A New Triterpenoid Saponin and Two Neolignans from *Ligularia veitchiana*

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Phytochemical investigation of the rhizomes and roots of *Ligularia veitchiana* afforded a new triterpenoid saponin and two known neolignans named liguveitoside B (**1**), citrusin A (**2**) and citrusin B (**3**), respectively. This is the first demonstration of the occurrence of a 8-*O*-4' neolignan and the second report on the isolation of triterpenoid saponin in the genus *Ligularia*. Their structures were elucidated on the basis of spectral data, particularly HSQC, HMBC and NOE experiments. Compound **1** exhibited weak cytotoxicity against a human acute promyelocytic leukemia cell-line (HL-60).

Key words: Ligularia veitchiana, Liguveitoside B, Neolignan, Saponin

Introduction

Ligularia species have long been used as folk remedies with antibiotic, antiphlogistic and antitumor activities [1]. *Ligularia veitchiana* (Hemsl.) Greenm, is grown in northwestern China and has been used in traditional Chinese medicines since ancient times. The presence of sesquiterpenes and several other widespread known compounds in this plant are well documented [2 – 6], but research on the polar section was limited. Up to now, only liguveitoside A was isolated by Zhao *et al*. [7]. In our systematic search for cytotoxic constituents of this plant, compounds **1**, **2**, **3** were isolated from the *n*-BuOH fraction. In the present paper, the isolation and structural elucidation of liguveitoside B (**1**), citrusin A (**2**), citrusin B (**3**) and their cytotoxic activities are described.

Results and Discussion

The *n*-BuOH soluble part of the EtOH extract of the plant afforded, through repeated chromatographic separation and HPLC-ELSD, **1** in low yield, besides the known neolignans **2** and **3**.

Compound **1** was obtained as amorphous white powder. Positive-mode HR-ESI mass of **1** established the molecular formula $C_{36}H_{60}O_9$. The ^{1}H and ^{13}C NMR spectra of **1** showed the presence of one β-D-glucopyranosyl unit from the signals at δ C 105.9 and 5.13 (1H, *d*, $J = 8.0$ Hz). The ^{13}C NMR and

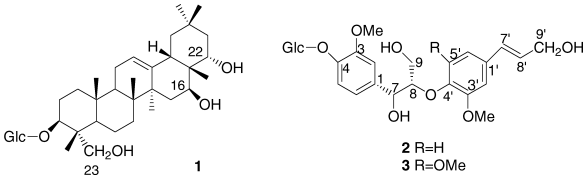

Fig. 1. Structure of **1**, **2** and **3**.

DEPT mode measurements determined a pentacyclic triterpenoid aglycone. The presence of two carbons at δ 123.0 and 143.2 indicated that the aglycone possessed an olean-12-ene skeleton. In addition, there were three hydroxylated methane resonances (δ 82.1, 78.0, 66.7) and one hydroxylated methylene resonance (δ 64.7) in its ^{13}C NMR spectrum of aglycone part. Thus, the aglycone structure was identified as tetrahydroxylated olean-12-ene.

The configuration at C18 in compound **1** was recognized to be 18β-series by inspecting the chemical shift of C12, C13 and C18 [8]. The oxy substitutions were considered at C-3, C-23, C-16 and C-22 on the basis of comparison with those related triterpenoids [8, 9]. The double doublet at $\delta = 4.90$ ($J = 11.2$, 5.0 Hz) supported the 16β-*O* substitution [10]. The pattern of hydroxyl substitution at C-22 was suggested by the strong similarities with the ^{13}C NMR resonance of ring E of olean-12-ene-3β, 16β, 22α-triol [11], and was further confirmed by NOE. The cross peak of H-22 with Me-28 in NOESY spectrum was observed.

Table 1. ^{1}H (HSQC) and ^{13}C NMR data, HMBC and ^{1}H-^{1}H COSY correlations of liguveitoside B (500 and 125 MHz, pyridine-d_5).

Carbon No.	^{1}H NMR δ H; mult.; J (Hz)	^{13}C NMR δ C	HMBC correlations	^{1}H-^{1}H COSY correlations
Aglycone part:				
1a	1.48 – 1.53	38.8		
1b				2a
2a	2.27	25.9		2b
2b	1.88 – 1.97			2a
3	4.27; *dd*; 4.5; 7.5	82.1	1', 23b	
4		43.5	23b, 3, 5	
5	1.63; *d*; 11.5	47.5		6b
6a	1.67 – 1.71	18.2	5	
6b	1.37; *m*			5
7	1.67 – 1.71	32.6	5	
	1.30			
8		40.3	15, 27	
9	1.67 – 1.71	47.2	5, 12	
10		36.7	5	
11	1.88 – 1.97	23.9	12	9, 12
12	5.30; *br s*	123.0		11
13		143.2	27	
14		42.9	12, 15, 18, 27	
15a	2.03; *t*; 12.5	36.5	27	15b, 16
15b	1.48 – 1.53			15a, 16
16	4.90; *dd*;11.2; 5.0	66.7	15, 18, 22, 28,	15a, 15b
17		41.3	18, 22, 28	
18	2.30	49.7	12, 28	19a,
19a	1.88 – 1.97	46.3	18	19b
19b	1.07			19a
20		32.0		
21	1.88 – 1.97	44.4		
	1.77; *m*			
22	4.02	78.0	21b, 28	
23a	4.35	64.7	3, 24	23b
23b	3.71; *d*; 11.0			23a
24	0.99; *s*	13.6	3, 5, 23b	
25	0.97; *s*	16.3	5	
26	1.05; *s*	17.1		
27	1.30; *s*	27.6	15	
28	1.44; *s*	19.8	16, 18, 22	
29	0.92; *s*	33.5		
30	0.97; *s*	25.0		
Glucose:				
1'	5.13; *d*; 8.0	105.9	3	2'
2'	4.04	75.9	1', 3'	1'
3'	4.16; *t*; 8.5	78.7		
4'	4.23	71.6	3'	
5'	3.90, *m*	78.4	1'	6'b
6'a	4.51; *d*; 10.0	62.8		
6'b	4.38; *m*			

These data permitted the deduction that the four oxygen atoms were located at positions 3β, 16β, 22α, and 23 of the olean-12-ene skeleton. The carbon resonances were associated with the corresponding proton signals using ^{1}H-^{1}H COSY, DEPT, HMBC, HSQC and the assignments are provided in Tables **1**. On the basis of above evidences, the new compound was assigned as 16β, 22α, 23-trihydroxyolean-12-ene-3β-D-glucopyranoside and was named liguveitoside B.

Compound **2** and **3** were identified as citrusin A and citrusin B by comparison of their $[\alpha]_D$, UV and NMR spectra data with the literature values [12, 13]. Their absolute configurations at C-7 and C-8 have not yet been determined, although the relative configuration of **2** was known as *erythro* form [14]. Here, we report the determination of their absolute stereo structures.

A ^{1}H NMR technique provided a convenient way to assign *threo* and *erythro* diastereomers of 8-*O*-4' neolignans. The small coupling constant of $J_{7,8}$ corresponded to the *erythro* derivatives [15]. However, the H-7 signal of **2** was overlapped with solvent signals. The problem could be overcome by using DMSO instead of CD_3OD as the solvent. The relative stereochemistry of 2 was shown to be *erythro* from the chemical shift value at 4.78 (1H, *d*, in DMSO) and the *J* coupling constant value (5.1 Hz) of H-7 [14]. In a similar way, H-7 (4.94, 1H, *d*, 5.4 Hz, in CD_3OD) of **3** also indicated the *erythro* form. Comparison of the negative CD curve in the 210 – 240 nm range of **2** and **3** with that of a series of 8-*O*-4' neolignan glycosides isolated by Matsuda and Kikuchi [16] indicated an 8*R* configuration. Thus, the absolute configurations of **2** and **3** were determined to be 7*S*, 8*R* on the basis of CD data and the coupling constant between the H-7 and H-8 protons [16]. Additionally, the ^{13}C NMR of citrusin B was corrected according to HMBC and HMQC (in CD_3OD) [12]. To our knowledge, this is the first report of a neolignan glycoside from the genus *Ligularia* and on the determination of absolute configuration of citrusin A and citrusin B.

Compounds **1**, **2** and **3** were tested cytotoxicity against HL-60, WM451, MDA231 and KB cells *in vitro*. Only compound **1** exhibited weak cytotoxicity against a human acute promyelocytic leukemia cell-line (HL-60) with an IC_{50} of 49 μg/ml.

Experimental Section

General

NMR: on a Varian INOVA-500 (**1**) and JEOL AL-300 (**2**, **3**), in C_5D_5N (**1**) and MeOH-d_6 (**2**, **3**), and TMS as int. standard; $[\alpha]_D$: Perkin-Elmer 243B polarimeter. mp: XT4A ESI Mass: MDS SCIEX QSTAR (ABI, USA). UV: Shimadzu 2401 CD: J-715 Spectropolarimeter. ESI-HRMS: Bruker APEX II spectrometer.

Plant material

Ligularia veitchiana was collected in July 2002 from Shennongjia of Hubei Province, China. The identification of the plant was performed by Prof. Hubiao Chen, School of Pharmaceutical Sciences, Peking University, Health Science Center. A voucher sample (No.F-2-5) is kept in Modern Research Center for Traditional Chinese Medicine, Peking University.

Extraction and isolation

The dried roots and rhizomes (7.4 kg) were powdered and successively percolated with 95% (88 l) and 50% ethanol (74 l) respectively. The combined extracts were submitted to liquid-liquid partition, resulting in petroleum ether, $CHCl_3$, EtOAc, *n*-BuOH and aqueous extracts.

The part of *n*-BuOH was solubled in water and subjected to CC (D101 resin), eluting with water, 10%, 30%, 50%, 70% and 95% ethanol, successively. The 70% eluate (1.58 g) was subjected to CC separation over silica gel (200 – 300 mesh, 90 g), eluting with $CHCl_3$-MeOH (20:1, 15:1, 12:1, 8:1, 6:1, 4:1, 3:1) and finally with CH_3OH. Four fractions were collected. Fraction B ($CHCl_3$-MeOH, 6:1) was further chromatographed over silica gel (200-300 mesh, 5.4 g) and eluted with $CHCl_3$-MeOH (6:1); fraction B2 (15 mg) was rechromatographed on HPLC using a Alltima column (C18 5 μm; 10.0×250 mm; CH_3OH: H_2O-80:20 v/v; flow rate: 3 ml/min; detection: ELSD: tube temp 75 °C, gas flow 2.0 l/min, Impactor Off, split ratio is 2.9:0.1) and afforded liguveitoside B (**1**, 2.8 mg).

30% eluate (4.53 g) was purification with Sephadex LH-20 column, eluting with water. Fourteen fractions were collected. Eighth fraction (200 mg) was further chromatographed on HPLC using a HR column (C18 5 μm; 7.8×300 mm; CH_3OH: H_2O-30:70 v/v; flow rate: 3 ml/min; detection: UV 230 nm) affording 26.4 mg A and 17.6 mg B. A and B were rechromatographed on HPLC (CH_3OH: H_2O-27:73 v/v; flow rate: 3 ml/min; detection: UV 230 nm) respectively, affording citrusin A (**2**, 16.7 mg) and citrusin B (**3**, 8.2 mg).

Spectroscopic data

liguveitoside B (**1**) was obtained as amorphous white powder, m.p. 233 – 235 °C, $[\alpha]_D^{17} + 18.0°$ (c 0.2, CH_3OH); UV (CH_3OH) λ_{max} 203 nm. The data of ^{1}H NMR, ^{13}C NMR are in Table 1. ESI-MS *m/z*: 637.4 $[M+H]^+$, 654.5 $[M+NH_4]^+$, 659.4 $[M+Na]^+$. HR-ESI-MS *m/z*: 659.4136 $[M+Na]^+$ ($C_{36}H_{60}O_9Na$ requires 659.4129).

Citrusin A (**2**): Amorphous powder. $[\alpha]_D^{17}$ −31.5° (c 0.45, CH_3OH); UV (CH_3OH) λ_{max}: 223, 272 nm. CD nm ($\Delta\varepsilon$): 224 (−8.60). The data of ^{1}H NMR, ^{13}C NMR is accordance with the literature values [12].

Citrusin B (**3**): Amorphous powder. $[\alpha]_D^{17}$ −34.2° (c 0.52, CH_3OH); UV (CH_3OH) λ_{max}: 223, 272 nm. CD nm ($\Delta\varepsilon$): 225 (−11.62). ^{1}H NMR (CD_3OD) δ: 3.84, 3.81 (3H, 6H, *s*, $OCH_3 \times 3$), 4.22 (2H, *dd*, $J = 5.4$, 1.2 Hz, H-9'), 4.94 (1H, *d*, $J = 5.4$ Hz, H-7), 6.31(1H, *dt*, $J = 15.6$, 5.4 Hz, H-8'), 6.53 (1H, *d*, $J = 15.9$ Hz, H-7'), 6.72 (2H, *s*, H-2' and H-6'), 6.91 (1H, *m*, H-6), 7.07(1H, *d*, $J = 1.8$ Hz, H-2), 7.12 (1H, *d*, $J = 8.5$ Hz, H-5); ^{13}C NMR (CD_3OD) δ: 137.5 (C-1), 112.3 (C-2), 150.4 (C-3), 147.2 (C-4), 117.5 (C-5), 120.8 (C-6), 73.7 (C-7), 87.3 (C-8), 61.4 (C-9), 134.7 (C-1'), 104.8 (C-2',6'), 154.5 (C-3',5'), 136.3 (C-4'), 131.4 (C-7'), 129.9 (C-8'), 63.6 (C-9'), 56.6 (OCH_3), 102.9 (C-1"), 73.7 (C-2"), 78.2 (C-3"), 71.4 (C-4"), 77.8 (C-5"), 62.5 (C-6").

Acid hydrolysis of **1**

Compound **1** and authentic D-(+)-glucose were spotted on a silica gel TLC plate and hydrolyzed *in situ* by exposure to HCl vapor at 80 °C for 50 min. The TLC plate was then developed with $CHCl_3$/MeOH/AcOH/H_2O (14:6:2:1) and sprayed with 10% H_2SO_4 for detection. D- (+)-glucose was detected with an R_f value of 0.2.

Cytotoxicity assay

The *in vitro* tests against HL-60, WM451, MDA231 and KB cells were essentially according to the method described as followed. Cells (1×10^4) were seeded in each well containing 100 μl of RPMI 1640 medium supplemented with 10% FBS in a 96-well microtiter plate and incubated overnight. The test samples, **1**, **2** and **3** were dissolved in dimethyl sulfoxide (DMSO) and were added in serial dilution (the final DMSO concentrations in all assays did not exceed 0.01%). Twenty-four hours after seeding, 100 μl new media or test samples were added, and the plates were incubated for 48 h. Cells were washed once before adding 50 μl FBS-free medium containing 5 mg/ml MTT. After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue which formed in the cells was replaced by adding 50 μl DMSO. Optical density was measured at 570 nm.

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