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Cytotoxic sesquiterpenes from *Ligularia platyglossa*

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ABSTRACT

Four sesquiterpene lactones including an eremophilenolide dimer, named as biligulaplenolide, **1**, 8β-hydroxy-1-oxo-(14α,15α eremophil-7(11),9(10)-dien-12,8α-olide, **2**, 1-hydroxy-2-oxo-(14α,15α eremophil-1(10),7(11),8(9)-trien-12,8-olide, **3**, 4α,8β,9α-trihydroxy- 5αH-7(11)-eudesmen-12,8α-olide, **4**, along with two known ones, 10α-hydroxy-1-oxo-eremophil-7(11),8(9)-dien-12,8-olide, **5**, and furano-eremophil-1(10)-ene-2,9-dione, **6**, were isolated from the underground organs of *Ligularia platyglossa* (Franch.) Hand.-Mazz. Their structures were elucidated by spectroscopic methods including single-crystal X-ray diffraction analysis (**2** and **3**). Their *in vitro* cytotoxicities against seven cancer cell lines (BGC-823, A549, HL-60, B16, SMMC-7721, BEL7402, Hela) were evaluated. Compounds **2**, **3**, **5** showed cytotoxic activities on HL-60 cancer cells with IC_{50} in the range of 24.0 to 51.1 μM, whereas compound **3** exhibited only weak cytotoxic activity against the B16, BEL7402 and Hela cancer cells. Flow cytometric analysis indicated that compound **3** induces Hela cells to apoptotic death after 48 h treatment with 0.38 mM of this compound.

1. Introduction

The genus *Ligularia* (Asteraceae) contains more than 110 species distributed in China, of which about 40 species have been used as Traditional Chinese Medicine or folk herbs with antibiotic, antiphlogistic and antitumor activities (Zhao et al., 1998). The most known chemical constituents of this genus are eremophilane-type sesquiterpenes (Wu et al., 2004a) and pyrrolizidine alkaloids (Asada et al., 1981; Tan et al., 2003a). Some eremophilane-type sesquiterpenes have demonstrated anti-HIV (Singh et al., 1999), antibacterial (Li et al., 2003) and cytotoxic activities (Wu et al., 2004a; Park et al., 2000; Zhao et al., 2002a, 2002b; Zhang et al., 2005). *Ligularia platyglossa* (Franch.) Hand.-Mazz is mainly distributed in southwest of China and local inhabitants have used its underground organs for a long time as a folk medicine to reduce phlegm and relieve cough. In our continuing chemical studies and screening of bioactive components from *Ligularia* species (Li et al., 2004a, 2004b; Tan et al., 2003b), six sesquiterpenes including four new ones were isolated from the ethanol extract of the underground parts of *L. platyglossa* and their *in vitro* cytotoxic activities against seven cancer cell lines were investigated. This paper reports on the structural elucidation of new compounds, **1–4** (Fig. 1) and cytotoxic activities of these isolated sesquiterpenes.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder and its molecular formula was established as $C_{30}H_{34}O_4$ by HR-TOFMS spectrum ($[M+H]^+$ found: *m/z* 459.2529, calc: 459.2529). The ^1H NMR spectrum showed three pairs of methyl signals (δ 1.87, *s*, 1.92, *br.s*; δ *0.77, s*, 0.90, *s*; δ *0.94, d*, 0.93, *d*) and five olefinic proton signals (δ 5.46, 5.87, 5.98, 5.84, 5.66). The ^{13}C NMR spectrum contained 30 resonances, which were assigned to six methyls, four methylenes, eight methines and twelve quaternary carbons including six double bonds and two lactone groups (Table 1). This indicated compound **1** was a dimer of an eremophil-7(11)-ene-olide sesquiterpene. The strong ion fragment peak at *m/z* 229 in ESI-MS/MS suggested that compound **1** had two units with the same mass. Comparison of the ^1H and ^{13}C NMR spectroscopic data between compound **1** and a known compound, ligularenolide (Ishizaki et al., 1970; Jenniskens and de Groot, 1998) indicated that one unit of **1** was ligularenolide, containing two olefinic proton signals (δ 5.46, *d*, *J* = 4.5 Hz, H-1; δ 5.87, *s*, H-9). However, the methylene resonance at δ_C 26.1 (C-2) of ligularenolide was replaced in **1** with a methine signal at δ_C 42.5 (C-2). The doublet signal of H-1 at δ 5.46 in **1** instead of the triplet resonance of H-1 at δ 5.79 in

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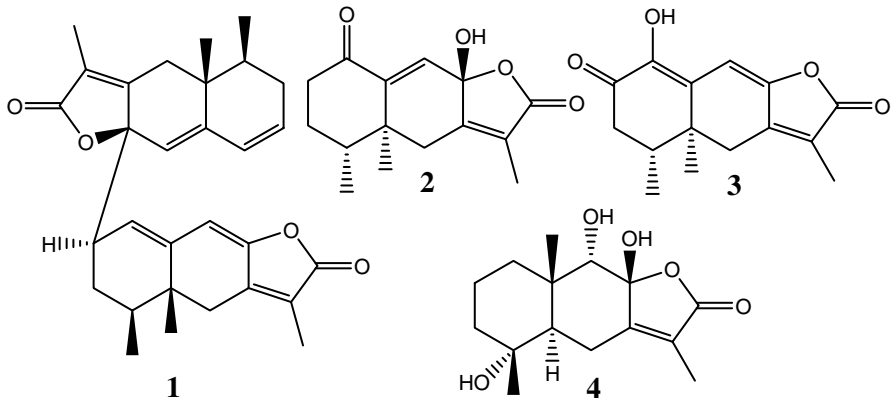


Fig. 1. Structures of compounds **1**–**4**.

Table 1
NMR spectroscopic data of compound **1** (measured in $CDCl_3$)

No	δ_H(*mult*, *J* Hz)	δ_C	Key NOE	No	δ_H(*mult*, *J* Hz)	δ_C	Key NOE
1	5.46(*d*, 4.5)	127.4	1H–6′αH	1′	5.98(*br.d*, 9.7,1.9)	127.4	
2	α: 2.78(*m*)	42.5	2αH–3αH	2′	5.84(*ddd*, 9.7,5.4,2.2)	130.6	
3	β: 1.62(*m*)	26.6	3βH–15Me	3′	β: 2.16(*m*)	32.4	3′βH–15′Me
	α: 1.58(*m*)		3αH–4αH		α: 1.90(*m*)		3′αH–4′αH
4	α: 1.72(*m*)	36.0		4′	α: 1.72(*m*)	38.7	
5		37.7		5′		43.7	
6	β: 2.80(*d*, 16.5)	34.4		6′	β: 2.68(*d*, 12.3)	36.3	
	α: 2.21(*d*, 16.5)				α: 2.24(*d*, 12.3)		
7		146.8		7′		161.0	
8		148.6		8′		87.4	
9	5.87(*s*)	108.4		9′	5.66(*s*)	121.8	9′H–3βH
10		142.9		10′		146.1	
11		121.4		11′		123.2	
12		171.1		12′		173.2	
13	1.92(*br.s*, 1.5)	8.5		13′	1.87(*s*)	8.5	
14	0.90(*s*)	18.9	14Me–15Me	14′	0.77(*s*)	17.3	14′Me–15′Me
15	0.93(*d*, 6.6)	15.4		15′	0.94(*d*, 6.8)	14.6	

ligularenolide further confirmed the substitution of C-2. In the ^1H NMR spectrum, the remaining unit of **1** contained the other three olefinic proton signals (δ 5.98, *br.d*, J = 9.7, 1.9 Hz, C-1′; δ 5.84, *ddd*, J = 9.7, 5.4, 2.2 Hz, C-2′; δ 5.66, *s*, C-9′), which were assigned based on their coupling relationships and HMBC experiment (Fig. 2). Therefore, the second unit of **1** was identified as eremophil-1(2),7(11),9(10)-trien-12,8-olide. The substitution at the position C-8′ in **1** by a ligularenolide (C-8′ to C-2) reduces the methine to a hemi-ketal quaternary carbon at δ 87.4. In addition the HMBC spectrum also supported **1** was a dimer of the above two sesquiterpenes units linked at C-2/C-8′ positions.

Typically, the relative configurations of Me-14, 14′ and Me-15, 15′ are in β orientations by reference to ligularenolide, and H-2 should be in α orientation based on the NOESY cross-peak between H-2 and H-3α. The structural stereo-model generated by the software (Chem 3D Ultra 8.0, CambridgeSoft Corporation) indicated that H-1/H-6′α and H-3β/H-9′ should be in close contact (<3 Å) if **1** had a 8′β lactone moiety. The NOESY correlations of H-1/H-6′α and H-3β/H-9′ were observed, so **1** should have an 8′β lactone moiety. Therefore, compound **1** was confirmed as a novel dimer of eremophilenolides linked at C-2β/C-8′α; this is different from other reported dimers that are typically linked at C-8/C-8′ positions (Bohlmann and Van, 1978; Fu et al., 2002; Wu et al., 2004a), 3,4a,5-trimethyl-7-(3,4a,5-trimethyl-2-oxo-2,4,4a,5,6,9a-hexahydronaphtho[2,3-b]furan-9a-yl)-4a,5,6,7-tetraydronaphtho[2,3-b]-furan-2(4H)-one (Fig. 1), which was named as biligulaplenolide.

Compound **2** was obtained as a colourless gum and its molecular formula was established as $C_{15}H_{18}O_4$ by HR-EIMS spectrum ($[M]^+$ found: *m/z* 262.1241, calc: 262.1205). The IR bands (ν_{max} 1720 and 1625 cm^{-1}) and UV absorption (λ_{max} 315.5 nm) indicated compound **2** was an α, β-unsaturated γ-lactone. The IR spectrum suggested the presence of a hydroxyl group (3264 cm^{-1}) and a keto group (1696 cm^{-1}). The ^1H, ^{13}C NMR and HSQC data indicated that **2** possessed three methyls, three methylenes, two methines and seven quaternary carbons including two double bonds, a lactone group and a keto group (Table 2). Comparison of the ^1H and ^{13}C NMR spectroscopic data between compound **2** and 8α-hydroxy-1-oxo-eremophil-7(11)-en-12,8β-olide (Wang et al., 1988) indicated a close structural similarity except for additional signals of a double bond between C-9 and C-10 in compound **2**. In HMBC experiments, the correlations between the olefinic proton resonance at δ 6.43 with carbon signals at δ 201.6 (C-1), 45.7 (C-5) and 156.6 (C-7) established that the olefinic proton was located at C-9. The cross-peaks between the hydroxyl proton signal at δ 6.54 with the carbon resonances at δ 156.6 (C-7), 98.6 (C-8) and 125.8 (C-9) confirmed the location of hydroxyl group at C-8, while the correlations between proton resonances at *δ 1.70–1.76, 1.85–1.88 (2 × H-3), 6.43 (H-9) with the keto group signal at δ* 201.6 indicated the keto group located at C-1. Typically, although the relative configurations of Me-14 and Me-15 are biogenetically in β orientations, single-crystal X-ray diffraction analysis showed that both Me-14 and Me-15 are in α orientation and C_8-OH is in β orientation (Fig. 3). Thus, compound **2** was confirmed as 8β-hydroxy-1-oxo-(14α,15α)eremophil-7(11),9(10)-dien-12,8α-olide.

Compound **3** was obtained as yellow needles and its molecular formula was established as $C_{15}H_{16}O_4$ by HR-EIMS spectrum ($[M]^+$ found: *m/z* 260.1038, calc: 260.1049). The IR bands (ν_{max} 1779, 1665, 1650 and 1632 cm^{-1}) and UV absorption λ_{max} 374.5 nm) indicated compound **3** was also an α, β-unsaturated γ-lactone possessing an extended conjugated system. The IR bands at ν_{max} 3358 and 1765 cm^{-1} suggested **3** had a hydroxyl group and a keto group. The ^1H, ^{13}C NMR and HSQC data (Table 2) indicated that **3** possessed three methyls, two methylenes, two methines and eight quaternary carbons including three double bonds, a lactone group and a keto group. The ^1H and ^{13}C NMR spectroscopic data of **3** were similar to those of 2-oxo-eremophil-1(10),7(11),8(9)-trien-12,8-olide (Jenniskens and de Groot, 1998) except for an additional

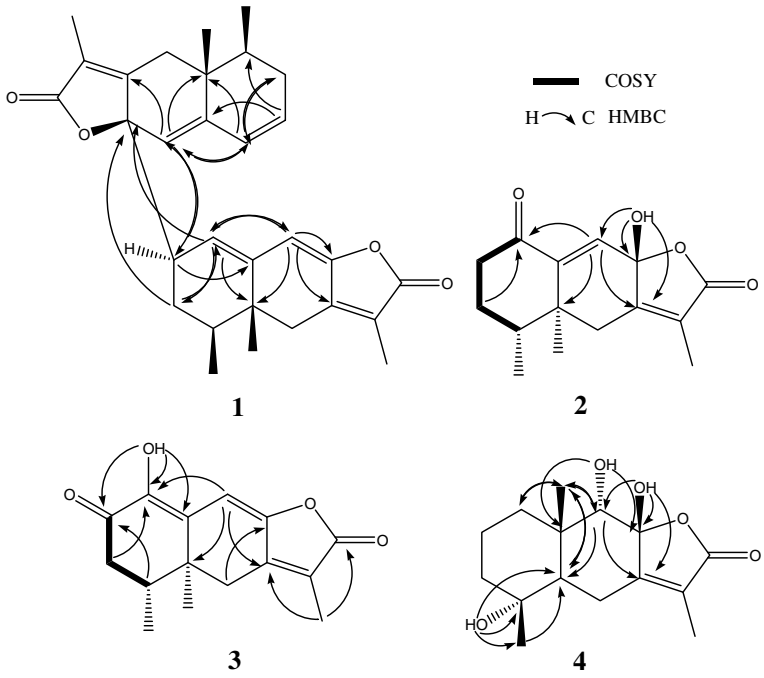


Fig. 2. Key NMR HMBC correlations of compounds **1**–**4**.

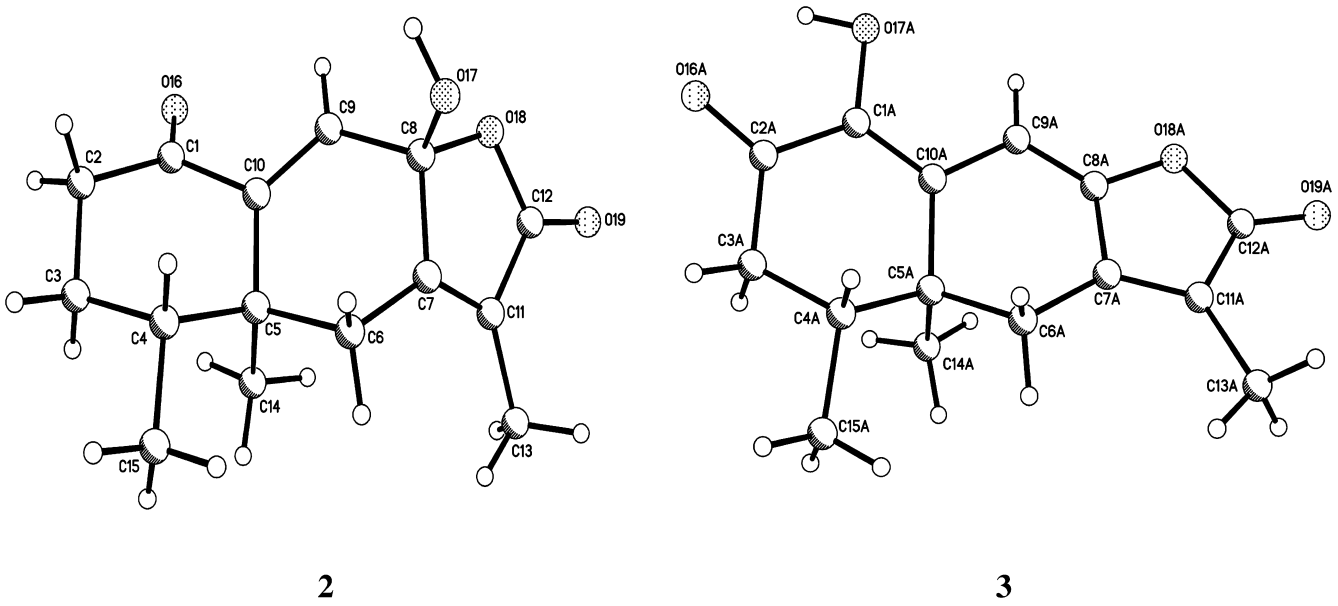
Table 2
NMR spectroscopic data of compounds **2–4** (measured in acetone-d_6)

No	**2** δ_H(*mult*, *J* Hz)	**2** δ_C	**3** δ_H(*mult*, *J* Hz)	**3** δ_C	**4** δ_H(*mult*, *J* Hz)	**4** δ_C
1		201.6	OH:7.62(*s*)	145.6	0.96(*m*) 1.73(*m*)	34.2
2	β: 2.44(*m*) α: 2.61(*m*)	39.9		192.6	1.47(*m*)	19.2
3	β: 1.86(*m*) α: 1.73(*m*)	27.7	2.44(*m*)	41.1	1.21(*m*) 1.55(*m*)	42.4
4	β: 2.09(*m*, 6.8)	40.2	2.31(*m*, 6.8)	39.3	OH: 4.14(*s*)	70.6
5		45.7		38.9	1.50(*m*)	48.2
6	β: 2.59(*br.d*, 12.6, 1.3) α: 2.74(*d*, 12.6)	35.3	2.51(*d*, 16.7) 3.14(*d*, 16.7)	35.0	2.02(*dd*,12.8, 12.8) 2.80(*dd*,12.8, 2.8)	21.0
7		156.6		151.5		159.6
8	β OH: 6.54(*s*)	98.6		146.7	OH: 7.12(*s*)	105.3
9	6.43(*s*)	125.8	6.51(*s*)	102.4	3.28(*d*, 5.4) OH: 5.29(*d*, 5.4)	79.1
10		148.5		132.0		39.5
11		123.5		123.7		121.4
12		171.2		170.8		172.5
13	1.87(*br.s*, 1.3)	7.8	1.95(*br.s*, 2.1)	8.6	1.69(*s*)	8.1
14	0.82(*s*)	17.5	1.14(*br.s*, 0.8)	18.7	1.04(*s*)	18.9
15	1.08(*d*, 6.8)	14.5	1.11(*d*, 6.8)	15.1	1.04(*s*)	22.8

hydroxyl resonance at δ 7.62 and a downfield oxygenated vinyl quaternary carbon signal at δ 145.6 in **3** replacing the vinyl methine resonances at δ_H 5.98 and δ_C 128.4 in 2-oxo-eremophil-1(10),7(11),8(9)-trien-12,8-olide. In the HMBC experiment, the correlations between hydroxyl signal at δ 7.62 with the carbon resonances at δ 192.6 (C-2), δ 132.0 (C-10) and δ 145.6 (C-1) supported the location of hydroxyl group at C-1, while the correlations between the olefinic proton resonance at δ 6.51 with carbon resonances at δ 38.9 (C-5) and δ 151.5 (C-7) established the olefinic proton located at C-9. In HMBC experiment, the correlations between H-6/C-8 (δ 146.7), and H-13(CH_3)/C-7 (δ 151.5) had been found, which confirmed that C-7 has a large chemical shift, which can be explained by the more strongly deshielding effect of the conjugated carbonyl group. Single-crystal X-ray diffraction analysis showed that the configurations of Me-14 and Me-15 in compound **3** are in α orientations (Fig. 3). Compound **3** was hence identified as 1-hydroxy-2-oxo-(14α,15α eremophil-1(10),7(11),8(9)-trien-12,8-olide.

Compound **4** was obtained as a colourless gum and its molecular formula was established as $C_{15}H_{22}O_5$ by HR-TOFMS spectrum ($[M+H]^+$ found: *m/z* 283.1541, calc: 283.1540). The ^{1}H NMR spectrum indicated that **4** possessed three free hydroxyl groups at δ *4.14, s*, 5.29, *d*, 7.12, *s*, which was further confirmed by the ion fragment peaks at *m/z* 265 $[M+H-H_2O]^+$, 247 $[M+H-2H_2O]^+$, 229 $[M+H-3H_2O]^+$ and from the D_2O exchange experiment. The ^{1}H, ^{13}C NMR and HSQC data (Table 2) indicated that **4** possessed three methyls, four methylenes, two methines, six quaternary carbons including a double bond and a lactone group. This suggested **4** was either an eremophil-7(11)-ene-olide or an eudesman-7(11)-en-olide sesquiterpene. The ^{13}C NMR spectrum showed a hemi-ketal signal at δ 105.3, indicates that one of the three hydroxyl groups was located at C-8. The low-field shift of C-4 (70.6 ppm) and the singlet resonance of Me-15 at δ 1.04 in ^{1}H NMR spectrum indicated that C-4 was bonded to a hydroxyl oxygen. In the HMBC experiments, the correlations between the same hydroxyl proton δ 4.14 with carbon signals at δ 70.6 (C-4) and 22.8 (Me-15) confirmed that this hydroxyl group was at C-4. The evident cross-peak between the hydroxyl resonance at δ 4.14 (OH-4) with the methine resonance at δ 48.2 (C-5) suggested that **4** was not an eremophil-7(11)-ene-olide, but an eudesman-7(11)-en-olide sesquiterpene. The locations of the other two hydroxyl groups (δ 7.12, δ 5.29) were also established by HMBC experiments (Fig. 2). In the NOESY spectrum, the correlations between Me-14 (δ 1.04) with H-9 (δ 3.28), OH-8 (δ 7.12) with H-9 (δ 3.28), OH-4 (δ 4.14) with H-5 (δ 1.50), suggesting OH-9 and H-5 have α orientations, while OH-8 has β orientations. Therefore, compound **4** was identified as 4α,8β,9α-trihydroxy-5αH−7(11)-eudesmen-12,8α-olide.

The known compounds **5** and **6** were identified based on their spectroscopic data and their structures were confirmed by



2 **3**

Fig. 3. X-ray crystal structures of compounds **2** and **3**.

Table 3
Cytotoxic activities of compounds **2**, **3**, **5** and previously isolated eremophilenolides from the same plant against HL-60 carcinoma cells

Compounds	IC_{50} (μM) (mean ± SD, n = 6)
2	24.0 ± 1.25
3	28.1 ± 1.34
5	51.1 ± 2.52
Eremophil-1(10),7(11),(9)-trien-12,8-olide	16.1 ± 0.83
9β,9′α-Bis-1,8-dihydroligularenolide	10.8 ± 1.35
9β,9′β-Bis-1,8-dihydroligularenolide	21.4 ± 1.65
8β-Hydroxy-eremophil-7(11),9(10)-dien-12,8α-olide	36.3 ± 0.64
2-Oxo-eremophil-1(10),7(11),8(9)-trien-12,8-olide	23.0 ± 2.38
10-Hydroxy camptothecin	0.49 ± 0.22
Cisplatin	0.33 ± 0.14

comparison with published literature data (**5**, Wu et al., 2004b; **6**, Mei et al., 2001; Bohlmann et al., 1974).

The compounds **2**–**5** and five previously isolated eremophilenolides (see Table 3) by the authors from the same plant (Liu et al., 2005a, 2005b) were evaluated for their cytotoxicity against seven carcinoma cell lines (BGC-823, A549, HL-60, B16, SMMC-7721, BEL7402 and Hela), with **4** only being tested using B16, BEL7402 and Hela by MTT assay (Mosmann, 1983) (see Table 3). The purities of the tested compounds were more than 98%, as estimated by HPLC analysis. All of the tested eremophilolides exhibited medium inhibitory activities against HL-60 carcinoma cells with IC_{50} values in the range of 10.8–51.1 μM, but none showed inhibitory activities against six other carcinoma cells. Compound **3** exhibited weak cytotoxic activities against B16, BEL7402 and Hela carcinoma cells with IC_{50} values of 167.7, 271.5 and 203.5 μM, respectively, and compound **4** showed no inhibitory activities against B16, BEL7402 and Hela carcinoma cells.

The results indicated that these eremophilolides were primarily activive against the HL-60 cell line. Compound **3** also exhibited weak cytotoxicities against B16, BEL7402 and Hela carcinoma cells and induced apoptosis of these cells, as evaluated by flow cytometric analysis. The comparatively wide bandwith of cytotoxicities of **3** may possibly be due to its structural features of an enolic hydroxyl and keto groups adjacent to each other, which provide an efficient hydrogen bonding capability comparing to the other tested eremophilolides in this study.

Table 4
Apoptosis induced by compound **3** in B16, BEL7402 and Hela cancer cells

Cancer cell	Apoptosis % (control)	Apoptosis % (0.38 mM)
B16	0.44 ± 0.02	4.83 ± 1.54**
BEL7402	0.09 ± 0.00	9.06 ± 1.26**
Hela	0.09 ± 0.01	27.04 ± 3.85***

** $p < 0.01$.
*** $p < 0.001$ compared with control (Untreated with compound **3**) (mean ± SD, n = 3).

These results indicated that compound **3** induces Hela cells apoptotic death after 48 h treatment with 0.38 mM of the compound (apoptosis up to 27.04%) (see Table 4).

3. Conclusion

Four new compounds were isolated from *L. platyglossa* and fully characterized. A dimer of eremophilenolides (**1**) linked at C-2β /C-8′α was isolated for the first time from *L. platyglossa*. Compounds **2**, **3** and **5** have an additional keto and hyydroxy functional group in the structure and yet only **3** shows good cytotoxic activities against HL-60, B-16, BEL7402 and Hela cancer cells.

4. Experimental

4.1. General experimental procedures

Melting points were determined on an XT_4-100A(Shanghai Jicheng Analytical Instrument Co., Ltd. China)micro-melting point apparatus and are uncorrected. Optical rotations were measured on a PE-241MC polarimeter(PerkinElmer, Inc., USA). Silica gel 200–300 mesh, Sephadex LH20 and Rp-C_{18} were used for column chromatography (CC), while precoated silica GF_{254} plates were used for TLC. Spots were visualized by UV (λ 254 nm and 365 nm) and 10% H_2SO_4-EtOH solution. IR spectra were obtained on a NICOLET Impact 410 spectrometer (Thermo Nicolet Corporation, USA) with KBr pellets. UV spectra were recorded on a Shimadzu

UV-2501PC UV–VIS spectrometer (Shimadzu Corporation, Japan). One- and two-dimensional NMR spectra were recorded on a Bruker AV-500 instrument (Bruker BioSpin Group, Switzerland)operating at 500 and 125 MHz for ^1H and ^{13}C NMR, respectively. Chemical shifts are reported in δ value in ppm using the solvent as reference. HR-EIMS spectra were recorded on a Micromass GCT mass spectrometer. (Micromass UK Ltd, UK) HR-TOFMS spectra were recorded on an Agilent TOF mass spectrometer (Agilent Technologies, Inc., USA). EI-MS spectra were recorded on a VG ZAB-HS mass spectrometer (VG Instrument Inc., UK) at 70 eV. Single-crystal diffraction measurements were made on a Siemens P4 diffractometer (Siemens Analytial X-ray Instruments Inc., Germany) (**2**,**3**).

4.2. Plant material

The underground parts *L. platyglossa* were collected from Lijiang Prefecture of Yunnan Province, PR China, in August 1999, and authenticated by Dr. Main Zhang. A voucher specimen (No. 990009) is deposited in the Herbarium of China Pharmaceutical University.

4.3. Extraction and isolation

The air-dried, pulverized underground parts of *L. platyglossa* (4.1 kg) were extracted three times (each 9 L, 4 h) with 90% EtOH under condition of reflux, yielding a residue (670 g) after evaporation under reduced pressure. The residue was extracted with 1 N H_2SO_4 several times to get the acid-insoluble and acid-soluble fractions, respectively. The acid- insoluble fraction was suspended in H_2O and partitioned with EtOAc, yielding the EtOAc solubles (210 g). The acid-soluble fraction was extracted with $CHCl_3$ three times (each 1 L). The acidic aqueous solution was then adjusted to pH 10 with 20% NH_4OH and extracted with $CHCl_3$ (3×1.5 L) to give a crude alkaloid fraction (3 g). The alkaline aqueous solution was neutralized with 50% H_2SO_4, then extracted with *n*-BuOH to yield the *n*-BuOH solubles (50 g) after evaporation under reduced pressure.

The EtOAc solubles (210 g) were subjected to silica gel CC (2.0 kg), eluted with gradients of petroleum ether-EtOAc (each 400 mL, 25:1, 10:1, 5:1, 2:1, 1:1, 0:1, labeled as eluates A–F). A total of 514 fractions were collected and regrouped on the base of TLC monitoring to afford ten combined fractions labeled as Fr.A 1–22, Fr.A 23–25, Fr.A 26–40, Fr.A 41–114, Fr.B 1–32, Fr.B 33–100, Fr.C, Fr.D, Fr.E, Fr.F, respectively. Fr.B 1–32 gave compound **1** (12 mg), as a white amorphous powder, which was further purified by preparative TLC (Precoated silica GF_{254} plates were used. The solvent system was $CHCl_3$-acetone (20:1, v/v), and spots were visualized by UV, λ 365 nm, $R_f = 0.48$). Fr.B 33–100 was subjected to silica gel CC, eluted with gradients of petroleum ether-EtOAc (25:1, 10:1, 5:1, 1:1, 0:1, each 150 mL, v/v), then compound **5** (80 mg) was obtained from the fraction of petroleum ether-EtOAc (10:1) and further purified by re-crystallization in EtOAc. The mother liquid of petroleum ether-EtOAc (10:1) was sequentially separated by silica gel CC, eluted with gradients of $CHCl_3$-acetone (1:0, 10:1, 0:1, each 150 mL). The combined fraction of $CHCl_3$-acetone (1:0) was sequentially subjected to silica gel CC, eluted with gradients of petroleum ether-EtOAc (each 150 mL) to give compounds **3** (45 mg) and **6** (13 mg) respectively from the fraction of petroleum ether-EtOAc (5:1). Compounds **3** and **6** were further purified by recrystallization in EtOAc and acetone, respectively. Fr.C gave compound **2** (100 mg), which was purified by recrystallization in EtOAc.

The *n*-BuOH solubles (50 g) were subjected to silica gel CC, eluted with gradients of $CHCl_3$-MeOH (20:1, 10:1, 100:13, 5:1), then four combined fractions were obtained on the base of TLC analysis. The combined fraction of $CHCl_3$–MeOH (10:1) was subjected to silica gel CC, eluted with gradients of $CHCl_3$–MeOH (50:3, 10:1, 5:1). Then, the obtained fraction of $CHCl_3$–MeOH (50:3) was subjected to Sephadex LH 20 CC, eluted with $CHCl_3$–MeOH (1:1) to give compound **4** (16 mg), purified by recrystallization in MeOH.

4.4. Cytotoxycity assay and flow cytometric analysis of apoptosis

4.4.1. Materials and cell culture conditions

Seven cancer cell lines have been used, including human stomach gland carcinoma BGC-823 cells, human lung carcinoma A549 cells, human leucocythemia carcinoma HL-60 cells, mouse melanoma B16 cells, human liver carcinoma SMMC-7721 and BEL7402 cells, human cervical carcinoma Hela cells. All cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco-BRL life Technologies Inc, Gaithersbutg MD. MTT was purchased from Sigma (St Louis, MO).

All cancer cells were cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated at 37 °C with 5% CO_2 in a humidified air atmosphere.

4.4.2. Cytotoxicity assay

The cytotoxicity of the compounds against BGC-823, A549, HL-60, SMMC-7721, B16, BEL7402 and Hela tumor cells were evaluated using the MTT method (Mosmann, 1983). Cells were seeded in 96-well microplates at a density of 10^4 per well and were cultured in cell culture medium (RPMI1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) for 12 h, then treated with the test compounds added from DMSO dissolved stock solution. The final DMSO concentration never exceeded 0.2% (v/v). Previous experiments showed that DMSO at this concentration did not modify the cell activities. After 48 h in culture, cells were incubated with MTT (0.5 mg/ml, 4 h) and subsequently resolved in DMSO. The absorbance in control and drug-treated wells was measured in an automated microplate reader (Bio-Rad 550) at 570/630 nm. The cytotoxycity was expressed as IC_{50} values (50% inhibitory concentration).

4.4.3. Flow cytometric analysis of apoptosis

Apoptosis was identified and quantified by flow cytometry with PI staining. Both adherent and floating cells were collected after treatment with compound 3 (0.38 mM), washed with ice-cold PBS, and fixed with 70% ice-cold ethanol overnight at 4 °C. Fixed cells were washed twice with PBS and treated with 1 mg/ml RNase for 30 min at 37 °C. Cellular DNA was stained with 50 μg/ml PI in PBS, containing 0.05% NP40. Cells were then analyzed by FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The percentages of cells in different cell cycle phases were evaluated. Cells with DNA content less than the G1 phase (sub-G1) were taken as apoptotic cells. Student *t*-test is used for the Statistical analysis.

4.5. Biligulaplenolide, ***1***

White powder; mp 176–177 °C; $[\alpha]_D^{20} = -128.3$ ($CHCl_3$; $c = 0.1$); UV(MeOH):

λ_{max}^{MeOH} nm (lgε): 328 (4.1); HR-TOF-MS: found 459.2529 $[M+H]^+$, calc for $C_{30}H_{35}O_4$ 459.2529; for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 1.

4.6. 8β-Hydroxy-1-oxo-(14α,15α)eremophil-7(11),9(10)-dien-12,8α-olide, ***2***

Colorless gum (EtOAc); mp 206–208 °C; $[\alpha]_D^{20} = -20.5$ (Acetone; $c = 0.1$); ν_{max}^{KBr} cm^{-1}: 3264, 2966, 2871, 1720, 1696, 1650, 1625,

1470, 1453, 1271, 1248, 1193, 1129, 976, 937, 889; λ_{max}^{MeOH} nm (lgε): 315. (3.5); HREI-MS: found 262.1241 $[M]^+$, calc for $C_{15}H_{18}O_4$ 262.1205; EI-MS (probe) 70 eV, *m/z* (rel. int): 262 $[M]^+$ (46), 247 $[M-CH_3]^+$ (20), 244 $[M-H_2O]^+$ (23), 234 $[M-CO]^+$ (33), 229 $[M-H_2O-CH_3]^+$ (25), 217 $[M-OH-CO]^+$ (79); for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 2.

4.7. 1-Hydroxy-2-oxo-(14α,15α)eremophil-1(10),7(11),8(9)-trien-12,8-olide, **3**

Yellow needles (EtOAc); mp 211–213 °C; $[\alpha]_D^{20} = -741.6$ (Acetone, $c = 0.1$); ν_{max}^{KBr} cm^{-1}: 3358, 2974, 1779, 1765, 1665, 1650, 1632, 1607, 1451, 1387, 1371, 1100, 996, 875; λ_{max}^{MeOH} nm (lgε): 374.5 (4.4); HREI-MS: found 260.1038 $[M]^+$, calc for $C_{15}H_{16}O_4$ 260.1049; EI-MS probe) 70 eV, *m/z* (rel. int): 260 $[M]^+$ (100), 245 $[M-CH_3]^+$ (27), 232 $[M-CO]^+$ (6), 217 $[M-CH_3-CO]^+$ (42); for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 2.

4.8. 4α,8β,9α -Trihydroxy-5αH−7(11)-eudesmen-12,8α -olide, **4**

Colorless gum (MeOH); mp 249–250 °C; $[\alpha]_D^{20} = +52.5$ (MeOH, $c = 0.1$); HR-TOF-MS: found 283.1541 $[M+H]^+$, calc for $C_{15}H_{23}O_5$ 283.1540; EI-MS (probe) 70 eV, *m/z* (rel. int): 282 $[M]^+$ (1), 264 $[M-H_2O]^+$ (12); for ^1H NMR and ^{13}C NMR spectroscopic data, see Table 2.

4.9. X-ray crystallographic studies

Compound **2:** $C_{15}H_{18}O_4$, $M = 262.29$, $T = 208(2)$ K, $\lambda = 0.71073$ Å, orthorhombic, $P2_12_12_1$, $a = 9.0435(18)$, $b = 10.034(4)$, $c = 14.543(4)$ Å, $V = 1319.77(5)$ Å^3, $z = 4$, $D_c = 1.320$ mg m^{-3}, (Mo Kα) = 0.095 mm^{-1}, $F(000) = 560$. Data were collected using a colourless block of size $0.40 \times 0.26 \times 0.14$ mm in the range $2.47° \leqslant \theta \leqslant 25.24°$ within the index range $-10 \leqslant h \leqslant 1$, $-1 \leqslant k \leqslant 12$, $-1 \leqslant l \leqslant 17$. 1884 reflections measured, 1392 unique reflections, $R_{int} = 0.0256$. Refinement by full-matrix least-squares on F^2 converged to give final R indices $R_1 = 0.0467$, $wR_2 = 0.0965$ $[I > 2\sigma(I)]$ and $R_1 = 0.0809$, $wR_2 = 0.1145$ (all data). Data/restraints/parameters = 1392/0/178, goodness-of-fit on $F^2 = 1.028$, largest difference peak and hole are 0.184 and −0.227 e Å^{-3}.

Compound **3**: $C_{15}H_{16}O_4$, $M = 260.28$, $T = 213(2)$ K, $\lambda = 0.71073$ Å, orthorhombic, $P2_12_12_1$, $a = 6.5553(13)$, $b = 19.256(3)$, $c = 20.007(3)$ Å, $V = 2525.5(7)$ Å^3, $z = 8$, $D_c = 1.369$ mg m^{-3}, (Mo Kα) = 0.099 mm^{-1}, $F(000) = 1104$. Data were collected using a yellow needle of size $0.50 \times 0.12 \times 0.10$ mm in the range $2.04° \leqslant \theta \leqslant 25.24°$ within the index range $-7 \leqslant h \leqslant 1$, $-23 \leqslant k \leqslant 1$, $-24 \leqslant l \leqslant 1$. 3441 reflections measured, 3232 unique reflections, $R_{int} = 0.0308$. Refinement by full-matrix least-squares on F^2 converged to give final R indices $R_1 = 0.0627$, $wR_2 = 0.1080$ $[I > 2\sigma(I)]$ and $R_1 = 0.1358$, $wR_2 = 0.1317$ (all data). Data/restraints/parameters = 3232/0/349, goodness-of-fit on $F^2 = 1.024$, largest difference peak and hole are 0.231 and -0.227 e Å^{-3}.

Supplementary material

CCDC 64433 (**2**) and CCDC 64434 (**3**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail:deposit@ccdc.cam.ac.uk).

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