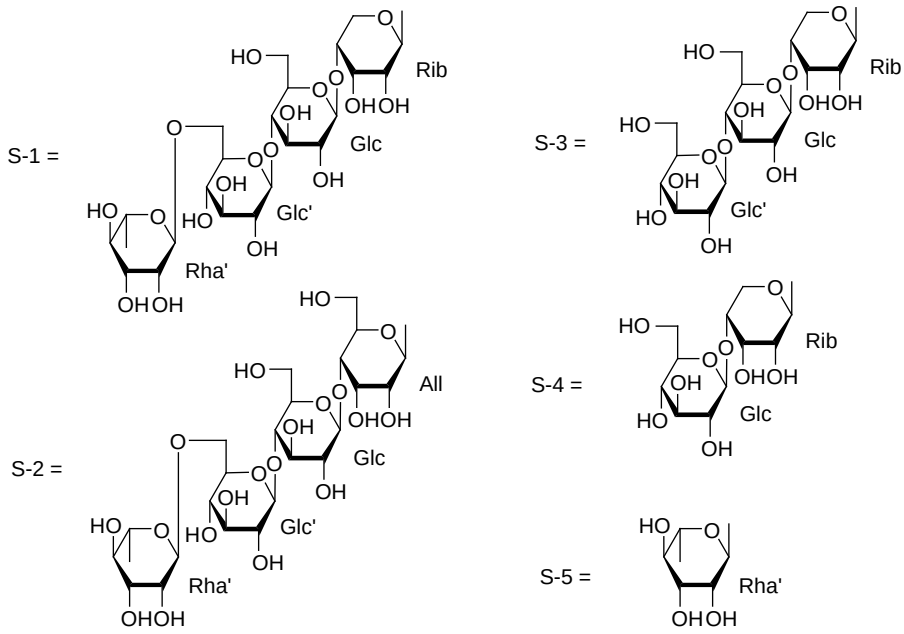

Figure 1. Chemical structures of compounds **1**–**5**.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder, and its positive results to both Liebermann–Burchard and Molish reactions indicated that **1** was a saponin. The IR spectrum of **1** showed a broad absorption band for hydroxyl groups at 3408 cm^{-1}, as well as absorption due to an ester carbonyl group at 1745 cm^{-1}. The HR-ESI-MS showed an accurate $[M + Na]^+$ ion at *m/z*

1683.7619, in accordance with the molecular formula of $C_{76}H_{124}O_{39}$, which was supported by the ^{13}C NMR and DEPT spectral data. The ^{1}H NMR spectrum showed seven tertiary methyl resonances at δ 0.89, 0.90, 0.90, 1.08, 1.14, 1.25, and 1.29 (each s) and a trisubstituted olefinic proton at δ 5.42 (t-like, $J = 3.6$ Hz), which was typical of the oleanolic acid skeleton. The resonances at δ 122.9 and 144.2 in the ^{13}C NMR spectrum also suggested that **1** possessed an oleanolic acid aglycone. The chemical shifts of C-3 (δ 88.8) and C-28 (δ 176.5) revealed that **1** was a bisdesmosidic glycoside. Of the 76 carbon signals observed in the ^{13}C NMR spectrum of **1** (Table 2), 30 were assigned to the aglycon and 46 to the oligosaccharide moieties. The ^{1}H and ^{13}C NMR spectra of **1** exhibited eight sugar anomeric protons at δ 4.86 (d, $J = 6.0$ Hz), 4.95 (d, $J = 7.9$ Hz), 5.04 (d, $J = 7.8$ Hz), 5.11 (d, $J = 7.8$ Hz), 5.44 (d, $J = 0.9$ Hz), 5.85 (d, $J = 5.2$ Hz), 6.24 (br s), and 6.25 (d, $J = 8.1$ Hz) and carbons at δ 95.7, 101.5, 103.2, 104.7, 105.0, 102.8, 105.2, and 105.3. The two methyl proton doublet signals at δ 1.54 (d, $J = 6.0$ Hz) and 1.60 (d, $J = 6.0$ Hz) and the methyl carbon signals at δ 18.5 and 18.6 indicated the presence of two deoxyhexopyranosyl units in **1**. Acid hydrolysis of **1** with 1 M HCl in dioxane–H_2O (1:1) afforded oleanolic acid, together with L-arabinose, L-rhamnose, D-ribose, and D-glucose. The monosaccharides were identified by HPLC analysis following conversion to the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives [14,15]. The exact sugar sequence and its linkage position to the aglycone were solved by detailed analysis of the 1D TOCSY and 2D NMR spectra. The ^{1}H NMR subspectra of the individual monosaccharide units were obtained by using selective irradiation of the easily identifiable proton resonances, as well as irradiation of other nonoverlapping proton resonances in a series of 1D TOCSY experiments. Subsequent analysis of the ^{1}H–^{1}H COSY spectrum resulted in the sequential assignments of all the proton resonances due to the eight monosaccharides, including identification of most of their multiplet patterns and coupling constants as shown in Table 1. The HSQC spectral data correlated the proton resonances with those of the corresponding one-bond coupled carbons and the HSQC–TOCSY spectra associated the anomeric protons with their respective carbon atoms, leading to unambiguous assignments of the carbons of all the individual monosaccharides. Comparison of the carbon chemical shifts thus assigned with those of the reference methyl glycosides [16], taking into account the known effects of *O*-glycosylation, indicated that **1** contained one L-arabinopyranosyl unit, one D-ribopyranosyl unit, two L-rhamnopyranosyl units, and four D-glucopyranosyl units. The β-anomeric configurations for the glucopyranosyl and ribopyranosyl moieties were identified from their $^{3}J_{H\text{-}1,H\text{-}2}$ coupling constants (5.2–8.1 Hz), and the arabinopyranosyl moiety was determined to have an α-anomeric configuration on the basis of the $^{3}J_{H\text{-}1,H\text{-}2}$ coupling constant (6.0 Hz). For the rhamnopyranosyl moieties, the anomeric protons were observed as a singlet. The ^{1}H nonsplitting pattern and the strong three-bond HMBC correlations from the anomeric protons to C-3 and C-5, the dihedral angles between H-1 and C-3 and between H-1 and C-5 about 180°, indicated that the anomeric protons were equatorial, thus possessing α-configurations. These configurations were also confirmed from the large $^{1}J_{H\text{-}1,C\text{-}1}$ values of the rhamnopyranosyl moieties (Rha, 172 Hz; Rha′, 170 Hz) [17]. The sequence of the glycan part connected to C-3 of the aglycon was deduced from the following HMBC correlations: the anomeric proton signals at δ 5.44 (Rha′), 5.11 (Glc′), 4.95 (Glc), 5.85 (Rib), 6.24 (Rha), and 4.86 (Ara) with C-6 of Glc′ at δ 68.6, C-4 of Glc at δ 81.9, C-4 of Rib at δ 76.6, C-3 of Rha at δ 82.1, C-2 of Ara at δ 75.6, and C-3 of the aglycone at δ 88.8, respectively (Figure 2). The disaccharide part at C-28 was established by the following HMBC

Table 1. ^{1}H NMR spectral data for the glycosidic moieties of compound **1** (500 MHz, C_5D_5N, δ in ppm).

No.	δ_H
Ara	
1	4.86 (d, $J = 6.0$ Hz)
2	4.57 (dd, $J = 6.7$, 6.0 Hz)
3	4.27 (hm)
4	4.27 (hm)
5a	4.32 (hm)
5b	3.83 (br d, $J = 10.3$ Hz)
Rha	
1	6.24 (br s)
2	4.88 (br s)
3	4.69 (dd, $J = 9.0$, 3.0 Hz)
4	4.43 (dd, $J = 9.5$, 9.0 Hz)
5	4.61 (dd, $J = 9.5$, 6.0 Hz)
6	1.54 (d, $J = 6.0$ Hz)
Rib	
1	5.85 (d, $J = 5.2$ Hz)
2	4.13 (hm)
3	4.68 (hm)
4	4.32 (hm)
5	4.30 (hm)
Glc	
1	4.95 (d, $J = 7.9$ Hz)
2	3.90 (dd, $J = 9.1$, 7.9 Hz)
3	4.18 (dd, $J = 9.2$, 9.1 Hz)
4	4.16 (dd, $J = 9.2$, 9.1 Hz)
5	3.90 (dd, $J = 9.1$, 2.8 Hz)
6a	4.45 (hm)
6b	4.28 (hm)
Glc′	
1	5.11 (d, $J = 7.8$ Hz)
2	4.03 (dd, $J = 9.8$, 7.8 Hz)
3	4.18 (dd, $J = 9.8$, 8.6 Hz)
4	3.96 (dd, $J = 9.8$, 8.6 Hz)
5	4.04 (dd, $J = 9.8$, 2.8 Hz)
6a	4.64 (hm)
6b	3.96 (hm)
Rha′	
1	5.44 (d, $J = 0.9$ Hz)
2	4.75 (hm)
3	4.55 (dd, $J = 9.2$, 3.7 Hz)
4	4.24 (dd, $J = 9.5$, 9.2 Hz)
5	4.28 (dd, $J = 9.5$, 6.0 Hz)
6	1.60 (d, $J = 6.0$ Hz)
Glc″	
1	6.25 (d, $J = 8.1$ Hz)
2	4.16 (hm)
3	4.23 (hm)
4	4.34 (hm)
5	4.11 (hm)
6a	4.72 (dd, $J = 12.5$, 2.6 Hz)
6b	4.32 (dd, $J = 12.5$, 4.4 Hz)
Glc‴	
1	5.04 (d, $J = 7.8$ Hz)
2	4.01 (dd, $J = 8.4$, 7.8 Hz)
3	4.20 (hm)
4	4.21 (dd, $J = 9.2$, 8.6 Hz)
5	3.89 (hm)
6a	4.47 (hm)
6b	4.35 (hm)

information: the correlations between H-1 of Glc‴ (terminal) at δ 5.04 and C-6 of Glc″ at δ 69.5 (inner) and between H-1 of Glc″ (inner) at δ 6.25 and C-28 of the aglycone at δ 176.5 (Figure 2). From the above evidence, the structure of 1 was established as 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside, named clematomandshurica saponin E.

Four known triterpenoid saponins, 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside (**2**) [3]; clematomandshurica saponin D, 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-β-D-allopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside (**3**) [9]; clematomandshurica saponin C, 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside (**4**) [9]; clematichinenoside

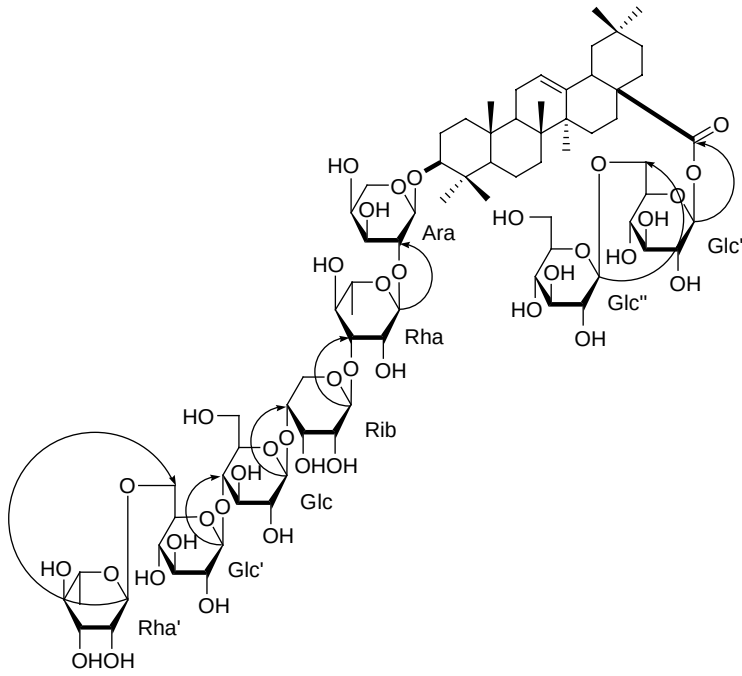

Figure 2. Key HMBC correlations of compound **1**.

C, 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside (**5**) [18], were also isolated and identified by comparison of their spectral data with those reported in the literature.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured by using a HORIBA SEPA-300 digital polarimeter. IR spectra were measured with a Perkin-Elmer FT-IR spectrometer as KBr pellets with absorption given in cm^{-1}. HR-ESI-MS and ESI-MS were obtained with a Bruck microTOFQ mass spectrometer. NMR spectra were measured on a Bruck AV-500 FT-NMR in C_5D_5N with TMS as internal standard. All chemical shifts (δ) are given in ppm. Column chromatography was performed with macroporous resin D101 (Haiguang Chemical, Ltd., Tianjin, China) and RP-18 reversed-phase silica gel (S-50 μm, YMC, Kyoto, Japan). TLC analysis was carried out on pre-coated TLC plates with silica gel 60 F_{254} and silica gel RP-18 60 F_{254} (Merck, Darmstadt, Germany, 0.25 mm). Detection was achieved by spraying with 10% H_2SO_4 in MeOH followed by heating. Preparative HPLC was performed on Shimadzu LC-6AD pump connected with a SHODEX RI-102 detector, using Shim Pak ODS column (250 mm × 21.2 mm, i.d., 5 μm, Shimadzu, Kyoto, Japan). The analytical HPLC was performed using a Shimadzu 2010A HT series liquid chromatographic system equipped with a quaternary solvent delivery system, an online degasser, an autosampler, a column temperature controller, and SPD-M10A photodiode-array detector coupled with an analytical workstation. The chromatographic separation was carried out on a Diamonsil C18 (5 μm, 250 mm × 4.6 mm, Dikma Technologies, Beijing, China). All

Table 2. ^{13}C NMR spectral data for compound **1** (125 MHz, C_5D_5N, δ in ppm).

No.	δ_C	No.	δ_C	No.	δ_C
1	39.0	Ara		Glc′	
2	26.7	1	105.2	1	105.0
3	88.8	2	75.6	2	74.9
4	39.6	3	74.6	3	78.2
5	56.1	4	69.2	4	72.0
6	18.6	5	65.5	5	76.8
7	33.2			6	68.6
8	39.9	Rha		Rha′	
9	48.1	1	101.5	1	102.8
10	37.1	2	72.0	2	71.8
11	23.8	3	82.1	3	72.6
12	122.9	4	72.8	4	74.1
13	144.2	5	69.9	5	69.9
14	42.2	6	18.5	6	18.6
15	28.3	Rib		Glc″	
16	23.4	1	104.7	1	95.7
17	47.1	2	72.5	2	73.9
18	41.7	3	69.5	3	78.8
19	46.3	4	76.6	4	71.0
20	30.8	5	62.0	5	78.0
21	34.1			6	69.5
22	32.6	Glc		Glc‴	
23	28.2	1	103.2	1	105.3
24	17.1	2	74.1	2	75.2
25	15.7	3	76.4	3	78.5
26	17.5	4	81.9	4	71.6
27	26.1	5	76.7	5	78.4
28	176.5	6	61.7	6	62.7
29	33.2				
30	23.7				

solvents used for the chromatographic separations were distilled before use.

3.2 Plant material

The roots and rhizomes of *C. mandshurica* were collected from Changbai Mountain, Jilin Province of China, in August 2008, and authenticated by Prof. Zhongkai Yan, Jilin Academy of Chinese Medicine Sciences, China. A voucher specimen (DTXL-2008-01) is deposited at the Institute of Phytochemistry, Jilin Academy of Chinese Medicine Sciences, China.

3.3 Extraction and isolation

The air-dried and ground roots and rhizomes (5 kg) of *C. mandshurica* were extracted twice with 70% ethanol under reflux for 2 h and the solvent was evaporated under reduced pressure to give a brown residue (1.2 kg). The residue (1.1 kg) was diluted with distilled water and subjected to D101 macroporous resin column chromatography and eluted with H_2O and 30 and 70% ethanol, successively.

The fraction eluted with 70% ethanol (2 g) was chromatographed over an RP-18 reversed-phase silica gel column (3 × 25, 100 g), eluting with a gradient of increasing MeOH (50–100%) in water and separated into six fractions (Fraction 1–6) on the basis of TLC analyses. Fraction 3 (0.71 g) was isolated by preparative HPLC (ODS column: 250 mm × 21.2 mm, i.d., 5 μm; flow rate: 2.2 ml/min) using MeOH–H_2O (65:35) as the mobile phase to yield compound **2** (199.9 mg, t_R = 75 min) and subfraction 3-I (0.26 g). Compound **3** (74.1 mg, t_R = 65 min) and compound **4** (69.6 mg, t_R = 74 min) were obtained from subfraction 3-I by preparative HPLC (RP-18 column: 250 mm × 21.2 mm, i.d., 5 μm; flow rate: 2.5 ml/min) employing MeCN–H_2O (30:70) as the mobile phase. Subfraction 4-I (0.31 g) was obtained from Fraction 4 (0.52 g) by preparative HPLC (ODS column: 250 mm × 21.2 mm, i.d., 5 μm; flow rate: 2.5 ml/min) employing MeCN–H_2O (30:70) as the mobile phase. Subfraction 4-I was purified by preparative HPLC (ODS column: 250 mm × 21.2 mm, i.d., 5 μm; flow rate: 2.2 ml/min) using MeOH–H_2O (65:35) as the mobile phase to yield compound **5** (73.6 mg, t_R = 154 min) and compound **1** (71.4 mg, t_R = 168 min).

3.3.1 *Clematomandshurica saponin E (**1**)*

White amorphous powder; $[\alpha]_D^{25}$ −34.8 (c = 0.42, MeOH); IR ν_{max} (KBr): 3408, 2930, 1745, 1060 cm^{-1}; ^1H NMR spectral data for the aglycone (500 MHz, C_6D_5N) δ: 5.42 (1H, t-like, J = 3.6 Hz, H-12), 3.29

(1H, dd, $J = 11.4$, 4.0 Hz, H-3), 1.29 (3H, s, H-23), 1.25 (3H, s, H-27), 1.14 (3H, s, H-24), 1.08 (3H, s, H-26), 0.90 (6H, s, H-29, 30), 0.89 (3H, s, H-25); ^{1}H NMR spectral data for the glycosidic moieties (500 MHz, C_6D_5N): see Table 1; ^{13}C NMR spectral data (125 MHz, C_6D_5N): see Table 2; HR-ESI-MS: *m/z* 1683.7619 $[M + Na]^+$ (calcd for $C_{76}H_{124}O_{39}Na$, 1683.7617).

3.3.2 Acid hydrolysis and determination of the absolute configuration of the monosaccharides

A solution of **1** (15 mg) in 1 M HCl (dioxane–H_2O, 1:1, 4 ml) was heated at 100°C for 2 h. After dioxane was removed, the solution was extracted with EtOAc (4 ml × 3). The extract was washed with H_2O, dried over $MgSO_4$, and evaporated to give oleanolic acid (4 mg). The aqueous layer was neutralized by passing through an Amberlite MB-3 ion-exchange resin (RohmandHass, Philadelphia, PA, USA) column and concentrated to furnish a monosaccharide residue. The sugar residue was dissolved in H_2O (1 ml), to which a solution of (*S*)-(−)-1-phenylethylamine (8 mg) and $Na[BH_3CN]$ (16 mg) in EtOH (1 ml) was added. The mixture was allowed to stand overnight, then acidified by the addition of glacial HOAc acid (0.3 ml) and evaporated to dryness. The resulting solid was acetylated with Ac_2O anhydride (0.5 ml) in pyridine (0.3 ml) at 100°C for 1 h. After codistillation with toluene, H_2O (2 ml) was added to the residue, and the crude mixture was passed through a Cleanert C18-SPE cartridge (Agela Technologies, Tianjin, China) and washed with H_2O–MeCN (4:1, 1:1, each 5 ml). The H_2O–MeCN (1:1) eluate contained a mixture of the 1-[(*S*)-*N*-acetyl-α-phenylethylamino]-1-deoxyalditol acetate derivatives of the monosaccharides, which were identified by co-HPLC analysis with standard sugars prepared under the same conditions. HPLC conditions: column, Diamonsil C18, 5 μm, 250 mm × 4.6 mm, i.d.; solvent, MeCN–H_2O (35:65); flow rate, 1.0 ml/min; detection, UV 230 nm; temperature, 40°C. The derivatives of D-glucose, D-ribose, L-rhamnose, and L-arabinose were detected with the retention times of 37.15, 24.32, 42.24, and 21.96 min, respectively, whereas the derivatives of L-glucose, L-ribose, D-rhamnose, and D-arabinose had the retention times of 35.43, 24.54, 42.02, and 20.85 min, respectively.

Acknowledgements

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