Chemical and Genetic Study of *Ligularia anoleuca* and *L. veitchiana* in Yunnan and Sichuan Provinces of China

by **Hajime Nagano***[a])[1]), **Midori Kanda**[a]), **Hiroka Yamada**[a]), **Ryo Hanai***[b])[2]), **Xun Gong***[c])[3]), and **Chiaki Kuroda***[d])[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.ac.jp)

[c]) Kunming Institute of Botany, Chinese Academy of Science, Kunming 650204, China (phone: +86-8715223625; e-mail: gongxun@mail.kib.ac.cn)

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

Chemical and genetic study of *Ligularia anoleuca* and *L. veitchiana*, which belong to section *Ligularia*, series *Speciosae*, was carried out. From *L. anoleuca* samples, collected in Yunnan and Sichuan Provinces of China, a new compound, furanoeremophil-1(10)-en-6α-ol, was isolated together with known 6β-{[2-(hydroxymethyl)prop-2-enoyl]oxy}furanoeremophil-1(10)-ene and 1β,10β-epoxy-6β-{[2-(hydroxymethyl)prop-2-enoyl]oxy}furanoeremophilane. From *L. veitchiana* samples, collected in Yunnan Province, euparin, 2-isopropenyl-5,6-dimethoxybenzofuran, and 6-hydroxy-3β-methoxytrementone were isolated. DNA Sequencing of the internal transcribed spacers of the ribosomal RNA gene showed that the two species are not particularly close despite morphological similarities, in agreement with the chemical results.

Introduction. – *Ligularia* CASS. (Asteraceae) in the Hengduan Mountains of China is highly diversified [1–3], 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 analysis [4–11]. To examine the chemical diversity, furanoeremophilanes and related compounds are analyzed, since the presence of the former can be easily detected by *Ehrlich*'s test on TLC plates [12]. To examine the genetic diversity, the DNA sequences of the two internal transcribed spacers (ITSs) of the ribosomal RNA gene in the nuclear genome and the *atpB-rbcL* intergenic region in the plastid genome are analyzed [13]. To date, we have revealed intra-specific diversity in a number of species in Yunnan and Sichuan Provinces. For example, *L. dictyoneura* (FRANCH.) HAND.-MAZZ. [4], *L. kanaitzensis* (FRANCH.) HAND.-MAZZ. [5], and *L. subspicata* (BUREAU & FRANCH.) HAND.-MAZZ. [6] have been found to be highly

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

diverse. *L. tsangchanensis* (FRANCH.) HAND.-MAZZ. [7] and *L. pleurocaulis* (FRANCH.) HAND.-MAZZ. [8] were found to be moderately diverse, and plants of each species could be separated into two distinct groups in accordance with geographic distribution. *L. virgaurea* (MAXIM.) MATTF. [9] was also separated into two types, but they were not geographically separated. *L. cymbulifera* (W. W. SM.) HAND.-MAZZ. [10] and *L. cyathiceps* HAND.-MAZZ. [11] were found to be uniform.

We have previously reported results on *L. latihastata* (W. W. SM.) HAND.-MAZZ. and *L. villosa* (HAND.-MAZZ.) S. W. LIU [14], morphologically close species belonging to section *Ligularia*, series *Speciosae* POJARK. [1]. They were found to produce benzofuran derivatives instead of eremophilane-type sesquiterpenoids, and to be chemically very similar as well. In the present study, we examined another pair of morphologically close species belonging to the same *Speciosae* series, *L. anoleuca* HAND.-MAZZ. and *L. veitchiana* (HEMSL.) GREENM. [1]. Chemical constituents of *L. veitchiana* have been reported by several groups [15–19]. The major components were eremophilane-type sesquiterpenoids in samples collected in northwestern China [15] and Henan Province [16]. Natural *Diels–Alder* products derived from eremophilane-type sesquiterpenoids were also obtained [17]. Guaiane and eudesmane-type sesquiterpenoids have also been isolated together with eremophilane-type sesquiterpenoids from a sample collected in Hubei Province [18]. To the best of our knowledge, there has been no report on the chemical constituents of *L. anoleuca*.

Results. – Samples of *L. anoleuca* were collected in 2009 at Cangshan Mountain, Dali City, Yunnan Province, and in Xiaojin County, Sichuan Province (samples *1* and *2*, resp.; *Fig.*). Three samples of *L. veitchiana* were collected in Jianchuan County, Yunnan Province (samples *3–5* in *Fig.*).

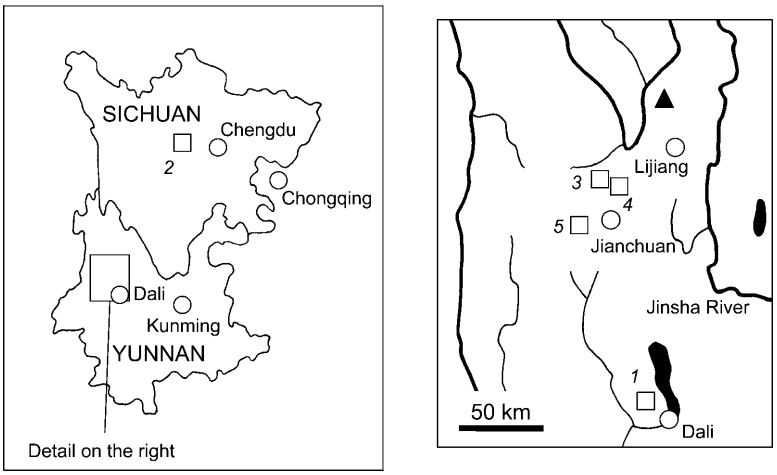
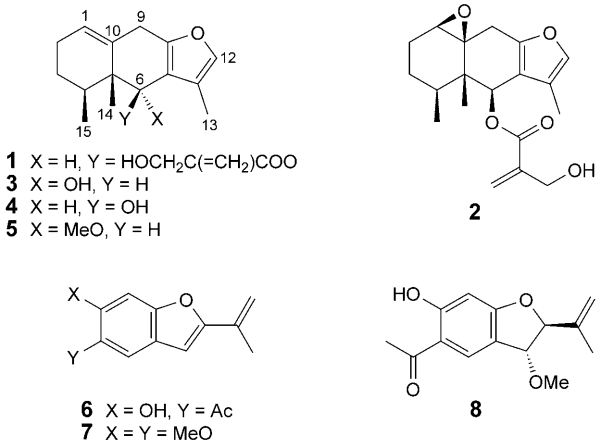


Figure. *Locations of sample collection of* L. anoleuca (samples *1* and *2*) *and* L. veitchiana (samples *3–5*). Rectangles, collection locations; circles, cities; triangle, Mt. Yulongxueshan.

Roots of the samples were extracted with EtOH. From the extract of a *L. anoleuca* sample (sample *1*), 6*β*-{[2-(hydroxymethyl)prop-2-enoyl]oxy}furanoeremophil-1(10)-

ene (**1**; R_f (hexane/AcOEt 7:3) 0.50; $[\alpha]_D^{24} = +21.0$ ($c = 0.77$, EtOH)) [4] and 1β,10β-epoxy-6β-{[2-(hydroxymethyl)prop-2-enoyl]oxy}furanoeremophilane (**2**; R_f 0.43) [20] were isolated by silica-gel column chromatography in 14 and 3.8% yields, respectively. A new compound, furanoeremophil-1(10)-en-6α-ol (**3**; R_f 0.65), was isolated as a minor component by repeated column chromatography in 1% yield. Compound **3** was detected as an *Ehrlich*-positive spot by TLC (purple in color; R_f (hexane/Et_2O 1:1) 0.61), slightly less polar than its epimer, furanoeremophil-1(10)-en-6β-ol (=6β-hydroxyeuryopsin; **4**; blue in color; R_f 0.59) [21]. The molecular formula of compound **3** was determined to be $C_{15}H_{20}O_2$ by HR-EI-MS (*m/z* 232.1458 (M^+); calc. 232.1463). IR Absorption at 3421 cm^{-1}, ^{1}H-NMR (δ 7.06 (*q*, $J = 1.0$, H−C(12)), 5.81–5.78 (*m*, H−C(1)), 4.32 (*d*, $J = 8.7$, H−C(6)), 2.04 (*d*, $J = 1.0$, Me(13)), 0.99 (*d*, $J = 6.8$, Me(15)), 0.82 (*s*, Me(14)), and ^{13}C-NMR (δ 150.2, 137.7, 134.3, 127.8, 120.1, 119.5, 68.0, together with the signals of three Me C-atoms at δ 17.5, 15.5, 7.8) spectra indicated the presence of an OH group and a C=C bond on the furanoeremophilane skeleton. A pair of geminally spin-coupled signals of CH_2(9) at δ 3.43 (*ddd*, $J = 19.5, 5.8, 2.9$, 1 H) and 3.23 (*d*, $J = 19.5$, 1 H) indicated the presence of a double bond between C(1) and C(10). These results suggested compound **3** to be furanoeremophil-1(10)-en-6-ol. The ^{1}H- and ^{13}C-NMR data of compound **3** were, however, different from those of furanoeremophil-1(10)-en-6β-ol (=6β-hydroxyeuryopsin; **4**) [21]. The diastereoisomeric structure of **3** was supported by mass spectroscopy, because **3** showed a fragment ion at *m/z* 109 (relative intensity 84%), whereas **4** was known to show a base peak at the same position [21a]. The shielding of Me(14) (δ 0.82; *cf.* δ 1.04 for **4**) in ^{1}H-NMR and the deshielding of Me(15) (γ-gauche effect) (δ 17.5; *cf.* δ 15.9 for **4**) in ^{13}C-NMR spectra of **3**, showing chemical-shift values similar to those of 6α-methoxyeuryopsin (**5**) (δ 0.76 (Me(14)) and 18.1 (Me(15))) [21b], indicated the α-configuration of the OH group at C(6). Finally, the configuration was confirmed by the observation of NOEs between H−C(6) and Me(14) and between H−C(6) and Me(15).



From the extract of the other *L. anoleuca* sample (sample *2*), furanoeremophilane derivatives **1** and **2** were isolated in 19 and 8.2% yields, respectively. Although the presence of alcohols **3** and **4** was suggested by *Ehrlich*'s test, their isolation in pure form

was not achievable due to their paucity and severe contamination by other chemical components.

The extracts of the *L. veitchiana* samples (samples *3*–*5*) showed no *Ehrlich*-positive spots on TLC plates. There were two major spots (R_f (hexane/AcOEt 7:3) 0.61 and 0.55) together with spots of minor components, when detected by coloring with MoO_3/H_3PO_4.

From the extract of sample *3*, euparin (**6**), (R_f 0.61) [22], 2-isopropenyl-5,6-dimethoxybenzofuran (**7**; R_f 0.55) [23], and 6-hydroxy-3β-methoxytrementone (**8**; $[\alpha]_D^{25} = -12$ ($c = 0.44$, $CHCl_3$); R_f 0.35) [24] were isolated in 5.4, 6.0, and 0.9% yields, respectively, by silica-gel column chromatography.

From the extract of sample *4*, benzofuran derivatives **6**–**8** were isolated in 8.6, 15, and 1.6% yields, respectively.

From the extract of sample *5*, euparin (**6**) [22] and 2-isopropenyl-5,6-dimethoxybenzofuran (**7**) [23] were isolated in 12 and 11% yields, respectively.

The nucleotide sequences of the ITS1-5.8S-ITS2 and the *atpB-rbcL* regions were determined. The sequences for *L. anoleuca* (sample *2*) and *L. veitchiana* (sample *5*) have been deposited with the database (AB557884–AB557887). A number of differences were found in the ITS1-5.8S-ITS2 sequence between the two species, as tabulated in *Table 1*. Variations within each species were fewer, as shown in *Table 2*. The sequence of the *atpB-rbcL* region was the same in the five samples except for the number of As in the stretch around the 510th base. In samples *1*, *2*, and *5*, the number was 9; in samples *3* and *4*, 10. The region has been found to harbor few inter-specific changes, and the intra-specific variation of the number of As has been found to be common in *Ligularia* [4–11].

Table 1. *Differences in ITS1-5.8S-ITS2 between* L. anoleuca *and* L. veitchiana[a])

	ITS1																ITS2				
						1	1	1	1	1	1	1		2	2	2				1	2
	1	1	4	4	9	0	1	2	2	2	6	7	8		2	3	4		7	9	8	0
	2	3	6	7	4	1	1	5	6	7	6	3	2	[b])	1	9	1	5	1	2	7	4
L. anoleuca[c])	W	T	C	R	Y	T	A	C	T	A	G	T	A	–	C	G	A	T	C	T	R	T
L. veitchiana[d])	A	A	T	G	C	C	C	T	C	C	R	C	–	C	G	A	G	C	–	C	T	C

[a]) Base numbering is based on the *L. anoleuca* sequence. R = A + G; W = A + T; Y = C + T; –: deletion. [b]) Insertion between the 216th and the 217th sites of the *L. anoleuca* sequence. [c]) Sample *2* (AB557884); 642 base-pairs in length. [d]) Sample *5* (AB557886); 641 base-pairs in length.

Discussion. – In this study, furanoeremophilane derivatives **1** [4], **2** [20], and **3** were isolated from *L. anoleuca*. From *L. veitchiana*, euparin (**6**) [22] and its derivatives **7** [23] and **8** [24] were obtained. Biochemically, sesquiterpenoids are synthesized from mevalonic acid, whereas euparin-type benzofurans are from shikimic acid [25]. Thus, the present samples of *L. anoleuca* and *L. veitchiana* appear biosynthetically quite separate despite their close morphological similarities. This separation finds support in the results of DNA analysis. When the ITS1-5.8S-ITS2 sequences of *L. anoleuca* and *L. veitchiana* were compared, 14 base substitutions, excluding sites with multiple bases,

Table 2. *Variations in ITS1-5.8S-ITS2 between* L. anoleuca *and* L. veitchiana *Samples*[a])

Sample	*L. anoleuca*						*L. veitchiana*				
	ITS1		ITS2				ITS1			ITS2	
	1	2	1	1	1	1	1	2	2		1
	6	0	1	7	8	9	6	1	4		0
	6	8	6	9	7	2	6	4	1	4	4
1	R	W	R	W	G	Y					
2	G	T	C	T	R	C					
3[b])							G	Y	G	G	C
4							G	C	K	R	Y
5							R	C	G	G	C

[a]) R = A + G; W = A + T; Y = C + T; K = G + T. [b]) Two sequences with a length difference of one base were superimposed with almost equal signal intensities. The longer one had four As in place of three at 184–186 in ITS1, and T in place of C at 214 in ITS1 (thus, listed as Y).

were found (*Table 1*). When the ITS1-5.8S-ITS2 sequence of *L. dictyoneura* (FRANCH.) HAND.-MAZZ. (AB299047) is compared with those of *L. anoleuca* and *L. veitchiana*, the number of base substitutions are 16 for *L. anoleuca* and 15 for *L. veitchiana*. Since *L. dictyoneura* belongs to section *Senecillis* and is considered to be in a lineage different from section *Ligularia* [3], these similar numbers indicate that *L. anoleuca* and *L. veitchiana* are not particularly close to each other. Thus, these results show that the present samples of *L. anoleuca* and *L. veitchiana* are chemically and genetically separate.

In this work, only euparin-type benzofurans were isolated from *L. veitchiana*. This result and literature suggest that the species is chemically diverse. Eremophilane-type sesquiterpenoids as well as euparin-type benzofurans have been isolated from *L. veitchiana* in other parts of China [15–18]. *Wang et al.* isolated eremophilane-type sesquiterpenoids from a sample collected in the Funiu Mountain area in Henan Province [16]; *Jia* and co-workers obtained both euparin (**6**) and eremophilane-type sesquiterpenoids from the species collected in northwestern China [15e]. Within the genus *Ligularia* in China, benzofuran derivatives have been isolated from various plants in section *Ligularia*, series *Speciosae*. Belonging to this series, *L. caloxantha* (DIELS) HAND.-MAZZ. [26], *L. przewalskii* (MAXIM.) DIELS [27], *L. intermedia* NAKAI [28], as well as *L. veitchiana*, produce both benzofuran and eremophilane derivatives. Benzofurans were also isolated from *L. latihastata* [14], *L. odontomanes* HAND.-MAZZ. [29], *L. villosa* [14], and *L. stenocephala* (MAXIM.) MATSUM. & KOIDZ. [30], but eremophilanes were not obtained. In contrast, only terpenoids were isolated from *L. fischeri* (LEDEB.) TURCZ. [31] and *L. dolichobotrys* DIELS [32]. Thus, this series is chemically highly diverse. Further studies on this series should yield clues to a mechanism of chemical evolution.

The root of *L. veitchiana* is used as a herbal medicine '*Shanziyuan*' ('*San-shion*' in Japanese) [2]. However, not only *L. veitchiana* but also *L. latihastata* [2], *L. franchetiana* (H. LÉV.) HAND.-MAZZ. [2], *L. sibirica* (L.) CASS. [33], *L. hodgsonii* HOOK. F. [33], and some more species [34] are registered as '*Shanziyuan*'. Our previous results [14][35] and literature data [15–19] indicate that the chemical compositions of

these species are different. Therefore, the active compounds in '*Shanziyuan*' may neither be eremophilane-type sesquiterpenoids nor benzofuran derivatives, but some other compounds commonly present in these species.

Conclusions. – Although *L. anoleuca* and *L. veitchiana* are morphologically very close to each other, their samples collected in the Hengduan Mountains area were chemically and genetically distinct. Chemical and genetic re-examination of *L. veitchiana* in other parts of China, from which eremophilanes have been isolated, might reveal the mechanism of chemical diversification in the species.

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Experimental Part

General. Column chromatography (CC): silica gel (*Kanto silica gel 60 N* (spherical neutral)). Anal. TLC: *Merck Kieselgel 60 F_{254}* (layer thickness, 0.25 mm). Optical rotations: *JASCO P-2200* polarimeter. IR Spectra: *Shimadzu FTIR-8700* spectrometer. ^{1}H- and ^{13}C-NMR spectra: *JEOL AL 400* or *GNM-AL 400* spectrometer, with $CDCl_3$ as solvent and Me_4Si as internal standard; δ in ppm, J in Hz. EI-MS (pos.): *JEOL JMS-700 MStation*; in *m*/*z*.

Plant Material. Samples of *L. anoleuca* were collected in Mt. Cangshan, Dali City (sample *1*; elevation 3200 m), Yunnan Province, and in Dawei, Xiaojin County (sample *2*; 3500 m), Sichuan Province, in August of 2009. Three samples of *L. veitchiana* were collected in Jianchuan County (sample *3*, at Laojunshan, 3300 m; sample *4*, at Jinhua, 2300 m; sample *5*, at Madeng, 3500 m), Yunnan Province, in August of 2008. Each plant was identified by *Xun Gong*, one of the authors.

Determination of DNA Sequences. DNA was prepared from dried leaves with *DNeasy Plant Mini Kit* (*QIAGEN*). After further purification with *GLASSMILK* (*Qbiogene*), the DNA was used as template for polymerase chain reaction (PCR) with *HotStarTaq plus* polymerase (*QIAGEN*). The primers used for the amplification of the ITS1–5.8S-ITS2 and the *atpB-rbcL* regions were as reported in [36]. PCR Products were subjected to electrophoresis in an agarose slab and purified with *High Pure PCR Product Purification Kit* (*Roche Diagnostics*). Sequencing reactions were carried out with *BigDye Terminator* v.3.1 *Cycle Sequencing Kit* (*Applied Biosystems*) and the primers reported in [36]. The reactions were analyzed on a *3100xl Genetic Analyzer* (*Applied Biosystems*).

Ehrlich*'s Test on TLC.* See [8–10].

Purification and Identification of Chemical Components of Sample 1. Dried root (23 g) of sample *1* was extracted with EtOH (197 ml) at r.t. to give an oily extract (631 mg). Part of the extract (411 mg) was subjected to CC (SiO_2 (15 g); hexane/AcOEt 500:1 to 5:1) to give furanoeremophilanes **1** (58 mg, 14%) and **2** (15 mg, 3.8%). Alcohol **3** (4.2 mg, 1.0%) was isolated by repeated CC.

Purification and Identification of Chemical Components of Sample 2. Dried root (22 g) of sample *2* was extracted with EtOH (250 ml) at r.t. to give an oily extract (524 mg). Part of the extract (280 mg) was subjected to CC (SiO_2 (20 g); hexane/AcOEt 50:1 to 5:1) to give **1** (52 mg, 19%) and **2** (23 mg, 8.2%).

Purification and Identification of Chemical Components of Sample 3. Dried root (27 g) of sample *3* was extracted with EtOH (360 ml) at r.t. to give an oily extract (638 mg). Part of the extract (360 mg) was subjected to CC (SiO_2 (18 g); hexane/AcOEt 400:1 to 30:1) to give benzofuran derivatives **6** (19.6 mg, 5.4%), **7** (21.7 mg, 6.0%), and **8** (3.4 mg, 0.9%); $[\alpha]_D^{25} = -12$ ($c = 0.44$, $CHCl_3$), [24a]: $[\alpha]_D = -3.7$ ($c = 0.02$, $CHCl_3$).

Purification and Identification of Chemical Components of Sample 4. Dried root (5.3 g) of sample *4* was extracted with EtOH (110 ml) at r.t. to give an oily extract (67 mg). Part of the extract (34 mg) was subjected to CC (SiO_2 (2.0 g); hexane/AcOEt 20:1) to give **6** (4.1 mg, 12%) and **7** (3.8 mg, 11%).

Purification and Identification of Chemical Components of Sample 5. Dried root (5.0 g) of sample *5* was extracted with EtOH (98 ml) at r.t. to give an oily extract (56 mg). The extract was subjected to CC

(SiO_2 (3.0 g); hexane/AcOEt 500:1 to 30:1) to give **6** (4.8 mg, 8.6%), **7** (8.5 mg, 15%), and **8** (0.9 mg, 1.6%).

Furanoeremophil-1(10)-en-6α-ol (=*(4*R*,4a*R*,5*S*)-4,4a,5,6,7,9-Hexahydro-3,4a,5-trimethylnaphtho[2,3-*b*]furan-4-ol*; **3**). $[\alpha]_D^{25} = -1.5$ ($c = 0.38$, EtOH). IR (neat): 3421. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.06 (*q*, $J = 1.0$, H–C(12)); 5.81–5.78 (*m*, H–C(1)); 4.32 (*d*, $J = 8.7$, H–C(6)); 3.43 (*ddd*, $J = 19.5$, 5.8, 2.9, 1 H, CH_2(9)); 3.23 (*d*, $J = 19.5$, 1 H, CH_2(9)); 2.47–2.37 (*m*, 1 H); 2.04 (*d*, $J = 1.0$, Me(13)); 0.99 (*d*, $J = 6.8$, Me(15)); 0.82 (*s*, Me(14)). ^{13}C-NMR (100 MHz, $CDCl_3$): 150.2; 137.7; 134.3; 127.8; 120.1; 119.5; 68.0; 43.7; 31.7; 29.8; 27.6; 26.0; 17.5; 15.5; 7.8. EI-MS: 232 (100, M^+), 217 (12, $[M - Me]^+$), 199 (31, $[M - Me - H_2O]^+$), 161 (33), 149 (37), 135 (30), 122 (33), 109 (84). HR-EI-MS: 232.1458 (M^+, $C_{15}H_{20}O_2^+$; calc. 232.1463).

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