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Diversity in Eremophilane Components of *Ligularia dictyoneura* in Yunnan and Sichuan Provinces of China

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Abstract

The chemical composition of 3 samples of *Ligularia dictyoneura* was studied. Nine furanoeremophilanes **1** to **9** and bakkenolide A (**10**) were isolated, among which compound **6** was a new furanoeremophilane-15,6-olide. These results, together with our previous results on 20 *L. dictyoneura* samples, show that furanoeremophilane is the major compound in the peripheries of the plant's distribution area, while eremophilan-8-one, in the center. DNA analysis suggests that a furanoeremophilane producer in the center, an exception, is introgressed; namely, the ability to produce furanoeremophilane in the sample may have its origin in some other plant.

Keywords

Ligularia dictyoneura, diversity, furanoeremophilane, internal transcribed spacer (ITS)

We are studying the chemical and genic diversity in *Ligularia* (Asteraceae) in the Hengduan Mountains area of China using root chemicals and evolutionarily neutral base sequences as the indices.[1,2] Eremophilane-type sesquiterpenoids have been isolated from most of the major *Ligularia* species and many *Ligularia* species have been found to harbor intraspecific diversity in their root chemicals. On the basis of the isolated chemicals and our observation on the abundance of the plants in the field, we have proposed a hypothesis that furanoeremophilane-producing species or intraspecific groups are ecologically advantageous over those producing eremophilan-8-ones.[1]

Ligularia dictyoneura (Franch.) Hand.-Mazz. grows in grassy slopes and scrubs in northwestern Yunnan and southwestern Sichuan Provinces.[3] Some eremophilane sesquiterpenes have been isolated from this species.[4-7] We isolated eremophilan-8-one derivatives from samples collected in Shangrila (formerly Zhongdian) area, Yunnan, and furanoeremophilanes from samples collected in its surrounding areas (Figure 1).[8] On the basis of the TLC patterns of their root extracts, furanoeremophilane-producing *L. dictyoneura* samples were subgrouped into 6 chemotypes (types 1-6).[8] Because furanoeremophilanes are believed to be generated from eremophilan-8-ones[9] (Scheme 1), furanoeremophilane-producing populations of *L. dictyoneura* may have evolved from those producing eremophilan-8-ones.

Here we report the results of analyses on 3 samples of *L. dictyoneura*. They were collected further afield from our previous *L. dictyoneura* samples. Samples A to C were collected in Yanbian, Yanyuan, and Litang counties, Sichuan, respectively (Table 1 and Figure 1). One of the compounds isolated from sample A was new. The compounds from samples B and C were only listed previously[1] and here we describe the details of their analyses.

The EtOH extract of each root sample was subjected to Ehrlich's test, which is a facile method to detect furanoeremophilanes.[10,11] The 3 samples were positive to the test. The TLC pattern of sample A was similar to that of the previous type-2 samples and the chemotype of this sample was judged to be type 2 (Table 1).[8] Sample B showed 2 large pink spots at R_f = 0.59 and 0.65 (hexane-EtOAc 7:3). In addition, 3 small pink spots (R_f = 0.43, 0.76, and 0.88) and an orange spot of ligularone (R_f = 0.80) were observed. Sample C showed the same 2 large spots; however, of the 4 minor spots, the orange spot and the least polar spot were absent. These TLC patterns were different from that of any of the previously analyzed samples of types 1 to 6, and, therefore, new chemotypes 7 and 8 were assigned to samples B and C, respectively (Table 1).[1]

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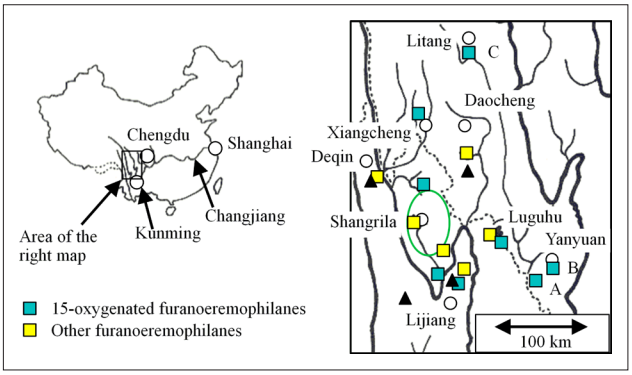
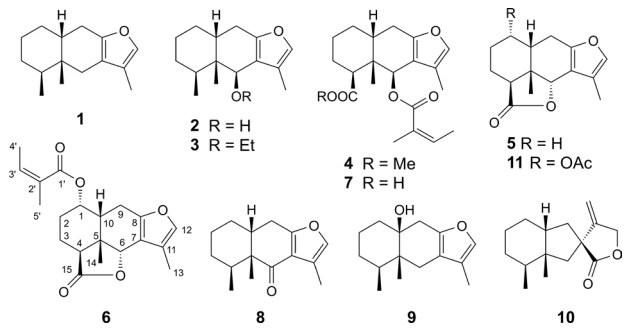
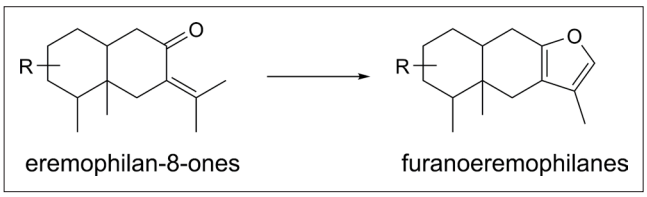


Figure 1. Collection locations of *L. dictyoneura* (squares). Circles and triangles indicate major cities and peaks, respectively. Solid and dotted lines indicate rivers and province boundaries, respectively. The green circle indicates the area of 8 samples with eremophilan-8-one.[8]



Compounds in each sample were isolated by use of silica gel column chromatography (CC) and HPLC. From sample A, compounds **1**,[12] **2** (ligularol),[13] **3**,[14,15] **4**,[16] **5**,[17,18] and a new compound **6** were isolated. From sample B, **1** to **5**, **7**,[19] and **8** (ligularone)[13,20] were isolated. From sample C, **1** to **3**, **5**, **7**, **8**, **9** (tetradymol),[21] and **10** (bakkenolide A)[22,23] were isolated. The structure of the new compound **6** was determined as follows.

The molecular formula of **6** was determined to be $C_{20}H_{24}O_5$ from the high-resolution CIMS (*m*/*z* 345.1697) and ^{13}C NMR (4 CH_3, 3 CH_2, 6 CH, and 7 quaternary C) data. The IR spectrum showed the presence of a γ-lactone (1780 cm^{-1}) and a conjugated ester carbonyl group (1711 cm^{-1}). The ^1H and ^{13}C NMR spectra showed the signals typical of furanoeremophilane [δ_H 6.86 (br s, H-12), 1.93 (d, H_3-13), 0.77 (s, H_3-14)] with an angeloyl moiety [δ_H 5.71 (qq, H-3′), 1.79 (quint, H_3-5′), and 1.93 (dq, H_3-4′); δ_C 166.4 (C-1′) and 128.0 (C-2′)] (Table 2). Two oxygenated methines [δ_H 5.11 (td, H-1) and 4.46 (s, H-6)] and another carbonyl [δ_C 174.0 (C-15)] were observed. These data, as well as the lack of a 15-Me signal in the ^1H NMR spectrum, suggested that **6** was a furanoeremophilan-15,6-olide with an angeloyloxy group. The position of the angeloyloxy group was determined to C-1α from the COSY spectrum (H-4/H-3/H-2/H-1/H-10/H-9) and the splitting pattern of H-1 (td, 5.3, 11.6 Hz). The planar structure was established by HMBC correlations (Figure 2). The configurations of the stereogenic centers were determined from the NOESY spectrum showing correlations between Me-14 and H-10 and between Me-14 and H-1 (Figure 2). Thus, the structure of compound **6** was established as depicted.



Scheme 1. Biosynthesis of furanoeremophilanes.

The base sequence of the ITS1-5.8S-ITS2 region was determined for samples A to C. Samples 3 to 14 and 16 to 19 from our previous study on *L. dictyoneura*[8] were also reanalyzed. The adoption of a new set of primers[24] and a new sequencing reaction kit has improved the quality of data and allowed us to determine the sequences that had remained unknown in these samples. The results, as well as those previously determined for samples 1, 2, 15, and 20, are shown in Table 3. Subjecting the data to a phylogenetic analysis program MEGA X[25] returned no phylogenetic tree with a significant bootstrapping value, implying that the samples have not differentiated very much genetically. (See below for discussion on sample 9.)

Nine furanoeremophilanes were isolated from the 3 *L. dictyoneura* samples. Of them, 4 were 15-oxygenated derivatives (**4-7**) and 3 were ligularol derivatives (**2**, **3**, and **8**). Two of the compounds isolated from sample A, ligularol (**2**) and its lactone **5**, were previously isolated from a type-2 sample (sample 3)[8]; however, the acid part of the 1α-acyloxyfuranoeremophilan-15,6α-olide was different between sample A and sample 3: angelate in **6** from sample A and acetate in **11** from sample 3.[8] Thus, there is variation among type-2 samples. Although samples B and C were given different chemotypes,[1] the compounds in them were very similar (Table 1). Our chemotyping of *L. dictyoneura* has relied on TLC, which is facile and should be useful for diversity studies[1,8]; however, the present results of compound isolation indicate that the chemical spectrum of *L. dictyoneura* is rather continuous.

The eremophilan-8-one type of *L. dictyoneura* is found in the Shangrila area, where the plant is most abundant (Figure 1, green circle); the furanoeremophilane type, in the surrounding areas. The only exception is sample 9 in our previous study: Although it was surrounded by eremophilan-8-one producers, furanoeremophilanes **1** and **2** were isolated from its extract. We surmised that the high diversity in *L. dictyoneura* may have arisen from hybridization of previously separate populations.[1,8] In this context, it is noteworthy that sample 9 contained 13 multiple-base sites and 2 indels in the ITS1-5.8S-ITS2 region; in no other sample, the former number was as large (Table 3). (Information of this kind cannot be handled by MEGA X.) This may suggest an introgression that

Table 1. Collection Localities and Isolated Compounds of *Ligularia dictyoneura* Samples.

Sample no.	Specimen no.	Locality[a]	Elevation (m)	Isolated compounds	Chemotype
A	2012-52	Gesala (Yanbian)	2500	**1-6**	Type 2
B	2007-25	Gongmushan (Yanyuan)	2700	**1-5, 7, 8**	Type 7
C	2007-103	Jiawa (Litang)	3900	**1-3, 5, 7-10**	Type 8

[a]County in parentheses. All in Sichuan Province.

resulted in the production of furano compounds in sample 9. The mechanism(s) which led *L. dictyoneura* in the peripheral areas to produce furano compounds is not known. However, an ultimate explanation for the phenomenon could be that the environments in these areas are not suitable to *L. dictyoneura* and the production of furano compounds helps the plant survive.

The characteristic components in 4 of the 8 furanoeremophilane-producing types (types 2, 4, 7, and 8) are 15-oxygenated derivatives. 15-Oxygenated furanoeremophilanes have been isolated from *L. tongolensis*,[26] *L. cymbulifera*,[26] *L. vellerea* (Shangrila-type),[19] and *L. hookeri*.[27] Because the distribution areas of these plants and *L. dictyoneura* almost overlap (Shangrila County and the surrounding areas, see Figure 1), the production of similar compounds in these species may not be a coincidence.

Table 2. ^{1}H and ^{13}C NMR Data of New Compound **6** (C_6D_6).

Carbon no.	δ_H	δ_C
1	5.11 (td, 5.3, 11.6)	70.3
2	1.00 (dq, 5.2, 12.5) 1.55 (m)	25.8
3	1.20 (dq, 4.2, 13.1) 1.52 (m)	17.6
4	1.65 (dd, 3.5, 12.6)	40.5
5	-	42.0
6	4.46 (s)	79.9
7	-	115.2
8	-	150.4
9	2.25 (dd, 10.0, 17.6) 2.37 (dd, 7.5, 17.5)	19.7
10	2.08 (ddd, 5.2, 7.8, 10.0)	40.2
11	-	120.4
12	6.86 (br s)	138.9
13	1.93 (d, 1.2)	8.3
14	0.77 (s)	19.5
15	-	174.0
1'	-	166.4
2'	-	128.0
3'	5.71 (qq, 1.3, 7.2)	138.5
4'	1.93 (qd, 1.3, 7.2)	15.9
5'	1.79 (quint, 1.3)	20.7

Experimental

General

NMR, JEOL ECX-400 (400 MHz for ^{1}H; 100 MHz for ^{13}C) or JEOL AL 400 spectrometer; IR, JASCO FT/IR-230 spectrometer; MS, JEOL JMS-700 MStation or CMATE II. CC was performed on silica gel (Wakosil C-200 or C-300 or Kanto silica gel 60 N [spherical neutral]). Analytical TLC was carried out on Merck Kieselgel 60 F254, 0.2 mm thickness, using either Ehrlich's reagent (*p*-dimethylaminobenzaldehyde and HCl) or *p*-anisaldehyde/AcOH/H_2SO_4 as the visualizing agent. HPLC was carried out using either a Shimadzu LC-20AT pump with a SPD-20A Prominence UV/VIS detector or a GL Sciences GL-7410 pump with a GL-7450 UV detector, and a Hitachi D-2500 Chromato-Integrator or a Shimadzu C-R8A Chromatopac, with either a GL Sciences Inertsil PREP-ODS column (20 × 250 mm) or a Kanto Mightysil Si60 (10 × 250 mm) column. DNA was purified from dried leaves using DNeasy Plant Mini Kit (Qiagen). A 30-cycle amplification of the ITS1-5.8S-ITS2 region by polymerase chain reaction was carried out with LC5 and LC6 primers[24] and HotStarTaq plus Master Mix Kit (Qiagen). The amplified DNA was separated by agarose gel electrophoresis and purified with High Pure PCR Product Purification Kit (Roche Diagnostics). Sequencing reactions were carried out with LC1 to LC4 primers[24] and BigDye Terminator Cycle Sequencing Kit Ver. 3.1 (Applied Biosystems). Sequence determination was carried out on a 3130xl Genetic Analyzer (Applied Biosystems).

Plant Materials

Samples were collected at locations shown in Table 1 and Figure 1 in August 2012 (sample A) and 2007 (samples B and C) and identified by X. G. (author). Voucher specimen

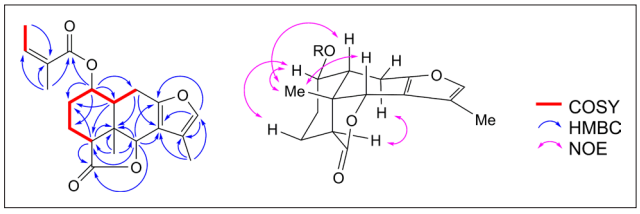


Figure 2. Selected COSY, HMBC, and NOESY correlations of compound **6**.

Table 3. Differences in the ITS1-5.8S-ITS2 Region Among *Ligularia dictyoneura* Samples.[ab]

	ITS1																									5.8S										ITS2																						
												1	1	1	1	1	1	2	2	2	2	2	2	2	2		1	1	1	1	1	1	1	1	1											1	1	1	1	1	1	1	1	1	2	2	2
	1	2	2	4	4	4	5	6	6	7	8	3	3	3	6	7	8	1	1	1	2	2	2	3	4	1	0	1	1	2	2	3	3	4	5			1	1	2	2	3	4	5	7	9	0	0	1	6	6	7	8	8	9	0	1	2
Sample no.[c]	1	2	6	1	7	8	0	7	8	3	9	2	5	9	7	3	1	2	8	9	0	3	5	5	6	2	7	0	2	2	4	3	6	0	1	4	8	4	9	5	7	2	2	6	2	3	4	8	6	0	9	9	0	2	9	2	4	0
A	C	G	T	A	G	G	C	T	C	T	C	T	C	T	C	C	A	T	T	T	T	G	G	G	C	C	G	C	C	C	G	T	C	G	C	G	C	C	C	C	C	G	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
B[e]	C	G	T	A	G	S	C	T	C	T	C	T	C	T	C	C	A	T	T	T	T	G	G	G	C	C	G	C	C	C	G	T	C	G	C	R	C	C	C	C	C	G	A	T	A	G	T	G	G	T	A	C	T	C	Y	C	C	T
C[e]	C	G	T	A	G	G	C	T	C	T	C	T	C	T	C	C	W	T		d		G	G	G	C	C	G	C	C	C	G	T	Y	G	C	G	C	Y	C	C	C	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
1	C	G	T	A	G	G	T	T	C	T	C	T	C	T	C	C	A	T	T	T	T	G	R	R	C	C	G	C	C	C	G	T	C	R	C	G	Y	C	C	Y	C	G	A	W	A	G	T	R	G	T	A	C	T	C	C	C	C	T
2	C	G	T	A	R	G	Y	T	C	T	Y	T	C	T	C	C	A	T	T	T	T	G	G	G	C	C	G	C	C	C	G	T	C	G	C	G	C	C	C	C	C	G	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
3	C	G	T	A	G	G	C	T	C	T	C	T	C	T	C	C	A	T		d		G	G	G	C	C	G	C	C	C	G	T	Y	G	C	G	C	C	C	C	C	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
4	C	G	T	A	G	G	Y	T	C	T	C	T	C	T	C	C	A	T		d		G	R	G	C	C	G	C	C	C	G	T	Y	G	Y	G	C	C	C	Y	C	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
5	C	G	T	A	G	G	C	T	C	T	C	T	C	T	C	C	W	T		d		G	G	G	C	C	G	C	C	C	G	T	Y	G	C	G	C	C	C	C	C	R	A	T	A	G	T	R	G	T	A	C	T	C	C	C	C	T
6	C	G	T	A	G	G	C	T	C	T	C	T	C	T	C	C	A	T		d		G	G	G	C	C	G	C	C	C	G	T	Y	G	C	G	C	C	C	C	C	R	A	T	A	G	T	G	G	T	A	Y	T	C	C	C	C	T
7	C	G	T	A	G	G	C	T	C	T	C	T	C	T	C	C	A	T		d		G	G	G	C	C	G	C	C	C	G	T	Y	G	C	G	C	C	C	C	C	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
8	C	G	T	W	G	G	Y	T	C	T	C	T	C	T	C	C	W	Y		d		G	G	G	C	C	G	C	C	Y	G	T	Y	G	Y	G	C	C	C	C	C	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
9[f]	C	G	Y	A	G	G	Y	T	S	T	C	Y	Y	T	Y	C	W	T		d		G	G	G	C	C	G	C	C	C	S	T	Y	G	C	G	C	Y	C	C	C	R	A	T	A	R	T	G	R	T	A	C	T	C	C	C	C	T
10	C	G	T	A	G	G	C	T	S	T	C	T	C	T	C	C	A	T		d		G	G	G	C	C	G	S	S	Y	G	T	Y	G	C	G	C	C	Y	C	C	R	A	T	R	G	T	G	G	T	A	C	T	C	C	C	C	T
11	C	G	T	A	G	G	C	T	S	T	C	T	C	T	C	C	W	T		d		G	G	G	Y	C	G	C	C	Y	G	T	Y	G	C	G	C	C	C	C	C	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
12	C	G	T	A	G	G	C	T	C	T	C	T	C	T	C	C	A	T		d		G	G	G	C	C	G	C	C	C	G	T	Y	G	C	G	C	C	C	C	C	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
13	C	G	T	A	G	G	C	T	C	T	C	T	C	T	C	Y	W	T		d		G	G	G	C	C	G	C	C	C	G	T	Y	G	C	G	C	C	C	C	C	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
14	C	G	T	A	G	G	C	T	C	T	C	T	C	T	C	C	A	T		d		G	G	G	C	C	G	C	C	C	G	T	Y	G	C	G	C	C	C	C	Y	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
15	Y	G	T	A	G	G	T	T	C	T	Y	T	C	T	C	C	A	T	T	T	T	R	G	G	C	C	G	C	C	C	G	T	T	G	C	G	C	C	C	C	C	G	A	T	A	G	T	G	G	T	A	C	T	C	C	Y	C	T
16	C	G	T	A	G	G	Y	T	C	T	Y	T	C	T	C	C	A	T		d		G	G	G	C	Y	G	C	C	C	G	T	C	G	C	G	C	C	C	C	C	G	A	T	A	G	T	G	G	T	R	C	T	C	C	C	C	T
17[g]	C	G	Y	A	G	G	T	T	C	T	C	T	C	T	C	C	A	T		d		G	R	G	C	C	-	C	C	C	G	T	Y	G	C	G	C	C	C	C	C	G	A	T	A	G	T	G	G	Y	A	C	T	C	C	C	C	T
18	C	G	T	A	G	G	Y	T	B	T	C	T	C	T	C	C	A	T		d		G	G	G	Y	C	G	C	C	Y	G	T	Y	G	C	K	C	C	C	C	C	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
19	C	G	T	A	G	G	C	T	C	T	C	T	C	T	C	C	A	T		d		G	G	G	Y	C	G	C	C	C	G	T	Y	G	C	G	C	C	C	C	C	R	A	T	A	G	T	G	G	T	A	C	T	C	C	C	C	T
20	C	R	C	A	G	G	T	T	C	C	C	C	C	G	C	C	A	T	T	T	T	G	G	G	C	C	G	C	C	C	G	C	C	G	C	G	C	C	C	C	C	G	G	T	A	G	Y	G	G	T	A	C	Y	T	C	C	T	C

[a]K = G + T; R = A + G; S = C + G; W = A + T; Y = C + T; - = deletion.

[b]The base numbering based on the sequence of sample 15, which had been deposited in the database (AB299047).

[c]Samples A to C, the present work; samples 1 to 20, our previous work.[8]

[d]A sequence with TTTT was also present.

[e]A sequence with AAA in place of AA at 252 to 253 of ITS1 was also present.

[f]A sequence without AC at 163 to 164 of 5.8S was also present.

[g]A sequence with T in place of GGA at 170 to 172 of ITS2 was also present.

numbers are 2012-52, 2007-25, and 2007-103, respectively (Kunming Institute of Botany).

Isolation of Compounds

Extraction of dried root of sample A (22.4 g) with EtOH afforded an extract (2.08 g). Silica-gel CC (*n*-hexane-EtOAc, gradient) of the extract afforded 5 fractions. CC (*n*-hexane-EtOAc) of Fr. 1 (eluted with *n*-hexane) afforded 7 fractions (Fr. 1-1 to 1-7). From Frs. 1-2 and 1-3, **1** (17.1 mg) and **3** (63.4 mg) were obtained, respectively, without further purification. From Fr. 1-5, **4** (6.2 mg) was isolated after purification with HPLC (*n*-hexane-EtOAc 80:20). From Fr. 1-6, **2** (1.9 mg) was isolated with HPLC (*n*-hexane-EtOAc 90:10). CC (*n*-hexane-EtOAc) and HPLC (*n*-hexane-EtOAc 75:25) of Fr. 4 (eluted with *n*-hexane-EtOAc 80:20) afforded **5** (10.4 mg) and **6** (30.3 mg)..

CC (SiO_2, *n*-hexane-EtOAc, gradient) of a part (182.8 mg) of the EtOH extract of dried root of sample B (7.7 g) afforded 4 fractions. From Fr. 1 (eluted with *n*-hexane), **1** (0.4 mg), **3** (1.4 mg), **4** (5.1 mg), and **8** (6.7 mg) were isolated by repeated CC (*n*-hexane-EtOAc). Fr. 2 consisted of **2** (27 mg). From Frs. 3 and 4, **5** (5.0 mg) was obtained by repeated CC (*n*-hexane-EtOAc). Compound **7** (11 mg) was isolated from another part of the root extract (200.1 mg) by CC (*n*-hexane-EtOAc, 20:1).

CC (SiO_2, *n*-hexane-EtOAc, 20:1) of a part (166.7 mg) of the EtOH extract of dried root of sample C (7.7 g) afforded 4 fractions. From Fr. 1, **1** (0.8 mg), **3** (5.9 mg), and **8** (1.1 mg) were isolated by repeated CC (*n*-hexane/EtOAc 100:1 and 80:1). From Fr. 2, **10** (4.5 mg) was obtained by CC (*n*-hexane/EtOAc 40:1). From Fr. 3, **2** (6.1 mg), **5** (5.9 mg), and **9** (16 mg) were obtained by repeated CC (*n*-hexane/EtOAc 20:1 and *n*-hexane/Et_2O 80:1). From Fr. 4, **5** (7.2 mg) was obtained by CC (*n*-hexane/EtOAc 20:1). Compound **7** (12 mg) was obtained from another part of the root extract (194.5 mg) by repeated CC (*n*-hexane-EtOAc, 20:1).

Compound **6**

An oil.

$[a]_D^{25}$ +67.2 (*c*, 0.361, MeOH).

IR (neat/NaCl): 1780, 1711, 1645, 1234, 1157, 945, 756 cm^{-1}.

^1H and ^{13}C NMR (C_6D_6): see Table 2.

MS (CI, CH_4): *m/z* (%) = 345 [$M+H^+$] (95), 245 [$M+H^+$-AngOH] (100), 199 (51).

HRMS-CI *m/z* [$M+H^+$] calcd for $C_{20}H_{25}O_5$: 345.1702; found: 345.1697.

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