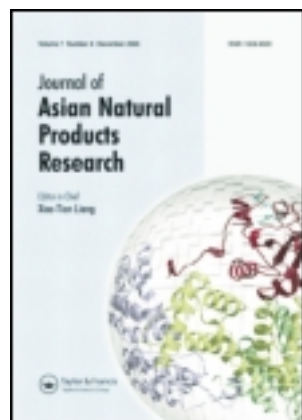


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Cytotoxic triterpene saponins from *Clematis mandshurica*

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Two new triterpene saponins, mandshunosides A and B (**1** and **2**), were isolated from the roots and rhizomes of *Clematis mandshurica*. Their structures were elucidated on the basis of spectroscopic evidence and hydrolysis products. Compounds **1** and **2** showed inhibitory activities against two colorectal human cancer cells HCT 116 (IC₅₀ 2.1 μM for **1** and 2.5 μM for **2**) and HT-29 (IC₅₀ 3.7 μM for **1** and 3.3 μM for **2**).

Keywords: *Clematis mandshurica*; triterpene saponins; mandshunoside A; mandshunoside B; cytotoxicity

1. Introduction

The root and rhizome of *Clematis mandshurica* Rupr. (Ranunculaceae) are a main source of traditional Chinese medicine 'Weilingxian', which is widely used as an anti-inflammatory, antitumor, analgesic agent [1]. Previous phytochemical research revealed that it was rich in triterpene saponins. The corresponding aglycones were oleanolic acid (Ole) and hederagenin (Hed). A trisaccharide chain can be linked to the aglycone at the C-28, and a complex long oligosaccharides chain at the C-3. Some of them showed anti-inflammatory and antitumor activity [2,3]. As a continuation of study on this plant, we report on the isolation and structural elucidation of two new saponins mandshunosides A and B (Figure 1), as well as their cytotoxicity.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder. The HR-ESI-MS

(positive-ion mode) experiment revealed a pseudo-molecular ion peak $[M + Na]^+$ at m/z 1403.6614, corresponding to a molecular formula of C₆₉H₁₀₄O₂₈. The monosaccharides obtained after aqueous acid hydrolysis of **1** were identified as glucose, rhamnose, arabinose, and ribose by TLC comparison with authentic samples. The absolute configurations of the monosaccharides were determined to be D for glucose and ribose and L for rhamnose and arabinose by GC analysis of chiral derivatives of the monosaccharides in the hydrolysate of each compound (see Section 3). The relatively large coupling constants (5.0–8.0 Hz) for the anomeric protons in the ¹H NMR spectrum (see Section 3) of **1** suggested that the arabinopyranosyl moiety has an α-configuration and the glucopyranosyl and ribopyranosyl moieties have a β-configuration. The α-configurations of the rhamnopyranosyl moieties were determined from the broad singlets for the anomeric protons. The ¹H NMR spectrum showed

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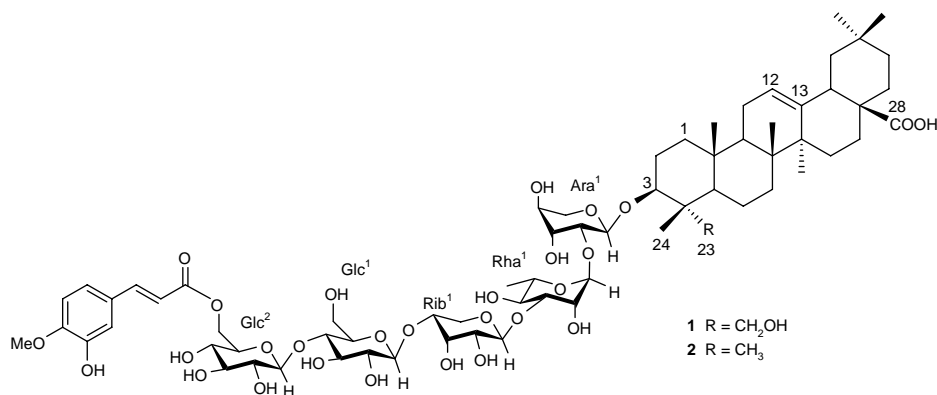


Figure 1. The structures of compounds **1** and **2**.

six tertiary methyl resonances at δ 0.83, 0.84, 0.93, 1.06, 1.07, and 1.13 and an olefinic proton at δ 5.37, which were typical signals of the Ole skeleton. The resonances at δ 123.0 and 145.1 in the ^{13}C NMR spectrum also suggested that **1** possessed an Ole aglycone. The aglycone of **1** was identified as Hed by comparison of its ^1H and ^{13}C NMR data obtained in 2D NMR experiments with those reported in the literature [4]. The downfield chemical shift at δ_{C} 80.9 (Aglycone-3) in the ^{13}C NMR spectrum of **1** (Table 1) indicated that a sugar chain was linked to C-3 of aglycone. Alkaline hydrolysis of **1** afforded a prosapogenin (**1a**), which was identified as CP₁₀. This was achieved by direct comparison of ^1H and ^{13}C NMR spectral data of **1a** with those of CP₁₀ reported in the literature [5]. The ^1H NMR

spectrum of **1** exhibited five anomeric proton resonances at δ 6.27 (1H, br s), 5.80 (1H, d, $J = 5.0$ Hz), 5.14 (1H, d, $J = 8.0$ Hz), 5.05 (1H, d, $J = 6.5$ Hz), and 4.90 (1H, d, $J = 8.0$ Hz), respectively. The three-proton doublets observed at 1.51 (3H, d, $J = 6.0$ Hz) suggested the presence of a deoxyhexopyranosyl unit in **1**. The spin-spin coupling system of individual monosaccharide units was identified by analysis of 1D TOCSY and 2D NMR spectra. ^1H NMR spectral data of individual monosaccharide units were obtained by selective irradiation of the anomeric protons or methyl groups of rhamnose units in a series of 1D TOCSY experiments. The analysis of the ^1H - ^1H COSY spectrum resulted in sequential assignment of all proton resonances of the five monosaccharide units, including the

Table 1. ^{13}C NMR spectral data for the aglycone moieties of **1** and **2** (125 MHz, in $\text{C}_5\text{D}_5\text{N}$).

No.	1	2	No.	1	2	No.	1	2
1	39.0	39.0	11	24.0	23.8	21	34.3	34.1
2	26.3	26.5	12	123.0	122.8	22	33.4	33.5
3	80.9	88.6	13	145.1	145.1	23	63.7	28.0
4	43.5	39.4	14	42.0	42.0	24	16.5	17.1
5	47.6	56.1	15	28.2	28.2	25	15.7	15.6
6	18.0	18.1	16	23.6	23.2	26	16.5	16.4
7	32.6	33.0	17	46.7	46.7	27	20.8	20.8
8	39.8	39.8	18	42.2	42.2	28	180.1	180.1
9	48.1	48.0	19	46.7	46.7	29	33.0	33.0
10	36.8	36.8	20	31.1	31.0	30	23.6	23.6

Table 2. ^1H NMR spectral data for the sugar moieties of compounds **1** and **2** (500 MHz, in $\text{C}_5\text{D}_5\text{N}$).

No.	1 (<i>J</i> in Hz)	2 (<i>J</i> in Hz)	No.	1 (<i>J</i> in Hz)	2 (<i>J</i> in Hz)
Ara ¹			Glc ¹		
1	5.05 d (6.5)	4.82 d (6.5)	1	4.90 d (8.0)	4.92 d (8.0)
2	4.54 dd (8.0, 6.5)	4.52 dd (8.0, 6.5)	2	3.86 dd (9.0, 8.0)	3.85 dd (9.0, 8.0)
3	4.01 dd (8.0, 4.5)	4.16 m	3	4.13 m	4.12 m
4	4.11 m	4.16 m	4	4.25 m	4.24 m
5a	4.20 d (9.0)	4.22 m	5	3.64 m	3.64 m
5b	3.62 d (9.0)	3.76 m	6a	4.23 m	4.24 m
Rha ²			6b	4.12 m	4.12 m
1	6.27 br s	6.27 br s	Glc ²		
2	4.83 br s	4.92 br s	1	5.14 d (8.0)	5.14 d (7.5)
3	4.63 m	4.71 m	2	3.92 m	3.92 m
4	4.38 dd (9.0, 9.0)	4.39 dd (9.0, 9.0)	3	4.11 m	4.11 m
5	4.62 m	4.60 m	4	4.14 m	4.13 m
6	1.51 d (6.0)	1.51 d (6.0)	5	4.01 m	4.01 m
Rib ¹			6a	5.17 m	5.16 m
1	5.80 d (5.0)	5.79 d (5.0)	6b	4.66 m	4.65 m
2	4.09 m	4.07 m			
3	4.58 m	4.57 m			
4	4.30 m	4.30 m			
5a	4.28 m	4.27 m			
5b	4.28 m	4.27 m			

identification of most of their multiple splitting patterns and coupling constants, as shown in Table 2. In the HSQC experiment, proton resonances were correlated with those of the corresponding carbons, and associated anomeric protons were correlated with their respective carbon atoms from HSQC–TOCSY data, leading to unambiguous assignments of the carbons in each monosaccharide unit (see Table 3). By comparison of the carbon chemical shifts thus assigned with those of reference methyl glycosides [6], and taking into account the known effects of *O*-glycosylation, **1** was found to contain one L-arabinopyranosyl unit (Ara), one D-ribosepyranosyl unit (Rib), one L-rhamnopyranosyl unit (Rha), and two D-glucopyranosyl units (Glc). In the HMBC spectrum, the anomeric proton signals at δ_{H} 5.05 (Ara¹-H-1), 6.27 (Rha¹-H-1), 5.80 (Rib¹-H-1), 4.90 (Glc¹-H-1), and 5.14 (Glc²-H-1) showed cross-peaks with the carbon signals at δ_{C} 80.9 (Agly-C-3), 75.3 (Ara¹-C-2), 82.0 (Rha¹-C-3), 76.4 (Rib¹-C-4), and 81.8 (Glc¹-C-4), respectively. These

signals provide ample evidence to determine the linkages between the sugars, as well as between the sugar and the aglycone. Further analysis of the NMR

Table 3. ^{13}C NMR spectral data for the sugar moieties of compounds **1** and **2** (125 MHz, in $\text{C}_5\text{D}_5\text{N}$).

No.	1	2	No.	1	2
Ara ¹			Glc ¹		
1	104.6	105.3	1	103.1	103.1
2	75.3	75.3	2	74.3	74.2
3	75.1	74.8	3	76.4	76.6
4	69.6	69.4	4	81.8	81.8
5	66.2	65.5	5	76.4	76.4
Rha ²			6	61.5	61.5
1	101.3	101.4	Glc ²		
2	71.8	71.9	1	105.1	105.1
3	82.0	82.1	2	72.3	72.3
4	72.5	72.5	3	78.6	78.6
5	69.6	69.7	4	71.2	71.2
6	18.4	18.4	5	75.5	75.6
Rib ¹			6	64.2	64.2
1	104.7	104.7			
2	72.6	72.6			
3	69.6	69.8			
4	76.4	76.4			
5	61.5	61.6			

Table 4. ^1H and ^{13}C NMR spectral data for the isoferuloyl moieties of compounds **1** and **2** (500 MHz for ^1H NMR, 125 MHz for ^{13}C NMR, in $\text{C}_5\text{D}_5\text{N}$).

No.	1		2	
	^1H (J in Hz)	^{13}C	^1H (J in Hz)	^{13}C
1		167.1		166.6
2	6.89 d (16.0)	116.3	6.90 d (16.0)	116.4
3	8.09 d (16.0)	145.6	8.12 d (16.0)	145.6
4		128.5		128.6
5	7.52 d (2.0)	115.4	7.53 d (2.0)	115.4
6		148.4		148.4
7		150.8		150.9
8	6.87 d (8.5)	112.0	6.87 d (8.5)	112.1
9	7.07 dd (8.5, 2.0)	121.4	7.09 dd (8.5, 2.0)	121.4
OCH_3	3.73 s	55.8	3.73 s	55.8

spectral data of **1** revealed the presence of a 3-hydroxy-4-methoxycinnamoyl group (isoferuloyl; Table 4). The typical protons of the isoferuloyl moiety resonated at δ_{H} 7.52 (1H, d, $J = 2.0$ Hz), 7.07 (1H, dd, $J = 8.5, 2.0$ Hz), 6.87 (1H, d, $J = 8.5$ Hz), 8.09 (1H, d, $J = 16.0$ Hz), 6.89 (1H, d, $J = 16.0$ Hz), and 3.73 (3H, s), and at δ_{C} 116.3, 121.4, 112.0, 145.6, 115.4, 150.8, 148.4, 128.5, 167.1, and 55.8. The correlation observed between Glc²-H-6 (δ 5.17 and 4.66) and the carbonyl carbon (δ 167.1) of the isoferuloyl moiety in the HMBC spectrum indicated that the isoferuloyl moiety is linked to the sugar chain on Glc²-C-6 (δ 64.2). From the above evidence, the structure of **1** was established as 3-*O*-[(6-*O*-isoferuloyl)- β -D-glucopyranosyl]-(1 \rightarrow 4)- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-ribosepyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl Hed, which has been named mandshunoside A.

Compound **2** was isolated as a white amorphous powder. The HR-ESI-MS (positive-ion mode) experiment revealed a pseudo-molecular ion peak $[\text{M} + \text{Na}]^+$ at m/z 1387.6659, suggesting a molecular formula of $\text{C}_{69}\text{H}_{104}\text{O}_{27}$. The spectroscopic properties of **2** were closely related to those of **1**, however, the molecular formula of **2** ($\text{C}_{69}\text{H}_{104}\text{O}_{27}$) had one oxygen atom lower

than that of **1**. Comparison of ^1H and ^{13}C NMR spectral data for **1** with those for **2** revealed that they differed only in the aglycone part. The upfield shift of Agly-C-23 by 28.0, and the correlation between signals at δ 28.0 (Agly-C-23) and δ 1.22 (3H, s) in the HSQC spectrum indicated the presence of the methyl (Agly-C-23). In addition, a set of carbon signals for **2** at δ 88.6 (Agly-C-3) and 56.1 (Agly-C-5) were observed, different from the corresponding carbons at δ 80.9 (Agly-C-3) and 47.6 (Agly-C-5) in **1**. Further comparison of its ^1H and ^{13}C NMR spectroscopic data with those reported values confirmed that the aglycone of **2** is Ole [7]. Acid hydrolysis of **2** yielded Ole, L-arabinose, D-glucose, L-rhamnose, and D-ribose. Signals of the arabinopyranosyl moiety connected to the aglycone were observed at δ 4.82 (Ara-H-1) and 105.3 (Ara-C-1) for **2**. The correlation observed between Ara-H-1 at δ 5.05 and Agly-3 at δ 88.6 confirmed that the arabinopyranosyl moiety was linked to the C-3 of aglycone. Thus, the structure of **2** was determined as 3-*O*-[(6-*O*-isoferuloyl)- β -D-glucopyranosyl]-(1 \rightarrow 4)- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-ribosepyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl Ole (mandshunoside B).

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241-MC digital polarimeter. UV spectra were obtained on a TU-1901 spectrometer. IR spectra were recorded on a Perkin-Elmer 577 spectrometer. NMR spectra were recorded on a Bruker INOVA 500 spectrometer with TMS as an internal standard. HR-ESI-MS was obtained on a IonSpec 4.7 T Fourier Transform Mass Spectrometry in m/z . GC was obtained on an Agilent 6890N gas chromatograph. Column chromatography was carried out on silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Ltd, Qingdao, China) and C18 silica gel (150–200 mesh, Merck, Darmstadt, Germany). HPLC separation was carried out on an Octadecylsilanized (ODS) column (YMC-pack ODS-A, 250 × 10 mm, i.d. 5 μm YMC, Kyoto, Japan) with an Agilent evaporative light scattering detector.

3.2 Plant material

The roots and rhizomes of *C. mandshurica* were collected in September 2010 in Jinzhou City, Liaoning Province, China. The identification of the plant was done by one of the authors (Y.-X.H.). A voucher specimen (CM 201009) is maintained in the herbarium of Bioengineering College of Xihua University.

3.3 Extraction and isolation

The dried roots and rhizomes (5 kg) of *C. mandshurica* were extracted with 50% EtOH. After removing the solvent, the residue (1262 g) was suspended in H₂O and extracted with EtOAc and *n*-BuOH. The *n*-BuOH extract (120 g) was subjected to silica gel column chromatography and eluted with CHCl₃–MeOH–H₂O (10:1:0, 4:1:0.2, 1:2:0.5) to afford fractions 1–3 (29, 31, and 18 g, respectively). Fraction 3 (18 g) was subjected to C₁₈ silica gel column chromatography and eluted with

MeOH–H₂O in a gradient of MeOH (MeOH–H₂O, 30:70–100:0%, to afford subfractions 3-1 to 3-7 (1.2, 3.0, 2.3, 1.4, 4.5, 0.8, and 4.8 g, respectively). Subfraction 3-6 (0.8 g) was isolated by preparative HPLC (CH₃CN–H₂O, 28:72, 2.0 ml/min) to yield compound **1** (41 mg) and compound **2** (37 mg).

3.3.1 Mandshunosides A (**1**)

A white amorphous solid, $[\alpha]_D^{20}$ –42, (c = 1.0, MeOH); UV (MeOH) λ_{\max} (log ϵ) 240 (2.31), 294 (sh), 324(4.20) nm; IR(KBr) ν_{\max} (cm^{–1}): 3412, 2931, 1710, and 1632; ¹H NMR (C₅D₅N, 500 MHz): δ 1.13 (3H, s, Me-27), 1.07 (3H, s, Me-24), 1.06 (3H, s, Me-26), 0.93 (3H, s, Me-25), 0.84 (3H, s, Me-29), 0.83(3H, s, Me-30), 4.25 (1H, dd, J = 12.0, 5.0 Hz, H-3), 4.23 (1H, d, J = 12.0 Hz, H-23a), 3.88 (1H, d, J = 12.0 Hz, H-23b), 5.37 (1H, br s, H-12); ¹³C NMR spectral data, see Tables 1 and 3; ¹H and ¹³C NMR spectral data for isoferuloyl moiety, see Table 4.

3.3.2 Mandshunoside B (**2**)

A white amorphous solid, $[\alpha]_D^{20}$ –37 (c = 1.0, MeOH); UV (MeOH) λ_{\max} (log ϵ) 242 (2.34), 294 (sh), 326 (4.24) nm; IR(KBr) ν_{\max} (cm^{–1}): 3406, 2933, 1714, and 1630; ¹H NMR (C₅D₅N, 500 MHz): δ 1.22 (3H, s, Me-23), 1.21(3H, s, Me-27), 1.09 (3H, s, Me-24), 1.03 (3H, s, Me-26), 0.86 (3H, s, Me-29), 0.85 (3H, s, Me-30), 0.82 (3H, s, Me-25), 3.22 (1H, dd, J = 12.0, 5.0 Hz, H-3), 5.36 (1H, br s, H-12); ¹³C NMR spectral data, see Tables 1 and 3; ¹H and ¹³C NMR spectral data for isoferuloyl moiety, see Table 4.

3.4 Acid hydrolysis

A solution of compound **1** or compound **2** (5.0 mg each) in 2 M trifluoroacetic acid (1 ml) was heated at 110°C for 2 h, and then dried by N₂ gas. After cooling, the reaction mixture was neutralized with

Amberlite IRA-400 resin (OH^- form) and the resin was filtered. After removal of the solvent under pressure from the filtrate, the residue was passed through a Sep-Pak C_{18} cartridge with H_2O and MeOH. The H_2O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (2.0 mg) in pyridine (1.0 ml) at 60°C for 2 h. After drying by N_2 gas, the residue was treated with *N*-(trimethylsilyl)imidazol (0.2 ml) at 60°C for 1 h. The reaction was stopped by adding water (1.0 ml), and extracted with cyclohexane (1.0 ml, three times). The cyclohexane layer was collected and concentrated to 1.0 ml for GC analysis. Separations were carried out on a HP-5 column ($28\text{ m} \times 0.32\text{ mm}$). Highly pure He was employed as carrier gas (1.0 ml/min flow rate), and the Flame Ionization Detector detector operated at 260°C (column temp 180°C). The retention times of the monosaccharide derivatives were as follows: L-Rha, 5.75 min; D-Glc, 11.90 min; D-Rib, 5.43 min; and L-Ara, 5.12 min. The retention times of standard monosaccharide derivatives were L-Rha, 5.74 min; D-Glc, 11.89 min; D-Rib, 5.43 min; and L-Ara, 5.11 min.

3.5 Alkaline hydrolysis

Pure compound **1** (10 mg) was refluxed in 5% KOH solution (pH 12–13) at 90°C for 1 h. The reaction mixtures were neutralized with 5% HCl solution and then concentrated to dryness. The residues were extracted with *n*-BuOH, and the organic layers of pure compounds were analyzed by NMR spectroscopy.

Hydrolysis of **1** afforded a known compound identified as CP_{10} .

3.6 MTT cytotoxicity assay

The bioassay was carried out according to a previously described method, against two human colorectal cancer cell lines (HCT 116 and HT-29) [8]. Paclitaxel was used as a positive control with IC_{50} values of 3.5 nM (HCT 116) and 3.4 nM (HT-29).

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