Phytochemical Investigation and Cytotoxic Evaluation of the Components of the Medicinal Plant *Ligularia atroviolacea*

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A phytochemical investigation of the roots of *Ligularia atroviolacea* resulted in the isolation of 24 compounds including seven new eremophilanoids named eremophila-3,7(11),8-triene-12,8;14,6α-diolide (**1**), 3β-(angeloyloxy)eremophil-7(11)-en-12,8β-olid-14-oic acid (**2**), 1α-chloro-10β-hydroxy-6β-(2-methylpropanoyloxy)-9-oxo-7,8-furoeremophilane (**3**), (10βH)-8-oxoeremophila-3(4),6(7)-diene-12,14-dioic acid (**4**), (10αH)-8-oxoeremophila-3(4),6(7)-diene-12,14-dioic acid (**5**), 8β-[eremophila-3′,7′(11′)-diene-12′,8′α;14′,6′α-diolide]eremophila-3,7(11)-diene-12,8α;14,6α-diolide (**6**), and ligulatrovine A (**7**), eleven known eremophilanoids, **8**–**18**, four steroids, one glucose derivative, and one fatty acid. The structures of these compounds were elucidated by spectroscopic methods including 2D-NMR experiments. The structure of **3** was also established by an X-ray diffraction study. The *in vitro* cytotoxicity evaluation of selected compounds was performed on seven cultured tumor cell lines, *i.e.*, KB, BEL-7404, A549, HL-60, HeLa, CNE, and P-388D1. The preliminary taxonomy of this species was also discussed, and the possible biogenesis of a dimer possessing a new noreremophilanoid type skeleton, **7**, is presented in a preliminary form.

Introduction. – *Ligularia* species (Asteraceae) are mainly distributed in damp, shaded areas of western China, especially in the provinces of Gansu, Sichuan, and Yunnan. They have been traditionally used in folk medicine for the treatment of asthma, hemoptysis, hepatitis, and pulmonary tuberculosis [1]. The widespread compounds in this genus include furoeremophilanes [2–5] and pyrrolizidine alkaloids [6–8]. *Ligularia atroviolacea* (Franch.) Hand.-Mazz. is a yellow-flower plant scattered abundantly in the Lijing region of the Yunnan plateau [9]. Its roots have been utilized as one of the herbal medicines for the treatment of hepato-diseases. During a genetic study of *Ligularia* species in the Hengduan mountains of China, the title species collected in Lijiang region has been reported to possess furoeremophilanes as typical characteristic of *Ligularia* species, which was further evidenced by the molecular-biological tools such as nucleotide sequence assay, *etc.* [10]. As an extension of our serial researches on the chemistry and biodiversity studies of the genus of

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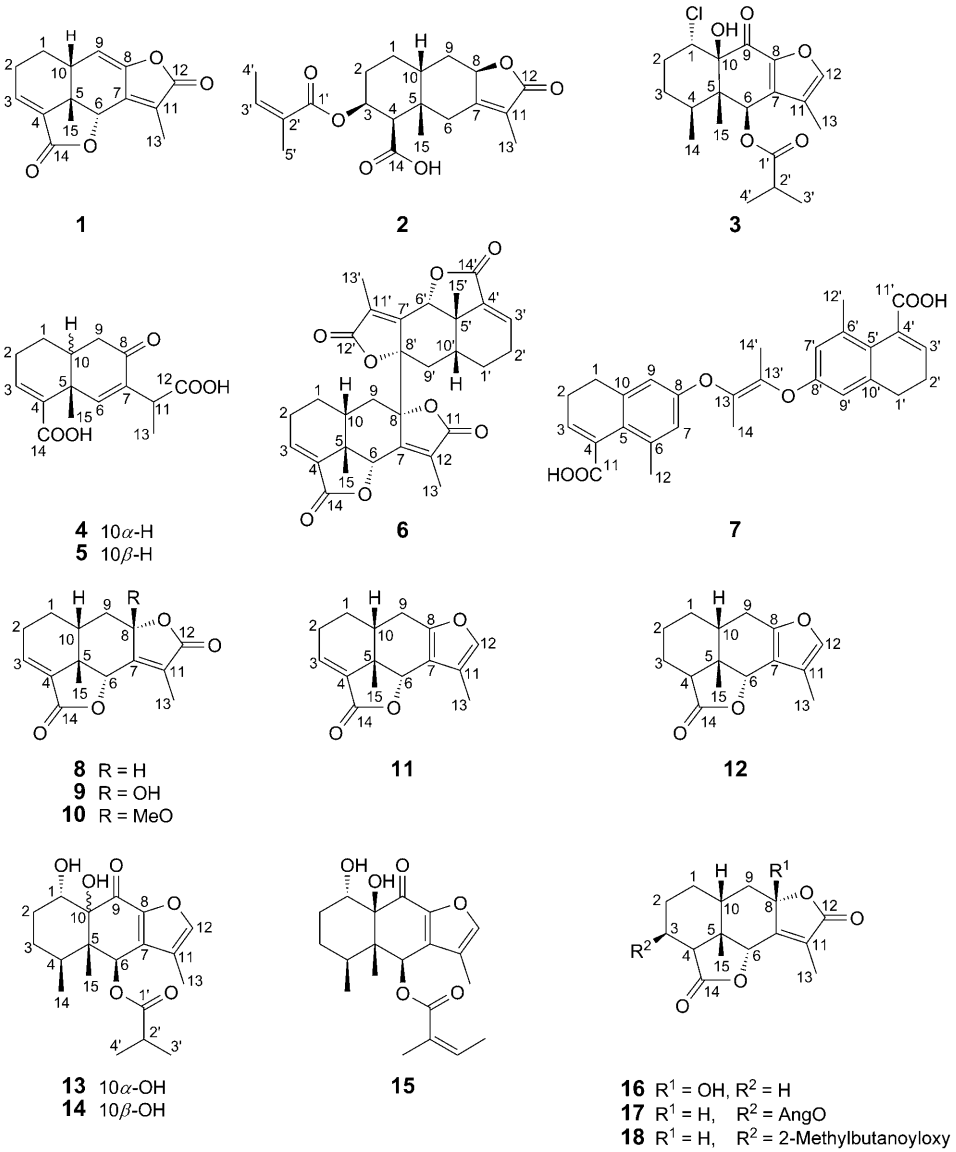
Ligularia [11–13], the roots of *L. atroviolacea* collected from Lijiang county was re-investigated. It was found that most of the isolated compounds are furoeremophilanolides or the scarce eremophiladiolides. Since the eremophilane type diolides from *L. tongolensis* was reported to possess cytotoxicities against human cell lines SMMC-7721 and HL-60 [2], five eremophilanolides, **1**, **2**, **8**, **9**, and **11**, including four diolides from the title plant were subjected to a broad-spectrum cytotoxicity assay against KB, BEL-7404, A549, HeLa, CNE, HL-60, and P388D1 cells. Among them, the hepatoma cell line BEL-7404 is related to the folk application of the title plant, while the others are the most prevalent carcinomas among Chinese. The isolation, structural elucidation, cytotoxic assays of selected eremophilanoids, as well as the discussion of the biogenesis of the two rare dimers were reported herein. The chemotaxonomy of the title species, including other *Ligularia* species, is also presented.

Results and Discussion. – 1. *Chemistry.* Air-dried roots of *L. atroviolacea* were extracted with 95% EtOH, and the concentrated EtOH extract was partitioned with solvents of increasing polarity. The AcOEt extract was separated by various chromatographic procedures, including column chromatography (CC) on normal silica gel and *Sephadex LH-20* to afford 24 compounds including **1**–**18**.

The IR spectrum of compound **1** indicated the presence of a saturated γ-lactone (1772 cm^{-1}) and an α,β-unsaturated γ-lactone unit (1756 and 1681 cm^{-1}). The molecular formula of **1**, $C_{15}H_{14}O_4$, was determined by the ^{13}C-NMR and DEPT data in accordance with the molecular-ion peak at *m/z* 259.0936 ($[M+H]^+$) in the HR-ESI-MS spectrum. Furthermore, the ^{1}H- and ^{13}C-NMR and DEPT spectra of **1** (*Tables 1* and *2*) revealed the presence of two Me groups, two CH_2 groups, two olefinic CH groups, and two sp^3-hybridized CH groups, one being oxygenated. Signals of seven quaternary C-atoms including two lactone CO groups and three C=C bonds were also observed (*Table 2*). The NMR data of **1** were similar to those of (8βH)-eremophila-3,7(11)-diene-12,8α;14,6α-diolide (**8**) [2], except for the presence of an additional C(8)=C(9) bond in the case of **1** (*Tables 1* and *2*). The localization of the C=C bond at C(9) could be deduced from the HMBC spectrum, in which H–C(9) (δ(H) 5.45) showed a long-range coupling with C(10) (δ(C) 36.4) and C(8) (δ(C) 141.7). The disappearance of the signal of the oxygenated C-atom (δ(C) 81.7 in the case of **8**) in **1** also confirmed the existence of this C=C bond. Therefore, compound **1** was assigned eremophila-3,7(11),8-triene-12,8;14,6α-diolide.

Compound **2** was obtained as white powder. Its molecular formula, $C_{20}H_{26}O_6$, was deduced from the ^{1}H- and ^{13}C-NMR, and DEPT spectra. The IR spectrum of **2** displayed characteristic signals of a COOH group (2985 and 1706 cm^{-1}) and of an α,β-unsaturated γ-lactone (1768 and 1654 cm^{-1}), further supported by the UV maximum at 224 nm. The ^{13}C-NMR and DEPT spectra indicated that compound **2** possesses two Me groups, four CH_2 groups, four CH groups, five quaternary C-atoms, as well as an angeloyl (=2-methylbut-2-enoyl) substituent. Moreover, the ^{1}H-NMR spectrum of **2** (*Table 1*) indicated the presence of two Me groups (at δ(H) 1.64 (*s*, Me(15)) and δ(H) 1.77 (*d*, *J*=1.2, Me(13))). Furthermore, the downfield shifted signal of H–C(3) (δ(H) 5.58) suggested that C(3) was substituted by an O-bearing group. In addition, H–C(4) (δ(H) 2.64 (*d*, *J*=3.0)) was shifted downfield by the adjacent COOH group, which is consistent with a COOH group attached to C(4) of **2**. From a biogenetic point of view,



the Me substituent at C(5) and the COOH substituent at C(4) should be both β-configured for an eremophilane. Furthermore, by the NOE difference experiment, irradiation of H–C(14) enhanced the signal of H–C(10), whereas no enhancement of H–C(4) was observed, thus indicating that H–C(4) adopts α-orientation, while H–C(10) possesses β-orientation. The localization of the (angeloyl)oxy moiety at C(3) was deduced from the HMBC spectrum of **2** in which H–C(3) (δ(H) 5.58 (q, J = 3.0)) showed a diagnostic long-range coupling with C(1′) (δ(C) 174.2). The coupling pattern

Table 1. *^{1}H-NMR Data of Eremophilanoids* **1**–**5**. At 400 MHz; δ in ppm, J in Hz.

Position	**1**[a])	**2**[b])[c])	**3**[a])[d])	**4**[a])	**5**[a])
1	2.36–2.42 (*m*), 1.92–1.98 (*m*)	1.87–1.91 (*m*), 1.38–1.44 (*m*)	3.94 (br. *s*)	1.22–1.26 (*m*), 1.50–1.60 (*m*)	1.33–1.42 (*m*), 1.58–1.64 (*m*)
2	2.13–2.25 (*m*)	1.70–1.75 (*m*)	1.76 (*dd*, *J*=14.0, 2.0), 2.59 (*t*, *J*=14.0)	2.22–2.26 (*m*), 2.29–2.32 (*m*)	2.22–2.27 (*m*), 2.29–2.32 (*m*)
3	6.81 (*t*, *J*=3.6)	5.58 (*q*, *J*=3.0)	1.44 (*d*, *J*=13.6), 2.47 (*t*, *J*=14.0)	7.17 (*t*, *J*=4.0)	7.37 (*t*, *J*=4.0)
4		2.64 (*d*, *J*=3.0)	1.63–1.70 (*m*)		
6	5.30 (br. *s*)	2.07 (*d*, *J*=14.4), 1.68 (*dd*, *J*=14.4, 1.2)	7.06 (*s*)	7.37 (*s*)	7.24 (*s*)
8		4.87 (*dd*, *J*=9.6, 4.2)			
9	5.45 (*d*, *J*=2.4)	2.21–2.26 (*m*), 1.84–1.88 (*m*)		2.36 (*dd*, *J*=16.4, 4.8), 2.50 (*dd*, *J*=16.4, 4.8)	2.36 (*dd*, *J*=16.4, 4.8), 2.51 (*dd*, *J*=16.4, 4.8)
10	2.91–2.95 (*m*)	2.15–2.20 (*m*)	–	1.90–2.02 (*m*)	1.84–1.96 (*m*)
11				3.85 (*q*, *J*=7.2)	3.49 (*q*, *J*=7.2)
12			7.50 (*s*)		
13	2.05 (br. *s*)	1.77 (*d*, *J*=1.2)	1.94 (*s*)	1.27 (*d*, *J*=7.2)	1.31 (*d*, *J*=7.2)
14			1.14 (*d*, *J*=7.6)		
15	1.47 (*s*)	1.64 (*s*)	1.05 (*s*)	1.51 (*s*)	1.52 (*s*)

[a]) Recorded in $CDCl_3$. [b]) Recorded in CD_3OD. [c]) C(3)–OAng: 6.11 (*qq*, *J*=7.2, 1.6, H–C(3′)), 1.98 (*dq*, *J*=7.2, 1.6, H–C(4′)), 1.92 (br. *s*, H–C(5′)). [d]) C(6)–Isobutyroxy: 1.28 (*d*, *J*=7.2, H–C(3′)), 1.30 (*d*, *J*=7.2, H–C(4′)), 2.73 (*qq*, *J*=7.2, 7.2, H–C(2′)).

observed for H–C(3) (a triple *doublet* with $J(3\alpha,2\alpha)=J(3\alpha,2\beta)=J(3\alpha,4\alpha)=3.0$ Hz) implied that H–C(3) was an equatorial H-atom and should be α-orientated [13]. In addition, a homoallylic coupling ($J=1.2$ Hz) between the olefinic Me group H–C(13) and H_α–C(6), typically attributable to an eremophil-7(11)-en-12,8β-olide derivative [14], was evident in the ^{1}H-NMR spectrum of **2**. Consequently, compound **2** was elucidated as 3β-[(2-methylbut-2-enoyl)oxy]eremophil-7(11)-en-12,8β-olid-14-oic acid.

The molecular formula of **3** was established as $C_{19}H_{25}ClO_5$ on the basis of its ESI-MS and ^{13}C-NMR data (*Exper. Part* and *Table 2*). Its IR absorption bands suggested the presence of an OH group (3500 cm^{-1}), an ester C=O group (1736 cm^{-1}), and an α-furano-ketone system (1680, 1634, and 1560 cm^{-1}). The ^{1}H-NMR spectrum of **3** displayed signals of three Me groups (δ(H) 1.05 (*s*); 1.14 (*d*, *J*=7.6); 1.94 (*s*)) and one isobutyroxy (=(2-methylpropanoyl)oxy) group (δ(H) 1.28, 3 H; 1.30, 3 H; 2.73, 1 H) (*Table 1*). Except for the isobutyroxy moiety, the ^{13}C-NMR and DEPT spectra exhibited 15 C-atom signals ascribed to six C-atoms, and four CH, two CH_2, and three Me groups (*Table 2*). Based on the above-mentioned data, as well as biogenetic considerations, compound **3** was suggested to be a furoeremophilane, similar to 6β-(angeloyloxy)-1α,10β-dihydroxy-9-oxofuroeremophilane [15]. However, the differ-

Table 2. *^{13}C-NMR Data of Eremophilanoids* **1**–**5**. At 100 MHz; δ in ppm.

Position	**1**[a])	**2**[b])[c])	**3**[a])[d])	**4**[a])	**5**[a])
1	22.4	22.7	62.0	22.3	22.7
2	23.4	35.7	24.3	22.7	23.5
3	137.2	71.6	23.3	142.9	143.8
4	129.9	45.6	31.9	135.9	135.2
5	41.4	39.7	50.1	38.1	38.5
6	82.2	25.4	68.2	152.8	152.8
7	148.1	163.2	139.6	132.9	133.2
8	141.7	81.8	145.9	197.3	198.3
9	107.7	38.4	186.3	39.3	39.4
10	36.4	41.1	80.5	36.4	39.7
11	127.2	122.1	121.8	40.2	40.5
12	168.5	168.3	147.4	180.5	179.4
13	9.3	8.1	8.53	17.0	15.6
14	169.8	177.2	15.9	171.9	171.2
15	26.5	25.8	16.0	27.2	26.6

[a]) Recorded in $CDCl_3$. [b]) Recorded in CD_3OD. [c]) C(3)–OAng: 174.2 (C(1′)), 129.3 (C(2′)), 138.9 (C(3′)), 20.9 (C(4′)), 15.8 (C(5′)). [d]) C(6)–Isobutyroxy: 176.5 (C(1′)), 34.1 (C(2′)), 18.6 (C(3′)), 19.5 (C(4′)).

ence between the two molecules could be found on the substituents at C(1) and C(6). In the HMBC spectrum of **3**, the cross-peak between H–C(6) (δ(H) 7.06 (*s*)) and C(1′) (δ(C) 176.5; *Tables 1* and *2*) confirmed the location of an isobutyroxy group at C(6), therefore indicating the presence of the Cl-atom at C(1). In general, H–C(14) and H–C(15) are, based on biogenetic considerations, β-oriented [16]. In the NOESY experiments, NOEs were observed between H–C(1) and H–C(15), and H–C(4) and H–C(6), which indicated their *cis*-relationships. Therefore, the structure of **3** was deduced as 1α-chloro-10β-hydroxy-6β-[(2-methylpropanoyl)oxy]-9-oxofuroeremophilane. The 3D structure of **3** was established by an X-ray diffraction experiment (*Fig. 1*).

Isomers **4** and **5** were obtained as a mixture (*ca.* 2:3 ratio as estimated from the ^{1}H- and ^{13}C-NMR spectral data). The NMR data showed close similarities with those of dimethyl 8-oxoeremophil-6-ene-12,14-dioate, a known sesquiterpenoid isolated from *L. przewalskii* [17]. However, the MeO signals of the known eremophilanoid were absent in the case of **4** and **5**, suggesting the presence of COOH groups instead. The disappearance of the H–C(4) signal, and the apparently downfield-shifted signals of H–C(3) at δ(H) 7.17 (for **4**) and 7.37 (for **5**) disclosed the presence of a C(3)=C(4) bond in **4** and **5**. A careful examination of the ^{1}H- and ^{13}C-NMR data revealed slight differences between **4** and **5** (*Tables 1* and *2*). Comprehensive comparisons of the differences unambiguously disclosed that compounds **4** and **5** are *C(10)*-epimers.

Compound **6** was obtained as optically active colorless crystals. The IR absorption bands of **6** at 1765, 1746, and 1675 cm^{-1} suggested the presence of saturated γ-lactone and α,β-unsaturated γ-lactone groups. Furthermore, a *quasi*-molecular-ion peak at *m*/*z* 536.2276 ($[M + NH_4]^+$; calc. 536.2284) in the HR-ESI-MS indicated the molecular formula to be $C_{30}H_{30}O_8$. However, the base peak appeared at *m*/*z* 259, suggesting that the molecule was composed of two parts with identical molecular weights. Further-

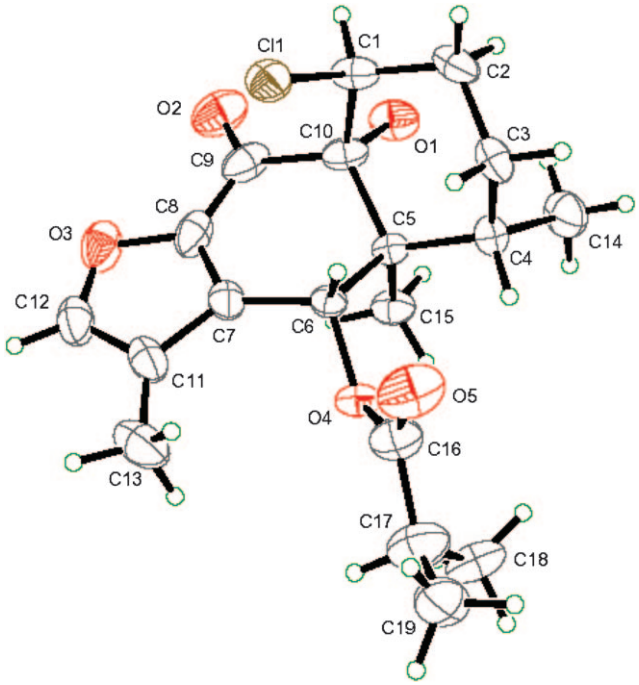


Fig. 1. *Molecular structure of compound* **3**, *showing the atom-labelling scheme.* Displacement ellipsoids drawn at the 30% probability level.

more, the ^{13}C-NMR spectrum of **6** contained only 15 signals including those of two Me, three CH_2, and three CH groups, as well as of seven quaternary C-atoms according to a DEPT spectrum (*Table 3*). Therefore, compound **6** should be made up of two identical sesquiterpenolide units [18], the two units being linked to each other through a C–C bond containing a C_2-axis. Structural elucidation of the half unit of **6** could be performed by analysis of the ^{1}H- and ^{13}C-NMR spectra (*Table 3*). The presence of a tertiary Me group (δ(H) 1.48 (*s*), δ(C) 26.2) and an olefinic Me group (δ(H) 1.97 (*d*, $J=2.4$), δ(C) 10.1) were characteristic of an eremophilanolide skeleton. These data suggested that the half unit of **6** was also an eremophilenolide similar to compound **8**, except for the absence of a CH signal (δ(H) 4.68 (*dd*, $J=9.3$, 4.2), δ(C) 77.4). Therefore, the structure of **6** was determined as a dimer of compound **8**. Furthermore, the diagnostic sp^3 signal at δ(C) 87.3 in the case of **6** implied that the two identical half units should be connected at C(8) and C(8′) positions to form the dimer. In addition, comprehensive analyses of the NMR data of **6** led to the deduction of the configuration at C(8) as β, which could be confirmed by the homoallylic coupling ($J=2.4$ Hz) between H–C(6) and Me(13) (*Table 3*). Therefore, the structure of **6** was elucidated as 8β-[eremophila-3′,7′(11′)-diene-12′,8′α;14′,6′α-diolide]eremophila-3,7(11)-diene-12,8α;14,6α-diolide. The results of the 2D-NMR investigation (*Fig. 2*) were in agreement with the proposed structure.

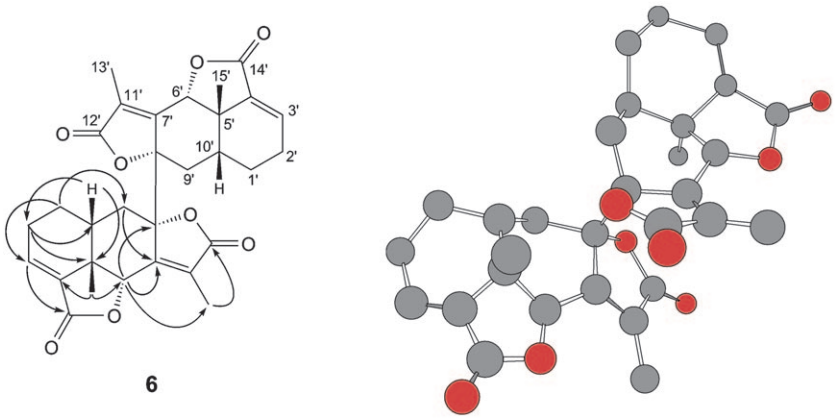


Fig. 2. *Key HMBC data and energy-minimized 3D structure of dimer* **6**

Table 3. *^{1}H- and ^{13}C-NMR Data of Compounds* **6** *and* **7**. At 400 and 100 MHz, respectively; δ in ppm, J in Hz.

Position	**6**[a])		**7**[b])	
	δ(C)	δ(H)	δ(C)	δ(H)
1, 1′	21.2	2.12–2.20 (*m*), 2.32–2.36 (*m*)	31.0	2.76 (*t*, *J*=7.2)
2, 2′	21.9	1.68–1.72 (*m*), 2.02–2.09 (*m*)	23.8	2.20–2.31 (*m*)
3, 3′	136.7	6.83 (*t*, *J*=3.2)	138.9	7.10 (*dt*, *J*=12.8, 6.4)
4, 4′	129.9		134.4	
5, 5′	44.3		127.1	
6, 6′	82.9	5.12 (br. *s*)	117.6	
7, 7′	157.4		142.3	7.48 (*d*, *J*=1.6)
8, 8′	87.3		155.4	
9, 9′	35.2	1.27–1.34 (*m*), 2.37–2.41 (*m*)	108.6	7.19 (*d*, *J*=1.6)
10, 10′	32.1	2.57–2.61 (*m*)	136.9	
11, 11′	128.5		169.6	
12, 12′	170.9		18.3	2.38 (*d*, *J*=1.2)
13, 13′	10.1	1.97 (*d*, *J*=2.4)	129.7	
14, 14′	168.4			
15, 15′	26.2	1.48 (*s*)	11.1	2.51 (*s*)

[a]) Recorded in CD_3COCD_3. [b]) Recorded in $CDCl_3$.

Different mechanisms of dimerization have been proposed for this kind of eremophilanes; for instance, the hypotheses regarding the involvement of free radicals [19][20] or an acid/base catalysis [21]. It is also conceivable to hypothesize that dimerization of this kind is catalyzed by plant enzymes. Detailed investigations on the formation of eremophiladiolide dimers are, however, not yet available.

Ligulatrovine A (**7**) crystallized as colorless plates, and its HR-ESI-MS analysis (*m*/*z* 461.1957 ($[M+H]^+$); calc. 461.1964) resulted in the molecular composition $C_{28}H_{28}O_6$. The IR spectrum of **7** showed absorption bands for a COOH group (3161 and 1726 cm^{-1}) and a C=C bond (1649 cm^{-1}), as well as for an aromatic ring (1596,

1540, and 1456 cm^{-1}). The ^{13}C-NMR spectrum (*Table 3*) of **7** displayed only 14 C-atom resonances including those of two Me, two CH_2, and three CH groups, as well as seven quaternary C-atoms, assigned by a DEPT experiment. With respect to the molecular formula, compound **7** was predicted to represent another symmetric dimer. In the upfield region of the NMR spectrum of **7**, there were two Me signals: δ(H) 2.38 (*d*, *J* = 1.2, Me(12), Me(12′)), 2.51 (*s*, Me(14), Me(14′)) and δ(C) 18.3, 11.1 (olefinic Me groups). Two allylic CH_2 groups were assigned to the signals at δ(H) 2.76 (*t*, *J* = 7.2, 2 H−C(1), 2 H−C(1′)) and 2.24 (*m*, 2 H−C(2), 2 H−C(2′)). Furthermore, the downfield region of the NMR spectrum of compound **7** indicated the presence of a 1,2,3,5-tetrasubstituted aromatic ring (δ(H) 7.19 (*d*, *J* = 1.6, H−C(9), H−C(9′)), 7.48 (*d*, *J* = 1.6, H−C(7), H−C(7′)), and δ(C) 127.1, 117.6, 142.3, 155.4, 108.6, and 136.9). The signals of a conjugated olefinic H-atom at δ(H) 7.10 (*dt*, *J* = 12.8, 6.4, H−C(3), H−C(3′)) and at δ(C) 138.9, 134.4, and of a C=O group at δ(C) 169.6 were also visible in the downfield part of **7**. Moreover, a single olefinic C-atom signal at δ(C) 129.7 (C(13)/C(13′)) in the ^{13}C-NMR spectrum suggested that the two units were linked to each other with a C=C bond. Additionally, the $^{1}H,^{1}H$-COSY spectrum of **7** showed correlations of H−C(2) with H−C(1) and H−C(3), and of H−C(7) with H−C(9). Combined with the observed HMBC of H−C(1) with C(9), C(5), and C(3), of H−C(2) with C(10) and C(4), of H−C(3) with C(1), C(5), and C(11), of H−C(7) with C(5), C(9), and C(12), of H−C(9) with C(7), C(1), and C(5), of H−C(12) with C(5) and C(7), and of H−C(14) with C(13) (*Fig. 3*), the single unit of the dimer as well as the subsequential whole structure of **7** could be unambiguously deduced. Compound **7** was named ligulatrovine A.

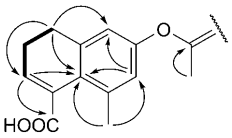


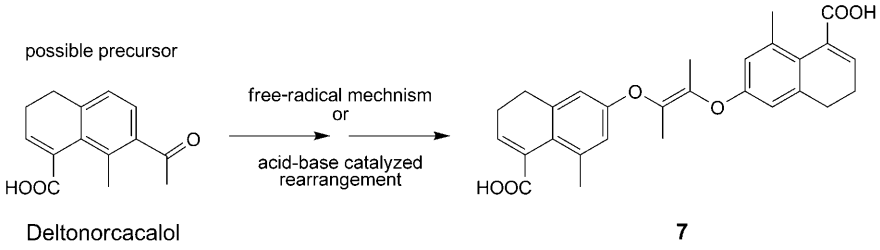
Fig. 3. *Partial structure, and key $^{1}H,^{1}H$-COSY* (bold lines) *and selected HMBC data* (arrows) *of ligulatrovine A* (**7**)

Considering the partial similarities of **7** with those of the known benzofuran noreremophilane, deltonorcacalol [22], the biogenesis of **7** is preliminarily assumed to be as follows: the possible precursor deltonorcacalol passes through a multistep oxidative rearrangement due to either a free radical-initiated mechanism [19][20] or an acid/base-induced process [21] (*Scheme*). However, this proposal needs further confirmation.

Compound **8** was obtained as colorless needles, the ^{1}H- and ^{13}C-NMR data were identical to those of an eremophilanodiolide recently reported by *Han et al.* [2]. In addition, 2D-NMR spectra were analyzed to unambiguously assign the H- and C-atom resonances of this diolide. Correlations in the $^{1}H,^{1}H$-COSY spectrum suggested the presence of a $=CHCH_2CH_2CHCH_2CH$ fragment. The β-orientations of H−C(6), H−C(8), and H−C(10) were confirmed by the NOESY experiment. Combined with the correlations in the HMBC data, the skeleton and the configuration of compound **8** was thus established.

Based on the comparison of the NMR data as well as the physicochemical properties of the known compounds, the following compounds were identified as

Scheme. *Possible Biosynthetic Pathway of Ligulatrovine A* (**7**)



follows: 8β-hydroxyeremophila-3,7(11)-diene-8α,12(6α,14)-diolide (**9**) [23], 8β-methoxyeremophila-3,7(11)-diene-8α,12(6α,14)-diolide (**10**) [23], furoeremophil-3-en-14,6α-olide (**11**) [24], furoeremophilan-14β,6α-olide (**12**) [25], 1α-hydroxy-6β-isobutyroxy-9-oxo-(10βH)-furoeremophilane (**13**) [26], 1α-hydroxy-6β-isobutyroxy-9-oxo-(10αH)-furanoeremophilane (**14**) [26][27], 6β-(angeloyloxy)-1α,10β-dihydroxy-9-oxofuranoeremophilane (**15**) [15], 8β-hydroxyeremophil-7(11)-ene-12,8α;14,6α-diolide (**16**) [28], 3β-(angeloyloxy)-(8βH)-eremophil-7(11)-ene-12,8α;14,6α-diolide (**17**) [29], 3β-[(2-methylbutanoyl)oxy]-(8βH)-eremophil-7(11)-ene-12,8α;14,6α-diolide (**18**) [30], β-sitosterol, stigmasterol, 8β,Δ^4-stigmasten-3-ol [31], daucosterol, 6-*O*-acetyl-α-D-glucopyranoside [32], and cerotic acid.

2. *Taxonomy.* From a chemotaxonomic point of view, the *Ligularia* species is rich in eremophilanes. However, the diolides have been rarely isolated from this genus compared to other more prevalent eremophilanes such as eremophilanolides and furoeremophilane derivatives. Impressively, these highly oxidized secondary metabolites possessing diolide ring systems were obtained from the title plant collected from Yunnan Province (altitude of sampling spot *ca.* 3560 m) as well as from *L. tongolensis* (FRANCH.) HAND.-MAZZ collected at Muli, Sichuan Province, at an altitude of 3600 m [2]. This reflected a possible biogenetic fact that the unique local vegetal environment at such high altitude may influence the biodiversity of its secondary metabolites. It could be inferred that the scarce oxygen supply might compensatively initiate the enzymes, especially the oxidases, of the plant to produce these metabolites and thus make the biospecificity of these *Ligularia* species. Furthermore, the diolide **8** was isolated both from *L. tongolensis* and from *L. atroviolacea*. This finding might suggest that this kind of diolide might be assumed as one of the characteristic components of *Ligularia* species scattered in plateau regions (over 3400 m) of western China. It should also be mentioned that dimers appeared more frequently in the genus *Ligularia* [33][34]. The unusual dimeric compound **7** described here might be formed from a rearranged derivative of a degraded benzofuran-type eremophilanoid, deltonorcacalol. The findings reported in the present article could further support an explanation of the rich biodiversity of the eremophilane family.

In addition, the identification of a certain amount of eremophilane derivatives from the title plant further evidenced that *L. atroviolacea* should be classified into *Ligularia* genus, tribe *Senecioneae*, Asteraceae, which is in full agreement with the traditional taxonomy and most of our previous investigations [35], except for the recent investigation on *L. caloxantha* (DIELS) HAND.-MAZZ [36].

3. *Cytotoxicity Assay.* Among the reported bioactivities of eremophilanes, cytotoxicity is one of the most intensively studied. *Han et al.* reported the *in vitro* cytotoxicity of diolide **8** against SMMC-7721 and HL-60 cell lines with IC_{50} values of 399 and 448 μg/ml, respectively [2]. More recently, the eremophiladiolides **9** and **10**, isolated from *L. laphthifolia*, were reported to possess moderate cytotoxicity against HEP-G2 and S-180 cell lines [23]. To extend the information on the bioactivities of these eremophilanes, the cytotoxicity evaluation of the five representative eremophilanoids from the title plant, **1**, **2**, **8**, **9**, and **11**, were performed against seven cultured tumor cell lines including KB, BEL-7404, A549, HeLa, CNE, HL-60, and P388D1 with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide (MTT) assay.

According to the assay results, none of the tested eremophilanolides exhibited apparent cytotoxicities against these seven tumor cell lines (*Table 4*). Compounds **1** and **8** were inactive against all of the seven tumor cell lines; therefore, *Table 4* does not include the data for these two compounds. Compound **2** displayed a weak inhibitory effect against P388D1 cells with an IC_{50} value of 62.3 μM, while compound **9** showed an IC_{50} value of 73.2 μM against KB cells. Nevertheless, it should be noticed that compound **9** exhibited a broad cytotoxicity spectrum against all of the seven tumor cell lines (*Table 4*), a result which might be due to the presence of an active 8*β*-hemiacetal functionality in the molecule. In addition, the sole furoeremophilanolide obtained from this species, substance **11**, demonstrated relatively weak cytotoxicities. The two detectable IC_{50} values for **11** were 293 and 130 μM against KB and P388D1 cells, respectively (*Table 4*). To our knowledge, it is the first report on the cytotoxicity of eremophiladiolides against KB, BEL-7404, A549, HeLa, CNE, and P388D1 cell lines. These results might afford some reference data for future systematic pharmacological screenings and SAR investigations on sesquiterpenoic diolides of this type.

Table 4. *Cytotoxicity of Compounds* **2**, **9**, *and* **11** *against Seven Tumor Cell Lines.* IC_{50} Values [μM] are expressed as mean ± SEM. KB, Human oral epithelial cell line; BEL-7404, human hepatoma cell line; A549, human lung adenocarcinoma cell line; HL-60, human promyelocytic leukemia cell line; HeLa, human cervical carcinoma cell line; CNE, nasopharyngeal carcinoma cell line; P388D1, murine macrophage-like cell line.

Compd.	KB	BEL-7404	A549	HL-60	HeLa	CNE	P388D1
2	99.0 ± 8.2	> 300[a])	> 300	208 ± 28	89.3 ± 7.2	167 ± 8.7	62.3 ± 5.5
9	73.2 ± 3.1	111 ± 17	106 ± 16	197 ± 27	98.6 ± 4.3	191 ± 37	85.4 ± 5.8
11	293 ± 45	> 300	> 300	> 300	> 300	> 300	130 ± 17
DDP[b])	0.4 ± 0.02	3.4 ± 0.3	8.3 ± 1.3	2.4 ± 0.3	2.0 ± 0.1	4.5 ± 0.3	3.6 ± 0.4

[a]) IC_{50} Values > 300 μM were considered as inactive. [b]) Positive control: cisplatin.

Further systematic phytochemical, pharmacological, and taxonomical investigations of diverse Yunnan Asteraceae plants scattered in plateau regions of western China are in progress.

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Experimental Part

General. Column chromatography (CC): *Sephadex LH-20* (*Pharmacia Biotech*, Sweden). TLC: precoated GF_{254} silica-gel plates (SiO_2; 10–40 μ, *Qingdao Marine Chemical Co.*, Qingdao, P. R. China), spots were detected by UV at 254 nm before I_2 vapor exposure and heating after spraying with 5% (*v*/*v*) H_2SO_4 in EtOH. M.p.: *X-4* digital melting-point instrument; uncorrected. Optical rotations: *Polax-2L* polarimeter. UV Spectra: *Shimadzu UV-240* UV spectrometer; λ_{max} (log ε) in nm. IR Spectra: *Bruker Vector-22* spectrometer; $\tilde{\nu}$ in cm^{-1}. ^{1}H-, ^{13}C-, and 2D-NMR Spectra: *INOVA* NMR spectrometer, at 400 and 100 MHz, resp.; δ in ppm rel. to Me_4Si. ESI-MS: *Bruker Esquire 3000+* mass spectrometer; in *m*/*z*. HR-ESI-MS: *Bruker Bio Apex 70eV FT-ICR* mass spectrometer; in *m*/*z*.

Plant Material. The roots of *Ligularia atroviolacea* (Franch.) Hand.-Mazz. were collected from Lijiang, Yunnan Province in August, 2001. A voucher specimen (LSP200108-04) was deposited with the Department of Traditional Chinese Medicine and Natural Drug Research, College of Pharmaceutical Sciences, Zhejiang University and was identified by *H. P.* at the Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. Air-dried roots of *L. atroviolacea* (5.0 kg) were powdered and extracted with 95% EtOH (3 × 50 l) for 7 d at r.t. The solvents were removed under reduced pressure to give an extract (462 g). The residue was suspended in H_2O and successively partitioned with petroleum ether (PE), AcOEt, and BuOH.

The PE extract (25 g) was subjected to CC (300 g of SiO_2; PE/AcOEt 1:0→1:1). Four fractions (*Frs. 1–4*) were obtained under TLC examination of the eluants. *Fr. 2* (2.1 g) was subjected to CC (45 g of SiO_2; PE/AcOEt 6:1, 4:1, 3:1, and 2:1). β-Sitosterol (11 mg) and stigmasterol (10 mg) were purified from the 4:1 eluant after repeated recrystallization from Me_2CO. The 3:1 eluant was combined (107 mg) and subjected to CC (100 g of *Sephadex LH-20*; MeOH) to afford 8β,Δ^4-stigmasten-3-ol (11 mg). *Fr. 3* (1.9 g) was purified by CC (100 g of *MCI*, MeOH/H_2O 1:4→1:0): **2** (26 mg) was recrystallized with MeOH from the 3:1 eluant. *Fr. 4* (0.8 g) was directly subjected to CC (100 g of *Sephadex LH-20*; MeOH) leading to 16 mg of pure cerotic acid.

The AcOEt extract (89 g) was subjected to CC (500 g of SiO_2; $CHCl_3$/MeOH 50:1, 20:1, 10:1, 9:1, 8:2, 7:3, 6:4, 5:5, and 0:1). Based on the differences exhibited by TLC monitoring, 14 fractions (*Frs. A–N*) were obtained. *Fr. C* (25.9 g) was separated by CC (130 g of SiO_2; PE/Me_2CO 10:1→0:1), the eluant of 8:2 (5.2 g) was recrystallized to afford **11** (1.8 g), and the eluant of 5:5 (3.6 g) afforded finally **8** (140 mg) and **12** (86 mg) after recrystallization from Me_2CO. The eluant of 7:3 (0.95 g) was re-chromatographed (5.0 g of SiO_2; PE/Me_2CO 4:1 and 3:1) to afford **9** (15 mg), **10** (19 mg), and **1** (12 mg). The eluant of 6:4 (0.28 g) was further purified by CC (50 g of *Sephadex LH-20*; MeOH) to give 21 mg of **16**. *Fr. D* (11 g) was submitted to CC (250 g of SiO_2; PE/AcOEt 8:1→2:1) to provide five main fractions (*Frs. D_1–D_5*). Compound **17** (19 mg) and **18** (12 mg) were purified by prep. TLC (SiO_2 GF_{254}; PE/AcOEt 6:1) from *Fr. D_2* (102 mg). Vacuum liquid chromatography (VLC) of *Fr. D_3* (850 mg; 16 g of Si *H*, PE/AcOEt 4:1→2:1) afforded **4** and **5** (21 mg). *Fr. E* (19 g) was subjected to CC (400 g of SiO_2; PE/Me_2CO 10:1→0:1) to afford seven main fractions (*Frs. E_1–E_7*). *Fr. E_1* (0.2 g) afforded **3** (14 mg) after recrystallization. *Fr. E_4* (1.6 g) was subjected to CC (60 g of SiO_2; CH_2Cl_2/Me_2CO 10:1→0:1), **13** (54 mg) was obtained after recrystallization from the 5:1 eluant (192 mg), while **14** (29 mg) was obtained after recrystallization from the 3:1 eluant (123 mg). *Fr. G* (21 g) was fractionated by CC (100 g of *MCI*; H_2O/MeOH 15:1→0:1), the 50% MeOH eluant (900 mg) was re-chromatographed (100 g of *Sephadex LH-20*; MeOH) and gave **15** (42 mg). The 70% MeOH eluant (4.8 g) was rechromatographed (100 g of SiO_2; PE/Me_2CO 8:1→4:1), the 6:1 eluant (492 mg) gave **6** (29 mg), while the 4:1 eluant (231 mg) afforded **7** (8 mg), after recrystallization. *Fr. H* (1.6 g) was submitted to CC (100 g of *Sephadex LH-20*; MeOH) to afford 6-*O*-acetyl-α-D-glucopyranoside (28 mg).

10 g of the BuOH extract (80 g) was subjected to CC (100 g of *MCI*; H_2O/MeOH 15:1→0:1), recrystallization of the 90% MeOH eluant from MeOH afforded daucosterol (150 mg). The known compounds were identified by comparing their properties (m.p., MS, IR, and NMR) with literature values [15][23–32].

Eremophila-3,7(11),8-triene-12,8;14,6α-diolide (**1**). Colorless needles. M.p. 233–235°. $[\alpha]_D^{20} = +29.0$ ($c = 0.30$, $CHCl_3$). UV ($CHCl_3$): 224 (3.96), 279 (3.74). IR (KBr): 1772, 1756, 1681. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. HR-ESI-MS: 259.0936 ($[M+H]^+$, $C_{15}H_{16}O_4^+$; calc. 259.0926).

3β-[(2-Methylbut-2-enoyl)oxy]eremophil-7(11)-en-12,8β-olid-14-oic Acid (**2**). White powder. M.p. 200–202°. $[\alpha]_D^{20} = -136.5$ ($c = 0.30$, MeOH). UV (MeOH): 224 (3.89). IR (KBr): 2985, 1768, 1714, 1706, 1654. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. HR-ESI-MS: 361.1742 ($[M-H]^-$, $C_{20}H_{26}O_6^-$; calc. 361.1729).

1α-Chloro-10β-hydroxy-6β-[(2-methylpropanoyl)oxy]-9-oxofuroeremophilane (**3**). Colorless needles. M.p. 107–108°. $[\alpha]_D^{20} = +0.9$ ($c = 0.20$, MeOH). UV ($CHCl_3$): 221 (3.83), 285 (3.69). IR (KBr): 3500, 1736, 1680, 1634, 1604, 1560, 1465. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. ESI-MS: 369 ($[M+H]^+$).

X-Ray Crystallographic Analysis of **3**. Measurements were performed on a *Rigaku RAXIS-RAPID* X-ray diffractometer using MoK_α radiation at 298 K. The structure was solved by direct methods using SHELXS-97. Crystal data and refinement details: $C_{19}H_{24}ClO_5$, M_r 367.85, orthorhombic, space group $P2_12_12_1$; $a = 8.557(2)$, $b = 14.545(5)$, and $c = 15.121(4)$ Å; $V = 1882.0(9)$ Å^3, $Z = 4$, $\lambda = 0.71075$ Å; $D_{calc.} = 1.298$ Mg/m^3; $F(000) = 780$, μ(MoK_α) = 0.228 mm^{-1}; crystal size: 0.50 mm × 0.47 mm × 0.42 mm. At convergence, $R_1 = 0.0484$ (based on F and 3465 data with $F > 4\sigma(F)$), $\omega R_2 = 0.1442$ (based on F^2 and all 4285 data), and $S = 1.006$ for 228 parameters. $\Delta\rho_{max}$ and $\Delta\rho_{min}$ were 0.58 and −0.49 e Å^{-3}, resp. The crystallographic data of **3** have been deposited with the *Cambridge Crystallographic Data Centre* with the deposition number CCDC-680796. Copies of the data can be obtained, free of charge, at http://www.ccdc.cam.ac.uk/data request/cif.

*(10α*H*)- and (10β*H*)-8-Oxoeremophila-3,6-diene-12,15-dioic Acids* (**4** and **5**, resp.). Colorless gum. IR (KBr): 3016, 1736, 1682, 1434, 1379. ^{1}H- and ^{13}C-NMR: see *Tables 1* and *2*, resp. ESI-MS: 279 ($[M+H]^+$).

8β-[Eremophila-3′,7′(11′)-diene-12′,8′α;14′,6′α-diolide]eremophila-3,7(11)-diene-12,8α;14,6α-diolide (*=[(5a*R*,6a*S*,9b*R*,9c*S*,5′a*R*,6′a*S*,9′b*R*,9′c*S*)-9,9c,9′,9′c-Tetramethyl-4,5,5a,6,9b,9c,4′,5′,5′a,6′,9′b,9′c-dodecahydro[6a,6′a]bi[1,7-dioxacyclopenta[*d*]acenaphthylenyl]-2,8,2′,8′-tetraone]*; **6**). Colorless plates. $[\alpha]_D^{20} = +39.1$ ($c = 0.20$, $CHCl_3$). UV ($CHCl_3$): 221(3.78). IR (KBr): 1765, 1746, 1675. ^{1}H- and ^{13}C-NMR: see *Table 3*. HR-ESI-MS: 536.2276 ($[M+NH_4]^+$, $C_{30}H_{34}NO_8^+$; calc. 536.2284).

Ligulatrovine A (*=(*E*)-6,6′-(But-2-ene-2,3-diylbis(oxy))bis(8-methyl-3,4-dihydronaphthalene-1-carboxylic Acid)*; **7**). Colorless plates. IR (KBr): 3161, 1726, 1649, 1596, 1540, 1456. ^{1}H- and ^{13}C-NMR: see *Table 3*. ESI-MS: 461 ($[M+H]^+$).

Cytotoxicity Assays. Cytotoxicities of the test compounds against cultured KB, BEL-7404, A549, HeLa, CNE, HL-60, and P388D1 tumor cell lines were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide (MTT) colorimetric assay [37]. It was performed by the assessment of remaining number of viable cells after exposure of a given number of cells in culture to a particular concentration of the test compounds. Cell viability was assessed using MTT (*Sigma*, St. Louis, MO, USA). Briefly, the different cell lines were diluted in fresh complete medium, and seeded in 96-well plates, applying 10^4 cells/well. After 24 h of incubation, the cells were treated with the isolated compounds at various concentrations during 72 h. Then, MTT soln. (10 μl) was added to each well. The plates were in a CO_2 incubator (*Shel Lab*, Cornelius, OR, USA) for 4 h, and then lysed and incubated with DMSO. The plates were analyzed in a multi-well-plate reader (*Bio-Tek ELX800*, Winooski, VT, USA) at 570 nm. The IC_{50} value was the compound concentration required to reduce the MTT signal by 50% compared with untreated control cultures, which was generated graphically from the dose–response curves. The measured IC_{50} values were expressed as the mean ± standard error of the mean (SEM) of three independent experiments.

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