Two New Asymmetric Sesquiterpene Dimers from the Rhizomes of *Ligularia muliensis*

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Two new asymmetric eremophilane-type sesquiterpene dimers, ligulamulienin A (1) and B (2), were isolated from the rhizomes of *Ligularia muliensis*. Their structures were determined based on their spectroscopic data, including IR, EI-MS, HR-electrospray ionization (ESI)-MS, 1D and 2D-NMR spectroscopy. The cytotoxicity of compounds 1 and 2 was measured in *in vitro* human carcinoma (MGC-803), human hepatoma (HEP-G2), and murine sarcoma (S-180) cell lines.

Key words *Ligularia muliensis*; sesquiterpene dimer; ligulamulienin A; ligulamulienin B; cytotoxic activity

Many *Ligularia* plants which relieve internal heat or fever, reduce phlegm and relieve coughs, invigorate the circulation of blood and stop pain, as well as having antibiotic, anti-inflammation and antitumour activities have been used as folk medicines in China.[1)] In order to explore many more plants with medical potential and bioactive compounds, we have focused on the chemical constituents of *Ligularia* species and reported the isolation of a number of new sesquiterpenoids and their interesting biological properties.[2—4)] As a part of our ongoing investigation on the identification of novel bioactive constitutents from *Ligularia* species, we have further investigated the rhizomes of *Ligularia muliensis* Hand.-Mazz. and two new asymmetric dimeric eremophilane-type sesquiterpenes, ligulamulienin A (**1**) and B (**2**), were isolated. Compounds **1** and **2** showed moderate cytotoxicity against human stomach carcinoma (MGC-803), human hepatoma (HEP-G2), and murine sarcoma (S-180) cell lines.

Compound **1** was obtained as colorless needles, mp 146—148 °C, $[\alpha]_D^{20}$ −99 (c=0.6, acetone). Its HR-electrospray ionization(ESI)-MS exhibited a quasi-molecular ion peak at m/z 477.2972 $[M+Na]^+$ (Calcd for $C_{29}H_{42}O_4Na^+$, 477.2975), corresponding to a molecular formula of $C_{29}H_{42}O_4$, requiring 9 degrees of unsaturation. The IR spectrum of **1** showed the absorptions for hydroxyl (3443 cm^{-1}), α,β-unsaturated carbonyl (1701 cm^{-1}), and double bond (1611 cm^{-1}) groups. Inspection of the richly detailed ^{1}H- and ^{13}C-NMR spectra (Tables 1, 2) sets the stage for a dimer of a sesquiterpene (unit I) and a nor-sesquiterpene (unit II). In the upfield region of the ^{1}H-NMR spectrum, three typical methyl signals at δ_H 0.98 (3H, s), 0.84 (3H, d, J=7.0 Hz), and 2.03 (3H, br s) indicated that unit I was a furan eremophilane.[5)] The rest of the methyl signals at δ_H 0.71 (3H, s), 0.84 (3H, d, J=7.0 Hz), and 2.04 (3H, s) showed that unit II was 12-norerermophilene and

Table 1. ^{1}H-NMR (300 MHz) Data for Compounds **1** and **2** in $CDCl_3$

Position	1	2
1	1.55—1.86 overlap	2.73 (dq, J=14.1, 7.8, 3.0 Hz), 2.61 (br d, J=9.0 Hz)
2	1.24—1.45 overlap	1.86 (m)
3	1.24—1.45 overlap	1.50 (m), 1.36 (m)
4	1.55—1.86 overlap	3.32 (dq, J=6.5, 3.0 Hz)
6	2.55 (d, J=9.6 Hz), 2.30 (m)	
9	3.70 (br s)	7.09 (s)
10	1.98 (m)	
13	2.03 (s)	2.20 (s)
14	0.98 (s)	2.29 (s)
15	0.84 (d, J=7.4 Hz)	1.33 (d, J=6.6 Hz)
1′	1.55—1.86 overlap	1.86 (m)
2′	1.24—1.45 overlap	1.51—1.68 (m)
3′	1.24—1.45 overlap	1.51—1.68 (m)
4′	1.55—1.86 overlap	1.66—1.70 (m)
6′	2.50 (m), 1.66—1.69 (m)	2.37 (m), 1.66—1.70 (m)
9′	2.50—2.55 (m), 1.66—1.69 (m)	2.37 (m), 1.66—1.70 (m)
10′	1.34—1.38 (m)	1.51—1.68 (m)
13′	2.04 (s)	2.01 (s)
14′	0.71 (s)	0.82 (s)
15′	0.84 (d, J=7.0 Hz)	0.88 (d, J=6.3 Hz)
OH-9	4.63 (br s)	

Table 2. ^{13}C-NMR (75 MHz) Data for Compounds **1** and **2** in $CDCl_3$

Position	1	2
1	25.2 t	25.4 t
2	19.8 t	18.7 t
3	29.5 t	27.3 t
4	34.0 d	29.5 d
5	39.7 s	125.4 s
6	32.3 t	130.6 s
7	115.1 s	126.5 s
8	149.4 s	152.1 s
9	68.3 d	116.7 d
10	39.4 d	130.9 s
11	119.2 s	110.7 s
12	148.4 s	151.1 s
13	9.0 q	8.54 q
14	15.3 q	20.0 q
15	16.0 q	21.1 q
1′	25.2 t	27.2 t
2′	20.3 t	19.9 t
3′	30.3 t	31.2 t
4′	34.0 d	39.6 d
5′	39.9 s	33.0 s
6′	30.9 t	31.1 t
7′	107.0 s	106.7 s
8′	184.3 s	186.2 s
9′	32.4 t	34.6 t
10′	40.5 d	40.5 d
11′	197.6 s	206.5 s
13′	24.5 q	23.8 q
14′	16.0 q	16.0 q
15′	17.3 q	17.4 q

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similar to 12-norerermophil-6(7)-en-8,11-dione.[6] The difference between unit II and 12-norerermophil-6(7)-en-8,11-dione was that the double bond between C-6 and C-7 and the ketone carbonyl at C-8 in 12-norerermophil-6(7)-en-8,11-dione changed into an enol form between C-7′ and C-8′ in unit II. Through the enol *O*-atom, unit II was joined to C-12 of unit I and formed a dimeric sesquiterpene. The absence of a characteristic proton H-C(12) of the furan eremophilane in unit I confirmed this deduction. The structure of **1** was further unambiguously assigned by ^1H–^1H correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) spectra (Fig. 2).

Stereochemically, the Me-14(14′) and Me-15(15′) were biogenetically β orientated.[7] On nuclear Overhauser effect (NOE) difference spectra, the irradiation of Me-15 enhanced the signals H-9 (8.10%) and H-10 (8.00%), and the irradiation of Me-14 enhanced the signal H-9 (2.58%), indicating H-9 and H-10 were in a β-orientation and the OH-9 in an α-orientation. Consequently, the structure of **1** was elucidated to be an asymmetric dimer of unit I and unit II with a unique linkage pattern of C-12 with C-8′ as shown in Fig. 1, and named ligulamulienin A. The molecular ion peak at *m*/*z* 454 and several important fragment ion peaks at *m*/*z* 436, 344, 326, 234, 220 and 216 in the EI-MS (Chart 1) confirmed the proposed structure.

Compound **2** was obtained as colorless needles with a molecular formula of $C_{29}H_{38}O_3$ determined by HR-ESI-MS at *m*/*z* 435.2899 $[M+H]^+$, and like **1**, **2** was also predicted to be an asymmetric sesquiterpenoid dimer. The ^1H-NMR spectrum of **2** was extremely similar to those of **1**, especially in unit II, which suggested that the two compounds contained an identical unit II carbon skeleton and a different unit I. The IR spectrum of **2** showed the absorption bands for a benzene ring (1613, 1460, 1433, 1154, 874 cm^{-1}) and its ^{13}C-NMR spectrum (Table 2) showed six aromatic carbon signals at δ_C 125.4 (C), 130.6 (C), 126.5 (C), 152.1 (C), 116.7 (CH), and 130.9 (C), which indicated that **2** contained an aromatic ring in unit I. However, only one aromatic proton signal at δ_H 7.09 (s) could be observed in its ^1H-NMR spectrum, which indicated the presence of a pentasubstituted benzene ring. The downfield shift of Me-14 to δ_H 2.29 (s) showed that the angular methyl Me-14 had migrated to a benzene ring and changed into the aromatic methyl group. The above evidence is consistent with unit I of compound **2** being a cacalioid sesquiterpene.[8] Therefore, the structure of **2** was proposed to be the asymmetric dimeric sesquiterpenoid, named ligulamulienin B.

The cytotoxic activities of compounds **1** and **2** were evaluated against human stomach carcinoma (MGC-803), human hepatoma (HEP-G2), and murine sarcoma (S-180) cell lines using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) colorimetric assay as previously reported.[9] The results of the cytotoxic studies, represented in terms of IC_{50} values (the concentration required for 50% inhibition), are summarized in Table 3. Compounds **1** and **2** exhibited moderate cytotoxic activity against MGC-803, HEP-G2, and S-180 cell lines, with the IC_{50} values ranging from 11.9—65.4 μM.

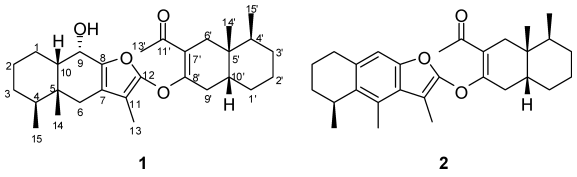

Fig. 1. Structures of the Compounds **1** and **2**

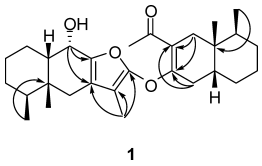

Fig. 2. Key HMBC Correlations for **1**

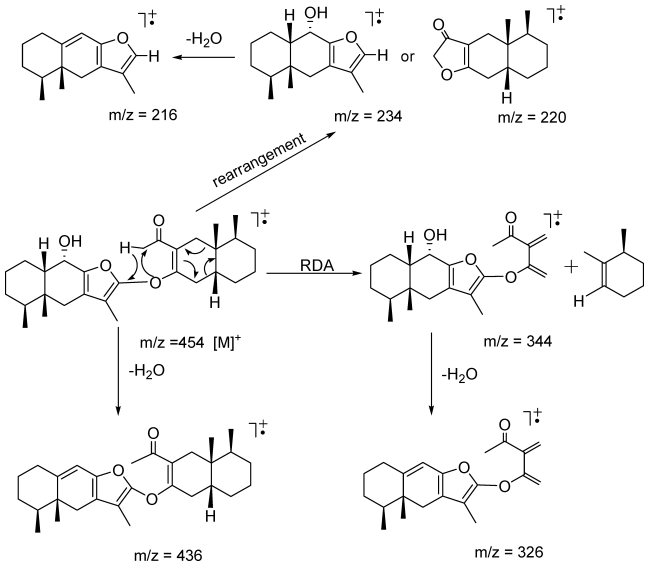

Chart 1. Major EI-MS Fragmentions of Compound **1**

Table 3. IC_{50} Values (μM) for Cytotoxicity of Compounds **1** and **2**

Compound	MGC-803	HEP-G2	S-180
1	20.2	65.4	52.5
2	11.9	12.2	35.8

Experimental

General Experimental Procedures Melting points were measured on a X-4 digital display micromelting point apparatus and were uncorrected. Optical rotations were measured on a Perkin Elmer 341 polarimeter. IR spectra were taken on a Nicolet NEXUS 670 FT-IR spectrometer. NMR spectra were recorded on a Varian Mercury-300BB NMR spectrometer with tetramethylsilane (TMS) as internal standard. EI-MS data were obtained on an HP5988A GCMS spectrometer. HR-ESI-MS data were measured on a Bruker Daltonics APEX II 47e spectrometer. Silica gel (200—300 mesh) used for CC and silica gel GF_{254} (10—40 μm) used for TLC were supplied by the Qingdao Marine Chemical Factory, Qingdao, P. R. China. Spots were detected on TLC under UV light or by heating after spraying with 5% H_2SO_4 in C_2H_5OH (v/v).

Plant Material The dried rhizomes of *L. muliensis* were collected from Muli autonomic county of Sichuan Province, P. R. China, in August 2003, and authenticated by Prof. Guoliang Zhang of the School of Life Science at Lanzhou University. A voucher specimen (No. LM20030816) was deposited in the College of Chemistry and Chemical Engineering, Lanzhou University.

Extraction and Isolation Dried and powdered rhizomes of *L. muliensis* (1.9 kg) were extracted four times (7 d each time) with petroleum ether–Et_2O–acetone (1 : 1 : 1) (6 l) and filtered. The combined filtrate was concentrated under vacuum at 55 °C to yield 66.7 g of extract. The extract was chromatographed over a silica gel column by gradient elution with pe-

troleum ether–acetone (1 : 0, 60 : 1, 10 : 1, 4 : 1, 2 : 1, 0 : 1) to give nine fractions (Fr. 1—Fr. 9). Fr.3 (18.5 g) was extensively chromatographed on a silica gel column (petroleum ether–AcOEt, 60 : 1—1 : 1) and then preparative TLC (petroleum ether–AcOEt, 15 : 1×2) to give compounds **1** (50 mg) and **2** (8 mg).

Ligulamulienin A (**1**): Colorless needles. mp 146—148 °C. $[\alpha]_D^{20}$ −99° (c=0.6, acetone). IR (KBr) ν_{max}: 3443, 2926, 2863, 1701, 1611 cm^{-1}. ^1H- and ^{13}C-NMR data: see Tables 1 and 2. HR-ESI-MS m/z 477.2972 $[M+Na]^+$ (Calcd for $C_{29}H_{42}O_4Na^+$, 477.2975). EI-MS m/z: 454 $[M]^+$, 436 $[M-H_2O]^+$, 411 $[M-Ac]^+$, 393 $[M-Ac-H_2O]^+$, 344 $[M-C_8H_{14}]^+$, 326 $[M-C_8H_{14}-H_2O]^+$, 311, 255, 234, 220, 216, 199, 177, 149, 124, 109, 84, 67, 55, 43.

Ligulamulienin B (**2**): Colorless needles. mp 186—188 °C. $[\alpha]_D^{20}$ −83° (c=0.3, acetone). IR (KBr) ν_{max}: 2924, 2859, 1460, 1433, 1154, 874, 788 cm^{-1}. ^1H- and ^{13}C-NMR data: see Tables 1 and 2. HR-ESI-MS m/z 435.2899 $[M+H]^+$ (Calcd for $C_{29}H_{39}O_3^+$, 435.2894).

Assays of Cytotoxocity MGC-803 (human stomach carcinoma), HEP-G2 (human hepatoma), and S-180 (murine sarcoma) were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in RPMI-1640 (Sigma, U.S.A.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, U.S.A.), penicillin (100 U/ml), and streptomycin (100 g/ml). Exponentially growing cells were used throughout the study. Cytotoxicity was determined by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) colorimetric assay.[10] Briefly, aliquots of MGC-803, HEP-G2, and S-180 cells containing 1×10^5 cells/ml were added to each well of 96-well flat-microtiter plates. After 24 h incubation, the cells were treated with compounds **1** and **2** at various concentrations (0.1, 1.0, 10, 100 mg/l), and with carboplatin at a concentration of 100 mg/l as a positive control. Six replicate wells were used in each point in the experiments. After 48 h of incubation, MTT solution (5 mg/ml in phosphate buffered saline (PBS)) stored at 4 °C in a dark bottle was added to each well and the plates were incubated for 4 h at 37 °C. Extraction buffer (10% SDS–0.01 M HCl) was added. After an overnight incubation at 37 °C, the optical densities (A) of 570 nm were measured using a Bio-Rad 550 ELISA microplate reader. The cytotoxicity was calculated as cytotoxicity (%)=[(A_{570} of untreated cells−A_{570} of treated cells)/A_{570} of untreated cells]×100%.

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