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Short communication

Intra-specific diversity of the chemical composition of *Ligularia lamarum* in the Hengduan Mountains, China: The structures of four new eremophilanes and a new seco-eremophilane

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ABSTRACT

Chemical compositions and internal transcribed spacer (ITS) sequences of five samples of *Ligularia lamarum* collected in Sichuan Province, China, were analyzed. Fourteen compounds, including four new eremophilanes and one new seco-eremophilane, were isolated and their structures were elucidated by spectroscopic methods. Intra-specific diversity in the chemical composition was found to be higher than previously known. The result of DNA analysis suggested that one of the samples was introgressed, although its chemical composition was typical of *L. lamarum*.

1. Introduction

Ligularia (Asteraceae) species in the Hengduan Mountains of China are highly diversified, thereby providing an opportunity for studying the diversity in secondary metabolites. We have studied the diversity of *Ligularia* using root chemicals and evolutionally neutral DNA sequences as indices, because the roots of *Ligularia* contain a variety of terpenoids. To date, we have revealed intra-specific diversity in many species with various modes. For example, many species consisted of several chemotypes (Kuroda et al., 2012, 2014), implying that the generation mechanism of chemical diversity is complex. Furanoeremophilanes have been isolated from most of the major species of *Ligularia*, and other compounds such as bisabolanes, benzofurans, and phenylpropanoids have also been obtained.

L. lamarum (Diels) C. C. Chang is widely distributed in the Hengduan Mountains, including the Yunnan, Sichuan, and Gansu provinces and the Xizang Autonomous Region of China and northwestern Myanmar (Liu and Illarionova, 2011). Taxonomically, the species is closely related to *L. subspicata* (Bureau and Franch.) Hand.-Mazz. The major morphological difference between *L. lamarum* and *L. subspicata* is the presence (*L. lamarum*) or absence (*L. subspicata*) of ray florets (Liu and Illarionova, 2011). We previously analyzed *L. lamarum* and *L. subspicata* collected in northwestern Yunnan and southwestern Sichuan, and showed that the two species were indistinguishable by our two indices (Saito et al., 2011). Eremophilanes were isolated from all of our collected samples, which were classified into two chemotypes: 1) a furanoeremophilane type and 2) an eremophilan-8-one type. Subspicatins (1β-acyloxyfuranoeremophilanes) are characteristic of the furanoeremophilane type, although not all furanoeremophilane-type samples produce subspicatin(s).

Here, we report the results of analyses of *L. lamarum* samples collected in northern Sichuan Province, which showed further diversity within the furanoeremophilane type. Five new compounds were isolated and their structures were determined.

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Table 1
Collection localities and chemical composition of *Ligularia lamarum* samples.

Sample no.	Specimen no.	Location[a]	Altitude (m)	Isolated compounds
1	2010 – 09	Zhegushan (Hongyuan)	3900	**1**, **4**, **5**, **13**
2	2010 – 10	Zhegushan (Hongyuan)	3900	**1**, **7**
3	2011 – 53	Queershan, east side (Dege)	4100	**1**, **2**, **8**, **9**
4	2011 – 54	Queershan, east side (Dege)	4100	**1**, **2**, **3**, **6**, **7**
5	2011 – 56	Queershan, west side (Dege)	4400	**1**, **2**, **6–8**, **10–12**, **14**
6[b]	2008 – 55	Laojunshan (Jianchuan)	4000	
7[b]	2009 – 95	Maerkang/Xianjin (Maerkang)	3900	

[a] County in parenthesis. Jianchuan County is situated within Yunnan Province, whereas the others are situated within Sichuan Province.
[b] Previously analyzed sample (Saito et al., 2011). Samples 6 and 7 correspond to samples 7 and 9, respectively, in the report.

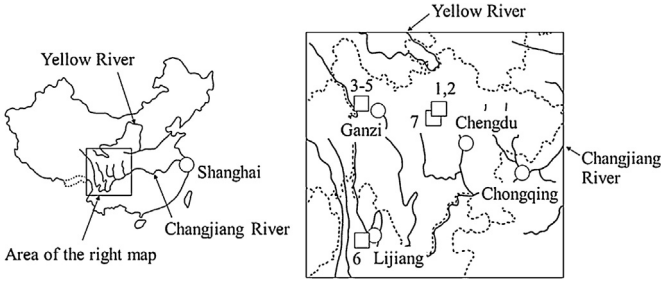

Fig. 1. Locations of the collected samples of *L. lamarum* (squares). Circles indicate major cities. Solid and dotted lines indicate rivers and boundaries of provinces, respectively.

2. Results

2.1. Samples

Five samples of *L. lamarum* were collected in northern Sichuan Province (Table 1 and Fig. 1): two in Hongyuan County (samples 1 and 2) and three in Dege County (samples 3–5). Samples 1 and 2 were less than 100 m apart. Sample 1 had typical ray florets, whereas sample 2 had very small ray florets. Samples 3 and 4 were collected sympatrically. The leaves of sample 3 were more cordate, whereas those of sample 4 were more sagittate. Sample 5 was collected approximately 10 km west of the locations of samples 3 and 4.

2.2. Chemical analysis

Compounds of the roots of each sample were extracted with EtOH immediately without drying and the extracts were subjected to Ehrlich's test on TLC plates. All five samples were Ehrlich-positive, suggesting the presence of furanoeremophilanes (Kuroda et al., 2004; Kuroda and Nishio, 2007). Although samples 1 and 2 were collected close to each other, they showed different TLC patterns. Sample 1 showed a blue spot at R_f = 0.48 (hexane/EtOAc 7:3), whereas sample 2 showed two purple spots: one at R_f = 0.73 and one at 0.39. Samples 3–5 showed the same spots as in sample 2, suggesting that the major chemical constituents were very similar.

The extracts were analyzed by LC–MS. The total ion chromatograms (TICs) are shown in Fig. 2. The chromatograms of samples 2–5 were almost identical, in agreement with the TLC results. The EtOH extracts of two previous samples (samples 6 and 7 (Saito et al., 2011; see Table 1)) were also subjected to LC–MS. The TICs of samples 2–5 were very similar to that of sample 7, but differed from that of sample 6.

Fourteen compounds, including eremophilane-type sesquiterpenes **1–12**, α-bisabolol, and lupeol, were isolated from the dried roots (Fig. 3). From sample 1, **1** (Tori et al., 2008), **4**, **5**, and α-bisabolol were isolated (Table 1). From sample 2, **1** and **7** were isolated. From sample 3, **1**, **2** (Nagano et al., 1982), **8** (Shimizu et al., 2014a), and **9** were isolated. From sample 4, **1**, **2**, **3** (Tada et al., 1971), **6** (ligularone; Ishii et al., 1965; Koike et al., 1999), and **7** were isolated. From sample 5, **1**, **2**, **6**, **7**, **8**, **10** (Tori et al., 2008), **11** (Saito et al., 2015), **12**, and lupeol were isolated.

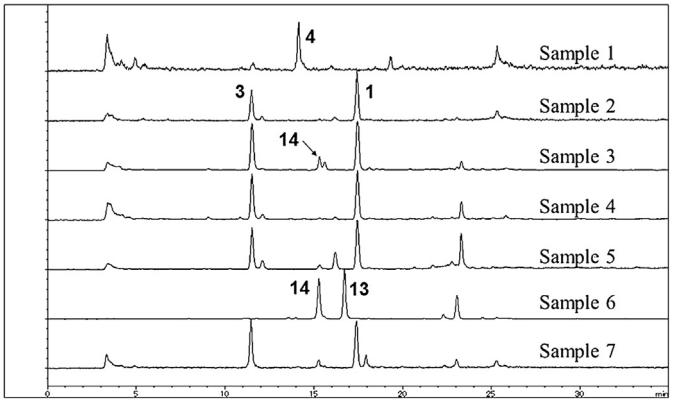

Fig. 2. LC profiles (total ion chromatograms) for samples 1–7. Mass spectra of **1**, **3**, **4**, **13**, and **14** were *m/z* 233 ($[M-OEt]^+$), 233 ($[M-OH]^+$), 329 ($[M-OH]^+$), 333 ($[M+H]^+$), and 235 ($[M+H]^+$), respectively.

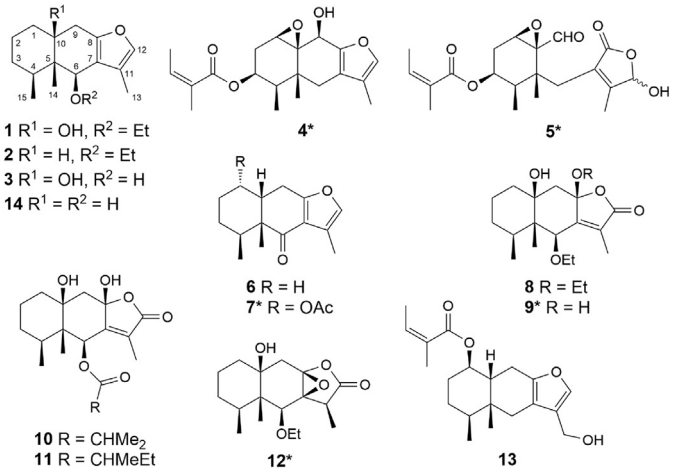

Fig. 3. Components of *L. lamarum* (asterisks indicate new compounds).

Of the 14 compounds, **4**, **5**, **7**, **9**, and **12** were new. Their structures were determined as detailed below.

The molecular formula of **4** was determined to be $C_{20}H_{26}O_5$ from high-resolution CI-MS (HRCIMS) (*m/z* 346.1773; M^+) and ^{13}C NMR data. The IR spectrum showed the presence of a hydroxy group (3510 cm^{-1}) and a conjugated ester carbonyl group (1712 cm^{-1}). The ^1H and ^{13}C NMR spectra showed typical signals of furanoeremophilane [δ_H 7.20 (q, 1.1 Hz, H-12), 1.95 (d, 1.1 Hz, Me-13), 1.36 (s, Me-14), and 1.11 (d, 7.2 Hz, Me-15); δ_C 140.4 (C-12), 8.0 (C-13), 23.7 (C-14), and 9.8 (C-15)] as well as those of an angeloyl group [δ_H 6.10 (qq, 7.3, 1.4 Hz, H-3′), 2.00 (dq, 7.3, 1.4 Hz, Me-4′), and 1.89 (quint, 1.4 Hz, Me-5′); δ_C 167.4 (C-1′), 127.8 (C-2′), 138.4 (C-3′), 15.8 (C-4′), and 20.6 (C-5′)] (Tables 2 and 3). Signals of three oxygenated methines were also observed [δ_H 3.29 (d, 5.6 Hz, H-1), 5.21 (ddd, 11.5, 7.1, 4.2 Hz, H-3),

Table 2
^{1}H NMR data of compounds **4**, **5**, **7**, **9**, and **12** ($CDCl_3$).[a]

Carbon no.	**4**	**5** (major isomer)	**5** (minor isomer)	**7**	**9**	**12**
1	3.29 (d, 5.6)	3.53 (d, 5.5)	3.48 (d, 5.4)	5.22 (dt, 11.0, 4.6)	1.70 (td, 13.7, 5.1) 1.41 (br d, 13.7)	1.64–1.70 (m) 1.35–1.42 (m)
2	2.12 (dd, 14.5, 11.5) 2.23–2.32 (m)	2.10 (dd, 14.5, 11.6) 2.19 (ddd, 14.5, 7.2, 5.5)	2.21–2.28 (m) 2.33–2.41 (m)	1.90 (dq, 13.5, 4.6) 1.69–1.78 (m)	1.57–1.62 (m) 1.29–1.33 (m)	1.67–1.72 (m) 1.46–1.54 (m)
3	5.21 (ddd, 11.5, 7.1, 4.2)	4.99 (ddd, 11.6, 7.2, 4.5)	4.84 (ddd, 11.2, 7.4, 4.4)	1.61–1.67 (m) 1.41–1.49 (m)	1.37 (br d, 12.7) 1.30–1.35 (m)	1.41–1.46 (m) 1.35–1.42 (m)
4	1.80 (qd, 7.2, 4.2)	1.52 (qd, 7.2, 4.5)	1.73–1.81 (m)	1.80–1.90 (m)	1.20–1.25 (m)	1.27–1.31 (m)
6	2.91 (br d, 16.5) 2.32 (d, 16.5)	2.35 (d, 15.0) 3.59 (br d, 15.0)	2.81 (d, 15.2) 3.10 (br d, 15.2)	–	4.42 (s)	3.80 (s)
9	4.15 (s)	8.67 (s)	8.72 (s)	3.01 (dd, 16.9, 11.0) 2.85 (dd, 16.9, 5.3)	2.46 (d, 14.5) 2.17 (d, 14.5)	2.76 (d, 15.9) 2.40 (d, 15.9)
10	–	–	–	2.50 (ddd, 11.0, 5.3, 4.6)	–	–
11	–	–	–	–	–	2.70 (q, 7.4)
12	7.20 (q, 1.1)	5.81 (br d, 12.5)	5.83 (br d, 11.4)[b]	7.08 (s)	–	–
13	1.95 (d, 1.1)	2.05 (br s)	2.07 (br s)	2.20 (s)	1.90 (s)	1.45 (d, 7.4)
14	1.36 (s)	1.40 (s)	1.55 (s)	1.23 (s)	1.19 (s)	1.05 (s)
15	1.11 (d, 7.2)	1.02 (d, 7.2)	0.96 (d, 7.2)	0.77 (d, 6.6)	0.82 (d, 6.6)	0.84 (d, 6.7)
1′	–	–	–	–	3.60 (dq, 8.6, 7.1) 3.31 (dq, 8.6, 7.1)	3.74 (dq, 7.9, 7.0) 3.31 (dq, 7.9, 7.0)
2′	–	–	–	2.01 (br s)	1.20 (t, 7.1)	1.24 (t, 7.0)
3′	6.10 (qq, 7.3, 1.4)	6.18 (qq, 7.3, 1.4)	6.15 (qq, 7.3, 1.4)	–	–	–
4′	2.00 (dq, 7.3, 1.4)	1.96 (dq, 7.3, 1.4)	1.94 (dq, 7.3, 1.4)[b]	–	–	–
5′	1.89 (quint, 1.4)	1.84 (quint, 1.4)	1.84 (quint, 1.4)[b]	–	–	–
OH	2.41 (br s)	5.45 (d, 12.5)	4.93 (d, 11.4)	–	4.48 (br s, 8-OH) 4.54 (s, 10-OH)	3.96 (s)

[a] *J* values are given in Hz.
[b] Overlapping signals. The *J* value was deduced from the coupling partner.

Table 3
^{13}C NMR data of compounds **4**, **5**, **7**, **9**, and **12** ($CDCl_3$).

	4	**5** (major isomer)	**5** (minor isomer)	**7**	**9**	**12**
1	61.8	58.5	56.1	71.3	34.4	32.9
2	25.2	23.9	24.2	25.8	21.8	21.7
3	68.6	68.6	68.6	28.1	29.5	28.8
4	40.9	40.8	37.6	30.4	33.4	32.2
5	36.3	38.9	38.4	50.1	47.4	44.9
6	32.9	33.5	31.6	197.7	78.6	77.3
7	120.3	128.5	127.1	118.3	153.9	61.7
8	146.6	172.2	171.6	164.1	103.7	86.0
9	68.1	195.6	197.5	21.2	45.0	31.5
10	66.2	63.5	66.2	43.5	75.0	72.8
11	119.6	158.8	159.4	119.8	128.8	42.7
12	140.4	99.7	98.7	139.4	170.8	175.6
13	8.0	11.9	12.4	9.1	8.7	10.8
14	23.7	24.8	23.0	14.2[a]	11.1	10.8
15	9.8	10.5	9.8	15.7	16.4	16.3
1′	167.4	167.9	167.5	170.3	65.6	66.8
2′	127.8	126.9	127.1	21.2	15.0	15.1
3′	138.4	141.2	140.3	–	–	–
4′	15.8	16.0	16.0	–	–	–
5′	20.6	20.3	20.4	–	–	–

[a] Very broad.

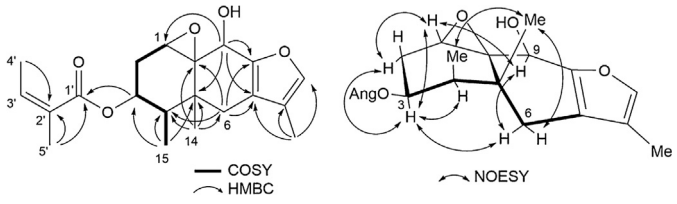

Fig. 4. Selected COSY, HMBC, and NOESY correlations of compound **4**.

and 4.15 (s, H-9); δ_C 61.8 (C-1), 68.6 (C-3), and 68.1 (C-9)], among which the δ and *J* values of the H-1 and H-3 signals were typical of 3β-acyloxy-1β,10β-epoxyfuranoeremophilane derivatives (Shimizu et al., 2014b). The planar structure, including the position of the hydroxy group, was determined by COSY (H-1/H_2-2/H-3/H-4/Me-15) and HMBC (from H-9 to C-1, 5, 7, 8; from Me-14 to C-4, 5, 6, 10; from Me-15 to C-3, 4, 5; from H-3 to C-1′) (Fig. 4). Relative configurations were determined by NOEs between H-9 and H-1, H-1 and H-3, H-3 and H-6α [δ_H 2.91], H-6α and H-9, H-6β [δ_H 2.32] and Me-14, and Me-14 and Me-15 (Fig. 4). The quasi-axial orientation for H-3 and the quasi-equatorial orientation for H-4 were supported by the *J*-values between H-2β [δ_H 2.12] and H-3 (11.5 Hz) and between H-3 and H-4 (4.2 Hz). Thus, the structure of compound **4** was established as depicted.

The molecular formula of **5** was determined to be $C_{20}H_{26}O_7$ from HRCIMS (*m/z* 379.1757; $[M+H]^+$) and ^{13}C NMR data. The IR spectrum showed the presence of carbonyl groups (1759, 1732, and 1716 cm^{-1}) and a hydroxy group (3420 cm^{-1}). Signals of ^{1}H and ^{13}C NMR were observed in pairs at a 4:1 ratio, indicating that the compound was a mixture of isomers (Tables 2 and 3). Three methyl signals of eremophilanes [δ_H 2.05/2.07 (br s, Me-13), 1.40/1.55 (s, Me-14), and 1.02/0.96 (d, 7.2 Hz, Me-15); δ_C 11.9/12.4 (C-13), 24.8/23.0 (C-14), and 10.5/9.8 (C-15)], as well as an angeloyl group [δ_H 6.18/6.15 (qq, 7.3, 1.4 Hz, H-3′), 1.96/1.94 (dq, 7.3, 1.4 Hz, Me-4′), and 1.84 (quint, 1.4 Hz, Me-5′); δ_C 167.9/167.5 (C-1′), 126.9/127.1 (C-2′), 141.2/140.3 (C-3′), 16.0 (C-4′), and 20.3/20.4 (C-5′)] and an aldehyde [δ_H 8.67/8.72 (s, H-9); δ_C 195.6/197.5 (C-9)], were observed. The COSY spectrum indicated proton connectivity for H-1/H_2-2/H-3/H-4/Me-15 and the planar structure with an 8,9-seco-eremophilane skeleton was determined by HMBC (from H-1 to C-9; from H-6 to C-4, 5, 7, 8, 10, 11; from H-9 (aldehyde) to C-5, 10; from Me-13 to C-7, 11, and 12) (Fig. 5). A related 8,9-seco-eremophilane was previously isolated from *L. virgaurea* (Saito et al., 2012); however, the position of the angeloyloxy group was different. Relative configurations were determined by

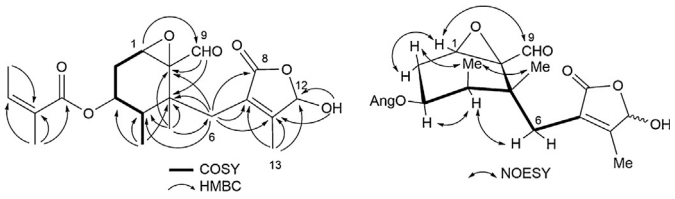

Fig. 5. Selected COSY, HMBC, and NOESY correlations of compound **5**.

NOEs between H-1 and H-9, H-2β [δ_H 2.10 (major isomer)] and Me-15, and Me-14 and Me-15 (Fig. 5). This compound may have been generated from **4** by oxidation of the furan ring, followed by cleavage between C-8 and C-9, as reported by Yaoita and Kikuchi (1996) and by us (Saito et al., 2012).

Compound **7** showed a molecular ion peak at *m/z* 290, and its molecular formula was determined to be $C_{17}H_{22}O_4$ from HREIMS (*m/z* 290.1526; M^+) and ^{13}C NMR data. The ^1H and ^{13}C NMR features were very similar to those of **6,** except for the presence of signals of an oxymethine [δ_H 5.22 (dt, 11.0, 4.6 Hz, H-1); δ_C 71.3 (C-1)] and an acetoxy group [δ_H 2.01 (s, Me-2′); δ_C 170.3 (C-1′) and 21.2 (C-2′)], indicating that **7** was an acetoxy derivative of **6**. The position of the acetoxy group was deduced to be at C-1 by the COSY (H_2-9/H-10/H-1/H_2-2/H_2-3) and HMBC (from H-1 to C-1′; from H-14 to C-4, 5, 6, 10) shown in Fig. 6. The *cis*-fused decalin ring with a steroidal conformation was revealed by the NOEs between H-10 and Me-14 and between H-2α [δ_H 1.69–1.78] and H-9α [δ_H 3.01]. The NOE between H-1 and H-3β [δ_H 1.41–1.49] and Me-14 indicated that the acetoxy group was α-oriented, which was supported by the *J* value of H-1. Therefore, the structure of **7** was established as depicted in Fig. 6.

The ^1H NMR spectrum of **9** was very similar to that of **8**. Because only one set of signals of an ethoxy group [δ_H 3.60 (dq, 8.6, 7.1 Hz, H-1′), 3.31 (dq, 8.6, 7.1 Hz, H-1′), and 1.20 (t, 7.1 Hz, Me-2′); δ_C 65.6 (C-1′) and 15.0 (C-2′)] was observed, **9** was suggested to be a mono-*O*-ethyl derivative of 6,8,10-trihydroxyeremophilan-12,8-olide, which was further supported by IR absorption at 1768 cm^{-1} (γ-lactone) and by HRCIMS data (*m/z* 311.1851, $[M+H]^+$). An HMBC correlation from H-6 to C-1′ indicated that the ethoxy group was attached to C-6 (Fig. 7). The NOE correlations between H-9β [δ_H 2.17] and OH, OH and Me-14, H-6 and Me-13, H-4 and H-9α [δ_H 2.46] (Fig. 7) indicated that all substituents were β-oriented.

The molecular formula of **12** was determined to be $C_{17}H_{26}O_5$ from HREIMS (*m/z* 310.1782; M^+) and ^{13}C NMR data. The IR spectrum showed the presence of a hydroxy group (3500 cm^{-1}). Absorption at 1805 cm^{-1} indicated the presence of an epoxy- or enol-lactone (Saito et al., 2011; Tori et al., 2006, 2008). The ^1H and ^{13}C NMR spectra showed the presence of an ethoxy group [δ_H 3.74 (dq, 7.9, 7.0 Hz, H-1′), 3.31 (dq, 7.9, 7.0 Hz, H-1′), and 1.24 (t, 7.0 Hz, Me-2′); δ_C 66.8 (C-1′) and 15.1 (C-2′)] and an oxymethine [δ_H 3.80 (s, H-6); δ_C 77.3 (C-6)] as well as a hydroxy proton [δ_H 3.96 (s)]. The 2D correlations shown in Fig. 8 indicated an eremophilanolide skeleton bearing oxygen functionalities at C-6, C-7, C-8, and C-10. The HMBC correlations from H-6 to C-1′ and from OH to C-1 and C-10 indicated that the ethoxy and hydroxy

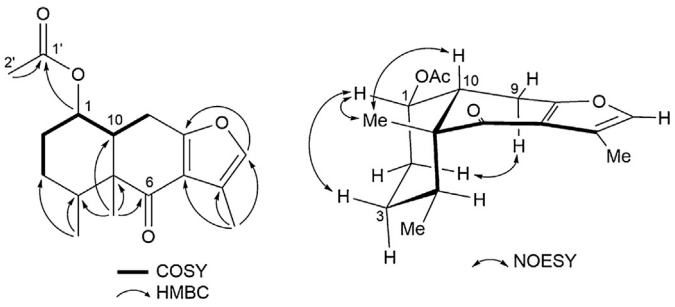

Fig. 6. Selected COSY, HMBC, and NOESY correlations of compound **7**.

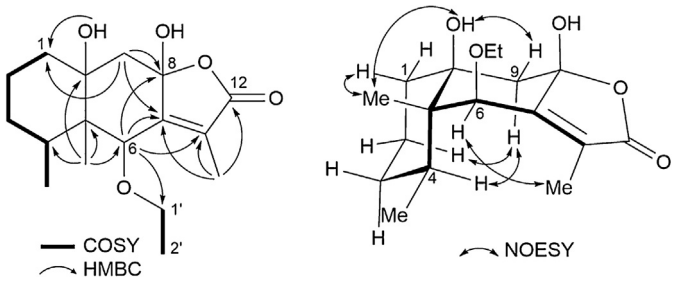

Fig. 7. Selected COSY, HMBC, and NOESY correlations of compound **9**.

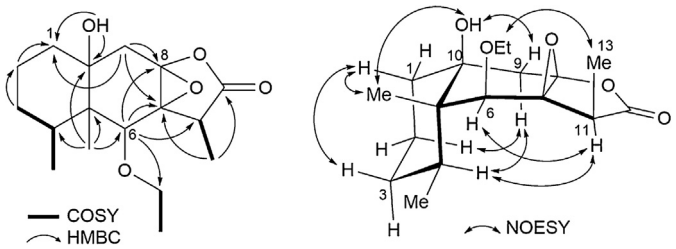

Fig. 8. Selected COSY, HMBC, and NOESY correlations of compound **12**.

groups were at C-6 and C-10, respectively. The NOE between Me-14 and OH indicated that the decalin ring was *cis*-fused. Furthermore, its steroidal conformation was supported by the NOEs between H-9α [δ_H 2.76] and H-4, H-1β [δ_H 1.64–1.70] and Me-14, and Me-14 and OH. The NOEs between H-11 and H-4, 6 and between Me-13 and H_2-1′ indicated that the configuration of the epoxide oxygen atom and Me-13 was β· Therefore, the structure of **12** was established as depicted in Fig. 8.

2.3. Genetic analysis

The DNA sequence of the ITS1 – 5.8S – ITS2 region of the ribosomal RNA (rRNA) gene cluster was determined. The results are shown in Table 4. Sample 3 showed a relatively large number of sites with multiple bases and variation in the number of repeated C at two sites, suggestive of introgression. A Basic Local Alignment Search Tool (BLAST) search suggested *L. sagitta* as a candidate of the hybridization partner: some of the sites with R, W, or Y (underlined in Table 4) and the presence of length variants in sample 3 could be explained by superposition of *L. lamarum* and *L. sagitta* sequences. Although samples 1 and 6 were chemically different from the others, the sequences of samples 1, 2, and 4–6 were very similar.

3. Discussion

Furanoeremophilanes, including **1**, were isolated from all five samples; however, the chemical composition of sample 1 was different from those of samples 2–5 in that 9-oxygenated derivatives, **4** and **5**, were isolated. LC–MS analysis of the isolated compounds indicated that **4** (t_R = 14.0 min) was the major component (Fig. 2). The major peaks of samples 2–5 were determined to be furanoeremophilane-6,10-diol (**3**, t_R = 11.4 min) and 6-ethoxyfuranoeremophilan-10-ol (**1**, t_R = 17.5 min). Compound **1** may be an artifact generated from **3** or 6-acyloxy derivative(s) during ethanol extraction (Kuroda et al., 2016; and references cited therein); thus, **3** or its 6-acylated derivatives may be the major component of these samples. Compound **3** was not isolated from samples 1–3 or 5, indicating **3** was converted to **1** during extraction. The TIC of sample 7 was also very similar to those of samples 2–5. Compound **3** was isolated as the major component of sample 7 (Saito et al., 2011). None of the present five samples showed a peak of subspicatin A (**13**, t_R = 16.8 min), a major component of sample 6 (Fig. 2). Ligularol (**14**, t_R = 15.3 min), another major

Table 4
DNA sequence of the ITS1–5.8S–ITS2 region.[a]

Sample	ITS1																		5.8S	ITS2											
	4	6	7	8	9	1 0	1 0	1 1	1 7	1 2	1 2	1 7	2 0	2 1	2 1	2 2	2 4	2 4	1 3			1	2	6	7	7	9	9	1 3	1 5	1 9
	6	8	0	7	5	0	8	2	7	2	2	7	0	7	8	4	1	4	2	5	8	9	7	3	0	7	1	6	6	5	6
1	T	C	T	G	G	G	G	A	T	C	A	T	A	[b]	[b]	T	G	R	T	C	C	Y	C	C	C	G	C	K	A	C	T
2	T	C	T	G	G	G	G	A	T	C	A	K	A	C	C	T	G	G	T	Y	C	C	C	C	C	G	C	G	A	C	T
3[c]	Y	C	Y	S	R	R	S	R	Y	C	A	G	W	[b]	[b]	Y	R	G	Y	Y	Y	C	C	C	[d]	R	Y	G	R	C	T
4	T	C	T	G	G	G	G	A	T	C	A	G	A	C	C	T	G	G	T	T	C	C	C	C	C	G	C	G	A	Y	Y
5	T	C	T	G	G	G	G	A	T	C	A	G	A	C	C	T	G	G	T	T	C	C	C	C	C	G	C	G	A	Y	Y
6	T	Y	T	G	G	G	G	A	T	Y	A	G	T	C	–	T	G	G	T	C	C	C	Y	Y	C	G	C	G	A	C	T
7[e]	T	C	T	G	G	G	G	A	T	C	A	R	A	C	C	T	G	G	T	Y	C	C	C	C	C	G	C	G	A	Y	Y
ref[f]	C	C	T	G	G	A	G	G	C	C	T	G	T	–	–	C	A	G	T	C	C	T	C	C	[g]	A	T	G	G	C	T

[a] Only the differences are shown. The base numbering is to samples 1, 2, 4, and 5. K = G + T; R = A + G; S = C + G; Y = C + T; W = A + T. −: deletion.
[b] Two variants with and without C were present. The variant with C was major in sample 1; the two variants had almost equal signal intensities in sample 3.
[c] The underlined multiple bases can be explained by the superposition of the sequences of other *L. lamarum* samples and the reference *L. sagitta* sequence (see text).
[d] Two variants with two Cs and three Cs at sites 69–70 were present with almost equal intensities.
[e] A minor variant without AC at 18–19 in ITS2 was also present (Saito et al., 2011).
[f] A *L. sagitta* sequence (database ID = **LC002205**).
[g] Three Cs at sites 69–70.

component of sample 6, was observed only for sample 3. Previously analyzed *L. lamarum* samples were grouped into two chemotypes: 1) a furanoeremophilane type and 2) an eremophilen-8-one type (Saito et al., 2011). The present results indicate the presence of at least three subtypes within the furanoeremophilane type: 1) a 9-hydroxy subtype (sample 1), 2) a 6,10-dihydroxy subtype (samples 2–5 and 7), and 3) a 1β-acyloxy subtype (subspicatins; sample 6 and most of the previously analyzed samples). Interestingly, the configuration of the 1-acyloxy group in **7** is different from that in subspicatins. Sample 1 is unique in that the major component is a furanoeremophilan-9-ol derivative, which is rare in *Ligularia*. Furanoeremophilanes with a 9-hydroxy (Fei et al., 2010; Saito et al., 2012) or a 9-acyloxy group (Bohlmann et al., 1977; Ponomarenko et al., 2014) have been isolated only as minor components.

Sample 3 appeared to have undergone hybridization, most likely with *L. sagitta*, which is abundant in the northwestern Sichuan to southeastern Qinghai area. However, the chemical composition of sample 3 is very similar to that of pure *L. lamarum* (Figure 2). We reported a similar instance in which a *L. lamarum* sample was introgressed with *L. cyathiceps*, whereas its chemical composition was typical of *L. lamarum/L. subspicata* (Shimizu et al., 2014a). Although furanoeremophilanes have not yet been isolated from *L. sagitta*, 6,10-oxygenated eremophilanolides (Li et al., 2003) and many other eremophilanes have been isolated. Hybridization is an important process in plant evolution (Riesberg and Carney, 1998). Introgression between *L. lamarum* and *L. sagitta* may have contributed to the chemical diversity in both species, which requires further examination of more samples.

4. Conclusion

Twelve eremophilane sesquiterpenes, including one seco-type, were isolated from five samples of *L. lamarum* collected in northern Sichuan Province. We previously reported that *L. lamarum* samples collected in Yunnan and southern Sichuan consisted of two chemotypes: 1) a furanoeremophilane type and 2) an eremophilan-8-one type (Saito et al., 2011). The present results showed diversity within the furanoeremophilane type. Introgression, presumably with *L. sagitta*, was also detected, adding to our previous observations of introgression of *L. lamarum* with *L. cyathiceps* (Shimizu et al., 2014a) and of hybridization between *L. nelumbifolia* and *L. subspicata*, a species that cannot be chemically or genetically distinguished from *L. lamarum* (Hanai et al., 2012).

5. Experimental

5.1. General experimental procedures

Column chromatography (CC) was conducted on silica gel (Wakogel C-200 or C-300 or Kanto silica gel 60 N (spherical neutral)) with hexane/EtOAc as an eluent. Analytical TLC was performed on silica gel (Merck Kieselgel 60 F_{254}, layer thickness 0.2 or 0.25 mm) using either Ehrlich's reagent (*p*-dimethylaminobenzaldehyde and HCl) (Kuroda et al., 2004; Kuroda and Nishio, 2007) or *p*-anisaldehyde/AcOH/H_2SO_4 as a visualizing agent. HPLC was conducted on a Shimadzu LC-20AT pump with an SPD-20A UV/VIS detector or a GL Sciences GL-7410 pump with a GL-7450 UV detector and a Hitachi D-2500 Chromato-Integrator, using a Kanto Mightysil Si60 (10 × 250 mm; 5 μm; 5.0 mL/min) column, Nacalai Tesque COSMOSIL 5SL-II (10 × 250 mm; 5 μm; 6.0 mL/min) column, or a Tosoh TSK-GEL G1000H_{HR} (7.8 × 300 mm; 5 μm; 1.0 mL/min) column. IR spectra were recorded on a JASCO FT/IR-230 or a FT/IR-410K spectrometer. ^{1}H and ^{13}C NMR spectra were taken on a JEOL ECX-400 (400 MHz and 100 MHz, respectively) or a Varian 500-MR (500 MHz and 125 MHz, respectively) spectrometer with $CDCl_3$ as the solvent and tetramethylsilane as an internal standard. Mass spectra (MS) were obtained on a JEOL CMATE II or an MStation JMS-700. LC–MS was measured on an Agilent 1100 series LC/MSD mass spectrometer (capillary voltage 3.5 kV; corona current 4 μA; capillary exit voltage (fragmentor) 90 V; drying temperature 330 °C; drying flow 9 L/min; nebulizer pressure 50 psig) with 5C18-MS-II (COSMOSIL; 4.6 × 150 mm; 5 μm octadecyl column) using a gradient system (MeOH/H_2O; 0 min (7:3)–20 min (10:0)–35 min (10:0)–40 min (7:3)–45 min (7:3); 0.5 mL/min) as the eluent. Optical rotations were measured with a JASCO DIP-370 or a P-1020 digital polarimeter. DNA sequencing was performed on 3130xl and 3500 Genetic Analyzers (Applied Biosystems). Purification of DNA from dried leaves, amplification of the ITS1–5.8S–ITS2 region by polymerase chain reaction, and DNA sequencing of the regions were conducted as previously described (Nagano et al., 2010).

5.2. Plant materials

Samples were collected in the Hongyuan and Dege counties of Sichuan Province (Table 1 and Fig. 1). Each sample was identified by X.G. (author). Voucher specimen numbers are listed in Table 1 (deposited in the Kunming Institute of Botany).

5.3. Extraction for Ehrlich's test and LC–MS

Extraction of fresh root of each plant (2–5 g) with EtOH was initiated immediately after harvest without drying. Solid plant material was removed after several days and the extract was subjected to TLC without evaporation of the solvent. See our previous report for details of Ehrlich's test (Kuroda et al., 2004; Kuroda and Nishio, 2007).

5.4. Extraction and purification

The dried roots of sample 1 (9.8 g) were extracted with EtOAc/EtOH (*ca.* 1:1), and the extract (282.7 mg) was roughly separated by silica-gel (12 g) CC using hexane/EtOAc (100:0, 98:2, 0:100) as the eluent to obtain three fractions. From the less polar fraction, **1** (6.4 mg) was isolated by further CC (silica gel, hexane/EtOAc) and HPLC (Mightysil, hexane/Et_2O 7:3). From the middle fraction, α-bisabolol (2.2 mg) was obtained by HPLC (hexane/EtOAc 4:1). From the polar fraction, **4** (6.9 mg) and **5** (2.1 mg) were obtained by HPLC (hexane/Et_2O 6:4).

The dried roots of sample 2 (9.0 g) were extracted with EtOAc/EtOH (*ca.* 1:1), and the extract (366.1 mg) was subjected to silica-gel (12 g) CC using hexane/EtOAc as the eluent. From the fraction eluted with 4% EtOAc, **1** (14.1 mg) was isolated by HPLC (Mightysil, hexane/EtOAc 4:1). From the fraction eluted with 10% EtOAc, **7** (2.8 mg) was obtained by HPLC (hexane/EtOAc 7:3).

The dried roots of sample 3 (4.5 g) were extracted with EtOH, and the extract (321.0 mg) was subjected to silica-gel (24 g) CC using hexane/EtOAc as the eluent. Compound **1** (19.4 mg) was obtained from the fraction eluted with 5–10% EtOAc. From the fraction eluted with 5% EtOAc, **2** (0.8 mg) was isolated by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 99:1, followed by TSK-GEL G1000H_{HR}, EtOAc). From the fraction eluted with 30% EtOAc, **8** (4.4 mg) was obtained by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 8:2, followed by TSK-GEL G1000H_{HR}, EtOAc). From the fraction eluted with 50% EtOAc, **9** (14.8 mg) was obtained by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 8:2).

The dried roots of sample 4 (13.5 g) were extracted with EtOH, and the extract (914.0 mg) was subjected to silica-gel (37 g) CC using hexane/EtOAc as the eluent. Compound **1** (127.1 mg) was obtained from the fraction eluted with 5–10% EtOAc. From the fraction eluted with 5% EtOAc, **2** (2.3 mg) and **6** (1.6 mg) were isolated by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 99:1, followed by TSK-GEL G1000H_{HR}, EtOAc). From the fraction eluted with 20–30% EtOAc, **7** (5.9 mg) was obtained by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 9:1, followed by TSK-GEL G1000H_{HR}, EtOAc). From the fraction eluted with 50% EtOAc, **3** (1.2 mg) was obtained by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 8:2).

The dried roots of sample 5 (6.1 g) were extracted with EtOH, and the extract (344.7 mg) was subjected to silica-gel (24 g) CC using hexane/EtOAc as the eluent. From the fraction eluted with 5% EtOAc, **2** (4.2 mg) and **6** (10.4 mg) were isolated by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 99:1, followed by TSK-GEL G1000H_{HR}, EtOAc). From the fraction eluted with 5–10% EtOAc, **1** (29.0 mg) was obtained by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 19:1). From the fraction eluted with 20% EtOAc, **7** (5.1 mg) and lupeol (4.4 mg) were obtained by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 9:1, followed by TSK-GEL G1000H_{HR}, EtOAc). From the fraction eluted with 30% EtOAc, **8** (2.0 mg) and **12** (3.0 mg) were obtained by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 8:2, followed by TSK-GEL G1000H_{HR}, EtOAc). From the fraction eluted with 50% EtOAc, **10** (2.0 mg) and **11** (0.1 mg) were obtained by HPLC (COSMOSIL 5SL-II, hexane/EtOAc 6:4, followed by TSK-GEL G1000H_{HR}, EtOAc).

5.5. Characterization of new compounds

5.5.1. 3β-Angeloyloxy-1β,10β-epoxyfuranoeremophilan-9β-ol (4)

Oil; $[\alpha]_D^{25}$ + 7.7 (*c* 0.15, MeOH); IR (neat) ν_{max} 3510, 1712, 1648, 1234 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) see Table 2; ^{13}C NMR (100 MHz, $CDCl_3$) see Table 3; CIMS (CH_4) *m/z* 346 $[M]^+$ (80), 329 $[M-OH]^+$ (100), 247 (79), 229 (97); HRCIMS *m/z* 346.1773 $[M]^+$ (calcd for $C_{20}H_{26}O_5$, 346.1781).

5.5.2. 3β-Angeloyloxy-1β,10β-epoxy-12-hydroxy-9-oxo-8,9-secoeremophil-7(11)-en-8,12-olide (5)

Oil; $[\alpha]_D^{25}$ − 17.6 (*c* 0.056, MeOH); IR (neat) ν_{max} 3420, 1759, 1732, 1716, 1650 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) see Table 2; ^{13}C NMR (100 MHz, $CDCl_3$) see Table 3; CIMS (CH_4) *m/z* 379 $[M+H]^+$ (47), 361 $[M-OH]^+$ (17), 279 (95), 235 (100); HRCIMS *m/z* 379.1757 $[M+H]^+$ (calcd for $C_{20}H_{27}O_7$, 379.1757).

5.5.3. 1α-Acetoxyfuranoeremophilan-6-one (7)

Oil; $[\alpha]_D^{27}$–48.3 (*c* 0.18, $CHCl_3$); IR (ATR) ν_{max} 1736, 1670 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) see Table 2; ^{13}C NMR (100 MHz, $CDCl_3$) see Table 3; EIMS (70 eV) *m/z* 290 $[M]^+$ (27), 230 (35), 83 (100); HREIMS *m/z* 290.1526 M^+ (calcd for $C_{17}H_{22}O_4$, 290.1519).

5.5.4. 6β-Ethoxy-8β,10β-dihydroxyeremophil-7(11)-en-12,8α-olide (9)

Oil; $[\alpha]_D^{25}$ + 126.2 (*c* 0.30, $CHCl_3$); IR (ATR) ν_{max} 3480, 1768 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) see Table 2; ^{13}C NMR (100 MHz, $CDCl_3$) see Table 3; CIMS (CH_4) *m/z* 311 $[M+H]^+$ (5), 293 (68), 247 (100); HRCIMS *m/z* 311.1851 $[M+H]^+$ (calcd for $C_{17}H_{27}O_5$, 311.1858).

5.5.5. 11αH-7β,8β-Epoxy-6β-ethoxy-10β-hydroxyeremophilan-12,8α-olide (12)

Oil; $[\alpha]_D^{27}$ − 4.1 (*c* 0.26, $CHCl_3$); IR (ATR) ν_{max} 3500, 1805, 1089 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) see Table 2; ^{13}C NMR (100 MHz, $CDCl_3$) see Table 3; EIMS (70 eV) *m/z* 310 $[M]^+$ (13), 236 (89), 109 (100); HREIMS 310.1782 $[M]^+$ (calcd for $C_{17}H_{26}O_5$, 310.1780).

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