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Diversity of Furanoeremophilane Composition in *Ligularia tongolensis*

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Abstract

The composition of root chemicals was studied for 7 samples of *Ligularia tongolensis* collected in Yunnan and Sichuan Provinces of China. The structures of 2 new 3β-angeloyloxy-6β-acyloxyfuranoeremophilan-15-oic acids were determined. It was found that the plant harbors chemical diversity in the acyloxy groups in 3,6-bis(acyloxy)eremophilan-15-oic acids. The presence of a 3-methylpentanoate moiety at C-3 appears geographically differentiated to a degree. Consistent with this low diversity, results of DNA analysis indicated little genetic differentiation, although introgression was inferred for one of the samples.

Keywords

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

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The genus *Ligularia* is highly diversified in the Hengduan Mountains area of China. We have been studying the chemical diversity within each species while referring to DNA data as an index of genetic diversification. To date, we have found various degrees of intraspecific diversity in many species.[1,2] Sesquiterpenoids of the eremophilane type have been found in many species; furanoeremophilanes, in particular, are the major components in major *Ligularia* species. *Ligularia tongolensis* (Franch.) Hand.-Mazz. is found in northwestern Yunnan, western Sichuan, and southeastern Tibet.[3] We previously reported that *L. tongolensis* in northwestern Yunnan Province (Shangrila County) and southwestern Sichuan Province (Daocheng County) produced 3,6-bis(acyloxy)furanoeremophilan-15-oic acids and 3-acyloxyfuranoeremophilan-15,6-olides.[4] Related eremophilane-(12,8),(15,6)-diolides were isolated by Han et al.[5] The chemical composition, judged from TLC patterns, was very similar among our *L. tongolensis* samples except for the presence of tetradymol in some samples collected in Shangrila County.[4] Hybrids of *L. tongolensis* and *L. cymbulifera* were also analyzed and the ability to produce tetradymol in *L. tongolensis* was inferred to have been introgressed from *L. cymbulifera*.[6]

In the course of our subsequent search in the field, we collected 32 additional samples of *L. tongolensis*. Seven of the samples (samples 1-7), collected in 2015 to 2017 at locations shown in Table 1 and Figure 1, were analyzed in detail. Samples 1 and 2 (4 km apart from each other) were collected in Wenchuan County, Sichuan, which is about 500 km from the collection area of our previous *L. tongolensis* samples[4] (Figure 1, red circle). Sample 3 was collected at Pachahai, Shangrila County, Yunnan, where the above hybrid samples were collected.[6] Sample 4 was collected at Tianchi, Shangrila County, where we collected various *Ligularia* hybrids, for which the chemical outcomes of hybridization were described.[7-9] Samples 5, 6 (4 km apart from each other), and 7 were collected in Muli County, Sichuan.

The EtOH extract of each root sample was subjected to Ehrlich's test on TLC, a facile method to detect furanoeremophilanes.[10,11] All the samples showed TLC spots similar to those of our previous samples, suggesting that the composition of furanoeremophilanes was similar. Sample 4 showed an additional strong Ehrlich-positive spot of tetradymol at R_f = 0.66 (hexane/EtOAc 7:3).

Compounds in each sample were separated with such standard methods as silica-gel column chromatography (CC) and

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Table 1. Collection Locales and Isolated Compounds of *Ligularia tongolensis* Samples.

Sample no.	Specimen no.	Locality[a]	Elevation (m)	Isolated compounds
1	2015-04	Balangshan (Wenchuan, Sichuan)	2900	**1a-1d, 3**
2	2015-05	Balangshan (Wenchuan, Sichuan)	3100	**1a, 1c, 1d, 2**
3	2016-220	Pachahai (Shangrila, Yunnan)	3600	**1b, 3, 5**
4	2016-221	Tianchi (Shangrila, Yunnan)	3600	**1a-1d, 4**
5	2017-02	Liziping (Muli, Sichuan)	3600	**1a-1d, 6, 7**
6	2017-06	Liziping (Muli, Sichuan)	4000	**5**
7	2017-27	Wachang (Muli, Sichuan)	3900	**1a-1d**

[a]County and province in parentheses.

HPLC, while air oxidation and decomposition were carefully avoided. From the Wenchuan samples (samples 1 and 2), **1a** to **1d** were obtained together with **2**[12-14] and **3**.[12] While compounds **1a**[12,15] and **1b**[12] were known, **1c** and **1d** were new. From sample 3, **1b**, **3**, and **5**[4] were isolated. From sample 4, tetradymol (**4**)[16] was isolated in addition to a mixture of **1a** to **1d**. From 2 of the Muli samples (samples 5 and 7), **1a** to **1d** were obtained. β-Bisabolene (**6**) and β-eudesmol (**7**) were also isolated from sample 5. In contrast, **5** was isolated as the major component from sample 6 (Figure 2).

Attempts to isolate the new compounds **1c** and **1d** in pure form by chromatography were unsuccessful. Their structures were deduced from the MS and NMR spectra of a mixture of **1a**, **1c**, and **1d**. ^{1}H and ^{13}C NMR signals of the furanoeremophilane skeleton and those of an angelate at C-3 of **1a**, **1c**, and **1d** almost overlapped, indicating that **1c** and **1d** were furanoeremophilanes with an angeloyloxy group at C-3β (Table 2). The difference among the compounds was in the acid part of the ester moiety at C-6. High-resolution LCMS (ESI) of the mixture showed 3 peaks of **1a**, **1c**, and **1d** (**1a**: m/z 467.2022, $C_{25}H_{32}O_7$; **1c**: m/z 469.2227, $C_{25}H_{34}O_7$; **1d**: m/z 481.2176, $C_{26}H_{34}O_7$) ($[M+Na]^+$). A fragment ion resulting from the elimination of ROH at C-6 was observed at m/z 367.1486 ($[M+Na\text{-}ROH]^+$) for each of **1a**, **1c**, and **1d**. The acyloxy group at C-6 (allylic) is eliminated more easily than that at C-3.[12] The molecular formula of **1c** indicated that the acyloxy group at C-6 was -$OCOC_4H_9$ and its structure was determined to be 2-methylbutanoate from the correlations shown in Figure 3. The acyloxy group in **1d** was similarly determined to be 3-methylpent-2-enoate (Figure 3). The geometry of the double bond was deduced to be the *E*-form from the δ value of H_3-6" (δ 2.12). The 2 methyl groups of the senecioate (= 3-methylbut-2-enoate) moiety in 6-senecioyloxy-1,10-epoxyfuranoeremophilane was observed at δ 2.13 (*Z*-Me) and 1.43 (*E*-Me) in C_6D_6.[17] HMBC correlation was observed between H-6 and C-1" for both **1c** and **1d**, supporting that **1a**, **1c**, and **1d** are variants of the acyloxy group at C-6.

The chemical composition was also analyzed for each sample by LCMS. The total ion chromatograms (TICs) of samples 1 to 7 are shown in Figure 4. In samples 1 and 2, **1a** and **1c** were the major components. Compound **2**, detected as another major peak, is a lactonization product of **1a** to **1d** (vide infra). In sample 3, **1b** and **5** were detected. In sample 4, **1a** to **1d** and tetradymol (**4**) were detected. In samples 5 and 7, another

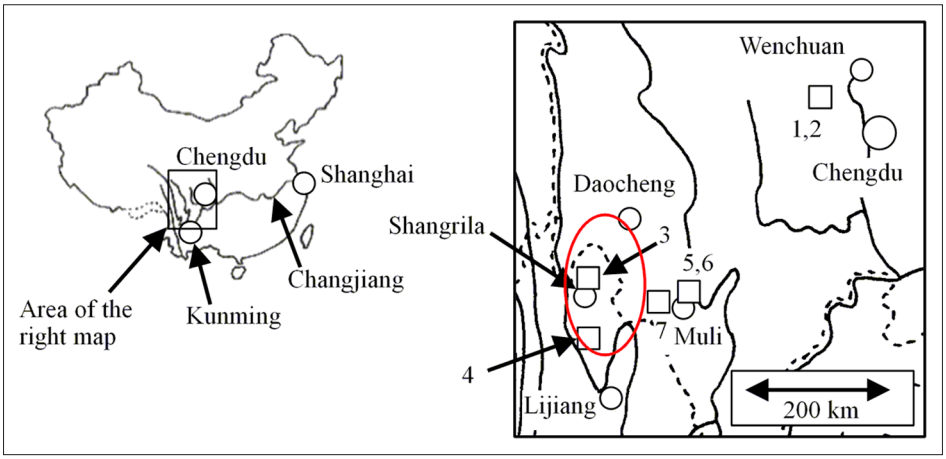


Figure 1. Collection locations of the present *Ligularia tongolensis* samples (squares). Circles indicate major cities. Solid and dotted lines indicate rivers and province boundaries, respectively. The red circle indicates the collection area of our previous 17 samples.[4]

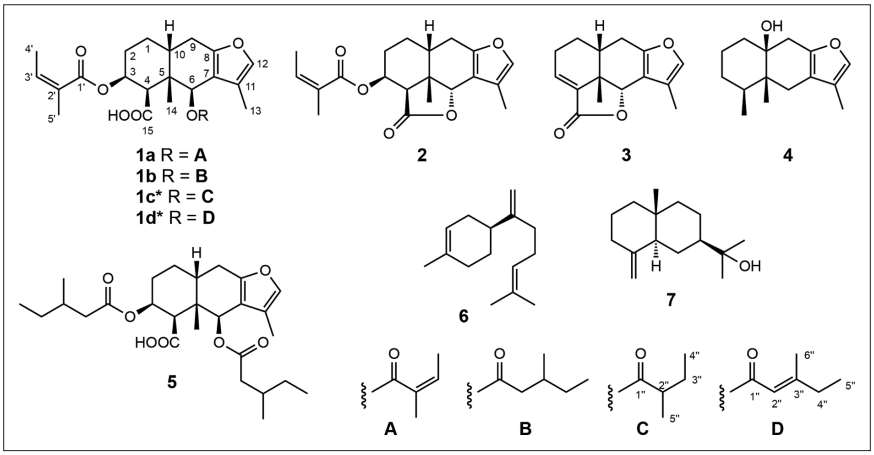


Figure 2. Isolated compounds from *Ligularia tongolensis*. The asterisks denote new compounds.

Table 2. ^{1}H and ^{13}C NMR of New Compounds **1c** and **1d** (C_6D_6).[a]

Carbon no.	**1c**		**1d**	
	δ_H	δ_C	δ_H	δ_C
1	0.97-1.08 (2H, m)[b]	26.4-26.5[b]	0.97-1.08 (2H, m)[b]	26.4-26.5[b]
2	1.82-1.92 (m)[b,c] 2.11-2.26 (m)[b,c]	26.9	1.90-2.00 (m)[b,c] 2.18-2.31 (m)[b,c]	26.9
3	5.65-5.75 (m)[b,c]	70.1	5.88 (br dt, 12.0, 5.8)	70.1
4	3.30-3.35 (m)[b,c]	49.9	3.41-3.45 (m)	50.1
5		41.6		41.6
6	6.48 (br s)	68.1	6.61 (br s)	67.4
7		114.7		115.1
8		150.3		150.1
9	1.95-2.04 (m)[b,d] 2.60-2.69 (m)[b,d]	26.4-26.5[b]	1.95-2.04 (m)[b,d] 2.60-2.69 (m)[b,d]	26.4-26.5[b]
10	2.37-2.50 (m)[b,d]	37.0	2.37-2.50 (m)[b,d]	36.9
11		119.9		120.0
12	6.91 (s)	139.1	6.92 (s)	139.1
13	1.88 (d, 1.1)	9.1	1.93 (d, 1.2)	8.9
14	1.13 (br s)[b,d]	19.4	1.13 (br s)[b,d]	19.5
15		179.0		179.0
1'		166.3		166.3
2'		127.8-128.4[b]		127.8-128.4[b]
3'	5.67 (br q, 7.3)[b]	138.5	5.67 (br q, 7.3)[b]	138.5
4'	1.95-2.00 (m)[b,d]	15.9	1.95-2.00 (m)[b,d]	15.9
5'	1.82-1.85 (m)[b,d]	20.7	1.82-1.85 (m)[b,d]	20.7
1"		176.3		167.0
2"	2.30 (quint d, 7.2, 6.5)	41.8	5.75-5.78 (m)	113.9
3"	1.34 (dqd, 13.5, 7.3, 6.5) 1.79-1.89 (m)[b,c]	26.5	-	163.8
4"	0.86 (t, 7.3)	12.1	1.66 (qd, 7.4, 1.0)	33.8
5"	1.20 (d, 7.2)	17.6	0.66 (t, 7.4)	11.1
6"			2.12 (d, 0.9)	19.1

[a]Determined on a mixture of **1a**, **1c**, and **1d**.
[b]The equivalent signals of **1a**, **1c**, and **1d** overlapped.
[c]Determined from the COSY spectrum.
[d]No exact value could be determined.

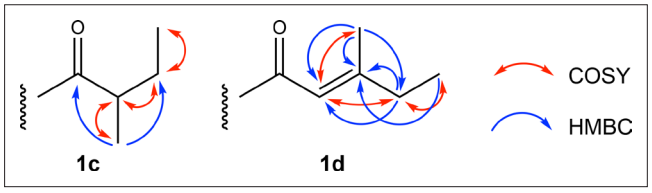


Figure 3. Key COSY and HMBC correlations for the acid part of **1c** and **1d** at C-6.

compound was detected at t_R = 19.4 minutes in addition to **1a** to **1d** (Figure 4, compound **A**). Its mass spectrum (*m*/*z* 345, 245) was very similar to those of **1a** to **1d**, suggesting that it was a related furanoeremophilan-15-oic acid; however, we could not identify the compound. In sample 6, **5** was detected as the most major component. Some additional peaks were observed in samples 6 (t_R about 18 minutes) and 7 (t_R 16-17 minutes); however, we were unable to identify the compounds of the peaks.

The LCMS data indicate that the ratio of 3,6-bis(acyloxy)-eremophilan-15-oic acids, **1a** to **1d** and **5**, was variable among the samples. These compounds are indistinguishable by TLC and the variation could not be uncovered in our previous study.[4] This raised the possibility that there may have been the variation among our previous samples as well. Therefore, we analyzed some of our previous samples collected in 2003, with newly assigned sample numbers of 8 to 21 (Supplemental Table S1 for the correspondence between the new and old sample numbers), and the 25 additional samples collected in 2004 to 2011 (samples 22-46) by LCMS. The TICs of samples 8 to 11 are shown in Figure 5 (Supplemental Figures S1-S6 for the others). Four peaks were detected at t_R = 8.2 (*m*/*z* 245; **3**), 13.1 (*m*/*z* 345, 245; **2**), 13.3 (*m*/*z* 347, 245), and 15.1 (*m*/*z* 361, 245) minutes. The absence of the 3,6-bis(acyloxy) compounds **1a** to **1d** and the concomitant presence of **2** and **3** were presumably due to lactonization and elimination during storage of the extracts in EtOH: **1a** to **1d** were converted into **2**[12] and then into **3** (Scheme 1). The structures of the compounds in

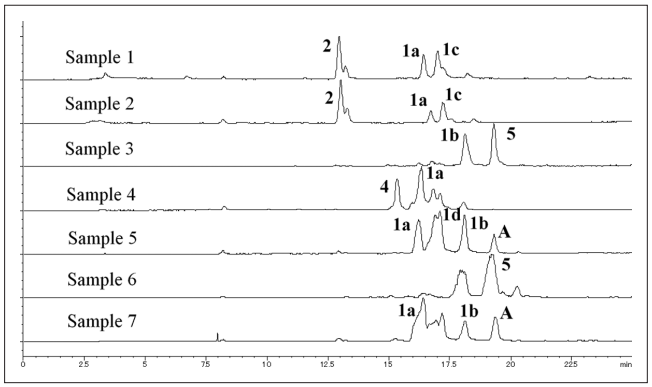


Figure 4. Total ion chromatograms of samples 1 to 7. The differences in the retention time among the samples are within experimental error.

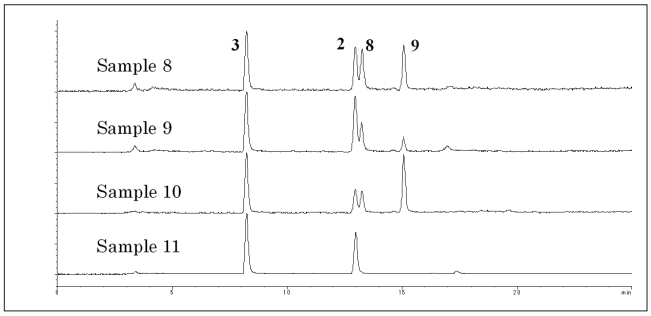
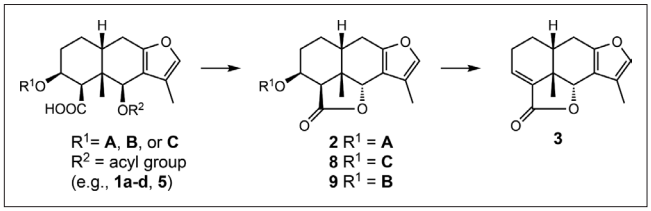


Figure 5. Total ion chromatograms of samples 8 to 11.

the peak at t_R = 13.3 and 15.1 were identified as **8**[13] and **9**,[4] respectively, on the basis of the LCMS data obtained with previously purified materials. Compound **8** has been isolated from *L. hookeri*[13] but not from *L. tongolensis*; however, its lactone derivative, an eremophilane-(12,8),(15,6)-diolide with a 2-methylbutanoate moiety at C-3, was isolated from *L. tongolensis* by Han et al.[5] Eremophilan-12,8-olides are likely to be derived from furanoeremophilanes.[18,19] Compound **9**, presumably generated from **5** or related compound(s), was previously isolated from *L. tongolensis*.[4] Compounds **8** and **9** also result in **3** through the elimination of the C-3 ester moiety (Scheme 1).

Supposing that the rate of the conversion of **2**, **8**, and **9** into **3** is the same, the variable ratios of **2**, **8**, and **9** in samples 8 to 11, seen in Figure 5, indicates a variation in the acyloxy group at C-3. Among the furanoeremophilanes obtained from *Ligularia*, compounds with 3-methylpentanoyloxy moiety at C-3 (**5** and/or **9**) have been isolated only from *L. tongolensis*[4,6] and *L. hookeri*.[13] On the criterion of the presence/absence of these characteristic compounds, all the 46 samples could be grouped into a **5**/**9**-producing and a nonproducing type. The producing type includes 16 samples from Yunnan (samples 3, 8-10, 13, 14, 16-19, 23-26, 31, and 46) and 3 samples from southern Sichuan (6, 35, and 37). The nonproducing type includes most of the other samples from Sichuan (samples 1, 2, 5, 7, 11, 20, 21, 28-30, 32-34, 36, 38-45) and the other 5 samples from Yunnan (samples 4, 12, 15, 22, and 27) (Figures 4, 5, S1-S6). This suggests some geographical differentiation in the presence of 3-methylpentanoic acid. Our previous study did not find **1a** to **1d** in our samples collected in 2002.[4] Unfortunately, we could not reanalyze the 2002 samples due to decomposition; however, the samples may have been of the



Scheme 1. Lactonization of 3,6-bis(acyloxy)furanoeremophilan-15-oic acids. See Figure 2 for **A**, **B**, and **C**.

5/9-producing type, because **5** and **9** were isolated as major components.

The base sequence of the ITS1-5.8S-ITS2 of the ribosomal RNA gene in the nuclear genome was determined for samples 1 to 11. The results are shown in Table 3. Sample 6 has an exceptionally large number (27) of multiple-base sites. As we discussed previously, it indicates hybridization with some other species.[6] Since the morphology of the sample is of *L. tongolensis*, it must have undergone backcrossing; namely, it is introgressed. The bases at these sites cannot be accounted for by the superposition of *L. tongolensis* and *L. cymbulifera* sequences (Table 3). The hybridization partner is currently unknown because database search using a putative introgressive sequence found no clear candidate with a very good agreement. The sequences of the other samples are essentially the same, indicating that they are not differentiated genetically.

Tetradymol (**4**) was detected in sample 4 as a major component. Tetradymol is a major compound of *L. cymbulifera*,[4] a species abundant in the Shangrila area, Yunnan, and it is considered ecologically useful as an allelopathic.[20] As described previously, the ability to produce tetradymol in *L. tongolensis* is likely to be acquired through introgression from *L. cymbulifera*.[6] However, no evidence of hybridization is seen in the ITS1-5.8S-ITS2 sequence of sample 4 (Table 3). One possible explanation is that backcrossing eliminated the *L. cymbulifera* sequence from the rRNA gene locus while the gene(s) for tetradymol production remained in sample 4.

In conclusion, *L. tongolensis* harbors some chemical diversity in the acyloxy groups of 3,6-bis(acyloxy)eremophilan-15-oic acid. This range of variation within the same terpenoid framework suggests that the diversity in *L. tongolensis* is limited, which appears consistent with DNA sequence data.

Experimental

General

NMR, JEOL ECX-400 spectrometer (400 MHz for ^{1}H; 100 MHz for ^{13}C) or Varian Unity Plus 500 spectrometer (500 MHz for ^{1}H; 125 MHz for ^{13}C); 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). Analytical TLC was carried out on Merck Kieselgel 60 F254, 0.2 mm thickness, with either Ehrlich's reagent (*p*-dimethylaminobenzaldehyde and HCl)[10,11] or *p*-anisaldehyde/AcOH/H_2SO_4 as visualizing agent. HPLC was carried out by use of either a Shimadzu LC-20AT pump with an SPD-20A Prominence UV/VIS detector, a GL Sciences GL-7410 pump with a GL-7450 UV detector, or a JASCO 880-PU pump with an 875-UV detector, and a Hitachi D-2500 Chromato-Integrator, a Shimadzu C-R8A Chromatopac, or a SOMA OPTICS MDL-102 recorder, with either a GL Sciences Inertsil PREP-ODS column (20 × 250 mm), a Kanto Mightysil Si60 (10 × 250 mm) column, or a Nacalai Tesque Cosmosil 5SL-II (10 × 250 mm) column. LCMS was measured on an Agilent 1100 series LC/MSD mass spectrometer. See Reference 21 for details. High-resolution LCMS (ESI) was measured on a Waters SYNAPT G2-Si HDMS with a UPLC-I Class system and an electrospray ionization interface. UPLC was carried out by use of an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, Waters).

Plant Material

Samples were collected in August 2015, 2016, and 2017 at locations shown in Table 1 and Figure 1. Each sample was identified by X. G. (author). Voucher specimen numbers are 2015-04, 2015-05, 2016-220, 2016-221, 2017-02, 2017-05, and 2017-27 for samples 1 to 7, respectively (Kunming Institute of Botany).

Isolation of Compounds

EtOH extraction of dried root of sample 1 (23.7 g) afforded an extract (2.24 g). CC (*n*-hexane-EtOAc, gradient) of the extract resulted in 9 fractions. CC (*n*-hexane-EtOAc) of fraction 5 (eluted with *n*-hexane-EtOAc 90:10) afforded a mixture of **1a, 1b, 1c** (121.5 mg, ratio 1:1:2). From fraction 6 (eluted with *n*-hexane-EtOAc 90:10 to 80:20), 3 mixtures of **1a, 1c, 1d** (108.2 mg, ratio 10:5:2; 17.8 mg, ratio 6:2:1; 43.7 mg, 8:3:1) and **3** (0.3 mg) were obtained by CC (*n*-hexane-EtOAc) and HPLC (*n*-hexane-EtOAc). From fraction 7 (eluted with *n*-hexane-EtOAc 80:20), 2 mixtures of **1a, 1c, 1d** (5.5 mg, ratio 7:4:1; 11.6 mg, ratio 5:1:1) were obtained by CC (*n*-hexane-EtOAc) and HPLC (*n*-hexane-EtOAc).

EtOH extraction of dried root of sample 2 (19.6 g) afforded an extract (1.25 g). CC (*n*-hexane-EtOAc, gradient) of the extract afforded 8 fractions. From fraction 4 (eluted with *n*-hexane-EtOAc 90:10), **2** (1.1 mg) was obtained by repeated HPLC (*n*-hexane-EtOAc). From fraction 5 (eluted with *n*-hexane-EtOAc 90:10 to 80:20), a mixture of **1a, 1c, 1d** (351.0 mg, ratio 7:5:1) was afforded by CC (*n*-hexane-EtOAc) and HPLC (*n*-hexane-EtOAc). From fraction 6 (eluted with *n*-hexane-EtOAc 80:20 to 60:40), **1a** (0.4 mg) was obtained by HPLC (*n*-hexane-EtOAc).

EtOAc extraction of dried root of sample 3 (1.9 g) afforded an extract (116.2 mg). CC (*n*-hexane-EtOAc, gradient) of the extract afforded 6 fractions. From fraction 3 (eluted with *n*-hexane-EtOAc 95:5 to 90:10), **1b** (2.2 mg), **3** (1.1 mg), and **5** (0.1 mg) were obtained by repeated HPLC (*n*-hexane-EtOAc).

EtOH extraction of dried root of sample 4 (6.9 g) afforded an extract (630.5 mg). CC (*n*-hexane-EtOAc, gradient) of the extract afforded 9 fractions. From fraction 3 (eluted with *n*-hexane-EtOAc 96:4 to 94:6), **4** (0.8 mg) was obtained by CC (*n*-hexane-EtOAc) and HPLC (*n*-hexane-EtOAc). From fraction 4 (eluted with *n*-hexane-EtOAc 94:6 to 92:8), a mixture of **1a, 1b, 1c, 1d** (69.9 mg, ratio 2:1:3:1) was obtained by HPLC (*n*-hexane-EtOAc). From fractions 5 (eluted with *n*-hexane-EtOAc 92:8 to 86:14) and 6 (eluted with *n*-hexane-EtOAc 86:14 to 80:20), mixtures of **1a, 1c, 1d** (216.3 mg, ratio 2:2:1; 51.5 mg, ratio 3:1:2) were obtained.

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

Sample no.	ITS1																												5.8S								ITS2																			
	6	11	12	21	25	26	62	68	92	101	103	104	110	112	116	127	132	144	166	167	178	198	210	215	231	237	238	239	246	1	5	34	122	124	136	157	164	3	15	19	20	27	28	36	45	101	107	111	115	174	182	188	209	213	215	220	221
1	A	C	C	C	C	C	C	C	G	C	T	C	T	G	T	A	C	T	G	C	G	K	C	T	T	A	C	A	C	C	C	C	Y	G	C	C	C	T	A	T	G	Y	G	A	G	C	C	C	C	G	Y	C	T	G	T	T	T
2	A	C	C	C	C	C	C	C	G	C	T	C	T	G	T	A	C	T	G	C	G	G	C	T	T	A	C	A	C	C	C	Y	Y	G	C	C	C	T	A	T	G	Y	G	A	G	C	C	Y	C	G	C	C	T	G	T	T	T
3	A	C	C	C	C	C	Y	C	G	C	T	C	T	G	T	A	C	T	R	C	G	G	C	T	T	A	C	A	Y	C	C	C	C	G	C	C	C	T	A	T	G	C	G	A	G	C	C	C	Y	G	C	C	T	R	T	T	T
4	A	T	C	Y	C	Y	C	C	T	C	T	C	T	G	T	A	T	T	G	C	T	G	C	T	T	A	C	A	C	C	C	C	C	A	C	Y	Y	C	R	T	G	C	G	T	G	C	Y	C	C	K	C	C	T	G	T	T	T
5[b]	W	C	C	C	Y	C	C	C	G	C	T	C	T	G	T	A	C	T	G	C	G	G	C	T	T	A	C	A	C	C	Y	C	C	G	C	C	C	T	A	T	G	C	K	W	G	C	C	Y	C	G	C	C	T	G	T	T	T
6	W	C	C	C	C	C	C	M	G	Y	W	M	K	R	Y	A	C	K	G	C	G	G	C	T	Y	A	Y	R	C	Y	C	C	C	G	Y	Y	C	T	A	Y	R	Y	K	W	R	Y	C	Y	C	G	Y	Y	Y	G	Y	Y	T
7	T	C	C	C	C	C	C	C	G	C	T	C	T	G	T	A	C	T	G	C	G	G	C	T	T	A	C	A	C	C	C	C	C	G	C	C	C	T	A	T	G	C	T	T	G	C	C	T	Y	G	C	C	T	G	T	T	T
8[c]	A	C	C	C	C	C	Y	C	G	C	T	C	T	G	T	A	C	T	R	C	G	G	C	T	T	A	C	A	Y	C	C	C	C	G	C	C	C	T	A	T	G	C	G	A	G	C	C	C	Y	G	C	C	T	R	T	T	T
9	A	C	C	C	C	C	Y	C	G	C	T	C	T	G	T	A	C	T	G	C	G	G	Y	T	T	A	C	A	C	C	C	C	C	G	C	C	C	T	A	T	G	C	G	A	G	C	C	C	C	G	C	C	T	G	T	T	T
10[c]	A	C	C	C	C	C	Y	C	G	C	T	C	T	G	T	A	C	T	G	C	G	G	C	T	T	A	C	A	Y	C	C	C	C	G	C	C	C	T	A	T	G	C	G	A	G	C	C	C	Y	G	C	C	T	G	T	T	T
11	A	T	C	Y	C	C	C	C	T	C	T	C	T	G	T	A	T	T	G	C	T	G	C	T	T	A	C	A	C	C	C	C	C	A	C	C	C	C	R	T	G	C	G	T	G	C	Y	C	C	G	C	C	T	G	T	T	T
Ref[d]	A	C	C	C	C	C	C	C	G	C	T	C	T	G	T	A	C	T	G	C	G	G	C	T	T	A	C	A	C	C	C	C	C	G	C	C	C	T	A	T	G	C	G	A	G	C	C	C	T	G	C	C	T	G	T	C	C
Ref[e]	A	C	T	C	C	C	C	C	G	C	T	C	T	A	C	C	T	A	G	A	G	G	C	C	C	T	C	G	C	C	C	C	C	G	C	C	C	C	A	C	G	C	G	T	A	C	C	C	C	T	C	C	C	G	T	C	C

[a]The base numbering is according to the database entry DQ272337 of *L. tongolensis*. K = G + T; M = A + C; R = A + G; W = A + T; Y = C + T.
[b]A sequence with C_6 in place of C_5 at 154 to 159 of ITS1 was also present.
[c]A sequence with T_3 in place of T2 at 156 to 157 of ITS2 was also present.
[d]A *L. tongolensis* sequence in the database (DQ272337).
[e]A *L. cymbulifera* sequence in the database (DQ272330).

EtOH extraction of dried root of sample 5 (13.0 g) afforded an extract (1021.0 mg). CC (*n*-hexane-EtOAc, gradient) of the extract afforded 13 fractions. From fraction 2 (eluted with *n*-hexane-EtOAc 96:4), **6** (24.4 mg) was obtained. From fraction 9 (eluted with *n*-hexane-EtOAc 70:30), **7** (4.8 mg) and **2** (0.6 mg) were isolated by repeated HPLC (*n*-hexane-EtOAc). From fraction 10 (eluted with *n*-hexane-EtOAc 60:40), **1c** (16.1 mg), mixtures of **1a, 1b, 1c** (22.0 mg, ratio 1:2:3), of **1a, 1c, 1d** (18.3 mg, ratio 2:2:3), and of **1c, 1d** (24.2 mg, ratio 1:1) were obtained by HPLC (*n*-hexane-EtOAc).

EtOH extraction of dried root of sample 6 (1.7 g) afforded an extract (54.1 mg). CC (*n*-hexane-EtOAc, gradient) of the extract afforded 2 fractions. From the polar fraction (eluted with *n*-hexane-EtOAc 80:20 to 50:50), **5** (43.4 mg) was obtained.

EtOH extraction of dried root of sample 7 (4.2 g) afforded an extract (122.8 mg). CC (*n*-hexane-EtOAc, gradient) of the extract afforded 4 fractions. From fraction 3 (eluted with *n*-hexane-EtOAc 91:9 to 86:14), a mixture of **1a, 1b, 1c, 1d** (27.8 mg, ratio 7:2:6:2) was obtained by HPLC (*n*-hexane-EtOAc).

Compounds 1c and 1d

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

HR-LCMS (ESI): **1c**: *m*/*z* 469.2227 [M+Na^+] (100) (calcd for $C_{25}H_{34}O_7Na$: 469.2203), 367.1529 [M-C_4H_9COOH+Na^+] (32); **1d**: *m*/*z* 481.2176 [M+Na^+] (100) (calcd for $C_{26}H_{34}O_7Na$: 481.2203), 367.1529 [M-C_5H_9COOH+Na^+] (28).

DNA Analysis

Purification of DNA from dried leaves, amplification of the ITS1-5.8S-ITS2 region with polymerase chain reaction, purification of the amplification product, and DNA sequencing were carried out as previously described.[22]

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Supplemental Material

Supplemental material for this article is available online.

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