# High Diversity of *Ligularia dictyoneura* in Chemical Composition and DNA Sequence

by **Hajime Nagano***[a])[1]), **Yukiko Iwazaki**[a]), **Mika Matsushima**[a]), **Masahiko Sato**[b]), **Xun Gong***[c])[2]), **Yuemao Shen**[c]), **Hiroshi Hirota**[d]), **Chiaki Kuroda***[e])[3]), and **Ryo Hanai***[b])[4])

[a]) Department of Chemistry, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan (phone: +81-359785348; fax: +81-359785715; e-mail: nagano.hajime@ocha.ac.jp)

[b]) Department of Life Science, Rikkyo University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan (phone/fax: +81-339852377; e-mail: hanai@rikkyo.ne.jp)

[c]) Kunming Institute of Botany, Chinese Academy of Science, Kunming 654204, P. R. China (phone: +86-8715223625; e-mail: gongxun@mail.kib.ac.cn)

[d]) RIKEN Genomic Sciences Center, Tsurumi-ku, Yokohama, 230-0045, Japan

[e]) Department of Chemistry, Rikkyo University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan (phone/fax: +81-339852396; e-mail: chkkuroda@grp.rikkyo.ne.jp)

The chemical composition of root extracts of the title species collected at 20 different places in the Hengduan Mountains of China was examined. From these samples, a total of 17 eremophilane derivatives were isolated, three of which were new franoeremophilane derivatives: 3$\beta$-acetoxy-6$\beta$-(angeloyloxy)furanoeremophilan-10$\beta$-ol, 1$\alpha$-acetoxyfuranoeremophilan-15,6$\alpha$-olide, and 6$\beta$-[2-(hydroxymethyl)prop-2-enoyloxy]furanoeremophil-1(10)-ene. Based on the chemical composition, the samples could be classified into as many as seven types: one type containing non-furanoeremophilane derivatives and the other six containing furanoeremophilane derivatives with different oxidation levels. Results of DNA sequencing of the *atpB-rbcL* region and the internal transcribed spacers of the ribosomal RNA gene also indicated a high diversity in the species.

**Introduction.** – *Ligularia* Cass. (Asteraceae) in the Hengduan Mountains of China is highly diversified [1] and suitable for the study of diversity and evolution of plant chemicals. We have been investigating the chemical diversity of *Ligularia* species in combination with genetic analyses [2–6]. To examine the chemical diversity, we have been analyzing furanoeremophilanes in root, since they have been found in many *Ligularia* species (see the references cited previously [2–5]), and since the presence/absence of furanosesquiterpenes can be easily examined by *Ehrlich*'s test on TLC [7]. As an index of diversity independent of the chemicals, we have been analyzing the DNA sequences of the *atpB-rbcL* intergenic region in the plastid genome and the two internal transcribed sequences (ITSs) of the ribosomal RNA gene in the nuclear genome. The regions are non-coding, and variations therein are mostly neutral to evolution [8]. Hence, they are routinely analyzed in the studies of plant diversity and

[1]) Corresponding author for chemical aspects of the work.
[2]) Corresponding author for taxonomy.
[3]) Corresponding author for general information.
[4]) Corresponding author for genetic aspects of the work.

phylogeny. Thus far, we have found that *1*) *L. pleurocaulis* (FRANCH.) HAND.-MAZZ. of northwestern Yunnan and that in southwestern Sichuan were different both chemically and genetically [3]; *2*) *L. tsangchanensis* (FRANCH.) HAND.-MAZZ. was similarly different between Yunnan and Sichuan [5]; *3*) *L. virgaurea* var. *virgaurea* (MAXIM.) MATTF. in Sichuan was also found to consist of at least two types; however, in contrast to the above two species, the two types were not geographically separated [4]; *4*) *L. cymbulifera* (W. W. SMITH) HAND.-MAZZ. was uniform, and the diversity in *L. tongolensis* (FRANCH.) HAND.-MAZZ. was small [2].

In this report, we describe the results of analyses on *L. dictyoneura* (FRANCH.) HAND.-MAZZ., which belongs to the section Senecillis [9]. This species grows at grassy slopes, banks, and forest understories of 2000–3500 m in altitude in northwestern Yunnan and southwestern Sichuan. A related species, *L. melanocephala*, also grows in the same area at the elevation of 3400–4000 m. Morphological differences between *L. dictyoneura* and *L. melanocephala* have been described with respect to the thickness of leaves, the breadth of wings on petioles, the number of ligulate florets, and the color of involucres [9]. However, in our observation, these differences were not distinct: some of our samples exhibited intermediate features (see *Results*). Because of this taxonomical ambiguity, we designate all our samples as *L. dictyoneura*.

Chemical constituents in the root of *L. dictyoneura* have been studied. *Tan et al.* isolated some eremophilanes as well as bakkenolide A from a sample collected near Lijiang city [10]. Coumarin derivatives were also isolated from a Chinese medicine '*Yantianma*', the roots of the species [11]. Nevertheless, we were convinced of finding different compounds in samples from different locales, because many *Ligularia* species harbor intraspecific diversity [2–6], and because morphological variation was observed in *L. dictyoneura* as mentioned above. Description of intraspecific variation in chemical composition is also important for medicinal and conservation purposes. Our results show that the intraspecific diversity of *L. dictyoneura* is extremely high.

**Results.** – Twenty samples of *L. dictyoneura* (*Table 1* and *Fig. 1*) were collected in northwestern Yunnan and southwestern Sichuan Provinces in 2002–2006. As mentioned in the *Introduction*, a large morphological variation was observed. For example, the leaf margin of samples *14* and *18* was dentate, while that of sample *4* was not. Some features attributable to *L. melanocephala* were also found in some samples (see *Footnote b* of *Table 1*).

*Ehrlich*'s test on TLC was carried out as described in our previous report [2]. For this experiment, the roots of each plant sample were extracted with EtOH immediately after harvesting without drying. About half of the 20 samples were *Ehrlich*-positive, and the rest were negative. Most samples collected near Zhongdian (Shanglila) city were *Ehrlich*-negative, except for sample *9*, while the samples obtained from the other places were *Ehrlich*-positive, except for sample *18*.

The *Ehrlich*-positive samples were grouped into six on the basis of the TLC pattern (*Table 1*, types 1–6). Type 1 (samples *1*, *4*, and *15*) showed many *Ehrlich*-positive spots on TLC, indicating the presence of several furanoeremophilane derivatives. Four large spots were observed at $R_f$ 0.74, 0.69, 0.63, and 0.38 (hexane/AcOEt 7:3). Type 2 (samples *2*, *3*, *16*, and *19*) showed three major *Ehrlich*-positive spots at $R_f$ 0.63, 0.58, and 0.44. Type 3 (sample *9*) showed two major *Ehrlich*-positive spots at $R_f$ 0.92 and 0.63.

Table 1. *Collection Details, Type of Components, and Base Sequence of Samples of* L. dictyoneura

| Sample[a])[b]) | Location | Altitude [m] | *Ehrlich*'s test | *atpB-rbcL*[c]) |
|---|---|---|---|---|
| *1* | Sandawan | 3200 | positive; type 1 | **B**, G-A10 |
| *2* | Yulongxueshan | 3000 | positive; type 2 | **B**, G-A8 |
| *3* | Hutiaoxia | 2200 | positive; type 2 | **B**, G-A9 |
| *4* | Baishuitai | 2700 | positive; type 1 | **B**, G-A10 |
| *5* | Xiaozhongdian | 3200 | negative | **A**, G-A11 |
| *6* | Xiaozhongdian | 3300 | negative | **A**, A-A11 |
| *7* | Dabaoshan | 3500 | negative | **A**, G-A11 |
| *8* | Tianshengqiao | 3400 | negative | **A**, G-A11 |
| *9* | Shikashan | 3400 | positive; type 3 | **A**, G-A9 |
| *10* | Nixi | 3400 | negative | **A**, G-A11 |
| *11* | Zhongdian | 3300 | negative | **A**, G-A11 |
| *12* | Gezan | 3200 | negative | **A**, G-A11 |
| *13* | Gezan | 3300 | negative | **A**, G-A11 |
| *14* | Xiaoxueshan | 3400 | positive; type 4 | **A**, G-A9 |
| *15* | Yongning | 3000 | positive; type 1 | **A**, G-A11 |
| *16* | Luguhu | 3300 | positive; type 2 | **B**, G-A8 |
| *17* | Rencun | 2900 | positive; type 5 | **B**, G-A10 |
| *18* | Reda | 3400 | negative | **A**, G-A9 |
| *19* | Reda | 3400 | positive; type 2 | **A**, G-A9 |
| *20* | Baimaxueshan | 4300 | positive; type 6 | **A′**, G-A10 |

[a]) Samples *5*, *10*, and *11* were collected in 2002; samples *14* and *17* were collected in 2003; samples *1–3*, *6*, *7*, *12*, *13*, *15*, and *16* were collected in 2004; samples *4*, *8*, *9*, *18*, and *19* were collected in 2005; sample *20* was collected in 2006. [b]) Morphological features of *L. melanocephala*, such as basal leaves with broadly winged petiole, 8–10 ligulate florets, and herbaceous leaves, were observed for samples *6*, *15*, and *18*, resp. Typical *L. dictyoneura* has leathery leaves with no or narrow wing on petiole, and the number of ligulate florets are 4–6 [9]. [c]) **A** = 245G, 301C, 469A, and T9; **A′** = 245G, 301C, 469A, and T8; **B** = 245T, 301T, 469C, and T11, in which T8, T9, and T11 indicate the number of a T-stretch around the 390th base. Combination of the 28th base and the number of A-stretch around the 510th base was designated such as G-A12; see our previous report [2].

Type 4 (sample *14*) showed two spots at $R_f$ 0.79 and 0.58. The color of the spot at $R_f$ 0.79 was orange, indicating the presence of an oxo group at C(6) [7b]. Type 5 (sample *17*) and type 6 (sample *20*) showed one strong spot ($R_f$ 0.62) and many spots, respectively. Samples of types 1 and 2 were not geographically separated. For example, two samples were collected in the Lijiang area (samples *1* and *2*), but their composition was different. A similar situation was observed for the samples from the Luguhu area (samples *15* and *16*).

For the *Ehrlich*-negative samples, components were detected on TLC by coloring with $Ce(SO_4)_2$, and their chemical composition was found to be the same. This indicated that the plant near Zhongdian city was almost uniform with respect to chemical composition.

The compounds from the roots of each type were isolated, and their structures were determined. Compounds **1**–**17** (*Fig. 2*) were isolated, among which compounds **9**, **11**, and **16** were new. From sample *7* (*Ehrlich*-negative), five eremophil-9-en-8-one

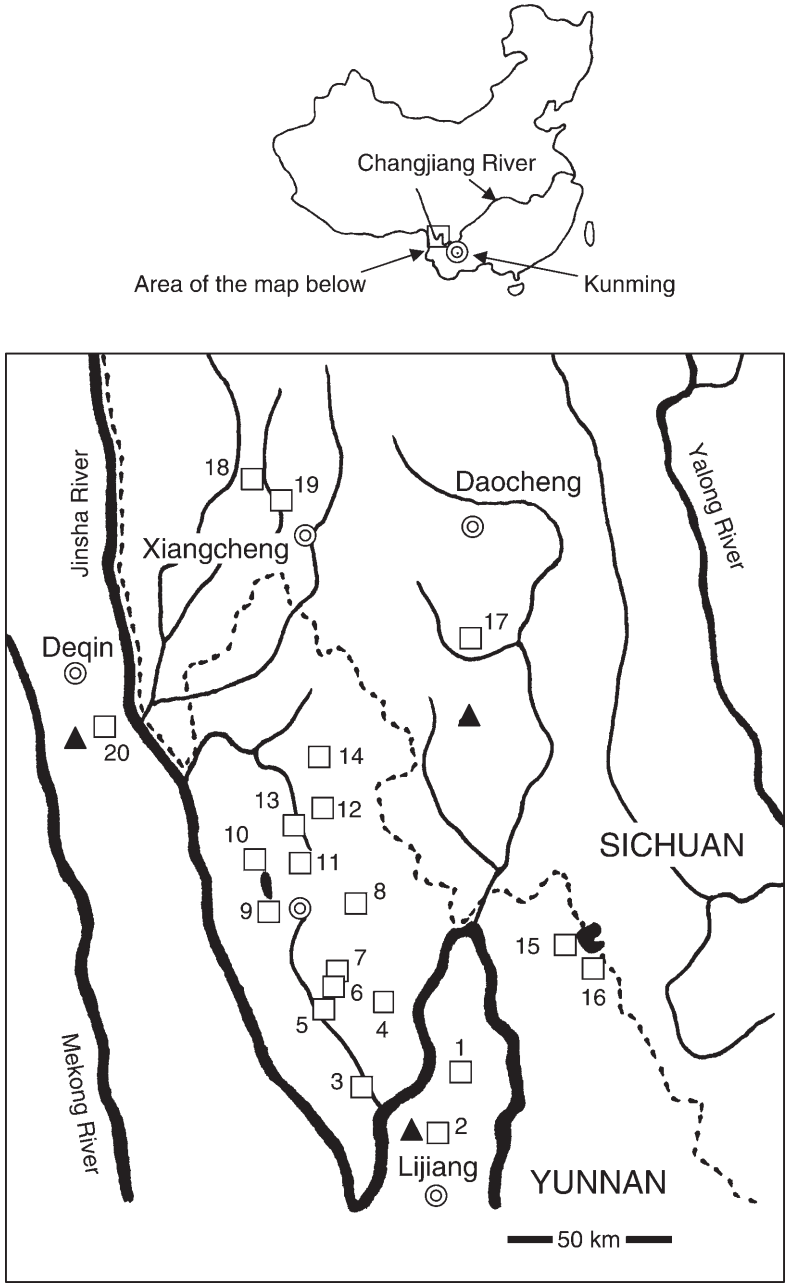



Fig. 1. *Locations where samples of* L. dictyoneura (open squares) *were collected*. Filled triangles and double circles indicate major peaks and major cities, resp.

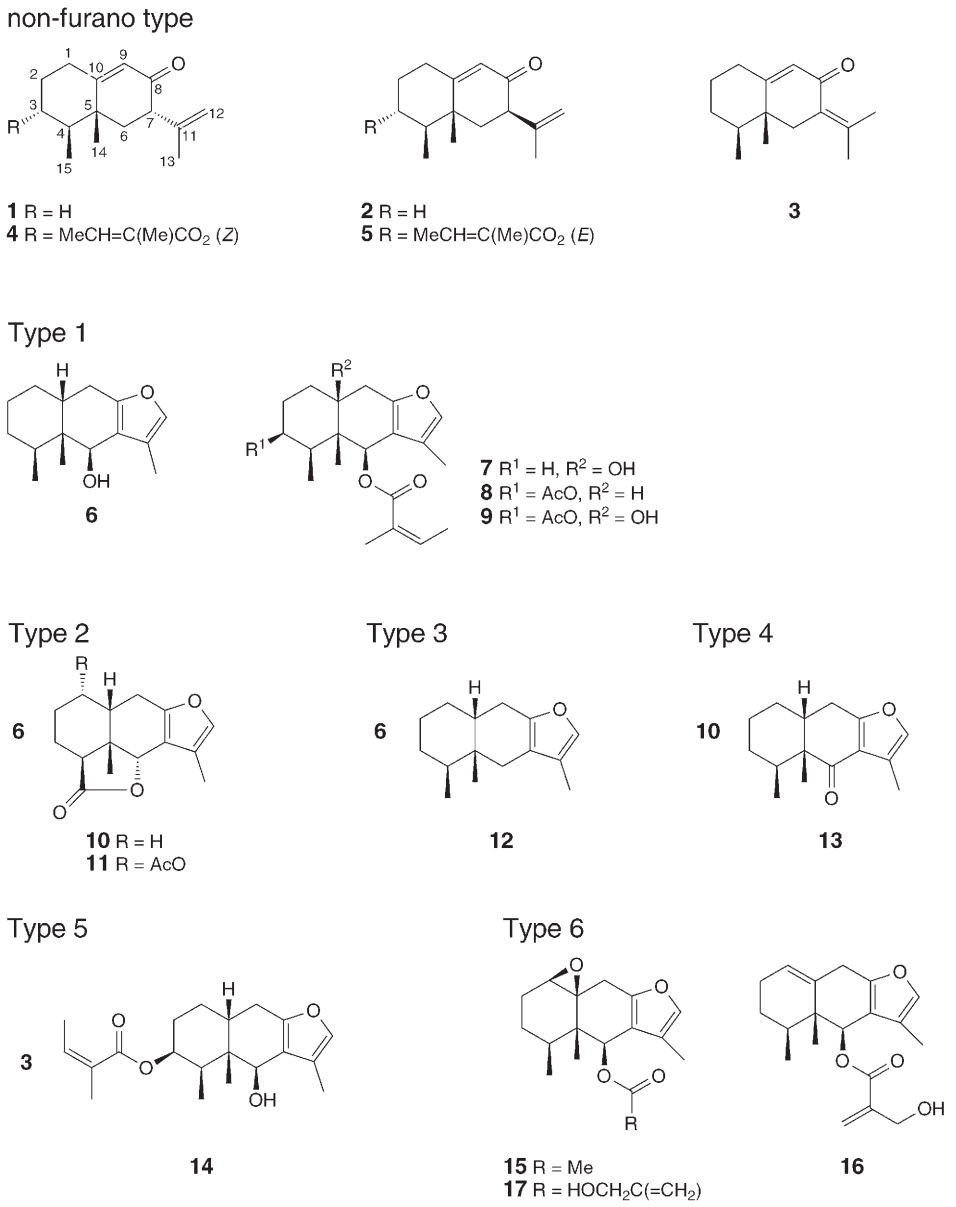



Fig. 2. *Chemical constituents of* L. dictyoneura

derivatives, **1** [12], alloeremophilone (**2**) [13], dehydrofukinone (**3**) [14], petasin (**4**) [13][15], and **5** [13][16] were isolated in 11, 2, 2, 4, and 2% yields, respectively.

From sample *4* (type 1), ligularol (= petasalbin; **6**) [17][18], 6$\beta$-(angeloyloxy)-furanoeremophilan-10$\beta$-ol (**7**) [19], 3$\beta$-acetoxy-6$\beta$-(angeloyloxy)furanoeremophilane (**8**) [20], and a new compound **9** were isolated in 2.1, 4.0, 3.3, and 14.3% yields,

respectively. The major product **9** was also isolated from sample *15* in 8.5% yield. Its molecular formula was determined to be $C_{22}H_{30}O_6$ by high-resolution (HR) EI-MS (*m*/*z* 390.2052). IR (3488 and 1713 $cm^{-1}$), $^1$H-NMR (1.84 (*s*, MeC=), 1.95 (*dq*, *J* = 6.8, 1.4, *Me*CH=), 2.05 (*s*, Ac), 4.97 (br. *s*, H−C(3)), 6.05 (*qq*, *J* = 6.8, 1.4, CH=), 6.22 (*s*, H−C(6))), and $^{13}$C-NMR (69.2, 72.6, 74.8, 166.7, 170.4) spectra suggested the presence of a OH, an AcO, and an angeloyloxy groups on the furanoeremophilane skeleton (*Tables 2* and *3*). The presence of the angeloyloxy group at C(6) was established by the observation of a *singlet* for the CH H-atom at $\delta$ 6.22 and of a retro-*Diels–Alder* fragment (*m*/*z* 206.0931, $C_{12}H_{14}O_3$; *Fig. 3*) in the mass spectrum. A pair of *doublet* signals of $CH_2$(9) at $\delta$ 2.73 (*d*, *J* = 18.1, 1 H) and 3.08 (br. *d*, *J* = 18.1 (half band width 39 Hz), 1 H) indicated the presence of a tertiary OH group at C(10) ($\delta$ 74.8). The lowering of the chemical shift of the *doublet* at $\delta$ 3.08 ($H_\beta$−C(9)) may be due to the vicinal OH group, as the signal of the corresponding H-atoms in the 3$\beta$,6$\beta$-diacetoxyfuranoeremophilan-10$\beta$-ol (**18**) [21] has been observed at $\delta$ 3.11. The steroidal conformation of the *cis*-decalin ring bearing the 10$\beta$-OH group was confirmed by a significant NOE enhancement (8.1%) of the Me(14) signal induced by the irradiation of $H_\alpha$−C(6). Comparison of the broad *singlet* (half band width: 17.2 Hz at room temperature and 12.6 Hz at 40°) of the AcO-bearing CH H-atom at $\delta$ 4.97 with the signals of $H_\alpha$−C(3) of **18** ($\delta$ 4.99) and 1$\alpha$,3$\beta$,6$\beta$-triacetoxyfuranoeremophilan-10$\beta$-ol (**19**) ($\delta$ 5.09, half band width: 10 Hz) [21] suggested that the AcO group was located

Table 2. *$^1$H-NMR Data of Compounds* **9**, **11**, *and* **16**

| H-Atom | **9** (in $CDCl_3$) | **11** (in $C_6D_6$) | **16** (in $CDCl_3$) |
|---|---|---|---|
| $H_\alpha$−C(1) | 1.55 (*m*) | – | 5.71 (*m*)[a]) |
| $H_\beta$−C(1) | 1.95 (*m*) | 5.09 (*ddd*, *J* = 11.7, 4.9, 4.9) | – |
| $H_\alpha$−C(2) | 1.85 or 1.90 (*m*) | 1.01 (*m*) | 1.95 (*m*) |
| $H_\beta$−C(2) | 1.90 or 1.85 (*m*) | 1.55 (*m*) | 2.06 (*m*) |
| $H_\alpha$−C(3) | 4.97 (br. *s*)[b]) | 1.14 (*m*) | 1.88 (*m*) |
| $H_\beta$−C(3) | – | 1.29 (*m*) | 1.47 (*m*) |
| $H_\alpha$−C(4) | 1.60 (*m*) | 1.69 (*m*) | 1.00 (*m*) |
| $H_\alpha$−C(6) | 6.22 (*s*) | – | 6.25 (*s*) |
| $H_\beta$−C(6) | – | 4.53 (*s*) | – |
| $H_\alpha$−C(9) | 2.73 (*d*, *J* = 18.1) | 2.29 (*dd*, *J* = 17.6, 7.8) | 3.02 (*d*, *J* = 17.1) |
| $H_\beta$−C(9) | 3.08 (br. *d*, *J* = 18.1)[c]) | 2.40 (*dd*, *J* = 17.6, 10.3) | 3.45 (*d*, *J* = 17.1) |
| $H_\beta$−C(10) | – | 2.15 (*m*) | – |
| H−C(12) | 7.08 (*s*) | 6.96 (*m*) | 7.04 (*s*) |
| Me(13) | 1.89 (*s*) | 2.02 (*d*, *J* = 1.5) | 1.79 (*s*) |
| Me(14) | 1.27 (*s*) | 0.83 (*s*) | 1.10 (*s*) |
| Me(15) | 0.98 (*d*, *J* = 6.8) | – | 0.93 (*d*, *J* = 6.8) |
| Ac | 2.05 (*s*) | 1.69 (*s*) | – |
| *Me*CH= | 1.95 (*dq*, *J* = 6.8, 1.4) | – | – |
| MeC*H*= | 6.05 (*qq*, *J* = 6.8, 1.4) | – | – |
| MeC= | 1.84 (*s*) | – | – |
| C*H*H= | – | – | 5.90 (*s*) |
| CH*H*= | – | – | 6.33 (*s*) |
| $HOCH_2$ | – | – | 4.39 (*s*) |

[a]) Olefinic H-atom (H−C(1)). [b]) Half band width 17 Hz. [c]) Half band width 39 Hz.

Table 3. *$^{13}$C-NMR Data of Compounds* **9**, **11**, *and* **16**

| C-Atom | **9** (in $CDCl_3$) | **11** (in $C_6D_6$) | **16** (in $CDCl_3$) |
|---|---|---|---|
| C(1) | 29.7[a]) | 70.5 | 124.7 |
| C(2) | 28.9 | 25.7 | 21.6 |
| C(3) | 72.6 | 17.6 | 26.4 |
| C(4) | 37.1 | 40.4 | 32.4 |
| C(5) | 45.2 | 41.9 | 42.6 |
| C(6) | 69.2 | 79.8 | 73.1 |
| C(7) | 115.0 | 115.2 | 116.9 |
| C(8) | 150.8[a]) | 150.3 | 151.2 |
| C(9) | 33.6[a]) | 19.6 | 31.2 |
| C(10) | 74.8 | 40.1 | 134.9 |
| C(11) | 119.3 | 120.4 | 119.6 |
| C(12) | 139.0 | 138.9 | 138.3 |
| C(13) | 8.1 | 8.3 | 8.6 |
| C(14) | 12.3[a]) | 19.5 | 16.6 |
| C(15) | 11.8[a]) | 173.9 | 15.3 |
| *Me*CO | 21.3 | 20.5 | – |
| Me*C*O | 170.4 | 169.1 | – |
| C=O | 166.7 | – | 166.2 |
| *Me*CH | 15.6 | – | – |
| Me*C*H | 138.9 | – | – |
| *Me*C= | 20.5 | – | – |
| Me*C*= | 127.2 | – | – |
| *C*=$CH_2$ | – | – | 139.2 |
| C=*C*$H_2$ | – | – | 126.0 |
| $CH_2$OH | – | – | 62.7 |

[a]) Broad signal.

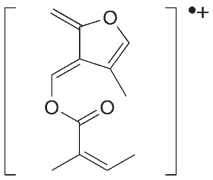


Fig. 3. *retro*-Diels–Alder *fragment of 3β-acetoxy-6β-(angeloyloxy)furanoeremophilan-10β-ol* (**9**)

at the 3$\beta$-position. The structure of **9**, including the location of the AcO and the angeloyloxy groups, was firmly established by NOESY, HMQC, and HMBC measurements (*Fig. 4*). The lowest-energy conformer of **9**, obtained by use of CONFLEX program [22], followed by semi-empirical molecular orbital calculations (PM3) of the resulting conformers using the Hamiltonian implemented in MOPAC 6.0. [23], is shown in *Fig. 5*.

From sample *3* (type 2), ligularol (**6**) [17][18] was isolated as the major component, which constituted 27% of the total extract. Furanoeremophilan-15,6$\alpha$-olide (**10**) [24] and a new compound **11** were also isolated in 6 and 1% yields, respectively. Compound **11** was determined to be 1$\alpha$-acetoxyfuranoeremophilan-15,6$\alpha$-olide as follows. The

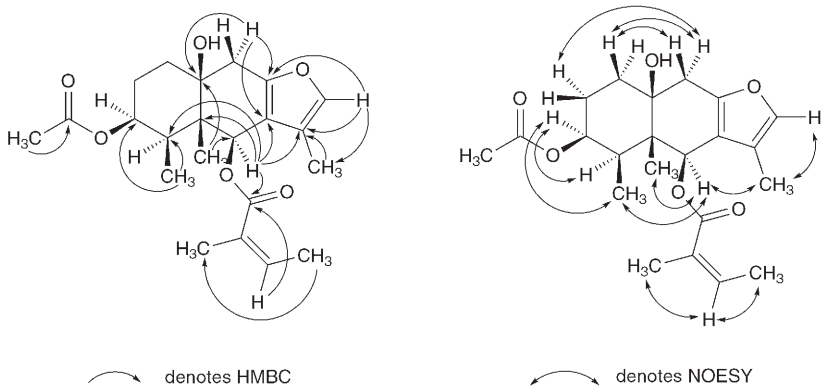



Fig. 4. *Selected NOESY and HMBC correlations of* **9**

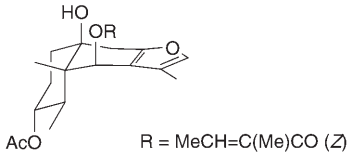



Fig. 5. *Lowest-energy conformation of* **9**

molecular formula, $C_{17}H_{20}O_5$, of **11** was obtained by the HR-EI-MS (*m*/*z* 304.1339). The IR (1733 $cm^{-1}$), $^1$H-NMR (1.69 (*s*, 3 H) and 5.09 (*ddd*, *J* = 11.7, 4.9, 4.9, 1 H)), and $^{13}$C-NMR (70.5 and 169.1) spectra of **11** resembled those of **10** except for the presence of an AcO group (*Tables 2* and *3*). The COSY spectrum in $C_6D_6$ showed the correlation between AcO-bearing CH H-atom ($\delta$ 5.09) and H−C(10) ($\delta$ 2.15 (*m*)), and between the signals of H−C(10) and $CH_2$(9) ($\delta$ 2.40 (*dd*, *J* = 17.6, 10.3) and 2.29 (*dd*, *J* = 17.6, 7.8)), indicating that the AcO group is located at C(1). The spin coupling of H−C(1) (*ddd*, *J* = 11.7, 4.9, 4.9) suggested the $\alpha$-equatorial configuration of the AcO group. Finally, the structure of **11** was firmly established on the basis of NOESY, HMQC, and HMBC spectra (*Fig. 6*).

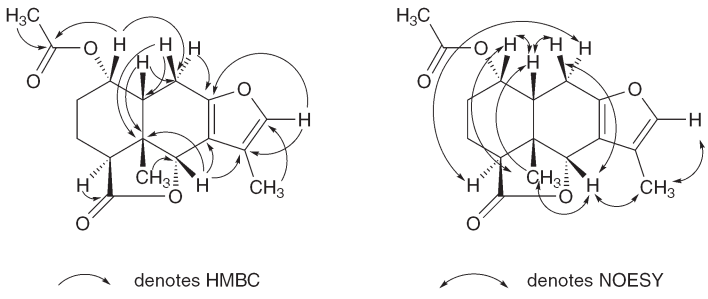


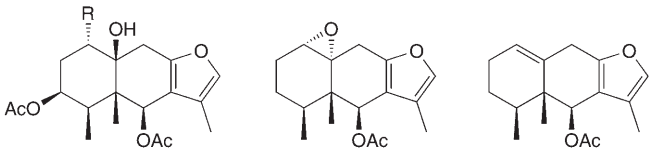

Fig. 6. *Selected NOESY and HMBC correlations of* **11**

From sample *9* (type 3), furanoeremophilane (**12**) [25] and ligularol (**6**) [17][18] were isolated in 31 and 7% yields, respectively.

From sample *14* (type 4), ligularone (**13**) [26] and furanoeremophilan-15,6$\alpha$-olide (**10**) [24] were isolated in 2.8 and 2.3% yields, respectively.

From sample *17* (type 5), 3$\beta$-(angeloyloxy)ligularol (**14**) [19b][27] and a non-furano eremophilane **3** [14] were isolated in 23 and 12% yields, respectively.

From sample *20* (type 6), compounds **15** [28–30], **16** [29], and **17** [31] were isolated in 4, 3, and 10% yields, respectively. The $^1$H-NMR data of **15** were identical with those of 6$\beta$-acetoxy-1,10-epoxyfuranoeremophilane reported by *Bohlmann et al.* [28] and by *Burgueno-Tapia et al.* [29]. *Bohlmann* and co-workers initially assigned the configuration of the epoxide to be 1$\alpha$,10$\alpha$, but they later revised the configuration to be 1$\beta$,10$\beta$ [30]. The $^1$H-NMR data of **15** were clearly different from those of 6$\beta$-acetoxy-1$\alpha$,10$\alpha$-epoxyfuranoeremophilane (**20**) reported by *Kitagawa et al.* [32]. The structure of 1$\alpha$,10$\alpha$-epoxide reported by *Burgueno-Tapia et al.* [29] should be revised to 1$\beta$,10$\beta$-epoxide, because their assignment was presumably based on the initial report of *Bohlmann et al.* [28]. Compound **16** was determined as 6$\beta$-{2-[(hydroxymethyl)prop-2-enoyl]oxy}furanoeremophil-1(10)-ene as follows. The molecular formula, $C_{19}H_{24}O_4$, of **16** was obtained by the HR-EI-MS (*m*/*z* 316.1694). The $^1$H- and $^{13}$C-NMR spectra of **16** resembled those of **21** [29] except for the presence of 2-(hydroxymethyl)prop-2-enoyloxy group (3420, 1713 cm$^{-1}$; $\delta$ 4.39 (*s*, 2 H), 5.90 (*s*, 1 H), 6.33 (*s*, 1 H)) instead of the AcO group in **21** [29] (*Tables 2* and *3*). The structure of **17** was assigned by comparing the $^1$H-NMR spectral data with those reported in [31].




**18** R = H
**19** R = AcO

**20**

**21**

The nucleotide sequences of the *atpB-rbcL* intergenic region in the plastid genome and the internal transcribed spacers (ITSs) of the ribosomal RNA gene in the nuclear genome were determined. Two different types were found for the *atpB-rbcL* sequence. In one type (**A** in *Table 1*), guanine (G), cytosine (C), and adenine (A) were at the 245th, the 301st, and the 469th positions, respectively, and the number of thymines (T) in a stretch around the 390th position was 9. This type was the same as in *L. cymbulifera* [2], *L. tongolensis* [2], *L. pleurocaulis* [3], and *L. virgaurea* var. *virgaurea* [4]. Within this type, the 28th base was A or G, and the number of As in a stretch around the 510th base was variable, as observed before [2–4]. Sample *20* had eight Ts in place of nine Ts, but otherwise the same as type **A**. Hence, it is designated as **A′** in *Table 1*. In the other type (**B** in *Table 1*), T, T, and C were at the 245th, the 301st, and the 469th positions, respectively, and the number of Ts was 11. Types **A** and **B** had been observed within *L. latihastata* [6] as well.

Variations were also observed in the ITSs, as summarized in *Table 4*. Most of the samples contained two ITS1 sequences of different lengths. In most of them, an extra T

Table 4. *Variations in the ITS1, the 5.8S RNA, and the ITS2 Sequences of the Ribosomal RNA Gene*[a])

| Sample | ITS1 | | | | | | | | | | | | 5.8S[b]) | | | | | ITS2 | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 11 | 12 | 26 | 47 | 50 | 73 | 89 | 132 | 139 | 223 | 255 | 235 | 122 | 123 | 136 | 140 | 151 | 48 | 58 | 118 | 125 | 127 | 132 | 142 | 156 | 172 | 104 | 108 | 169 | 179 | 180 | 182 | 202 | 214 | 220 |
| *1* | C | G | T | G | T | T | Y | T | T | G | R | R | C | T | C | R | C | G | Y | C | Y | C | G | A | W | G | T | R | A | C | T | C | C | C | T |
| *2* | C | G | T | R | Y | T | C | T | T | G | G | G | C | T | C | G | C | G | C | C | C | C | G | A | T | G | T | G | A | C | T | C | C | C | T |
| *3*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | C | G | C | C | C | C | R | A | T | G | T | G | A | C | T | C | C | C | T |
| *4*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | Y | G | C | C | Y | C | R | A | T | G | T | G | A | C | T | C | C | C | T |
| *5*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | C | G | C | C | C | C | R | A | T | G | T | R | A | C | T | C | C | C | T |
| *6*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | C | G | C | C | C | C | R | A | T | G | T | G | A | Y | T | C | C | C | T |
| *7*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | C | G | C | C | C | C | R | A | T | G | T | G | A | Y | T | C | C | C | T |
| *8*[c])[d]) | | | | | | | | | | | | | Y | T | Y | G | Y | G | C | C | C | C | R | A | T | G | T | G | A | C | T | C | C | C | T |
| *9*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | C | G | C | C | C | C | R | A | T | G | T | G | A | C | T | C | C | C | T |
| *10*[c])[d]) | | | | | | | | | | | | | Y | T | Y | G | C | G | C | Y | C | C | R | A | T | R | T | G | A | C | T | C | C | C | T |
| *11*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | C | G | C | C | C | C | R | A | T | G | T | G | A | C | T | C | C | C | T |
| *12*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | C | G | C | C | C | C | R | A | T | G | T | G | A | C | T | C | C | C | T |
| *13*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | C | G | C | C | C | C | R | A | T | G | T | G | A | C | T | C | C | C | T |
| *14*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | C | G | C | C | C | Y | R | A | T | G | T | G | A | C | T | C | C | C | T |
| *15*[e]) | Y | G | T | G | T | T | Y | T | T | R | G | G | C | T | T | G | C | G | C | C | C | C | G | A | T | G | T | G | A | C | T | C | Y | C | T |
| *16*[c])[d]) | | | | | | | | | | | | | C | T | C | G | C | G | C | C | C | C | G | A | T | G | T | G | R | C | T | C | C | C | T |
| *17*[f])[d]) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *18*[c])[d]) | | | | | | | | | | | | | Y | T | Y | G | Y | K | C | C | C | C | R | A | T | G | T | G | A | C | T | C | C | C | T |
| *19*[c])[d]) | | | | | | | | | | | | | C | T | Y | G | C | G | C | C | C | C | R | A | T | G | T | G | A | C | T | C | C | C | T |
| *20* | C | R | C | G | T | C | C | C | G | G | G | G | C | C | C | G | C | G | C | C | C | C | G | G | T | G | Y | G | A | C | Y | T | C | T | C |

[a]) K = G + T; R = A + G; W = A + T; Y = C + T. [b]) An additional ITS1 with an extra A after the 253rd position was present. [c]) An additional ITS1 sequence with an extra T after the 229th position was present. Because of superposition of signals from two sequences of different lengths, unambiguous sequence determination was not feasible. [d]) In samples with note b, c, or f, unambiguous sequence determination was possible only after the 121st base position. [e]) The entire base sequence of sample *15* has been deposited to the database (accession AB299047). [f]) An additional ITS1 with an extra A after the 253rd position was present. Besides, an additional ITS2 was present in which the 170th and 171st bases were deleted and the next base was changed from A to T.

was present after the 229th position in the longer sequence. In sample *16*, an extra A was present after the 253rd position in the longer one. Sample *17* not only contained an additional ITS1 with an extra T after the 220th but also contained an additional ITS2 in which the 170th and the 171st bases were missing, and the next base was changed from A to T. In the four samples for which the sequence of the entire ITS1–5.8S–ITS2 segment could be determined, sites with an additional base were not few. Besides, the sequences were different from one another.

**Discussion.** – In the present study, *L. dictyoneura* was examined with respect to the chemical composition and the nucleotide sequence. Compared with our previously studied species, quite a few more composition types, as many as 7, were identified, indicating that the intraspecific diversity in *L. dictyoneura* was extremely high. Nine samples contained no furano compounds, and eight of them were found in the Zhongdian area. All the nine had the **A** type of the *atpB-rbcL* sequence, and the sequence of the ITSs was similar (*Tables 1* and *4*). Thus, they seem to be similar both chemically and genetically. However, situations with other samples producing furano compounds were complex. For instance, type-1 and type-2 samples were collected at distant locations in a mixed manner. Such was the case in our previous results with *L. virgaurea* var. *virgaurea* [4], which had the two types that were not geographically separated. In the case of *L. virgaurea* var. *virgaurea*, there was a correlation between the composition and the ITS sequence. However, in the present case of *L. dictyoneura*, there appears no such correlation. The ITS sequence was dissimilar among type-1 samples. The same can be said of type-2 samples. It is also noteworthy that sample *20* had not only its own chemical composition but as many as eight base substitutions in the ITS/5.8S region. The sample was collected at an isolated location with a very high altitude (*Table 1* and *Fig. 1*). The isolation, and possibly adaptation to a different environment, may have rapidly drifted the population from others.

There were, largely speaking, two types of the *atpB-rbcL* sequence. The difference between them concerned four sites. As no intermediate type has been observed in either case, the presence of distinct variants suggests that hybridization has taken place [6]. The presence of multiple ITS sequences, manifestly seen in the length variants, also suggests hybridization [33]. Therefore, it is likely that some of the chemical and the morphological diversity in *L. dictyoneura* has resulted from hybridization.

Studies on rat cytochrome P450 have suggested that the furan ring in furanoeremophilanes is derived from eremophil-7(11)-en-8-one derivative [34]. For instance, **12** would be derived from **3**. In this regard, sample *9* is interesting. The sample produced a furanoeremophilane **12** as the major component (type 3), while samples from surrounding locations produced derivatives of 7(11)-en-8-one or its isomer, 11-en-8-one (*Table 1* and *Fig. 1*). At the location of sample *9*, Shikashan in the Zhongdian area, *L. dictyoneura* was very abundant, growing among scrubs on one whole hill. We could not see such a large habitat at the other sampling locations in the Zhongdian area. Although no exact biochemical/ecological function of furanoeremophilanes is currently known, furanosesquiterpenes are considered to be defensive materials [34][35]. Thus, it is conceivable that acquiring the ability to produce furanoeremophilane has given the Shikashan population an ecological advantage. The similarity of DNA sequence among the Zhongdian-area samples including sample *9* supports the notion

that the Shikashan population has evolved from the Zhongdian population that used to produce no furano compounds. *L. cymbulifera* [2] and *L. kanaitzensis* [36] produce furano compounds, and they are abundant in northwestern Yunnan and often found forming large exclusive colonies in open fields. This observation seems to support our hypothesis that production of furano compounds has an ecological advantage.

**Conclusions.** – *Ligularia dictyoneura* was found to be extremely diverse with respect to chemical composition, DNA sequence, as well as morphology. From 20 samples of the species, 17 eremophilane derivatives were isolated, including three new furanoeremophilane derivatives, 3$\beta$-acetoxy-6$\beta$-(angeloyloxy)furanoeremophilan-10$\beta$-ol (**9**), 1$\alpha$-acetoxyfuranoeremophilan-15,6$\alpha$-olide (**11**), and 6$\beta$-{2-[(hydroxymethyl)prop-2-enoyl]oxy}furanoeremophil-1(10)-ene (**16**). While several furanoeremophilan-15,6-olides are known, compound **11** is the first example of having an oxygen functionality at C(1). Seven types of chemical composition were identified in the 20 samples: one with non-furanoeremophilane derivatives and the others (types 1–6) with furanoeremophilane derivatives of different oxidation levels. Two distinct types were found for the DNA sequence of the *atpB-rbcL* intergenic region. The sequence of the ITSs/5.8S region was also very diverse. The presence of two distinct types of the *atpB-rbcL* sequence and multiple ITS sequences within individuals suggests hybridization in the past. No correlation between the chemical composition and the DNA sequence indicates the presence of complex mechanism in the evolution of *L. dictyoneura*. Our results on inter- and intraspecific diversity in *Ligularia* hitherto have shown that the genus is indeed continuing its rapid evolution and suggest that production of furano compounds confers an ecological importance.

The authors wish to thank Prof. *Sugong Wu*, Dr. *Qing Yin*, and Mrs. *Guowen Hu* of Kunming Institute of Botany, Miss *Yasuyo Yatsuhashi*, Miss *Masayo Yatsuhashi*, and Mr. *Shoji Yatsuhashi* for assistance in the field, and Dr. *Tsuneyoshi Kuroiwa* for use of his *Beckman* sequencer. This work was conducted as part of *Rikkyo Frontier Project* '*Adaptation and Evolution of Extremophile*', supported in part by the *Ministry of Education, Culture, Sports, Science and Technology of Japan*. Financial support was also obtained from *JSPS* (Grant-in-Aid for Scientific Research No. 16404008).

## Experimental Part

1. *General*. Column chromatography (CC): silica gel (*Merck Kieselgel 60*). Prep. TLC: silica gel (*Merck Kieselgel 60 $F_{254}$*, layer thickness 0.2 mm). Optical rotations: *JASCO DPI-181* polarimeter. IR Spectra: *SHIMADZU FTIR-8700* spectrometer; in $cm^{-1}$. $^1$H- and $^{13}$C-NMR spectra: *GNM-AL 400* ($^1$H: 400, $^{13}$C: 100.5 MHz) spectrometer with $CDCl_3$ or $C_6D_6$ as the solvent and TMS as an internal standard; $\delta$ in ppm, *J* in Hz. NOESY, HMQC, and HMBC spectra: *Bruker DRX-500* (500 MHz) spectrometer. EI-MS (pos.-ion mode): *JEOL JMS-700* mass spectrometer; in *m*/*z* (rel. %). DNA Sequencing was carried out with *DYEnamic ET Sequencing Kit* (*GE Health Science*) and *ABI Prizm 310* (*Applied Biosystems*), *DTCS Kit* (*Beckman*) and *CEQ2000* sequencer (*Beckman*), or *BigDye Terminator Kit* (*Applied Biosystems*) and *3130xl Genetic Analyzer* (*Applied Biosystems*). See our previous reports for *Ehrlich*'s test on TLC [2–5].

2. *Plant Material*. Samples of *L. dictyoneura* were collected in each August of 2002–2006 at locations indicated in *Table 1* and *Fig. 1*. Each plant was identified by *X. G.*

3. *Extraction for* Ehrlich*'s Test*. The roots of each plant (2–10 g) were harvested, and extraction with EtOH was started immediately without drying. Extraction was continued at r.t. for several days. After filtering, the soln. was subjected to TLC without concentration.

4. *Extraction for Structure Determination*. For the samples collected in 2003, the collected roots of *L. dictyoneura* were cut into small pieces without drying, and immediately extracted with EtOH at r.t. The extract was filtered and concentrated to afford an oily residue with an aq. phase. AcOEt was added to this oil/aq. mixture, and the org. layer was recovered. Evaporation of the solvent afforded an oily residue, to which water-soluble starch was added for handling purpose. For the samples collected in 2004–2006, the roots were dried and extracted with EtOH at r.t. Oily extracts were obtained by the standard method.

5. *Purification and Identification of Chemical Components of Sample* 7 (*Ehrlich*-negative type). The extract of sample *7* (940 mg) collected in Dabaoshan was subjected to CC ($SiO_2$ (20 g); hexane/AcOEt 10:1) to give a mixture of **1**, **2**, and **3** (201 mg, 21%, ratio 4.4:1:2.3), and a mixture of **4** and **5** (166 mg, 18% yield; ratio 1.2:1). The former mixture (104 mg) was separated by further CC ($CH_2Cl_2$/AcOEt 150:1) to give an inseparable mixture of **1** and **2** (62 mg, 12% yield, ratio 6.3:1), a mixture of **1–3** (25 mg, 5.2%), and **3** (9.7 mg, 2% yield). The mixture of **4** and **5** (76 mg) was subjected to CC (hexane/AcOEt; gradient) and then to prep. TLC (hexane/AcOEt 4:1; four times) to give **4** (17mg, 3.9% yield), a mixture of **4** and **5** (15 mg, 3.5%; 1:5), and **5** (9.9 mg, 2.3% yield).

6. *Purification and Identification of Chemical Components of Samples* 4 *and* 15 (type 1). The extract of sample *4* (276 mg) collected near Baishuitai was subjected to CC ($SiO_2$ (14 g); hexane/AcOEt 20:1) to give a mixture containing *ligularol* (**6**) (111 mg), a mixture of **6** and *3β-acetoxy-6β-(angeloyloxy)furanoeremophilane* (**8**; $R_f$ (hexane/AcOEt 7:3) 0.69; 25.8 mg), and *3β-acetoxy-6β-(angeloyloxy)furanoeremophilane-10β-ol* (**9**; $R_f$ 0.39; 39 mg, 14.3%). From the least polar mixture, **6** ($R_f$ 0.63) was isolated (1.8 mg, 0.65%). The mixture of **6** and **8** was subjected to prep. TLC (hexane/AcOEt 7:3) to give **6** (3.9 mg, 1.4%) and **8** (9.1 mg, 3.3%); total yield of **6**: 2.1%. Furthermore, the extract of sample *4* (361 mg) was submitted to CC (hexane/AcOEt 20:1) to give a mixture (81 mg), which was then purified by CC (hexane/AcOEt 25:1). Subsequent prep. TLC (hexane/AcOEt 4:1) gave the less polar compound **7** ($R_f$ 0.74; 14.6 mg, 4.0%).

*Compound* **9**: M.p. 95.5–96.5° (hexane). $[\alpha]_D^{31} = -56$ ($c = 0.16$, $CHCl_3$). IR (Nujol): 3488, 1713, 1456, 1378, 1276, 1229, 1160, 1035, 961. $^1$H- and $^{13}$C-NMR: *Tables 2* and *3*, resp. EI-MS: 390 (18, $M^+$), 290 (45), 206 (15), 83 (100), 55 (46), 43 (59). HR-EI-MS: 390.2052 ($M^+$, $C_{22}H_{30}O_6^+$; calc. 390.2042), 206.0931 (retro-*Diels–Alder* fragment (*Fig. 3*), $C_{12}H_{14}O_3^+$; calc. 206.0943).

The extract of sample *15* (97 mg) collected in Yongning was subjected to CC ($SiO_2$ (9 g); hexane/AcOEt 20:1) to give **9** (8.3 mg, 8.5% yield).

7. *Purification and Identification of Chemical Components of Sample* 3 (type 2). The extract of sample *3* (306 mg) collected in Hutiaoxia was subjected to CC ($SiO_2$ (15 g); hexane/AcOEt 30:1) to give a mixture of **6** and *furanoeremophilan-15,6α-olide* (**10**; 215 mg) and a mixture containing *1α-acetoxyfuranoeremophilan-15,6α-olide* (**11**; 54 mg). The mixture of **6** and **10** was separated by CC (hexane/AcOEt 50:1 then 40:1) to give **6** (83 mg, 27% yield) and **10** (18.1 mg, 6% yield). The mixture containing **11** was further purified by CC (hexane/AcOEt 30:1 and then $CH_2Cl_2$/AcOEt 50:1). Finally, the mixture was partly separated by prep. TLC (hexane/AcOEt 4:1; four times) to give **11** (3.6 mg, 1%). Oil. $[\alpha]_D^{30} = -33$ ($c = 0.11$, $CHCl_3$). IR (neat): 1780, 1733, 1260, 1240, 1090, 1030, 945, 802. $^1$H-NMR ($CDCl_3$): 1.34 (*s*, Me(14)), 2.03 (*d*, $J = 1.4$, Me(13)); 2.08 (*s*, COMe); 5.33 (*ddd*, $J = 11.7, 4.9, 4.9$, H–C(1)); 7.11 (*s*, H–C(12)). $^1$H- and $^{13}$C-NMR ($C_6D_6$): *Tables 2* and *3*, resp. EI-MS: 304 (39, $M^+$), 244 (98, [$M$ – AcOH]$^+$), 185 (70), 172 (100), 43 (39). HR-EI-MS: 304.1339 ($M^+$, $C_{17}H_{20}O_5^+$; calc. 304.1311).

8. *Purification and Identification of Chemical Components of Sample* 9 (type 3). The extract of sample *9* (204 mg) collected at Shikashan was subjected to CC ($SiO_2$ (10 g); hexane and hexane/AcOEt 10:1) to give *furanoeremophilane* (**12**; 63 mg, 31% yield) and a mixture containing **6**. The mixture was purified by prep. TLC ($CH_2Cl_2$) to give **6** (14 mg, 7%).

9. *Purification and Identification of Chemical Components of Sample* 14 (type 4). The extract of sample *14* (150 mg) collected at Xiaoxueshan was subjected to CC ($SiO_2$ (7 g); hexane and hexane/AcOEt 20:1) to give a mixture containing *ligularone* (**13**; 47 mg) and a mixture containing **10** (35 mg). The CC ($SiO_2$ (2 g); hexane/AcOEt 40:1) of the former mixture, followed by the prep. TLC ($CH_2Cl_2$/hexane 2:1) gave **13** (4.2 mg, 2.8%). The latter mixture was subjected to prep. TLC ($CH_2Cl_2$/AcOEt 20:1) to give a mixture (14 mg), which was further purified by CC ($SiO_2$ (1 g); hexane/AcOEt 30:1) to give **10** (3.5 mg, 2.3%).

10. *Purification and Identification of Chemical Components of Sample* 17 (type 5). The extract of sample *17* (43 mg) collected in Rencun was subjected to CC ($SiO_2$ (3 g); hexane/AcOEt 40 : 1 then 30 : 1) to give a crude mixture containing **3** and **14** (23.2 mg). The mixture was separated by CC ($CH_2Cl_2$/AcOEt 50 : 1 then 20 : 1) to give **3** (5.2 mg, 12%) and **14** (9.9mg, 23%).

11. *Purification and Identification of Chemical Components of Sample* 20 (type 6). The extract of sample *20* (144 mg) collected at Baimaxueshan was subjected to CC ($SiO_2$ (7 g); hexane/AcOEt 20 : 1) to give a mixture containing **15** (16 mg), a mixture containing **16** (29 mg), and epoxide **17** (15 mg; 10%). The mixture containing **15** was further purified by CC ($SiO_2$ (1 g); hexane/AcOEt 40 : 1) to give **15** (6 mg, 4% yield). The mixture containing **16** was further purified by CC ($SiO_2$ (1 g); hexane/AcOEt 20 : 1) to give *6β-{2-[(hydroxymethyl)prop-2-enoyl]oxy}furanoeremophil-1(10)-ene* (**16**; 4.4 mg, 3%). Oil. $[\alpha]_D^{28.5} = +17$ ($c = 0.22$, EtOH). IR (neat): 3420, 1713, 1160, 1049, 956. $^1$H- and $^{13}$C-NMR: *Tables 2* and *3*, resp. EI-MS: 316 (2.6, $M^+$), 214 (100), 199 (75), 172 (47), 149 (55). HR-EI-MS: 316.1694 ($M^+$, $C_{19}H_{24}O_4^+$; calc. 316.1674).

## REFERENCES


[1] S.-W. Liu, D.-S. Deng, J.-Q. Liu, *Acta Phytotaxonom. Sin.* **1994**, *32*, 514.

[2] R. Hanai, X. Gong, M. Tori, S. Kondo, K. Otose, Y. Okamoto, T. Nishihama, A. Murota, Y. Shen, S. Wu, C. Kuroda, *Bull. Chem. Soc. Jpn.* **2005**, *78*, 1302.

[3] H. Nagano, Y. Iwazaki, X. Gong, Y. Shen, C. Kuroda, R. Hanai, *Bull. Chem. Soc. Jpn.* **2006**, *79*, 300.

[4] M. Tori, K. Honda, H. Nakamizo, Y. Okamoto, M. Sakaoku, S. Takaoka, X. Gong, Y. Shen, C. Kuroda, R. Hanai, *Tetrahedron* **2006**, *62*, 4988.

[5] A. Torihata, R. Hanai, X. Gong, Y. Shen, C. Kuroda, *Chem. Biodiv.* **2007**, *4*, 500.

[6] C. Kuroda, K. Kiuchi, A. Torihata, K. Takeshita, X. Gong, Y. Shen, H. Hirota, H. Onuki, R. Hanai, *Chem. Biodiv.* **2007**, *4*, 2210.

[7] a) C. Kuroda, T. Ueshino, H. Nagano, *Bull. Chem. Soc. Jpn.* **2004**, *77*, 1737; b) C. Kuroda, E. Nishio, *Nat. Prod. Commun.* **2007**, *2*, 581.

[8] V. Savolainen, J.-F. Manen, E. Douzery, R. Spichiger, *Mol. Phylogenet. Evol.* **1994**, *3*, 27; D. E. Soltis, P. S. Soltis, in 'Molecular Systematics of Plants. II. DNA Sequencing', Eds. P. S. Soltis, D. E. Soltis, J. J. Doyle, Kluwer Academic Publishers, Boston, 1998, pp. 1–42.

[9] S.-W. Liu, 'Flora Reipublicae Popularis Sinicae', Science Press, Beijing, 1989, Vol. 77.

[10] A. Tan, H. He, H. Yang, M. Zhang, Z. Wang, X. Hao, *J. China Pharm. Univ.* **2002**, *33*, 104; A. Tan, H. Yang, Y. Li, Z. Bi, M. Zhang, Z. Wang, *Chin. Pharm. J.* **2004**, *39*, 498; A. Tan, H. He, H. Yang, M. Zhang, Z. Wang, X. Hao, *Acta Pharm. Sin.* **2003**, *38*, 924.

[11] S. Zhao, Y. Chen, *Zhongcaoyao* **1982**, *13*, 12; Y. Chen, S. Zhao, *Zhongcaoyao* **1985**, *16*, 277.

[12] H. Hagiwara, H. Uda, T. Kodama, *J. Chem. Soc., Perkin Trans. 1* **1980**, 963; M. Ishihara, T. Tsuneya, K. Ueyama, *Pytochemistry* **1993**, *33*, 1147.

[13] M. Neuenschwander, A. Neuenschwander, E. Steinegger, *Helv. Chim. Acta* **1979**, *62*, 627.

[14] S. Torii, T. Inokuchi, T. Yamafuji, *Bull. Chem. Soc. Jpn.* **1979**, *52*, 2640; P. Duhamel, G. Dujardin, L. Hennequin, J. M. Porrier, *J. Chem. Soc., Perkin Trans. 1* **1992**, 387; R. A. Schenato, E. M. dos Santos, B. S. M. Tenius, P. R. R. Costa, I. Caracelli, J. Zukerman-Schpector, *Tetrahedron: Asymmetry* **2001**, *12*, 579.

[15] M. Tori, M. Kawahara, M. Sono, *Phytochemistry* **1997**, *47*, 401; M. C. Witschel, H. J. Bestmann, *Synthesis* **1997**, 107.

[16] F. Bohlmann, K. H. Knoll, C. Zdero, P. K. Mahanta, M. Grenz, A. Suwita, D. Ehlers, N. Le Van, W. R. Abraham, A. A. Natu, *Phytochemistry* **1977**, *16*, 965.

[17] H. Ishii, T. Tozyo, H. Minato, *Tetrahedron* **1965**, *21*, 2605.

[18] K. Yamakawa, T. Satoh, *Chem. Pharm. Bull.* **1979**, *27*, 1747.

[19] a) H. Nagano, Y. Tanahashi, Y. Moriyama, T. Takahashi, *Bull. Chem. Soc. Jpn.* **1973**, *46*, 2840; b) F. Bohlmann, C. Zdero, M. Grenz, *Chem. Ber.* **1974**, *107*, 3928.

[20] F. Bohlmann, C. Zdero, D. Berger, A. Suwita, P. Mahanta, C. Jeffrey, *Phytochemistry* **1979**, *18*, 79.

[21] L. Novotny, M. Krojidlo, Z. Samek, J. Kohoutova, F. Šorm, *Coll. Czech. Chem. Commun.* **1973**, *38*, 739.

[22] H. Goto, E. Osawa, *J. Am. Chem. Soc.* **1989**, *111*, 8950; H. Goto, E. Osawa, *J. Chem. Soc.*, *Perkin Trans. 2* **1993**, 187.
[23] M. J. S. Dewar, E. G. Zoebisch, E. F. Healy, J. J. P. Stewart, *J. Am. Chem. Soc*. **1985**, *107*, 3902; J. J. P. Stewart, *J. Comp. Chem.* **1989**, *10*, 209.
[24] Y. Ishizaki, Y. Tanahashi, T. Takahashi, K. Tori, *J. Chem. Soc., Chem. Commun.* **1969**, 551; Y. Ishizaki, Y. Tanahashi, T. Tsuyuki, T. Takahashi, K. Tori, *Bull. Chem. Soc. Jpn.* **1979**, *52*, 1182; M. Tada, Y. Sugimoto, T. Takahashi, *Bull. Chem. Soc. Jpn.* **1980**, *53*, 2966; H.-M. Chen, M.-S. Cai, Z.-J. Jia, *Phytochemistry* **1997**, *45*, 1441.
[25] H. Ishii, T. Tozyo, H. Minato, *J. Chem. Soc. C* **1966**, 1545; K. Yamakawa, T. Satoh, *Chem. Parm. Bull*. **1978**, *26*, 3704.
[26] M. Tada, Y. Moriyama, Y. Tanahashi, T. Takahashi, *Bull. Chem. Soc. Jpn.* **1974**, *47*, 1999; M. Tada, T. Sato, T. Takahashi, K. Tori, I. Horibe, K. Kuriyama, *J. Chem. Soc.*, *Perkin Trans. 1* **1981**, 2695.
[27] H. Nagano, T. Takahashi, *Bull. Chem. Soc. Jpn.* **1972**, *45*, 1935; K. Naya, Y. Makiyama, T. Matsuura, N. Ii, H. Nagano, T. Takahashi, *Chem. Lett*. **1978**, 301.
[28] F. Bohlmann, C. Zdero, N. Rao, *Chem. Ber*. **1972**, *105*, 3523.
[29] E. Burgueno-Tapia, L. R. Hernandez, A. Y. Resendiz-Villalobos, P. Joseph-Nathan, *Magn. Reson. Chem*. **2004**, *42*, 887.
[30] F. Bohlmann, C. Zdero, *Phytochemistry* **1978**, *17*, 1135.
[31] T. Sato, Y. Moriyama, H. Nagano, Y. Tanahashi, T. Takahashi, *Bull. Chem. Soc. Jpn.* **1975**, *48*, 112.
[32] I. Kitagawa, H. Shibuya, M. Kawai, *Chem. Pharm. Bull.* **1977**, *25*, 2638.
[33] M. A. Koch, C. Dobeš, T. Mitchell-Olds, *Mol. Biol. Evol.* **2003**, *20*, 338, and refs. cit. therein.
[34] N. W. Gaikwad, K. M. Madyastha, *Biochem. Biophys. Res. Commun.* **2002**, *290*, 589.
[35] M. A. Schuler, *Plant Physiol.* **1996**, *112*, 1411.
[36] Y. Okamoto, K. Tachikawa, Y. Matsuo, K. Nakashima, M. Sono, M. Tori, R. Hanai, C. Kuroda, X. Gong, Y. Shen, 'Proceedings of the 50th Symposium on the Chemistry of Terpenes, Essential Oils, and Aromatics', Yokohama, Japan, 2006, pp. 355–357.

*Received June 11, 2007*