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Original article

Three new diterpenoids from *Leonurus japonicus*

Zhi-Ke Liu [a,c], Da-Rong Wu [b], Yi-Ming Shi [a,c], Ting Zeng [d], Shao-Hua Liu [b], Xue Du [a], Yong-Jun Dang [d,*], Wei-Lie Xiao [a,*], Han-Dong Sun [a]

[a] *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China*
[b] *Chengdu No. 1 Pharmaceutical Group Co., Ltd., Chengdu 610031, China*
[c] *University of Chinese Academy of Sciences, Beijing 100049, China*
[d] *Key Laboratory of Molecular Medicine, Ministry of Education, and Department of Biochemistry and Molecular Biology, Fudan University Shanghai Medical College, Shanghai 200032, China*

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ABSTRACT

Phytochemical investigation of the aerial parts of *Leonurus japonicus* led to the isolation of one unusual clerodane diterpenoid, leojaponin A (**1**), characterized by a C_4–C_7 oxa-bridge, and two new labdane diterpenoids, leojaponins B (**2**) and C (**3**). The structures of these new compounds were determined based on extensive 1D and 2D NMR spectroscopic data. To the best of our knowledge, compound **1** is the first clerodane diterpenoid obtained from *Leonurus. japonicus*. All of them were evaluated for their cytotoxicity.

1. Introduction

Leonurus japonicus, widely distributed in China, is an important folk medicine for the treatment of gynecological and obstetrical diseases, commonly known as "Motherwort" or "I-Mu Ts'ao" around the world and "Yi-Mu-Cao" in China [1,2]. The phytochemical investigation on this species has afforded numerous natural compounds with various structural patterns, such as alkaloids, iridoids, diterpenoids, triterpenoids, flavonoids, glycosides, and other compounds [3]. Among these compounds, labdane-type diterpenoids are major constituents of the species *L. japonicus*, some of which exhibit anti-platelet aggregation [4], anti-cholinesterase [5] and anti-inflammatory activities [6]. However, no clerodane-type diterpenoid has been isolated from this plant. Interestingly, our current research on the chemical constituents of *L. japonicus* led to the isolation of a new clerodane diterpenoid, leojaponin A (**1**), with a C4–C7 oxa-bridge and a double bond between C2 and C3, and two new labdane diterpenoids, leojaponins B–C (**2**–**3**). Herein, we report the isolation, structural elucidation and cytotoxicity of compounds **1**–**3**.

2. Experimental

The aerial parts of *L. japonicus* were collected in Xichang county, Sichuan Province, China, and identified by Prof. Xi-Wen Li. Voucher and specimens (KIB 20120601) were deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation: The dried aerial parts of *L. japonicus* (15.0 kg) were extracted with 95% EtOH (3 × 40 L) at room temperature, and the combined solvents were evaporated *in vacuo* to yield a residue (1.5 kg). This residue was chromatographed on a silica gel column eluting with $CHCl_3$–Me_2CO (1:0, 9:1, 8:2, 2:1, 1:1, 0:1) to afford fractions A–F. Fraction B (70.0 g) was subjected to MCI gel (90% MeOH–H_2O) and chromatographed on silica gel (petroleum ether-Me_2CO, 30:1–0:1) to afford subfractions B1–B7. Fraction B2 (10.0 g) was subjected to RP-18 column chromatography (20–100% gradient MeOH–H_2O) to afford subfractions B2.1–B2.9. Fraction B2.1 (0.03 g) was purified by semi-preparative HPLC (65% MeCN–H_2O) to give compound **1** (12 mg); Fraction B2.2 (0.15 g) was repeatedly chromatographed on Sephadex LH-20, and finally by semi-preparative HPLC (55%

* Corresponding author.
E-mail addresses: dangyogen@gmail.com (Y.-J. Dang), xwl@mail.kib.ac.cn (W.-L. Xiao).

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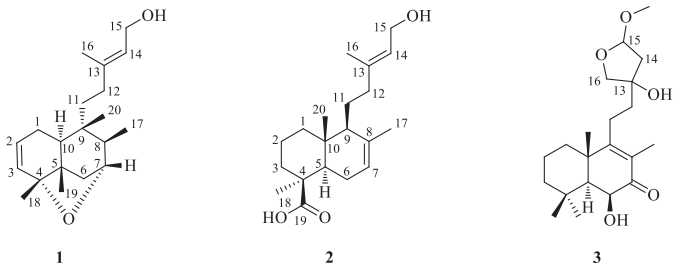

Fig. 1. Structures of compounds **1**–**3**.

MeCN–H_2O) yielding compound **2** (14 mg); Fraction B2.3 (0.07 g) was purified repeatedly by LH-20 and semi-preparative HPLC (40% MeCN–H_2O) to yield compound **3** (6 mg) (Fig. 1).

3. Results and discussion

Leojaponin A (**1**) was obtained as a colorless oil, [α]19.0 D −52.8 (*c* 0.21, MeOH) and UV (MeOH) λ_{max} (log ε) 202 (3.65) nm. The molecular formula of **1** was assigned as $C_{20}H_{32}O_2$ by HR-EI-MS (*m*/*z* 304.2402 [M]$^+$, calcd. 304.2402) with five degrees of unsaturation. The IR spectrum indicated the presence of a hydroxyl group (3440 and 3426 cm^{-1}). The ^{1}H NMR spectrum displayed signals of one secondary methyl and four tertiary methyl groups. Three olefinic proton signals at δ_H 5.75 (m), 5.53 (d, *J* = 8.0 Hz) and 5.40 (t, *J* = 6.9 Hz) suggested the presence of two double bonds (Table 1). The ^{13}C NMR and DEPT spectra exhibited signals for 20 carbons, including four quaternary carbons (including a single oxygenated one), six methines (including a single oxygenated one), five methylenes (including a single oxygenated one), and five methyls (Table 1). On the base of the HSQC spectrum, all protons were assigned unambiguously to their corresponding carbons. The above evidence implied that **1** was a clerodane diterpenoid, which was similar to the reported compound (3α, 4β, 13*E*)-4-ethoxyneoclerod-13-ene-3,15-diol [7]. The significant difference between **1** and (3α, 4β,13*E*)-4-ethoxyneoclerod-13-ene-3,15-diol was that **1** possessed two more degrees of unsaturation than (3α, 4β,13*E*)-4-ethoxyneoclerod-13-ene-3,15-diol. One degree of unsaturation was attributed to the double bond and the other one resulted from an additional ring based on the molecular formula. The location of the double bond was deduced to be between C-2 and C-3 by the HMBC correlations from H-10 to C-1, C-2; from H-1 to C-2, C-3 and the ^{1}H–^{1}H COSY correlations of H-10/H-1/H-2/H-3. C-7 was deduced to be an oxygenated methine based on the chemical shifts of C-7 and H-7, and the ^{1}H–^{1}H COSY of **1** supported the connectivities of H-8/H-7/H-6 (Fig. 2), which was confirmed by the HMBC correlations of H-8 with C-7 and C-6; of H-7 with C-6 and C-5 (Table 1). The location of the additional ring at C-4 and C-7 was determined by the HMBC correlations of H-7 with C-4, which suggested the connection of C-4 to C-7 through an oxygen bridge. The *E* geometry of the double bond between C-13 and C-14 was deduced from ROESY correlation of H-15 with Me-16 (Fig. 2).

The relative stereochemistry of **1** was deduced by the ROESY data. H-10 was supposed to be α-oriented on the basis of its reported analogs [8]. ROESY correlations of H-10 with H-11 and H-12, of H-1α with H-10 and H-12, of H-6α with H-11, suggested that Me-20 was β-oriented. ROESY correlations of H-1β with Me-20 and Me-18, of Me-19 with Me-18, of H-7 with Me-17, of H-17 with H-6β suggested that Me-18, Me-19, Me-17 and H-7 were all β-oriented (Fig. 2) (Figs. S1–10 in Supporting information).

Leojaponin B (**2**) was isolated as a colorless oil, [α]19.0 D −68.2 (*c* 0.16, MeOH) and UV (MeOH) λ_{max} (log ε) 202 (3.99) nm. Its molecular formula was determined as $C_{20}H_{32}O_3$ by its HR-EI-MS (*m*/*z* 320.2344 [M]$^+$, calcd. 320.2351), implying five degrees of unsaturation. The IR spectrum showed absorptions at 3449 and

Table 1
^{1}H and ^{13}C NMR data of compounds **1**–**3** in $CDCl_3$ (δ in ppm, *J* in Hz).

No.	**1**		**2**		**3**	
	[a]δ_C	[b]δ_H	[a]δ_C	[c]δ_H	[a]δ_C	[c]δ_H
1α	25.5 (t)	2.24 (m, 1H)	37.6 (t)	1. 68 (overlap, 1H)	37.5 (t)	1.32 (overlap, 1H)
1β		2.01 (m, 1H)		1. 76 (overlap, 1H)		1.92 (overlap, 1H)
2α	128.8 (d)	5.75 (m, 1H)	17.9 (t)	1.63 (overlap, 1H)	18.7 (t)	1.59 (overlap, 1H)
2β				1.63 (overlap, 1H)		1.79 (overlap, 1H)
3α	131.0 (d)	5.53 (d, 1H, 8.0)	35.3 (t)	1. 62 (overlap, 1H)	43.3 (t)	1.21 (overlap, 1H)
3β				1. 31 (overlap, 1H)		1.43 (overlap, 1H)
4	78.7 (s)		45.9 (s)		34.1 (s)	
5	41.6 (s)		37.5 (d)	2.11 (dd, 1H, 11.5, 5.5)	53.2 (d)	1.52 (d, 1H, 3.7)
6α	36.9 (t)	1.90 (m, 1H)	25.4 (t)	1.76 (t-like, 1H)	71.0 (d)	4.30 (d, 1H, 3.7)
6β		1.65 (m, 1H)		1.86 (m, 1H)		
7	81.0 (d)	4.02 (dd, 1H, 4.1, 6.4)	119.2 (d)	15.20 (br. S, 1H)	199.3 (s)	
8	45.1 (d)	1.84 (m, 1H)	136.6 (s)		170.2 (s)	
9	37.1 (s)		54.3 (d)	1.24 (overlap, 1H)	128.2 (s)	
10	48.2 (d)	1.44 (d, 1H, 5.4)	36.2 (s)		41.3 (s)	
11	41.4 (t)	1.34 (m, 2H)	29.8 (t)	1.64 (overlap, 1H)	25.0(t)	2.45 (t-like, 2H)
				1.37 (m, 1H)		
12	34.9 (t)	1.93 (overlap, 2H)	41.3 (t)	2.05 (t, 2H, 6.8)	36.4(t)	1.74 (t, overlap, 2H)
13	140.6 (s)		140.1 (s)		79.5 (s)	
14	122.9 (d)	5.40 (t, 1H, 6.9)	123.2 (d)	5.41 (t, 1H, 6.7)	44.9 (t)	2.11 (m, 1H)
						1.96 (overlap, 1H)
15	59.3 (t)	4.13 (d, 2H, 6.9)	59.4 (t)	4.16 (d, 2H, 6.7)	105.4 (d)	5.09 (br. d, 1H, 4.7)
16	16.5 (q)	1.67 (s, 3H)	16.4 (q)	1.68 (s, 3H)	80.1 (t)	4.01 (dd, m, 1H)
						3.84 (dd, m, 1H)
17	15.5 (q)	0.85 (d, 3H, 7.3)	23.3 (q)	1.64 (s, 3H)	11.4 (q)	1.83 (s, 3H)
18	25.3(q)	1.08 (s, 3H)	16.8 (q)	1.22 (s, 3H)	32.4 (q)	1.03 (s, 3H)
19	21.7 (q)	0.97 (s, 3H)	184.9 (s)		23.9 (q)	1.29 (s, 3H)
20	28.2 (q)	1.05 (s, 3H)	22.3 (q)	0.93 (s, 3H)	22.1 (q)	1.38 (s, 3H)
MeO					54.8 (q)	3.39 (s, 3H)

[a] Recorded at 100 MHz.
[b] Recorded at 500 MHz.
[c] Recorded at 400 MHz.

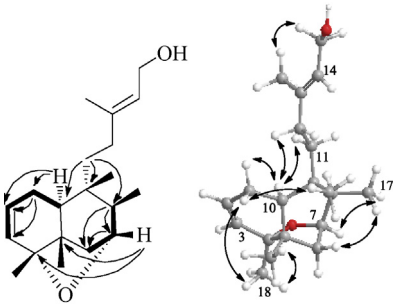

Fig. 2. Key HMBC (H→C), ^{1}H–^{1}H COSY (–) and ROESY (H ⟷ H) correlations of **1**.

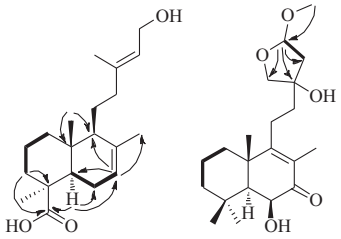

Fig. 3. Selected HMBC (H→C) and ^{1}H–^{1}H COSY (–) correlations of **2** and **3**.

1704 cm^{-1}, revealing the existence of hydroxyl and carbonyl groups. The ^{1}H NMR and ^{13}C NMR spectra (Table 1) of **2** were compared with those of the known labdane-type diterpenoid, villenol, and showed close structural similarities [9]. Careful comparison of their NMR data suggested that the main differences resulted from the hydroxymethyl (C-19, δ_C 64.7) in villenol oxidized to a carboxyl (C-19, δ_C 184.9) in **2**, which was confirmed by the HMBC correlations of H-3 and H-5 with C-19 (Fig. 3). The double bond between C-13 and C-14 of **2** was *E* geometry based on the ROESY correlation of H-15 with Me-16. The relative configurations of **2** were established by analysis of its ROESY data. Considering the structures of labdane-type diterpenoids previously isolated from the species *L. japonicus*, Me-20 was supposed to be β-oriented [10]. The correlations of Me-20 with H-11 and H-6β, of H-9 with H-5 and Me-18, and of H-6α with H-5 indicated that H-5, H-9 and Me-18 were all α-oriented (Figs. S11–20 in Supporting Information).

Leojaponin C (**3**), isolated as a colorless oil, [α]19.0 D +8.64 (*c* 0.22, MeOH), UV (MeOH) λ_{max} (log ε) 253 (3.68) nm, gave the molecular formula, $C_{21}H_{34}O_5$, from its HR-EI-MS (*m*/*z* 366.2420 [M]$^{+}$, calcd. 366.2406), requiring five degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl groups (3441 cm^{-1}) and double bonds (1646 cm^{-1}). The ^{1}H and ^{13}C NMR data (Table 1) of **3** were highly similar to those reported for (6β)-15,16-epoxy-15-ethoxy-6,13-dihydroxylabd-8-en-7-one [11], which was also one of labdane-type diterpenoids isolated in this plant. A careful comparison of their 1D NMR data, together with detailed HMBC and ^{1}H–^{1}H COSY analysis indicated that the difference was due to an ethoxyl group (δ_C 63.0, 15.2) in (6β)-15,16-epoxy-15-ethoxy-6,13-dihydroxylabd-8-en-7-one replaced by a methoxyl group (δ_C 54.8) in **3**. This was confirmed by HMBC correlations from MeO to C-15 (Fig. 3). In the ROESY spectrum, the correlations of Me-20 with Me-19 indicated that Me-19 was β-oriented. Correlations of Me-18 with H-5 and H-6 suggested that Me-18, H-5 and H-6 were on the same face as α-orientated (Figs. S21–30 in Supporting Information).

Compounds **1**–**3** were tested for their cytotoxicity against the HeLa cell line using the Alarmar-Blue assay [12]. All compounds were inactive with IC_{50} values greater than 40 μmol/L.

4. Conclusion

Three new diterpenoids, leojaponins A–C (**1**–**3**), were isolated from *L. japonicus* for the first time. To our knowledge, leojaponin A (**1**) is the first example of clerodane diterpenoid obtained from *L. japonicus*. This investigation should provide valuable information for further understanding of the chemical constituents of *L. japonicus*.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cclet.2014.01.047.

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