

Chemical and Genetic Study of *Ligularia tongolensis*, *Ligularia cymbulifera*, and *Ligularia atroviolacea* in the Hengduan Mountains of China

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Three *Ligularia* species (*L. tongolensis*, *L. cymbulifera*, and *L. atroviolacea*) were examined with respect to the chemical composition and nucleotide sequence. Furanoeremophilanes were found to be produced in the root of all samples of the three species collected in northwest Yunnan and southwest Sichuan. Eight furanoeremophilanes were identified, two of which were new. Most of the identified furanoeremophilanes were oxygenated at 3, 6, and 15-positions. The nucleotide sequence of the *atpB-rbcL* intergenic region was found to be essentially the same in the three species. These similarities imply that the three *Ligularia* species, all belonging to the section Corymbosae, are close to one another. The intra-specific diversity in the two widely distributed species in the Hengduan Mountains area, *L. tongolensis* and *L. cymbulifera*, were in contrast. Four out of 19 *L. tongolensis* samples contained a strongly Ehrlich-positive compound besides a number of positive compounds, and five variants of the *atpB-rbcL* sequence were found in these samples. In contrast, no variation was observed in 13 *L. cymbulifera* samples with respect to the furanoeremophilane composition or the *atpB-rbcL* sequence. The lack of diversity in *L. cymbulifera* probably resulted from the uniformity of its habitat.

Ligularia Cass., belonging to the family Compositae (Asteraceae), tribe Senecioneae, is a highly diversified genus, containing six sections and about 129 species, more than 100 species of which are distributed in the eastern Qinghai-Tibet Plateau and adjacent areas.[1] The species of *Ligularia* in the Hengduan Mountains, located in the eastern Qinghai-Tibet Plateau, are diverse in appearance, and occupy a great variety of habitats from forests to alpine meadows, ranging from 1000 to 4000 m in altitude. Thus, the Hengduan Mountains is considered to be the main centre of the on-going evolution and diversification of *Ligularia* species.[2] Morphological[2–4] and cytological studies[5–7] on the genus of the Hengduan Mountains have been reported. *Ligularia* species have also been studied with respect to secondary metabolites, and many sesquiterpenes of the furanoeremophilane type have been isolated from them.[8–12] However, no study has been carried out to investigate the relationship among morphological, genetic, and chemical variations in the genus.

In order to find correlates among these variations, and ultimately understand the diversity-generating mechanism of *Ligularia* species in the Hengduan Mountains, we initiated an extensive study that uses furanoeremophilanes as a chemical index and the DNA sequence as a genetic index. Furanoeremophilanes have been detected conventionally by Ehrlich's test,[13] which has been used in a search for novel natural products.[8–12,14,15] Since Ehrlich's test can be carried out on TLC plates, a primary statement of chemical compounds can be made without isolation. Thus, the method is suitable for studies of "intra-specific" chemical diversity, since many samples can be processed at once. On the other hand, a structure determination of the major components is necessary to discuss any "inter-specific" variation, since Ehrlich's test can only indicate the presence/absence of furano-compounds. As for the DNA sequence, we determined the nucleotide sequence of the *atpB-rbcL* intergenic region in the present study. The region is a segment between the *atpB* and the *rbcL* genes on the plastid DNA, and carries little genetic information. Consequently, mutations therein are likely to be neutral and have a larger probability of fixation. Besides, the region can be amplified in vitro by the polymerase chain reaction (PCR) with the same pair of primers for a wide range of plant species, because the abutting two genes are well conserved evolutionarily.[16] Variations among populations have been shown to exist in the region, although not in all species, and it has been used to study intra- and inter-specific diversity.[17]

This report focuses on *L. tongolensis* (Franch.) Hand.-Mazz. and *L. cymbulifera* (W. W. Smith) Hand.-Mazz., both belonging to the section Corymbosae.[18] These species are widely distributed in the Hengduan Mountains, and abundantly near Zhongdiang, Yunnan, and occupy a great variety of habitats

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Table 1. Collection Locality, Genetic Type, and Ehrlich's Test of *L. tongolensis* Samples

Sample	Locality	Elevation (m)	Genetic type[a)]	Ehrlich's test[b)]
1	Xiaozhongdian	3200	G-A9	−
2	Shuduhu (Shudu lake)	3500	G-A12	+
3	Bitahai (Bita lake)	3300	G-A9	−
4	South of Bitahai	3400	G-A12	−
5	South of Bitahai	3600	A-A9	−
6	South of Tianshengqiao	3400	G-A12	−
7	Tianshengqiao	3200	G-A11	+
8	Tianshengqiao	3200	A-A9	−
9	East of Zhongdian	3200	G-A12	−
10	Napahai (Napa lake)	3200	G-A12	−
11	North of Zhongdian	3300	G-A12	−
12	Zhongdian/Gezan	3400	G-A12	+
13	Gezan	3100	G-A12	−
14	Xiaoxueshan	3700	G-A11	−
15	Daxueshan	3800	G-A10	−
16	Daxueshan	3600	G-A12	+
17	Yading	3700	A-A9	−
18	Rencun	3700	A-A9	−
19	Rencun	3300	G-A12	−

a) See text for the designation. b) + and − indicate the presence and the absence of strongly Ehrlich-positive compound, respectively. All other Ehrlich-positive components were the same for the all samples.

ranging from 2000 to 4000 m in altitude. Chemical components were also examined for *L. atroviolacea* (Franch.) Hand.-Mazz., also belonging to Corymbosae, though only one sample could be collected.

Results

Nineteen samples of *L. tongolensis* (Table 1 and Fig. 1) and 13 samples of *L. cymbulifera* (Table 2 and Fig. 1) were collected in northwestern Yunnan and southwestern Sichuan. Since a preliminary test showed that Ehrlich-positive compounds are mostly contained in roots, but not in leaves, the roots of each samples were collected for a chemical study. Without drying, the plant materials were extracted with ethanol, and the extracted alcoholic solutions were subjected to Ehrlich's test on TLC. All of the 19 *L. tongolensis* extracts contained compounds positive to Ehrlich's test. Major Ehrlich-positive compounds were observed at $R_f = 0.57$ and 0.38–0.49 (hexane/AcOEt = 7:3), which were included in all of the samples. In addition, a strongly positive compound ($R_f = 0.61$) was present in samples 2, 7, 12, and 16 (Table 1). It was one of the major Ehrlich-positive compounds in samples 2 and 7 and a minor one in samples 12 and 16. In contrast, all of the thirteen *L. cymbulifera* samples contained the same Ehrlich-positive compounds, including a strongly positive compound ($R_f = 0.63$).

DNA was purified from leaves of the same samples and the nucleotide sequence of the *atpB-rbcL* region was determined. Five variant types were identified in the 19 samples of *L. tongolensis* (Table 1). The sequence of the major variant (for example, sample 2) is shown in Fig. 2 (The data have been deposited to GenBank,™ EMBL, and DNA Data Bank of Japan, accession number AB126944). Differences among the variants were observed at the 28th nucleotide and at an A stretch around the 510th base. At the former, the base was either A or G. At the latter, the number of the A bases was 9, 10, 11, or 12. The major variant shown in Fig. 2 had G and 12 As, and was thus designated as G-A12. The other variants under this designation were G-A9, G-A10, G-A11, and A-A9 (Table 1). The *atpB-rbcL* sequence was the same in the all *L. cymbulifera* samples, and A-A9 alone was found (Table 2). The sole sample of *L. atroviolacea*, collected near Lijiang,

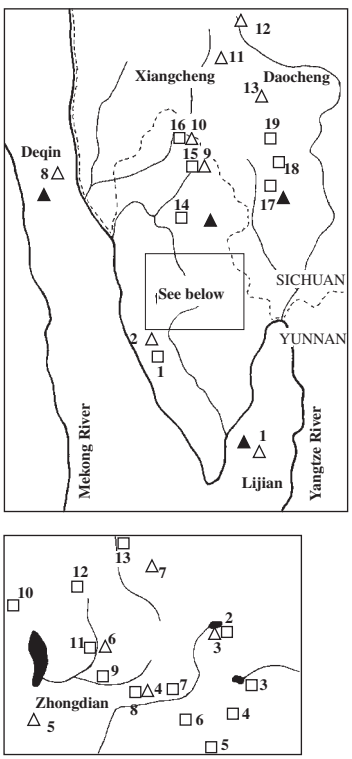

Fig. 1. Locations where samples of *L. tongolensis* (open squares) and *L. cymbulifera* (open triangles) were collected. Filled triangles indicate major peaks.

Table 2. Collection Locality, Genetic Type, and Ehrlich's Test of *L. cymbulifera* Samples

Sample	Locality	Elevation (m)	Genetic type[a)]	Ehrlich's test[b)]
1	Yulongxueshan	3600	A-A9	+
2	Xiaozhongdian	3500	A-A9	+
3	Shuduhu (Shudu lake)	3500	A-A9	+
4	Tianshengqiao	3200	A-A9	+
5	West of Zhongdian	3600	A-A9	+
6	North of Zhongdian	3300	A-A9	+
7	Gezan	3900	A-A9	+
8	Baimaxueshan	4300	A-A9	+
9	Daxueshan	3800	A-A9	+
10	Daxueshan	3600	A-A9	+
11	Wumingshan	3700	A-A9	+
12	Haizishan	3900	A-A9	+
13	Daocheng	4100	A-A9	+

a) See text for the designation. b) All samples contained the same Ehrlich-positive components.

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TCTCATAATATAATAATAAAGTAAAAAGTATCTAAAATTTTTGCGAAAATTATCGCATTC  60
AAAATAAATGTCCGATGGCAAGTTGATCGGTTAATTCAATAAGAAATGGCAGTTAGCACA 120
CGATTTTGTTAGTACCATCCAACCGAATCCAATTTAATTGTTTACTTATTCAATTTCAAT 180
GAGTGAATTTTCAAGTTCAACCAACCCATTTTCAAAATATCAAGTAGATGAATAAGAATC 240
TTGAGAAAATCTCTCATTTGTCTATCATTATAGACAATCCCATCTATATTATCTATGGAA 300
CTCGAACCTGAACTCTATTTACGATTCAGTATTTCTATATCATTGGCTCTTCTTATTTCT 360
ATTGATTTACGTCTAGCCTGTTGTTGTTTTTTTTTACCTTTTCATAGAAAAATTTCACAC 420
ATTTTCACATCTAGGATTTACATATACAACATAGTAAAGAGGAAATTTATTAGTATTTGA 480
GTGATTTTTAGGTATTTCGATTCAAAAAAAAAAAAATAAGAATAGGGTTGCGCCATATATA 540
TGAAAGAGTATACAATAATGATGTATTTGCCGAATCAAATACCATGGTCTTAATAATAAA 600
GCATTCTGATTAGTTGATAATTTTACTATTAGTTGGGAATTTTGTGAAAGGTTCCTGTAA 660
AAAGTTTCATTAACGCCTAATTCATGTCGAGTAGACCTTGTTGTTGTGAGAATTCTTAAT 720
TCATGAGTTGTAGGGAGGGATTT                                       743
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Fig. 2. Nucleotide sequence of the *atpB-rbcL* intergenic region of *Ligularia tongolensis*. The sequence is shown in the direction from the *atpB* to the *rbcL* gene. The major variant is shown and two variable locations are underlined.

Yunnan, was G-A11.

Next, the structures of the furanoeremophilane components were determined. Four Ehrlich-positive compounds, **1**–**4**, were isolated from *L. tongolensis*, which are common components in all *L. tongolensis* samples described above. The strongly Ehrlich-positive compound found in some samples, mentioned above, could not be isolated, because it was extremely unstable, and decomposed during filtration and concentration processes of the EtOH extract. Another coloring method on TLC was also carried out using $CeSO_4/H_2SO_4$ to find that **1**–**4** are the major components. The structure of two new compounds, **1** and **2**, was determined as follows.

Compound **1**, $[\alpha]_D^{21}$ −28.1 (*c* 0.42, $CHCl_3$), showed a quasi-molecular ion peak at *m/z* 499 in FABMS, corresponding to $C_{27}H_{40}NaO_7$; the molecular formula of **1** was $C_{27}H_{40}O_7$. IR absorption indicated the presence of carboxylic acid (3600–2500 cm^{-1}) and carbonyl groups (1730 and 1710 cm^{-1}). ^1H ($\delta = 6.92$) and ^{13}C NMR ($\delta = 114.8$, 119.8, 139.1, and 150.2) spectra indicated the presence of a trisubstituted furan ring. A furanoeremophilane skeleton with carboxylic acid at C-15 was established based on a careful analysis of ^1H–^1H COSY and HMBC (Fig. 3), which also showed the presence of two pairs of 3-methylpentanoate moieties. Correlations of H-3 and H-6 into ester carbonyl carbons at δ 172.9 (C-1′) and 171.4 (C-1″), respectively, indicated that two ester groups are attached to the C-3 and C-6 positions. The stereochemistry was determined by the NOESY spectrum, as shown in Fig. 4. Namely, a *cis*-fused decaline system was established from the observed NOE between H-14 and H-10. The NOE between H-3 ($\delta = 5.68$, axial) and H-6 indicates that the ester groups

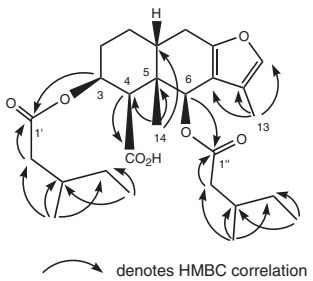


Fig. 3. Selected HMBC correlations of **1**.

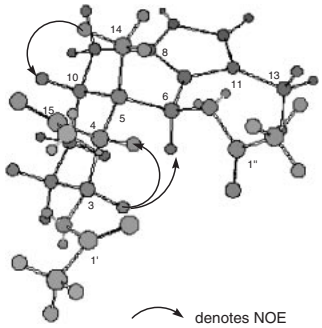


Fig. 4. Selected NOE correlations of **1**. Ester side-chains were replaced with acetate for clarity.

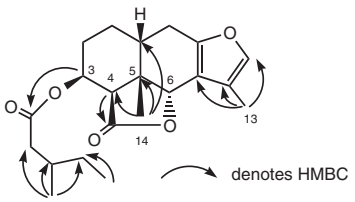


Fig. 5. Selected HMBC correlations of **2**.

at C-3 and C-6 are both β-orientations. The relative stereochemistry at C-3′ and C-3″ could not be determined. Therefore, compound **1** was established as 3β,6β-bis(3-methylpentanoyloxy)furanoeremophilan-15-oic acid.

The molecular formula of compound **2**, $[\alpha]_D^{21}$ +17.6 (*c* 0.81, $CHCl_3$), was determined by HRCIMS to be $C_{21}H_{28}O_5$. IR absorption indicated the presence of lactone (1780 cm^{-1}) and ester (1730 cm^{-1}) moieties. ^1H and ^{13}C NMR spectra indicated that the compound is a furanoeremophilane derivative. A careful analysis of the HMBC (Fig. 5) spectrum showed the presence of a 15,6-olide structure with a saturated ester at C-3. The ester should be 3-methylpentanoate. The relative stereochemistry was established by analyzing the NOESY spectrum shown in Fig. 6. Thus, compound **2** was established to be 3β-(3-methylpentanoyloxy)furanoeremophilan-15,6α-olide. The stereochemistry at C-3′ could not be established.

Spectral data of the other two compounds isolated from *L. tongolensis* were identical to those of 3β-angeloyloxyfuranoeremophilan-15,6α-olide (**3**)[19] and furanoeremophil-3-en-15,6α-olide (**4**).[20,21]

Five known compounds were isolated from *L. cymbulifera*: 3β-angeloyloxyfuranoeremophilan-15,6α-olide (**3**), furanoeremophil-3-en-15,6α-olide (**4**), furanoeremophilan-10β-ol (**5**),[22] 3-(methacryloyloxy)furanoeremophilan-15,6α-olide (**6**),[19] and eremophil-1(10)-en-11-ol (**7**).[23] Although another strongly Ehrlich-positive compound was detected on TLC, it was too unstable to isolate for a structure determination.

Compound **3** was also isolated from *L. atroviolacea*. Because other furanoeremophilanes from this plant were too labile to be isolated, LC-NMR was carried out in combination with LC-MS for a structure determination. Compounds in the extract were rapidly and roughly separated by silica-gel column chromatography avoiding higher temperatures for removing the solvent. Two major partially purified fractions were obtained, and were found to be highly polar by TLC, and the presence of carboxylic acid was indicated by the IR

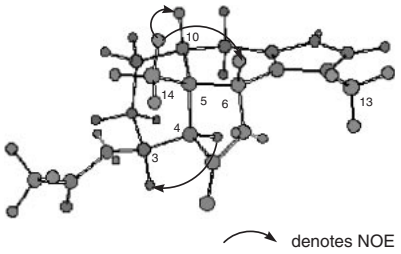


Fig. 6. Selected NOE correlations of **2**. Ester side-chain was replaced with acetate for clarity.

spectrum. Therefore, they were subjected to LC-NMR using CH_3CN–H_2O as a solvent with the column of Cosmosil ODS C-18 (4.6 × 150 mm). The fractions were analyzed by ^1H NMR and ^1H–^1H COSY, as well as the HSQC spectra, and were identified to be 3β,6β-bis(angeloyloxy)furanoeremophilan-15-oic acid (**8**)[20,24] as the component of the shorter retention time, and 3β-angeloyloxy-6β-(3-methylbutyryloxy)-furanoeremophilan-15-oic acid (**9**)[24] as the longer retention time, respectively.

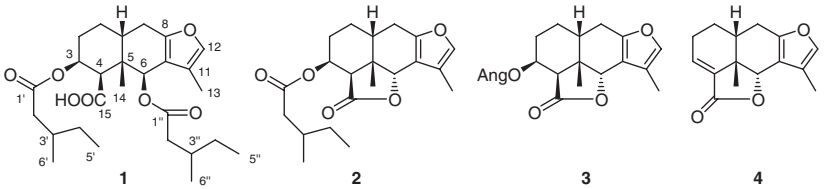
Discussion

In the present study, three *Ligularia* species (*L. tongolensis*, *L. cymbulifera*, and *L. atroviolacea*) were examined with respect to the composition of furanoeremophilanes and the nucleotide sequence of the *atpB-rbcL* region. Furanoeremophilanes were found to be produced by the three *Ligularia* species, and eight compounds were identified, two of which were new. One non-furano type of eremophilane was also isolated (Table 3).

The nucleotide sequence of the *atpB-rbcL* intergenic region was found to be essentially the same in the three species. The results of DNA sequencing of the regions of other *Ligularia* species have shown inter-specific variations at nucleotide positions other than the variable positions identified in this work, though not many (to be published elsewhere). Similarity among these species was also found in the components. All of the identified furanoeremophilanes, except **5**, were 15-oxygenated: compounds **1**–**4** from *L. tongolensis*, **3**, **4**, and **6** from *L. cymbulifera*, **3**, **8**, and **9** from *L. atroviolacea*. It is also interesting that all 15-oxygenated furanoeremophilanes isolated from these species were also oxygenated at both the 3- and 6-positions: **2**, **3**, and **6** are 3-acyloxyfuranoeremophilan-15,6α-olides; **1**, **8**, and **9** are 3,6-bis(acyloxy)furanoeremophilan-15-oic acids. Compound **4** is considered to be an elimination product from a 3-acyloxy derivative, since the hydrogen at C-4 is acidic. These similarities in DNA sequence and furanoeremophilane components imply that the three *Ligularia* species studied here are close to one another. Oxygenation at the 15-position of furanoeremophilanes is not common, and was found previously in *L. thyrsoidea*, belonging to section Stenostegia,[25] and *L. macrophylla*, belonging to Senecillis.[26] However, the 3-position of the previously isolated compounds were not oxygenated, suggesting that 3,6,15-trisoxygenated furanoeremophilanes are characteristic to Corymbosae. This type of furanoeremophilanes was reported to be obtained as the major Ehrlich-positive compound from *Syneilesis palmata*, which also belongs to tribe Senecioneae.[24]

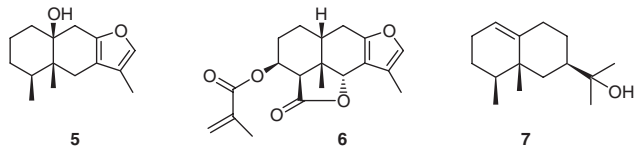
Table 3.

L. tongolensis



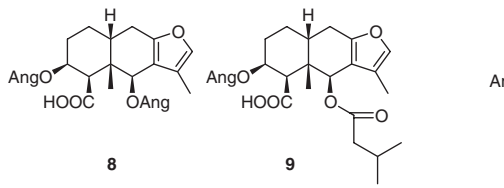
L. cymbulifera

3 4



L. atroviolacea

3



The results summarized in Tables 1 and 2 indicate the presence of intra-specific diversity in *L. tongolensis* and the absence in *L. cymbulifera*. Four of the 19 samples of *L. tongolensis* contained a strongly Ehrlich-positive compound that was absent in other samples. Five variants were found in the *atpB-rbcL* sequence. The presence of the strongly Ehrlich-positive compound and the *atpB-rbcL* sequence type did not coincide. With the two indexes, the 19 samples of *L. tongolensis* could be classified into 7 groups (Table 1). In contrast, no intra-specific diversity was found for *L. cymbulifera* in both the *atpB-rbcL* sequence and the chemical components.

All three studied species (*L. cymbulifera*, *L. tongolensis*, and *L. atroviolacea*) are included in section Corymbosae, series Lapathifolae.[18] The present results strongly support this taxonomical classification, because the *atpB-rbcL* sequence and the oxidation level of major furanoeremophilane were the same. A difference between *L. tongolensis* and *L. cymbulifera* was found in the presence/absence of intra-specific diversity. Although these two species are cosmopolite in the Hengduan Mountains region, their habitat diversity is different. *L. cymbulifera* usually grows in a moist meadow, while *L. tongolensis* grows in various habitats, such as meadows, dry and sunny hillsides, and forest fringes. The lack of chemical and genetic diversity in *L. cymbulifera* probably results from the uniformity of its habitat. *L. tongolensis* is probably adapting to various habitats, and is still in the process of differentiation.

Although biochemical or ecological roles of furanoeremophilanes are not known, it is conceivable that the presence of furanoeremophilanes reflects a selective evolutionary process to some extent. In contrast, variations of the nucleotide sequence in non-coding regions are thought to be neutral, and should simply reflect phylogeny. The present study has demonstrated that the combination of chemical and genetic approaches adds a new dimension to studies of plant diversity. We tend to believe that such combinations are meaningful because their response to the environments must be somewhat different.

Experimental

General. DNeasy Plant Mini Kit (Qiagen) was used to purify DNA from plant materials, and glass milk (Gentra Systems) was used to purify the DNA further. HotStarTaq polymerase (Qiagen) was used for the polymerase chain reaction (PCR). A High Pure PCR Product Purification Kit (Roche Diagnostics) was used to purify the PCR product after separation by agarose gel electrophoresis. DNA sequencing reactions were carried out with DYEnamic ET Sequencing Kit (Amersham) and analyzed on an ABI Prism 310 analyzer or with CEQ DTCS Kit (Beckman) and on a CEQ 2000 sequencer (Beckman). The primers used for PCR and nucleotide sequencing were: *ast-atpB*, 5′ GCTGTACCTCACAAGTCACATTAATTGGTTGACCA 3′; *ast-rbcL*, 5′ GGTTGAGGAGTTACTCGAAATGCTGCCAAGATATC 3′; *ast-atpB2*, 5′ TGAATTTTCAAGTTCAACCAACCCA 3′, and *ast-atpB3*, 5′ TTCAGATCTAGGATTTACATAT 3′.

Specific rotations were measured on a JASCO DIP-1000 auto recording polarimeter; IR spectra on a JASCO FT/IR-5300 spectrophotometer; ^{1}H and ^{13}C NMR spectra on a Varian Unity 600 (600 MHz) spectrometer. LC NMR was carried out on a Varian Unity 500 (500 MHz) with a column of Cosmosil ODS C_{18} (4.6 × 150 mm) using CH_3CN–H_2O as a solvent system at a flow rate of 0.7 cm^3/min (Varian LC systems). After a small quantity of sample was run to determine the retention time, a larger quantity was applied to measure the 2D-NMR spectra, including HSQC (overnight accumulation). Mass spectra, including high-resolution ones, were recorded on a JEOL JMS AX-500 mass spectrometer. Chemcopak Nucleosil 50-5 (10 × 250 mm or 4.6 × 250 mm) with a solvent system of hexane–ethyl acetate and Cosmosil ODS C_{18} (4.6 × 150 mm) with a solvent system of CH_3CN–H_2O were used for HPLC (Agilent 1100). Silica gel 60 (70–230 mesh, Fuji Sylisia) was used for column chromatography. Silica-gel 60 F_{254} plates (Merck) were used for TLC.

Plant Materials. TLC samples of *L. tongolensis* and *L. cymbulifera* were collected in August, 2002 and 2003 in northwestern Yunnan Province and southwestern Sichuan Province (Tables 1, 2, and Fig. 1). Each plant was identified by Xun Gong, one of the authors. For a structure determination, roots of *L. tongolensis* (25 g) and *L. cymbulifera* (155 g) were collected in August 2002 near Zhongdian, Yunnan. These are the combined samples of 2, 5, 8, 11, and 13 for *L. tongolensis* (Table 1 and Fig. 1), and 3 and 4 for *L. cymbulifera* (Table 2 and Fig. 2). Roots of *L. atroviolacea* (125 g) were collected in August 2001 near Lijian, Yunnan.

Ehrlich's Test. The root of each sample (10 to 40 g) was harvested, and extraction with ethanol was started immediately without drying. After filtering and without concentration, the alcoholic solution of extracts was subjected to TLC (Kieselgel 60 F254, layer thickness 0.2 mm) using hexane/ethyl acetate (7:3) as a solvent. The TLC plate was dipped in a 1% solution of *p*-dimethylaminobenzaldehyde in ethanol. The plate was dried, and then dipped in a 1 M solution of hydrochloric acid in aqueous ethanol, and the resultant coloring was photographed.

Determination of DNA Sequence. DNA was purified from dried leaves with a DNeasy Plant Mini Kit, and then with glass milk. PCR amplification of the *atpB-rbcL* intergenic region was carried out with primers *ast-atpB* and *ast-rbcL*. The primers were newly designed based on *atpB* and *rbcL* sequences of Asteraceae species in the data-base. In comparison with previously published primers,[16] the present ones were further inside the coding regions, which facilitated complete sequencing of the intergenic region. Forty cycles of amplification were carried out, with each cycle consisting of a 30-s denaturation at 95 °C, a 30-s annealing at 40 °C, and a 1-min extension at 72 °C. PCR products, ca. 1100 bp, were separated by agarose gel electrophoresis and purified with the High Pure PCR Product Purification Kit. Nucleotide sequencing was carried out with *ast-atpB*, *ast-rbcL*, *ast-atpB2*, and *ast-atpB3*.

Extraction for the Structure Determination. The collected roots of *L. tongolensis* and *L. cymbulifera* were cut into small pieces without drying, and immediately extracted with EtOH at room temperature. The extracted solution was filtered and concentrated to afford an oily residue together with an aqueous phase. AcOEt was added to this oil/aqueous mixture, and the organic layer was separated and concentrated to yield 3.0 g and 9.0 g of extracts for *L. tongolensis* and *L. cymbulifera*, respectively. For *L. atroviolacea*, the roots were partly dried for a few days, and extracted with AcOEt at room temperature. Filtration and evaporation by the standard method afforded 10.7 g of extract.

Purification. The extract of *L. tongolensis* (3.0 g) was subjected to silica-gel column chromatography using hexane–ethyl acetate as an eluent to give 6 fractions. The 3rd fraction (52 mg) was separated by HPLC (Nucleosil 50-5, hexane–ethyl acetate 10%, 2.0 cm^3/min) to afford **1** (9.9 mg), **2** (16.6 mg), **3** (3.2 mg), and **4** (0.8 mg).

A part of the extract of *L. cymbulifera* (1.5 g) was subjected to silica-gel column chromatography and elution with a mixture of hexane–ethyl acetate gave 7 fractions. The 4th fraction was further purified by HPLC (Nucleosil 50-5, hexane–ethyl acetate 20%, 2.0 cm^3/min) to afford **3** (3.2 mg), **4** (40 mg), **5** (176.3 mg), **6** (102.9 mg), and **7** (12.9 mg).

A part of the extract of *L. atroviolacea* (4.7 g) was subjected to silica-gel column chromatography (eluted with hexane–ethyl acetate) to give 5 fractions. The 3rd fraction was analyzed by HPLC (Cosmosil C-18, MeOH–H_2O 10%, 1.0 cm^3/min), which was subjected to LC-NMR and LC-MS (**8** and **9** were detected). The 4th fraction was further separated by HPLC (Nucleosil 50-5, $CHCl_3$–EtOAc = 98:2, 1.0 cm^3/min) to afford **3** (27.5 mg).

3β,6β-Bis(3-methylpentanoyloxy)furanoeremophilan-15-oic Acid (1): colorless gum, $[\alpha]_D^{21}$ −28.1 (*c* 0.42, $CHCl_3$). MS (FAB) *m/z* 499 $[M+Na]^+$, 476, 383, 361, 245 (base), 154, 99. HRMS (FAB) Obsd 499.2657 $[M+Na]^+$ Calcd for $C_{27}H_{40}NaO_7$ 499.2672. FT-IR (KBr) 3600–2400, 1730, 1710 cm^{-1}. ^{13}C NMR (150 MHz, C_6D_6) δ 9.0 (C-13), 11.3 (C-5′ or 5″), 11.4 (C-5′ or 5″), 19.3 (C-6′ or 6″), 19.4 (C-6′ or 6″), 19.5 (C-14), 26.3 (C-1 or 2 or 9), 26.4 (C-1 or 2 or 9), 26.7 (C-1 or 2 or 9), 29.3 (C-4′ or 4″), 29.4 (C-4′ or 4″), 31.8 (C-3′ or 3″), 32.0 (C-3′ or 3″), 36.4 (C-10), 41.5 (C-2′ and 2″), 41.6 (C-5), 49.7 (C-4), 68.2 (C-6), 69.8 (C-3), 114.8 (C-11), 119.8 (C-7), 139.1 (C-12), 150.2 (C-8), 171.4 (C-1″), 172.9 (C-1′), 177.5 (C-15). ^1H NMR (600 MHz, C_6D_6) δ 0.79 (3H, t, *J* = 7.4 Hz), 0.81 (3H, t, *J* = 7.4 Hz), 0.89 (3H, d, *J* = 6.6 Hz), 0.97 (3H, d, *J* = 6.6 Hz), 1.02 (1H, m), 1.03 (1H, m), 1.04 (1H, m), 1.05 (1H, m), 1.15 (3H, s), 1.27 (1H, m), 1.35 (1H, m), 1.84 (1H, m), 1.87 (3H, d, *J* = 1.4 Hz), 1.95 (1H, m), 1.99 (1H, dd, *J* = 14.8, 7.8 Hz), 2.01 (1H, m), 2.04 (1H, m), 2.09 (1H, dd, *J* = 14.6, 8.0 Hz), 2.15 (1H, dd, *J* = 14.8, 5.9 Hz), 2.24 (1H, m), 2.31 (1H, dd, *J* = 14.8, 5.9 Hz), 2.24 (1H, qd, *J* = 6.6, 5.2 Hz), 2.65 (1H, dd, *J* = 17.6, 4.1 Hz), 3.32 (1H, br d, *J* = 5.5 Hz), 5.68 (1H, dt, *J* = 12.1, 5.5 Hz), 6.48 (1H, s), 6.92 (1H, s).

3β-(3-Methylpentanoyloxy)furanoeremophilan-15,6α-olide (2): colorless gum, $C_{21}H_{28}O_5$. $[\alpha]_D^{21}$ +17.6 (*c* 0.81, $CHCl_3$). MS (CI) *m/z* 361 $[M+H]^+$, 285, 245 (base), 217, 117, 99. HRMS (CI) Obsd 361.2021 $[M+H]^+$ Calcd for $C_{21}H_{29}O_5$ 360.2015. FT-IR (KBr) 1780, 1730 cm^{-1}. ^{13}C NMR (150 MHz, C_6D_6) δ 8.3 (C-13), 11.4 (C-5′), 19.3 (C-6′), 22.2 (C-1), 22.7 (C-9), 23.1 (C-14), 24.9 (C-2), 29.4 (C-4′), 32.3 (C-3′), 36.8 (C-10), 40.7 (C-5), 41.9 (C-2′), 43.1 (C-4), 65.4 (C-3), 81.7 (C-6), 115.1 (C-7), 120.4 (C-11), 138.8 (C-12), 150.8 (C-8), 171.7 (C-15), 171.9 (C-1′). ^1H NMR (600 MHz, C_6D_6) δ 0.77 (1H, m), 0.79 (3H, t, *J* = 7.4 Hz), 0.89 (3H, d, *J* = 6.9 Hz), 1.06 (1H, m), 1.10 (1H, m), 1.23 (3H, s), 1.30 (1H, m), 1.59 (1H, m), 1.60 (1H, m), 1.61 (1H, m), 1.63 (1H, m), 1.87 (1H, m), 1.88 (1H, m), 1.93 (3H, d, *J* = 1.1 Hz), 1.98 (1H, dd, *J* = 14.4, 7.8 Hz), 2.10 (1H, dd, *J* = 17.4, 7.3 Hz), 2.14 (1H, dd, *J* = 14.4, 6.3 Hz), 4.58 (1H, s), 5.38 (1H, quint, *J* = 1.9 Hz), 6.85 (1H, s).

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References

1 C. Jeffrey and Y. L. Chen, *Kew Bull.*, **39**, 205 (1984).
2 S. W. Liu, D. S. Deng, and J. Q. Liu, *Acta Phytotaxon. Sin.*,

32, 514 (1994).
3 H. Handel-Mazzetti, *Bot. Jahrb.*, **69**, 95 (1938).
4 S. Y. Hu, *Quart. J. Taiwan Mus.*, **20**, 53 (1967).
5 X. Gong, Z. J. Gu, Y. X. Lu, and C. Q. Zhang, *Acta Bot. Yunnanica*, **23**, 216 (2001).
6 Y. Z. Pan, X. Gong, and Z. Y. Yang, *Acta Bot. Yunnanica*, **26**, 65 (2004).
7 J. Q. Liu, *Bot. J. Linn. Soc.*, **144**, 329 (2004).
8 H. Ishii, T. Tozyo, and H. Minato, *Tetrahedron*, **21**, 2605 (1965).
9 F. Patil, G. Ourisson, Y. Tanahashi, M. Wada, and T. Takahashi, *Bull. Soc. Chim. Fr.*, **1968**, 1047.
10 Y. Ishizaki, Y. Tanahashi, and T. Takahashi, *J. Chem. Soc., Chem. Commun.*, **1969**, 551 (1969).
11 M. Tada, Y. Moriyama, Y. Tanahashi, and T. Takahashi, *Bull. Chem. Soc. Jpn.*, **47**, 1999 (1974).
12 T. Sato, Y. Moriyama, H. Nagano, Y. Tanahashi, and T. Takahashi, *Bull. Chem. Soc. Jpn.*, **48**, 112 (1975).
13 C. Kuroda, T. Ueshino, and H. Nagano, *Bull. Chem. Soc. Jpn.*, **77**, 1737 (2004).
14 K. Naya, M. Nakagawa, M. Hayashi, K. Tsuji, and M. Naito, *Tetrahedron Lett.*, **1971**, 2961.
15 H. Nagano, Y. Tanahashi, Y. Moriyama, and T. Takahashi, *Bull. Chem. Soc. Jpn.*, **46**, 2840 (1973).
16 V. Savolainen, J. F. Mane, E. Douzery, and R. Spchiger, *Mol. Phylogenet. Evol.*, **3**, 27 (1994).
17 D. E. Soltis and P. S. Soltis, "Molecular Systematics of Plants II: DNA Sequencing," ed by P. S. Soltis, D. E. Soltis, and J. J. Doyle, Kluwer Academic Publishers, Boston (1998).
18 S. W. Liu, "Flora Reipublicae Popularis Sinicae," Science Press, Beijing (1989), Vol. 77.
19 F. Bohlmann and K.-H. Knoll, *Phytochemistry*, **17**, 461 (1978).
20 F. Bohlmann, C. Zdero, and M. Grenz, *Chem. Ber.*, **107**, 3928 (1974).
21 F. Bohlmann and A. Suwita, *Chem. Ber.*, **109**, 1230 (1976).
22 P. W. Jennings, S. K. Reeder, and J. C. Hurley, *J. Org. Chem.*, **39**, 3392 (1974).
23 H. Ishii, T. Tozyo, and H. Minato, *J. Chem. Soc. C*, **1966**, 1545.
24 C. Kuroda, T. Murae, M. Tada, H. Nagano, T. Tsuyuki, and T. Takahashi, *Bull. Chem. Soc. Jpn.*, **55**, 1228 (1982).
25 F. Bohlmann and C. Zdero, *Phytochemistry*, **19**, 1550 (1980).
26 F. Bohlmann and M. Grenz, *Phytochemistry*, **18**, 491 (1979).