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Isolation of new eremophilane-type sesquiterpenoids, subspicatins A–D and subspicatolide from *Ligularia subspicata*, and chemical and genetic diversity of the species

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ABSTRACT

New C-1 oxidized eremophilane-type sesquiterpenoids, subspicatins A, B, C, and D, and subspicatolide, were isolated from the root of *Ligularia subspicata* and their structures were established by spectroscopic and X-ray analyses. Subspicatins A and B were the major components. The species were found to be diverse both in the composition of the root chemicals and in the nucleotide sequences of the internal transcribed spacers (ITS) of the ribosomal RNA gene and the *atpB-rbcL* intergenic region, but the difference among the samples was not distinct but continuous.

1. Introduction

Ligularia Cass. (Asteraceae) in the Hengduan Mountains area provides us with materials suitable for studies of chemical and genetic diversity, since this genus is highly diversified in the area and is considered to be still evolving.[1,2] Our approach has been to analyze chemicals and supplement the results with neutral evolutionary information derived from the determination of base sequences. We have been analyzing furanoeremophilane and related sesquiterpenoids in roots and determining the nucleotide sequences of the plastid *atpB-rbcL* intergenic region and/or the internal transcribed spacers (ITSs) of the nuclear ribosomal RNA gene. We have detected intra-specific diversity in the chemical composition in most of the studied species of *Ligularia*.[3–12] For example, *Ligularia pleurocaulis* (Franch.) Hand.-Mazz.,[3] *Ligularia tsangchanensis* (Franch.) Hand.-Mazz.,[4] and *Ligularia virgaurea* (Maxim.) Mattf.[5] were grouped into two within each species on the basis of the terpenoid composition and the base sequences. *Ligularia kanaitzensis* (Franch.) Hand.-Mazz. is likely to be evolving from a type producing eremophilan-8-one to a type producing furanoeremophilane.[12] *Ligularia. dictyoneura* (Franch.) Hand.-Mazz. was found to be highly diverse.[6] These results have implied that the mechanisms of chemical evolution are various and complex.

In the present study, we focus on *Ligularia subspicata* (Bureau & Franch.) Hand.-Mazz., which belongs to the section *Ligularia*, series *Ligularia*.[13] The plant inhabits swamps, scrubs, and forest understories at altitudes ranging 3000–5000 m in northwestern Yunnan and southwestern Sichuan Provinces of China.[13,14] Although detection of pyrrolizidine alkaloids by LC–MS has been reported,[15] chemical constituents in the plant have not been fully investigated. In this report, we describe the identification of five new eremophilane sesquiterpenes and the diversity of the plant in the chemical composition and the nucleotide sequences.

2. Results

Twelve samples of *L. subspicata* were collected in Yunnan and Sichuan Provinces (Table 1 and Fig. 1). Ehrlich's test[16] of root

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† For structure determination.
‡ For taxonomy.
§ For general information.
¶ For genetic study.

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Table 1
Collection locality and chemical composition of *L. subspicata* samples

Sample[a]	Locality	Elevation (m)	Furanoeremophilanes[b]
1	Tianchi	3800	A+L
2	Tianchi	3900	A+L
3	Nixi	3700	A+L
4	Baimaxueshan	4200	B
5	Baimaxueshan	4100	B+L
6	Xiaoxueshan	3700	A
7	Daxueshan	3800	B
8	Reda	3800	B+L
9	Wumingshan	3800	D
10	Yading	4000	B+L
11	South of Litang	4000	D
12	Gaoersishan	4000	B+L

[a] Sample 4 was collected in 2002; samples 6, 7, 9, and 10 were collected in 2003; samples 1 and 2 were collected in 2004; samples 3, 8, 11, and 12 were collected in 2005; sample 5 was collected in 2006.
[b] A=subspicatin A (**1**); B=subspicatin B (**2**); L=ligularol (**6**) and its derivatives (**7**, **8**); D=furanoeremophilan-6β,10β-diol (**9**) and its derivatives (**10**, **11**, **13**).

extracts was carried out after development on TLC plates. All the 12 samples contained Ehrlich-positive components, indicating the presence of furanoeremophilane and/or related compounds. The samples, except for sample 6, showed a major Ehrlich-positive spot at R_f=0.60 (hexane/EtOAc 7:3); sample 6, at R_f=0.35. The spot pattern was different among the samples.

Compounds in each root extract were isolated and five new compounds, subspicatins A–D and subspicatolide, were identified. The structures of the new compounds were determined as follows.

Subspicatin A (**1**) showed a molecular ion peak at *m*/*z* 332 and the molecular formula was deduced to be $C_{20}H_{28}O_4$ by HRMS. The IR spectrum exhibited absorptions at 3500 and 1710 cm^{-1} attributable to a hydroxy and an ester functional groups, respectively. The presence of a hydroxymethyl group was supported by the ^1H and ^{13}C NMR spectra: two protons [δ 4.30 (s)] were attached to a carbon bearing the hydroxy group (δ 55.8). A furan ring was also

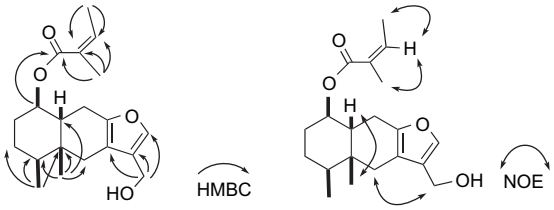


Figure 2. Selected HMBC and NOESY correlations in subspicatin A (**1**).

detected. The ester (δ 167.3) was deduced to be an angelate from the observation of a proton at δ 5.69 as a quartet of quartets. These assignments were further supported by the 2D NMR spectra, as depicted in Figure 2. In addition, the spectra indicated that the hydroxy group should be at C-13 and the angelate group at C-1 position. The stereochemistry of the compound was established by the NOESY spectrum. NOE between H-14 and H-10 indicated that rings A and B were cis-fused. The proton at C-1 appeared at δ 4.87 as a triplet (J=11.3 Hz) of doublets (J=4.4 Hz). This observation selected **1a** as the configuration and the conformation of the compound from among the possibilities **1a**–**d** (Fig. 3). Thus, subspicatin A was established to be 10β*H*-1β-angeloyloxyfuranoeremophilan-13-ol.

HRMS showed that subspicatin B (**2**) has the same molecular formula as **1**. Instead of the signals attributed to H-13 protons in **1**, a methyl signal at δ 1.99 and a methine signal at δ 4.74 as a singlet were observed for **2**. The ^1H and ^{13}C NMR spectra indicated the presence of 1-angeloyl and 6-hydroxy groups. A methine proton at δ 4.86 and its coupling as a triplet (J=10.4 Hz) of doublets (J=4.7 Hz) indicated that the angelate moiety at C-1 was in the β position, as in the case of subspicatin A. The configuration at the C-6 position was established to be β-OH by NOE between H-6α and H-1α.

Subspicatin C (**3**) showed a quasi-molecular ion peak at *m*/*z* 317 and the molecular formula was determined to be $C_{20}H_{28}O_3$.

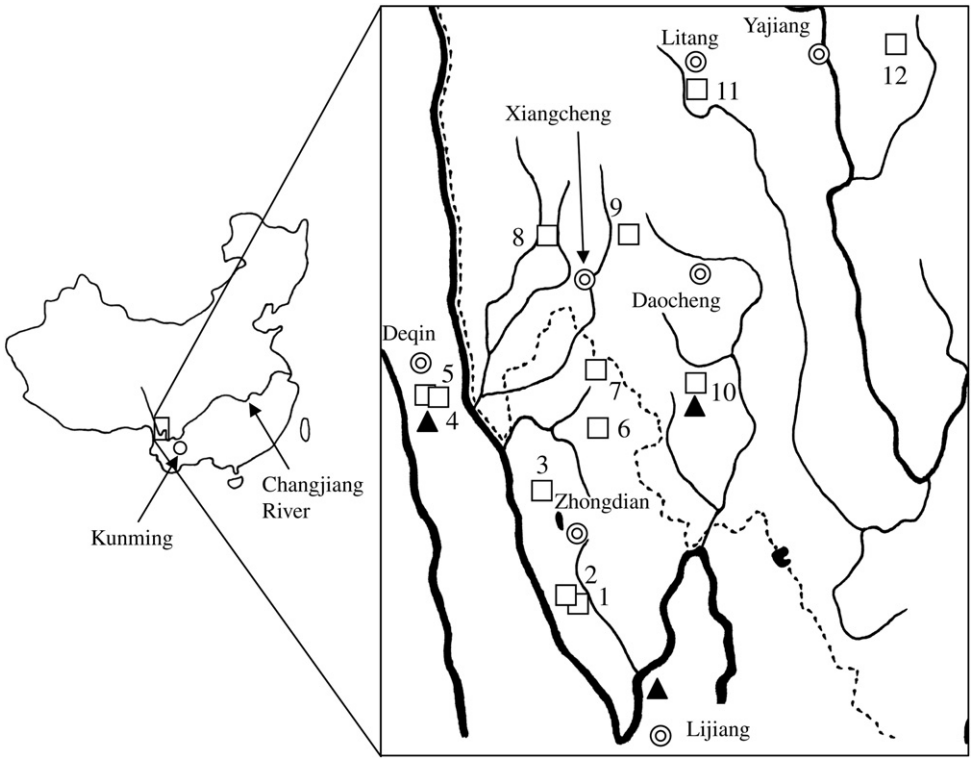


Figure 1. Locations where samples of *L. subspicata* (open squares) were collected. Filled triangles and double circles indicate peaks and cities, respectively.

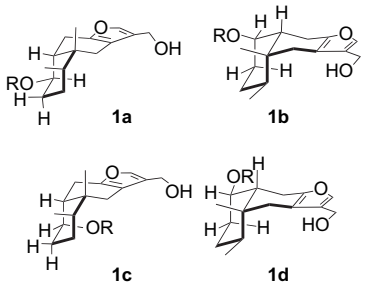


Figure 3. Four possible configurations and conformations in subspicatin A (**1**).

Absorption at 1720 cm^{-1} indicated the presence of an ester group. The ^1H and ^{13}C NMR spectra indicated the presence of an angelate moiety, a furan ring, five methyl groups, and an oxymethine proton. These indicated that this compound should be a deoxygenated derivative of subspicatin B, which was supported by the 2D NMR spectra. The stereochemistry at C-1 position was established by the coupling pattern of H-1, which was similar to those detected in compounds **1** and **2**.

Subspicatin D (**4**) exhibited a quasi-molecular ion peak at *m*/*z* 365 and its molecular formula was determined to be $C_{20}H_{28}O_6$. The IR spectrum indicated the presence of an epoxy-lactone or an enol-lactone by the absorption at 1800 cm^{-1} as well as an ester (1740 cm^{-1}) and a hydroxy (3500 cm^{-1}) groups. The ^1H NMR spectrum indicated the presence of three doublet and two singlet methyl groups, two oxymethine protons, and a proton attached to an olefinic carbon. The HMBC spectrum showed correlations between H-15 and C-3, C-4, and C-5, between H-14 and C-4, C-5, C-6, and C-10, between H-13 and C-7, C-11, and C-12 (Fig. 4). These correlations constituted an eremophilane skeleton substituted with oxygen functions at C-1 and C-6 positions. The ^{13}C chemical shifts of C-7 and C-8 were δ 66.6 and 86.7, respectively. Comparison of the chemical shifts with those of previously reported compounds[17–19] indicated the presence of an epoxide, not an enol, at the C-7 and C-8 positions with a doublet methyl at C-11. The stereochemistry at the C-1 position was established similar to compounds **1**, **2**, and **3**. NOE between H-14 and H-10 indicated the cis stereochemistry of rings A and B. NOE between H-6 and H-11 established the configuration at the C-11 position as 11α-H. According to the biosynthetic pathway discussed before,[17] and because the methyl group at C-11 is β, the epoxide ring should be β-configuration. Hence, the structure of **4** was established to be 10β*H*,11α*H*-1β-angeloyloxy-7β,8β-epoxy-6β-hydroxyeremophilan-12,8-olide.

The molecular formula of subspicatolide (**5**) was determined to be $C_{15}H_{22}O_4$ by HRMS. The IR spectrum showed absorptions at 3460, 1770, and 1710 cm^{-1}. The ^1H NMR spectrum indicated the presence of a singlet and two doublet methyl groups and an oxymethine proton at δ 4.16 as a singlet. The HMBC spectrum indicated the correlations shown in Figure 5, indicating an eremophilane skeleton. The compound was crystallized and analyzed by X-ray crystallography. Its ORTEP is shown in Figure 6. A lactone ring was

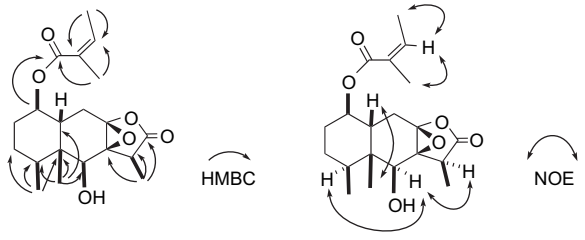


Figure 4. Selected HMBC and NOESY correlations in subspicatin D (**4**).

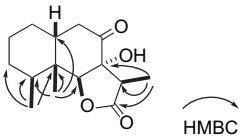


Figure 5. Selected HMBC correlations in subspicatolide (**5**).

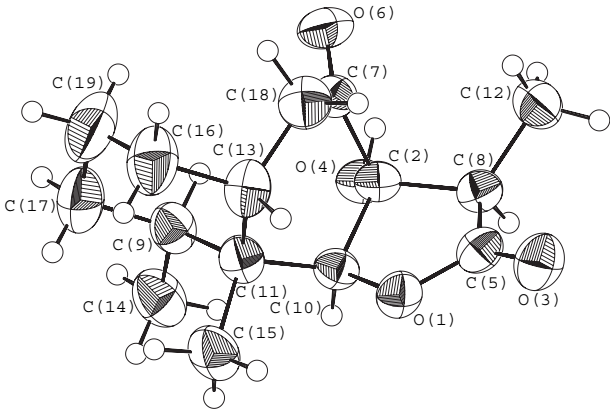
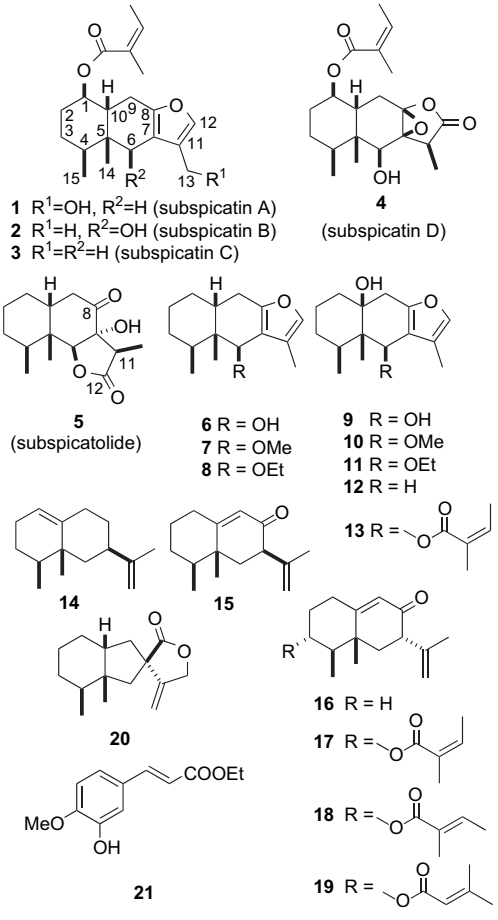


Figure 6. ORTEP drawing of subspicatolide (**5**).

present between C-12 and the oxygen at C-6. This molecule may have been derived from an α-epoxide **22**,[17] which was not isolated in this study, through opening of the lactone ring and reclosing of hydroxy acid **23** with the C-6 oxygen atom (Fig. 7).

The structures of compounds **6**,[20] **7**,[21] **8**,[22] **9**,[23] **10**,[24] **11**,[12] **12**,[25] **13**,[26] **14**,[27] **15**,[28,29] **16**,[30] **17**,[28] **18**,[12] **19**,[31] **20**,[32] and **21**[33] were determined spectroscopically.



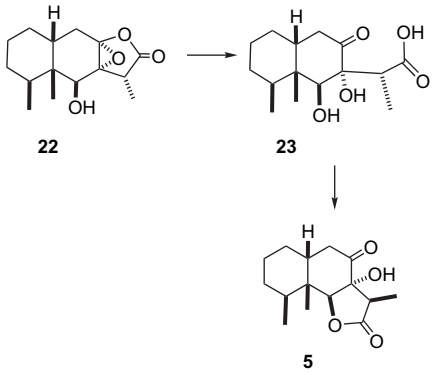


Figure 7. A plausible biogenetic pathway to subspicatolide (**5**).

The base sequences of the ITS1-5.8S-ITS2 and the *atpB-rbcL* regions were determined (Tables 2 and 3). All the samples contained sites with additional bases in the ITS1-5.8S-ITS2 region (Table 2). In samples 4, 6, 8, 11, and 12, sequences of different lengths were also present. The number of sites with additional bases was as large as 14 in sample 11, whereas it ranged from 2 to 7 in the other samples. The *atpB-rbcL* sequence is well conserved within most *Ligularia* species except for the 28th base site and the number of As in a stretch around 510th site.[2–7] In *L. subspicata*, the 409th base was also variable, as had been seen in *Ligularia tshangchanensis*.[4] The number of Ts around the 390th site varied as well (Table 3). In addition, sample 6 had a change at the 344th position; sample 11 had three changes at the 245th, the 301st, and the 469th positions. The fact that sample 11 had ITSs with many sites of additional bases and an *atpB-rbcL* with quite a few changes suggests that the individual has undergone a relatively recent hybridization event.

3. Discussion

Five new eremophilanes were isolated from *L. subspicata*. Four of them, subspicatins A–D, have an angeloyloxy group at C-1. Although various furanoeremophilanes have been isolated from *Ligularia* species of the Hengduan Mountains by our group[3,5–7,10,12] and other researchers,[34–37] C-1 oxidized compounds have been obtained only as minor components from a subset of samples of *L. dictyoneura*[6] and of *Ligularia vellerea*.[10] In contrast, most samples of *L. subspicata* produced subspicatin(s) as major components (Table 1).

Intra-specific diversity in *L. subspicata* was observed in the composition of the furanoeremophilane components (Table 1). However, the spectrum of the chemical composition in *L. subspicata* appears rather continuous, when compared with other widely distributed species, such as *Ligularia tongolensis*,[7] *L. pleurocaulis*,[3] *L. virgaurea*,[5] and *L. kanaitzensis*.[12] Within these species, the composition of the major components separated specimens into distinct groups. TLC analysis of extracts of fresh roots of *L. subspicata* indicated that ligularol (**6**) was present in all the samples except for sample 6, although the compound was not isolated from samples 4, 7, and 9 due to its paucity (Table 1). Thus, although the major components in samples 9 and 11 were furanoeremophilan-6β, 10β-diol derivatives, the chemical spectrum in *L. subspicata* samples appears continuous. Lastly, geographical correlation is noteworthy, as the samples producing subspicatin A (**1**) are found in the southern half of the collection area (Fig. 1), while those producing subspicatin B (**2**) in the northern half.

Standard bootstrapping analysis using ITS sequence data by the PAUP* program[38] did not separate the present *L. subspicata* samples

Table 2
ITS1-5.8S-ITS2 sequence of *L. subspicata* samples[a]

Sample	ITS1															5.8S		ITS2													
					1	1	1	1	1	1	2	2	2	2	2	1	1									1	1	2	2
	4	6	8	9	0	0	1	2	6	8	1	2	2	2	4	3	3			1	2	3	7	9	1	1	1	1
	6	8	2	4	0	8	2	7	5	6	2	3	3	4	5	0	2	6	5	8	3	7	0	7	1	0	8	4	7
1	T	C	T	C	G	G	A	T	T	G	A	C	T	G	G	G	T	C	C	C	C	Y	Y	G	C	C	G	T	C
2	T	Y	T	C	G	G	A	T	T	G	A	C	T	G	G	G	T	C	C	C	C	Y	T	G	C	C	G	T	C
3	T	Y	T	Y	G	G	A	T	T	G	A	C	T	K	G	G	T	C	C	C	Y	Y	T	G	C	C	G	Y	C
4[b]	T	C	Y	C	G	G	A	T	T	G	A	C	T	G	G	G	t	c	y	c	c	c	t	g	c	c	g	t	c
5	T	C	Y	C	G	G	A	T	T	G	A	C	T	G	G	G	T	C	Y	C	C	Y	T	G	C	C	G	T	C
6[c]	T	C	T	Y	G	G	A	T	T	r	a	c	t	g	g	g	T	Y	Y	C	C	Y	T	G	C	C	G	T	M
7	T	C	T	C	G	G	A	T	T	G	A	Y	T	G	K	G	T	C	C	C	C	Y	T	G	C	C	G	T	C
8[c]	t	c	t	c	g	g	a	t	y	g	a	y	t	g	g	g	T	C	C	C	C	C	T	G	C	C	G	T	C
9	T	C	T	C	G	G	A	T	T	G	A	Y	T	G	G	G	T	C	C	C	C	Y	T	G	C	C	G	T	M
10	T	C	T	C	G	G	A	T	T	G	A	Y	T	G	G	G	T	C	C	C	C	Y	Y	G	C	C	G	T	C
11[d]	Y	C	T	C	R	S	R	Y	T	G	w	y	y	g	g	r	y	c	c	y	c	y	t	r	y	g	t	C	C
12[e]	t	c	y	c	g	g	a	t	t	g	a	y	t	g	g	g	t	c	y	c	c	y	t	g	c	g	t	c	y
Ref.	T	C	T	T	G	G	A	T	T	G	A	C	T	G	G	G	T	C	C	C	C	C	T	G	C	T	T	C	C

[a] Only the differences from the reference sequence (accession DQ272338 by Pan and Gong) are listed. K=G+T; M=A+C; R=A+G; S=C+G; Y=C+T; W=A+T. Bases in lower-case letters were determined from data on only one strand because of the presence of two sequences of different lengths, making the base calling less accurate.
[b] An additional sequence lacking the 18th and the 19th base of the ITS2 was also present.
[c] An additional sequence with a C inserted after the 218th base of the ITS1 was also present.
[d] Besides the sequence in note c, another additional sequence with a C inserted after the 70th base of the ITS2 was also present.
[e] Besides the sequence in note c, another additional sequence lacking the 21st and the 22nd bases of the ITS2 was also present although at a small fraction.

Table 3
DNA sequence of *atpB-rbcL* intergenic region of *L. subspicata* samples[a]

Sample	Base position							
	28	245	301	344	409	469	Ts[b]	As[c]
1	G	G	C	T	A	A	9	10
2	G	G	C	T	T	A	10	9
3	G	G	C	T	A	A	8	10
4	G	G	C	T	T	A	9	9
5	G	G	C	T	A	A	9	10
6	A	G	C	G	A	A	9	9
7	G	G	C	T	T	A	9	9
8	G	G	C	T	T	A	9	9
9	G	G	C	T	A	A	8	10
10	G	G	C	T	T	A	9	9
11	G	T	T	T	A	C	11	9
12	G	G	C	T	T	A	9	9
Ref.	A/G	G	C	T	A	A	9	9–12

[a] The base numbering is according to the published sequence of *L. tongolensis*.[7] The bases at the other positions were the same as *L. tongolensis*.
[b] The number of thymines in a stretch around the 390th base.
[c] The number of adenines in a stretch around the 510th base.

with statistical significance. The data are still informative, as the presence of sites with additional bases in the ITSs is indicative of introgression in the species.[39] For example, the ITS and the *atpB-rbcL* sequences suggest that a hybridization event took place relatively recently in sample 11. Interestingly, the chemical compositions of samples 4 and 5 were slightly different, although they were collected within 100 m in the same grassland. The ITS sequence was also slightly different in that sample 4 contained a sequence with a base insertion in addition to the sequence of sample 5. Thus, it is plausible that the chemical diversity in *L. subspicata*, at least part of it, has been brought about by flow of genetic information.

4. Conclusion

L. subspicata was found to contain five new compounds: C-1 oxygenated furanoeremophilanes, subspicatins A–D, and subspicatolide. The plant was found to be diverse in the terpenoid composition and base sequences in neutral DNA regions. The difference among the samples was not distinct and the plant is the first example of such continuity in the *Ligularia* species we have examined. Compared with the distinct intra-specific differences in other widely distributed *Ligularia* species, the continuous nature of diversity in *L. subspicata* seems to suggest that the species is in its early stage of differentiation.

5. Experimental

5.1. General

See our previous report[5,7] for CD, IR, NMR, and mass spectra, X-ray crystallographic analysis, HPLC, column chromatography, and TLC. Ehrlich's test and the base sequence determination have been described previously.[3–7] Purification of DNA, polymerase chain reaction (PCR), purification of the PCR products, and DNA sequencing were carried out as described.[12] Four specialized sequencing primers were also designed and used to determine the sequences of parts of ITS1-5.8S-ITS2 for samples 6 and 11.

5.2. Plant materials

Samples of *L. subspicata* were collected in August, 2002–2006 at 12 locations (Table 1 and Fig. 1). Each plant was identified by Xun Gong, one of the authors, on the basis of published diagnostic characters.[13,14]

5.3. Extraction, purification, and structure determination

5.3.1. General procedure

For the samples collected in 2002 and 2003, the roots were cut into small pieces and immediately extracted with EtOH at room temperature. The extract was filtered and concentrated to afford an oily residue with an aqueous phase. AcOEt was added to this oil/aqueous mixture and the organic layer was recovered. Evaporation of the solvent afforded an oily residue, to which water-soluble starch was added to facilitate transportation. For the samples collected in 2004–2006, the roots were dried and extracted with EtOH at room temperature. Oily extracts were obtained by the standard method.

The EtOH extract (1.3 g) of sample 1 was separated by a silica gel column chromatography (hexane/AcOEt, in gradient) along with HPLC (Nucleosil 50-5, hexane–AcOEt) to isolate **1** (202.3 mg), **2** (2.1 mg), **3** (3.4 mg), **6**[20] (180.1 mg), **7**[21] (12.7 mg), **8**[22] (50.5 mg), and **11**[12] (38.0 mg).

The EtOH extract (835 mg) of sample 2 was separated similarly to isolate **1** (93.5 mg), **2** (3.1 mg), **6**[20] (50.4 mg), **7**[21] (6.0 mg), and **14**[27] (17.4 mg).

The EtOH extract (801 mg) of sample 3 was separated similarly to isolate **1** (5.2 mg), **6**[20] (15.9 mg), **15**[28,29] (4.6 mg), **16**[30] (6.1 mg), **17**[28] (4.4 mg), **18**[12] (17.5 mg), and **19**[31] (4.0 mg).

The EtOH extract (150 mg) of sample 4 was separated similarly to isolate **2** (1.4 mg).

The EtOH extract (602 mg) of sample 5 was separated similarly to isolate **2** (1.9 mg), **4** (8.4 mg), **5** (3.3 mg, after recrystallization from EtOAc), and **6**[20] (41.5 mg).

The EtOH extract (1.95 g) of sample 6 was separated similarly to isolate **1** (15.7 mg).

The EtOH extract (207 mg) of sample 7 was separated similarly to isolate **2** (2.5 mg) and **21**[33] (0.5 mg).

The EtOH extract (246 mg) of sample 8 was separated similarly to isolate **2** (3.4 mg) and **6**[20] (9.2 mg).

The EtOH extract (179 mg) of sample 9 was separated similarly to isolate **11**[12] (2.3 mg).

The EtOH extract (456 mg) of sample 10 was separated similarly to isolate **2** (2.3 mg), **6**[20] (17.2 mg), **20**[32] (2.1 mg), and **21**[33] (11.6 mg).

The EtOH extract (499.4 mg) of sample 11 was separated similarly to isolate **6**[20] (2.6 mg), **9**[23] (3.2 mg), **10**[24] (6.6 mg), **12**[25] (1.1 mg), and **13**[26] (7.5 mg).

The EtOH extract (724.3 mg) of sample 12 was separated similarly to isolate **2** (58.2 mg), **6**[20] (9.1 mg), **7**[21] (22.8 mg), and **10**[24] (6.5 mg).

5.3.2. Subspicatin A [10βH-1β-angeloyloxyfuranoeremophilan-13-ol] (**1**)

$[\alpha]_D$ −85.8 (*c* 0.87, EtOH); FTIR (KBr) 3500, 1710 cm^{-1}; MS (CI) *m*/*z* 332 $[M]^+$, 315 (base), 232; HRMS (CI) obsd *m*/*z* 332.2007 $[M]^+$, calcd for $C_{20}H_{28}O_4$ 332.1987; ^{13}C NMR (150 MHz, C_6D_6) δ 14.7 (C-15), 15.8 (C-4′), 20.8 (C-5′), 21.3 (C-9), 24.7 (C-14), 27.0 (C-2), 27.2 (C-3), 30.3 (C-6), 37.3 (C-5), 37.4 (C-4), 40.5 (C-10), 55.8 (C-13), 72.4 (C-1), 115.0 (C-7), 125.5 (C-11), 128.4 (C-2′), 137.6 (C-3′), 139.0 (C-12), 148.5 (C-8), 167.3 (C-1′); ^1H NMR (600 MHz, C_6D_6) δ 0.77 (3H, s, H-14), 0.83 (3H, d, *J*=7.1 Hz, H-15), 1.18 (1H, br d, *J*=12.6 Hz, H-3β), 1.32 (1H, m, H-4), 1.47 (1H, qd, *J*=11.3, 4.4 Hz, H-2α), 1.83 (3H, t, *J*=1.4 Hz, H-5′), 1.85 (1H, m, H-10), 1.86 (1H, m, H-3α), 1.92 (3H, dd, *J*=7.1, 1.4 Hz, H-4′), 1.95 (1H, m, H-6β), 1.96 (1H, m, H-2β), 2.47 (1H, br d, *J*=16.8 Hz, H-9β), 2.77 (1H, d, *J*=16.5 Hz, H-6α), 2.84 (1H, d, *J*=16.8 Hz, H-9α), 4.30 (2H, s, H-13), 4.87 (1H, td, *J*=11.3, 4.4 Hz, H-1), 5.69 (1H, qq, *J*=7.1, 1.4 Hz, H-3′), 7.12 (1H, s, H-12); CD [θ] 229 nm, −2470 (EtOH).

5.3.3. Subspicatin B [10βH-1β-angeloyloxyfuranoeremophilan-6β-ol] (**2**)

$[\alpha]_D$ −56.0 (*c* 0.23, EtOH); FTIR (KBr) 3500, 1720 cm^{-1}; MS (CI) *m*/*z* 332 $[M]^+$, 232 (base), 109; HRMS (CI) obsd *m*/*z* 332.1992 $[M]^+$, calcd for $C_{20}H_{28}O_4$ 332.1987; ^{13}C NMR (150 MHz, C_6D_6) δ 9.30 (C-13),

14.6 (C-15), 15.8 (C-4′), 18.8 (C-14), 20.8 (C-5′), 21.4 (C-9), 26.7 (C-2), 26.9 (C-3), 31.4 (C-4), 42.2 (C-5,10), 67.9 (C-6), 71.7 (C-1), 118.8 (C-7), 120.4 (C-11), 127.6 (C-2′), 137.7 (C-3′), 139.0 (C-12), 149.1 (C-8), 166.9 (C-1′); ^{1}H NMR (600 MHz, C_6D_6) δ 0.74 (3H, s, H-14), 0.77 (3H, d, J=7.1 Hz, H-15), 1.14 (1H, br d, J=14.4 Hz, H-3α), 1.45 (1H, tdd, J=13.2, 10.4, 4.4 Hz, H-2β), 1.69 (1H, m, H-3β), 1.79 (1H, m, H-4), 1.82 (3H, quint, J=1.4 Hz, H-5′), 1.93 (3H, dq, J=7.4, 1.4 Hz, H-4′), 1.94 (1H, m, H-2α), 1.98 (1H, m, H-10), 1.99 (3H, s, H-13), 2.52 (1H, ddd, J=17.0, 5.7, 1.6 Hz, H-9β), 2.71 (1H, d, J=17.0 Hz, H-9α), 4.74 (1H, s, H-6), 4.86 (1H, td, J=10.4, 4.7 Hz, H-1α), 5.66 (1H, dq, J=7.4, 1.4 Hz, H-3′), 6.93 (1H, s, H-12).

*5.3.4. Subspicatin C [10βH-1β-angeloyloxyfuranoeremophilane] (**3**)*

$[\alpha]_D^{21}$ −49.8 (*c* 0.34, EtOH); FTIR 1720 cm^{-1}; MS (CI) *m*/*z* 317 $[M+H]^+$ (base), 316, 217, 216; HRMS (CI) obsd *m*/*z* 317.2117 $[M+H]^+$, calcd for $C_{20}H_{29}O_3$ 317.2116; ^{13}C NMR (150 MHz, C_6D_6) δ 8.2 (C-13), 14.7 (C-15), 15.8 (C-4′), 20.9 (C-5′), 21.5 (C-9), 24.8 (C-14), 26.9 (C-2), 27.3 (C-3), 30.2 (C-6), 37.3 (C-5), 37.4 (C-4), 40.5 (C-10), 72.4 (C-1), 115.7 (C-7), 119.7 (C-11), 127.6 (C-2′), 137.4 (C-3′), 138.1 (C-12), 147.8 (C-8), 167.0 (C-1′); ^{1}H NMR (600 MHz, C_6D_6) δ 0.74 (3H, s, H-14), 0.81 (3H, d, J=7.4 Hz, H-15), 1.12 (1H, m, H-3β), 1.25 (1H, m, H-4α), 1.45 (1H, m, H-2β), 1.66 (1H, d, J=16.0 Hz, H-6β), 1.75 (3H, d, J=1.4 Hz, H-13), 1.79 (1H, m, H-3α), 1.84 (3H, quint, J=1.4 Hz, H-5′), 1.85 (1H, m, H-10), 1.94 (3H, dq, J=7.4, 1.4 Hz, H-4′), 2.01 (1H, m, H-2α), 2.49 (1H, br d, J=16.7 Hz, H-9β), 2.54 (1H, d, J=16.0 Hz, H-6α), 2.92 (1H, d, J=16.7 Hz, H-9α), 4.88 (1H, td, J=11.0, 4.7 Hz, H-1α), 5.66 (1H, qq, J=7.4, 1.4 Hz, H-3′), 7.00 (1H, s, H-12).

*5.3.5. Subspicatin D [10βH,11αH-1β-angeloyloxy-7β,8β-epoxy-6β-hydroxyeremophilan-12,8-olide] (**4**)*

$[\alpha]_D^{23}$ −29.4 (*c* 0.84, EtOH); FTIR 3440, 1800, 1690, 1650 cm^{-1}; MS (CI) *m*/*z* 365 $[M+H]^+$, 347, 265 (base), 247; HRMS (CI) obsd *m*/*z* 365.1973 $[M+H]^+$, calcd for $C_{20}H_{29}O_6$ 365.1964; ^{13}C NMR (150 MHz, C_6D_6) δ 11.5 (C-13), 14.2 (C-15), 15.9 (C-4′), 18.5 (C-14), 19.3 (C-9), 20.8 (C-5′), 26.4 (C-3), 26.7 (C-2), 31.3 (C-4), 39.1 (C-10), 40.5 (C-11), 42.1 (C-5), 66.58 (C-7), 66.61 (C-6), 71.2 (C-1), 86.7 (C-8), 128.2 (C-2′), 138.2 (C-3′), 166.9 (C-1′), 175.9 (C-12); ^{1}H NMR (600 MHz, C_6D_6) δ 0.49 (3H, s, H-14), 0.65 (3H, d, J=7.1 Hz, H-15), 1.01 (1H, m, H-3β), 1.24 (1H, m, H-2β), 1.37 (3H, d, J=7.4 Hz, H-13), 1.44 (1H, m, H-3α), 1.51 (1H, m, H-4α), 1.64 (1H, m, H-10β), 1.80 (3H, quintet, J=1.4 Hz, H-5′), 1.82 (1H, dd, J=15.7, 6.9 Hz, H-9β), 1.97 (3H, dq, J=7.4, 1.4 Hz, H-4′), 2.02 (1H, m, H-2α), 2.50 (1H, d, J=15.7 Hz, H-9α), 2.87 (1H, q, J=7.4 Hz, H-11), 3.76 (1H, d, J=6.0 Hz, H-6α), 5.10 (1H, td, J=11.5, 4.7 Hz, H-1α), 5.70 (1H, qq, J=7.4, 1.4 Hz, H-3′); CD [θ] 264 nm, +1300, 212 nm, −6100.

*5.3.6. 10βH,11αH-7α-Hydroxy-8-oxoeremophilan-12,6-olide (**5**)*

Colorless needle; mp: 148–149 °C (from EtOAc); $[\alpha]_D^{21}$ +59.7 (*c* 0.33, EtOH); FTIR 3460, 1770, 1710 cm^{-1}; MS (CI) *m*/*z* 267 $[M+H]^+$ (base), 249, 109; HRMS (CI) obsd *m*/*z* 267.1599 $[M+H]^+$, calcd for $C_{15}H_{23}O_4$ 267.1596; ^{13}C NMR (150 MHz, C_6D_6) δ 9.3 (C-13), 16.4 (C-15), 17.0 (C-14), 20.6 (C-2), 28.9 (C-1), 29.8 (C-3), 31.2 (C-4), 36.2 (C-10), 39.1 (C-5), 39.9 (C-9), 46.5 (C-11), 81.4 (C-7), 88.3 (C-6), 174.0 (C-12), 212.3 (C-8); ^{1}H NMR (600 MHz, C_6D_6) δ 0.63 (3H, d, J=6.6 Hz, H-15), 0.79 (1H, m, H-1), 0.82 (3H, d, J=7.4 Hz, H-13), 0.84 (1H, m, H-3), 0.85 (3H, s, H-14), 0.97 (1H, m, H-3), 1.01 (1H, m, H-2), 1.06 (1H, m, H-4), 1.12 (1H, m, H-2), 1.18 (1H, m, H-1), 1.85 (1H, dd, J=16.7, 4.7 Hz, H-9), 1.88 (1H, dd, J=16.7, 11.5 Hz, H-9), 2.09 (1H, m, H-10), 2.54 (1H, q, J=7.4 Hz, H-11), 3.67 (1H, br s, OH), 4.16 (1H, s, H-6); CD [θ] 297 nm, +7000, 219 nm, −9100.

Crystal data: Mo Kα radiation (λ=0.71073), monoclinic, $P2_1$, a=9.8540(9) Å, b=6.4480(4) Å, c=11.3850(14) Å, α=90.00°, β=92.739 (4)°, γ=90.00°, V=722.56 (12) Å^3, Z=2, 2570 measured reflections, 2570 independent reflections, 2270 observed reflections, θ_{max}=25.78°; refinement on F^2, full matrix least squares refinement, R(all)=0.0723, wR(ref)=0.1541, 2570 reflections, extinction correction: *SHELXL*, extinction coefficient=0.53 (4). Crystallographic data for compound **5** have been deposited at the Cambridge Crystallographic Data Center as supplementary publication number CCDC 688349. Copies of the data can be obtained, free of charge, via www.ccdc.cam.ac.uk/data_request/cif or by mailing to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 1223 336033 or e-mail: data_request@ccdc.cam.ac.uk].

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