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Diversity of *Ligularia kanaitzensis* in sesquiterpenoid composition and neutral DNA sequences

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Dedicated to Professor Kunio Iwatsuki, who has received an honorary distinction from the Japanese government for his achievements in the field of plant systematics

Abstract

Twenty-six eremophilane-type sesquiterpenoids, including six new compounds, were isolated from the title species. One of the new compounds, kanaitzensol, suggested the presence of an enzyme for the conversion of eremophil-7(11)-en-8-one derivative to furanoeremophilanes. The plant was collected at 15 locations in Yunnan Province of China and found to be diverse with respect to its sesquiterpenoid composition. DNA sequencing also revealed a large intraspecific diversity of the plant.

Keywords: *Ligularia kanaitzensis*; Furanoeremophilane; Internal transcribed spacers; Diversity

1. Introduction

Furanoeremophilanes and related sesquiterpenes are often found in plants of *Ligularia* (Asteracea) and related genera and have been studied since 1960s.[1–7] Although hundreds of furanoeremophilanes have been known to date, it is not known how the diversity of these compounds has been generated through plant evolution. We have been delineating intraspecific diversity of compounds in root extract of *Ligularia* in order to understand the mechanism(s) of generation of chemical diversity.[8–13] *Ligularia* in the Hengduan Mountains is suitable for such studies because it is highly diversified and is believed to be still evolving.[14,15] Besides, because some *Ligularia* species produce furanoeremophilanes, survey of root chemicals in many samples can be easily carried out by TLC and by visualization of furans with Ehrlich's reaction on TLC plates.[16,17] We have been also analyzing DNA sequences of non-coding regions in order to complement the results of chemical analyses with phylogenetic information.[8–13] Combination of these independent approaches has enabled us to describe diversity in more detail and offered some insight into chemical evolution in some species.

We have isolated furanoeremophilanes from all our samples of *Ligularia pleurocaulis* (Franch.) Hand.-Mazz.,[9] *Ligularia virgaurea* var. *virgaurea* (Maxim.) Mattf.,[10] *Ligularia tongolensis* (Franch.) Hand.-Mazz.,[8] and *Ligularia cymbulifera* (W.W. Smith) Hand.-Mazz.,[8] and from some samples of *Ligularia*

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‡ For taxonomy.
§ For general information.
¶ For genetic study.

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dictyoneura (Franch.) Hand.-Mazz.[13] The composition of furanoeremophilanes and related sesquiterpenes, as well as DNA sequence, was found to be diverse within most *Ligularia* species. Each of *L. pleurocaulis*,[9] *Ligularia tsangchanensis* (Franch.) Hand.-Mazz.,[11] and *L. virgaurea*[10] can be grouped into two types. The two types of *L. pleurocaulis*[9] and *L. tsangchanensis*[11] were geographically separated, while those of *L. virgaurea* were not.[10] Intraspecific diversity was particularly high in *L. dictyoneura*.[13] In contrast, *L. cymbulifera* was uniform.[8] These results indicated that complex mechanisms of evolution are at work to generate the diversity in *Ligularia*, which led us to investigate more species to obtain a fuller picture of the diversity.

In the present study, we focus on *Ligularia kanaitzensis* (Franch.) Hand.-Mazz., which belongs to the section *Ligularia*, series Racemiferae, and inhabits grassy slopes, swamps, and scrubs of around 3000 m in altitude.[18,19] The species are divided into two varieties, var. *kanaitzensis* and var. *subnudicaulis* (Hand.-Mazz.) S.W. Liu, based on morphological differences such as the shape of foliose bract.[18,19] However, in our observation, the difference was not distinct but continuous. Therefore, we do not distinguish the two varieties in the present report. Wang and co-workers obtained two eremophilane derivatives,[20] as well as lignan,[21] but no furanoeremophilane from *L. kanaitzensis* collected near Lijiang city. Here we report that *L. kanaitzensis* harbors chemical and generic diversity and plants from other locations produce furanoeremophilanes.

2. Results

Fifteen samples of *L. kanaitzensis* were collected in northwestern Yunnan Province of China in August of 2002–2006 (Table 1 and Fig. 1). The roots of each sample were extracted with ethanol and the compounds therein were subjected to Ehrlich's test on TLC plates. The pattern of the TLC spots that were positive to Ehrlich's test was mostly the same, especially for the samples from the eastern Zhongdian area (samples 5–8), indicating that the major furanoeremophilane composition of these samples was the same.

Table 1
Collection locality, chemical composition, *atpB-rbcL* and ITS sequence of *L. kanaitzensis* samples

Sample[a]	Locality	Elevation (m)	Composition[b]	*atpB-rbcL*[c]	ITS[d]
1	Jianchuan	2800	**N**	—	**B**
2	Niujie	2400	**N**	505A-A10	**B**
3	Sandawan	3200	**N**	—	**A**
4	Yulongxueshan	2900	**N**	605T	**B**
5	Baishuitai (Annan)	3000	**F**	—	**A**
6	Baishuitai (Annan)	3000	**F**	—	**A**
7	Baishuitai (Jiulong)	3600	**F**	—	**A**
8	Baishuitai (Jiulong)	3600	**F**	—	**A**
9	Luguhu	2700	**F**	—	**B**
10	Lidiping	3300	**N**	—	**B**
11	Lidiping	3200	**F**	—	**B**
12	Lidiping	3300	**F**	—	**B**
13	Labadi	2900	**F**	—	**B**
14	Jiushijiulongtan	3500	**N**	—	**B**
15	Dali	2400	**F**	505A-A10	**B**

[a] Samples 2 and 7 were collected in 2002; sample 1 was collected in 2003; samples 3 and 9 were collected in 2004; samples 4–6, 8, and 14 were collected in 2005; samples 10–13 and 15 were collected in 2006.
[b] Major components: **F**, furanoeremophilanes; **N**, non-furano eremophilanes.
[c] '—': Same as deposited under accession AB281495. See text for the others.
[d] See Table 2 and text for details.

Chemical constituents of typical samples were examined. Dried roots (samples collected in 2004–2006) were extracted with EtOAc and fresh roots (samples collected in 2002 and 2003) were extracted with EtOH. The extracts were separated by silica-gel column chromatography and HPLC. Twenty-six eremophilanes **1**–**26**, including six new compounds **1**–**6**, were isolated along with β-bisabolol (**27**),[22] valeranone (**28**),[23,24]

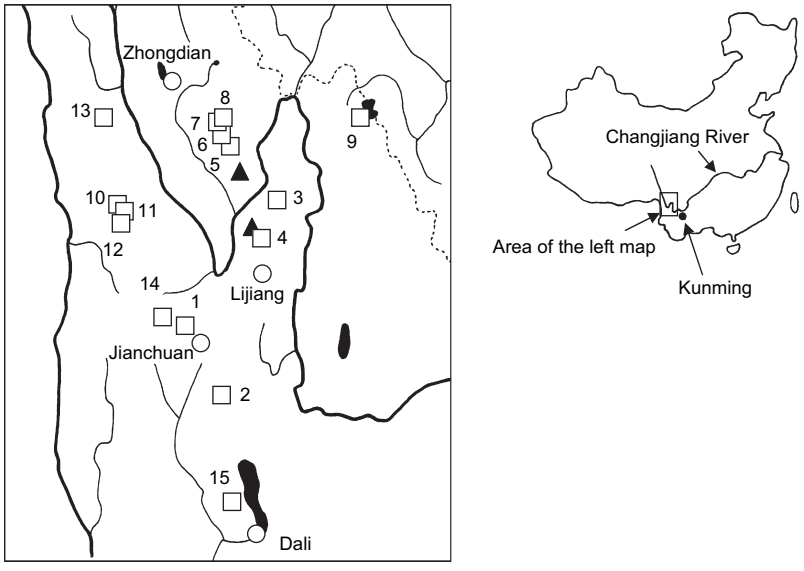
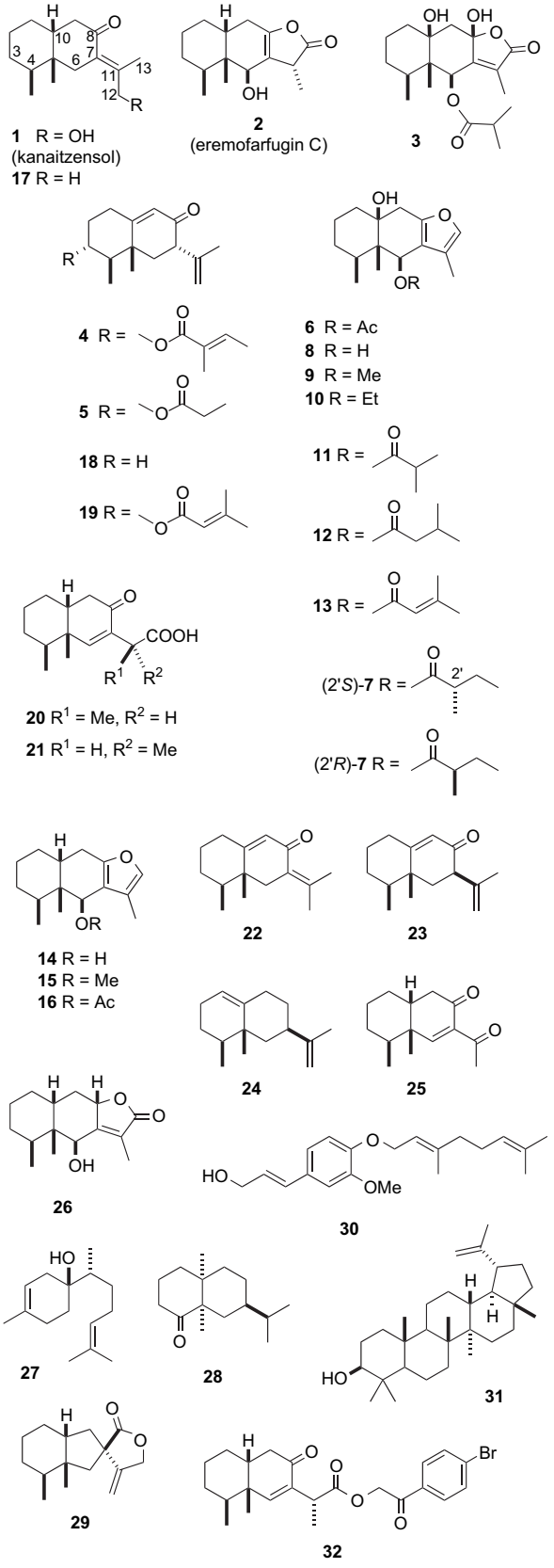


Figure 1. Locations where samples of *L. kanaitzensis* species (open squares) were collected. Filled triangles and open circles indicate major peaks and major cities, respectively.



bakkenolide (**29**),[25,26] *O*-geranylconiferyl alcohol (**30**),[27–29] and lupeol (**31**).[30,31] Compounds **6**, **7**,[5] **8**,[4] **9**,[5] **10**, **11**,[32] **12**,[33] **13**,[33] **14**,[1,34] **15**,[35] and **16**[36] were furanoeremophilanes, and compounds **1**–**5**, **17**,[37] **18**,[38,39] **19**,[40] **20**,[41] **21**,[41] **22**,[42] **23**,[39,43] **24**,[44,45] **25**,[10,46,47] and **26**[7] were eremophilanes without a furan ring, among which **25** has only 14 carbons (nor-eremophilane). Although compound **10** seems to be an artifact due to extraction with ethanol, the compound has not been reported.

From samples 1–3 and 14, only non-furano type of eremophilane compounds were isolated except for β-bisabolol (**27**).[22] Samples 4 and 10 contained similar compounds, together with a small amount of furanoeremophilane(s). Samples 5–9 and 13 contained furanoeremophilanes as major components. While enough amount of sample could not be collected for samples 11, 12, and 15, TLC analysis indicated that these samples also include furano compounds as major components. Wang and co-workers previously collected a *L. kanaitzensis* sample in the Lijiang county, presumably not far from our collection location of sample 3 or 4.[20] Although they obtained 11-hydroperoxyeremophila-6,9-dien-8-one and 6-hydroxyeremophil-7(11)-en-8,12-olide, these compounds were not isolated in the present study. This difference may have arisen from the difference in the extraction procedure and/or in the sampling location.

The structures of the new compounds were determined as follows.

Kanaitzensol (**1**) showed the presence of a hydroxy (3400 cm^{-1}) and a carbonyl (1700 cm^{-1}) group in the IR spectrum. The CIMS spectrum exhibited the quasi-molecular ion peak at *m/z* 237 and the molecular formula was deduced to be $C_{15}H_{24}O_2$ by HRCIMS. The ^1H NMR spectrum showed the presence of a doublet methyl, a singlet methyl, and a singlet methyl attached to an sp^2 carbon atom as well as two sets of doublet signals at δ 3.78 and 3.83 (each one proton). The HMBC spectrum clearly indicated correlations between H-15 and C-3, 4, and 5, between H-14 and C-4, 5, 6, and 10, between H-13 and C-7, 11, and 12, and between H-6 and C-5, 7, 8, and 11 (Fig. 2). Therefore, the planar structure was deduced to be 12-hydroxyeremophil-7(11)-en-8-one. Because the NOESY spectrum showed correlation between H-14 and H-10, rings A and B were judged to be cis-fused. The geometry of the 7(11)-double bond was determined to have the *E* configuration by the presence of NOE between H-12 and H-6 (Fig. 2).

Eremofarfugin C (**2**) exhibited absorptions at 3460 and 1800 cm^{-1} in the IR spectrum, indicating the presence of a hydroxy and an enol lactone groups, respectively. Because signals in the ^1H NMR spectrum were very broad at ambient temperature, measurement was carried out at 60 °C in C_6D_6. The ^1H NMR spectrum was very similar to those of eremofarfugins A[48] and B.[10] The HMBC spectrum at the elevated temperature clearly showed the correlations illustrated in Figure 3, indicating the eremophilane skeleton with an enol lactone moiety. The A/B-cis stereochemistry with the C-11α methyl group was also

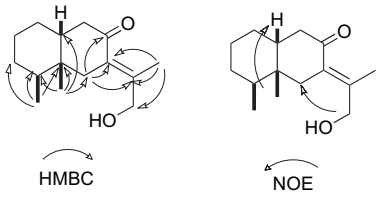


Figure 2. Selected HMBC and NOE correlations detected for kanaitzensol (**1**).

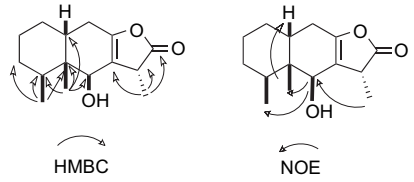


Figure 3. Selected HMBC and NOE correlations detected for eremofarfugin C (**2**).

established by the NOESY spectrum (Fig. 3). Thus, it was named eremofarfugin C.

Compound **3** exhibited a quasi-molecular ion peak at *m/z* 353 and its molecular formula was deduced to be $C_{19}H_{28}O_6$ by HRMS. The IR spectrum indicated the presence of a hydroxy group at 3350 cm^{-1}, an ester at 1730 cm^{-1}, and a lactone at 1760 cm^{-1}. The ^1H NMR spectrum showed one proton singlet at δ 5.66, which is characteristic of a proton at the C-6 position. The spectrum indicated the presence of five methyl groups, three of which were doublet. The ^{13}C NMR spectrum indicated signals of five methyls, four methylenes, three methines, and seven quaternary carbons. Because the ^{13}C NMR spectrum showed a signal of a quaternary carbon at δ 103.5, the C-8 position was deduced to have a hydroxy group. The HMBC spectrum showed connectivities between H-15 and C-3, 4, and 5, between H-14 and 4, 5, 6, and 10, between H-13 and C-7, 11, and 12, between H-3′ or 4′ and C-1′ and 2′, and between H-6 and C-1′ (Fig. 4). An isobutyryl group was found to be at the C-6 position, because the HMBC spectrum indicated correlation between H-6 and C-1′. These results led to the conclusion that the compound should be 8β,10β-dihydroxy-6β-isobutyryloxyeremophil-7(11)-en-12,8-olide. Its stereochemistry was established by the NOESY spectrum as depicted in the formula (Fig. 4).

Compound **4** had no furan ring but a 9-en-8-one system, judged by NMR. Compound **19** and an angelate derivative were reported by Bohlmann.[40] A doublet methyl at the C-4′ position was detected instead of a singlet methyl of **19**. An olefinic proton was detected at δ 6.95 as a quartet of quartets. The methyl groups at δ 1.42 and 1.82 had NOE cross peaks. Therefore, the compound was determined to be a tiglate derivative.

Compound **5** exhibited a quasi-molecular ion peak at *m/z* 291 and the molecular formula was deduced to be $C_{18}H_{26}O_3$, indicating six degrees of unsaturation, by HRMS. The ^1H NMR spectrum contained signals due to an ethyl group, a singlet and a doublet methyl groups as well as a singlet methyl attached to the double bond. Because two double bonds and two carbonyl groups were identified by the ^{13}C NMR spectrum, the compound was judged to be bicyclic from the six degrees of unsaturation.

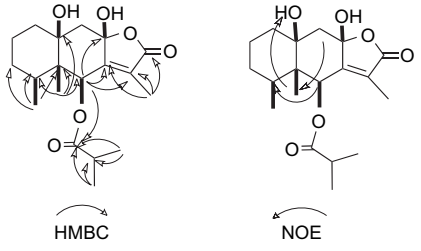


Figure 4. Selected HMBC and NOE correlations detected for compound **3**.

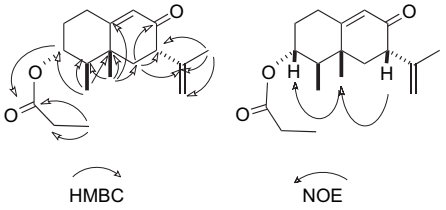


Figure 5. Selected HMBC and NOE correlations detected for compound **5**.

The ^1H NMR spectrum was similar to those of **4**, **18**, and **19**. The analysis of 2D NMR spectra (Fig. 5) led to the conclusion that it was a propionyloxy derivative of **18** as depicted in the formula including the stereochemistry.

Compound **6** had a molecular formula of $C_{17}H_{24}O_4$ and its ^1H NMR spectrum showed it to be an acetate. Two doublet signals at δ 2.99 and 2.71 with a coupling constant J=17.4 Hz suggested the presence of a hydroxy group at the C-10 position. Analysis of the 2D NMR spectra indicated that this compound was furanoeremophilane-6β,10β-diol 6-*O*-acetate, which was known as a derivative of 6,10-diol,[4] although no report of isolation from a natural source was found in the literature.

Compound **10** seems to be an artifact due to extraction with EtOH. However, the data of the compound are given in Section 5, since this compound was not found in the literature.

Although compound **7** had been reported by Tada and coworkers[5] the absolute configuration of the ester side chain was not determined. Therefore, we synthesized both (2′*R*)- and (2′*S*)-**7** from diol **8**. First, (2′*S*)-**7** was derived from **8** and (*S*)-2-methylbutyric anhydride. The doublet methyl group at the C-2′ position of (2′*S*)-**7** was observed at δ 0.98. Then, diol **8** was treated with *rac*-2-methylbutyric acid to afford a mixture of (2′*R*)-**7** (δ 0.95, H-5′) and (2′*S*)-**7** (δ 0.98, H-5′). The H-5′ of **7** from *L. kanaitzensis* was at δ 0.98 and the compound was established to be (2′*S*)-**7**.

Although the carboxylic acids **20** and **21** were isolated in pure, spectroscopic evidence was insufficient to assign their isomeric configurations. Therefore, **21** was treated with *p*-bromophenacyl bromide to give a crystalline compound **32**. The structure was solved by the direct method and refined by full matrix least-square on F^2. The refinement converged to a R(all) value of 0.0438 using all data and R(gt) value of 0.0410 for observed reflections. The ORTEP drawing is shown in Figure 6 with the absolute structural

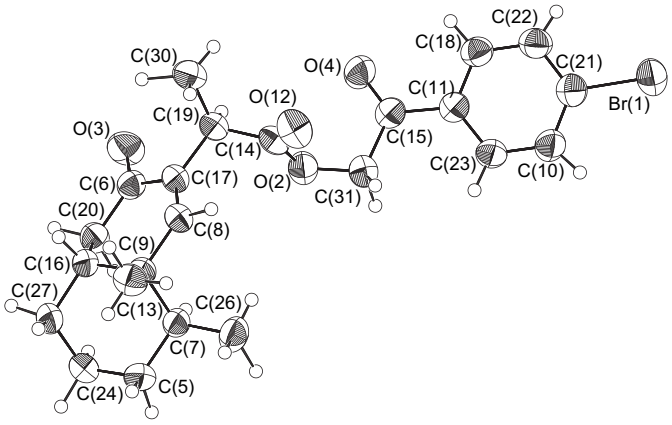


Figure 6. ORTEP drawing of compound **32**.

parameter of −0.018. Therefore, compound **21** was established to be 4*S*,5*R*,10*R*,11*R*-8-oxoeremophil-6-en-12-oic acid.

The other compounds were identified by comparing spectral data with those in the literature or by analysis of 2D NMR data.

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. Most of the samples had one same sequence of the *atpB-rbcL* region and it has been deposited to the database (accession AB281495). The common sequence was mostly the same as the one we reported earlier for *L. tongolensis*.[8] It differed from the *L. tongolensis* sequence at five sites: the 245th base is thymine (T) in place of guanine (G); the 301st base is T in place of cytosine (C); the 469th base is cytosine (C) in place of adenine (A); the number of Ts in a stretch around the 390th position is 11 not 9; the number of As in a stretch around the 510th position is 9 not 11 (the base numbering is based on the *L. tongolensis* sequence).[8] This type of *atpB-rbcL* sequence with Ts at the 245th and the 301st, C at the 469th, and 11Ts has been seen in some samples of *Ligularia latihastata*,[12] *Ligularia villosa*,[12] and *L. dictyoneura*.[13] Three samples had variations in the sequence (Table 1). In samples 2 and 15, the 505th base was A in place of C, and the number of A stretch around the 510th position was 10 not 9. In sample 4, the 605th base was T in place of C.

The results of sequencing the ITSs with 5.8S rRNA gene in-between (ITS1−5.8S−ITS2 region) are summarized in Table 2. A number of samples contained sequences of different lengths. Therefore, we were unable to unambiguously determine the sequences in them. Still, the sequences clearly separated into two groups. One consisted of samples 3 and 5−8 (type **A** in Table 1) and the other, the rest of the samples (type **B**). *L. kanaitzensis* sequences in the database (DQ272335 and AY458837) were type **B**. This separation was supported by the neighbor-joining cladistic analysis using the PAUP* program suite with a bootstrap value of 100%.[49]

3. Discussion

Six new compounds, kanaitzensol (**1**), eremofarfugin C (**2**), and compounds **3**−**6**, were obtained from *L. kanaitzensis* in addition to many known compounds. Among the six new compounds, kanaitzensol (**1**) isolated from sample 3 is especially noteworthy. A study with rat cytochrome P450 has suggested that a furanoeremophilane can be enzymatically synthesized from eremophil-7(11)-en-8-one via a (*Z*)-12-hydroxy derivative.[50] Compound **1** is a geometrical isomer of the proposed intermediate with respect to the 7(11)-double bond. Compound **1** would not form a furanoeremophilane because of geometric mismatch, and in fact, no furan was isolated from sample 3.

The spectrum of terpenoids isolated from the samples appears more or less continuous. However, the samples can be grouped roughly into two with respect to the major components (Table 1). Samples of one group (**F** in Table 1) contained furanoeremophilanes as major components, such as furanoeremophilane-6β,10β-diol (**8**) and its 6-alkoxy (**9** and **10**) and 6-acyloxy derivatives (**6**, **7**, and **11**−**13**) as well as furanoeremophilan-6β-ol (=ligularol or petasalbin, **14**) and its derivatives (**15** and **16**). Samples of the other group (**N** in Table 1) contained non-furano eremophilanes as major components, such as eremophilan-8-one derivatives **17**, **22**, and **23**. We have been observing that production of a furan(s) appears to be related to the ecological niche of the plant. For example, *L. cymbulifera*, which produces furanoeremophilan-10β-ol as the major component, makes extensive colonies in the Zhongdian area.[8,51] Similarly, we found that populations of *L. kanaitzensis* that produce furanoeremophilane-6β,10β-diol derivatives (samples 4−8, 11−13, and 15) were occupying a predominant niche, whereas the other populations of the plant (samples 1−3, and 14) were growing intermingling with other plants (the population size at the locations of samples 9 and 10 was very small). Although the exact biochemical role(s) of furanoeremophilanes has yet to be elucidated, this observation reinforces our idea that production of furanoeremophilanes has conferred an ecological advantage(s).[13] It also accords with an idea that plants that produce furanoeremophilanes must be evolutionarily more advanced.

Analysis of the ITS1−5.8S−ITS2 region revealed nucleotide sites with additive bases in most samples and sequences of different lengths in quite a few samples. Because DNA sequencing was carried out using a single leaf of one individual, multiple ITS sequences result from differences among the copies of the rRNA gene that exist in the thousands in higher plants.[52] The presence of multiple ITS sequences is believed to be a result of past hybridization.[53] Because we have seen multiple ITS sequences in many samples of *Ligularia* species that we have determined the sequence for[10−13] hybridization appears quite common in *Ligularia*, which suggests reticulate evolution in the genus and possibly in closely related genera.[54] Besides that past hybridization is clearly evident in most of the samples of *L. kanaitzensis*, the present samples can be unambiguously divided into two groups. Moreover, when the database is searched with Blast[55] for sequences similar to the ITS1−5.8S−ITS2 sequence of sample 6 (type **A**), sequences of *L. virgaurea*,[10] *L. tsangchanensis*,[11] *Ligularia vellerea*,[56] and other plants were found to be more similar than the type **B** sequence. Thus, the separation between the two types appears as large as that expected to be observed between species. Further studies are necessary to determine whether they constitute separate species.

Correlation between the chemical composition and the DNA sequence has been seen in some *Ligularia* species, most notably in *L. virgaurea*,[10] *L. dictyoneura*,[13] and *L. vellerea*,[56] which indicated that the chemical diversity was likely to have been brought about by genetic alteration. In the present case of *L. kanaitzensis*, no clear correlation was observed, as was the case with *L. dictyoneura*,[13] which was found to be highly diverse both in chemicals and DNA sequences. No correlation was observed between the DNA sequences and morphology of the plants. We believe further accumulation of data on other *Ligularia* species and on related plants will reveal the structure of the evolution of eremophilanes.

Table 2
ITS1−5.8S−ITS2 base sequence of *L. kanaitzensis* samples[a]

	ITS1																				5.8S									ITS2																							
	2	13	25	60	81	84	85	86	87	90	94	109	125	126	127	177	196	216	236	255	98	123	133	134	136	137	148	151	157	5	11	19	32	61	71	72	91	92	93	101	105	116	168	191	192	195	203	204	205	210	218	221	222
1[b]	c	a	c	a	t	g	a	t	g	t	c	g	t	c	C	g	g	c	g	c	c	c	c	g	c	c	c	c	c	c	t	c	g	c	c	t	a	t	a	a	c	c	t	C	c	t	t	c	t	c	t	c	c
2	C	A	C	A	T	G	A	T	G	T	C	G	T	C	C	G	G	C	G	C	Y	C	C	G	C	C	C	C	C	C	T	C	G	C	C	T	A	T	A	A	C	C	T	S	Y	T	T	C	K	Y	T	C	C
11	C	A	C	A	T	G	A	T	G	T	C	G	T	C	C	G	G	C	G	C	C	C	C	G	C	S	C	C	C	C	T	Y	G	C	C	T	A	T	A	A	C	C	T	C	Y	T	T	C	T	C	T	C	C
9	C	A	C	A	T	G	A	T	G	T	C	G	T	C	C	R	G	C	G	C	C	C	C	G	C	C	C	C	C	C	T	C	G	C	C	T	A	T	A	A	C	C	T	C	C	T	T	C	K	C	T	Y	C
4	C	A	C	A	T	G	A	T	G	T	C	G	T	C	C	G	G	C	G	C	C	C	C	G	Y	C	C	Y	C	C	T	C	G	C	C	T	A	T	A	A	C	C	T	C	C	T	T	C	Y	C	T	C	C
13	C	A	Y	A	T	G	A	T	G	T	C	G	T	C	C	G	G	C	G	C	C	C	C	G	Y	C	C	C	C	C	T	C	G	C	C	T	A	T	A	A	C	C	T	M	C	T	T	C	T	C	T	C	C
10[c]	C	A	C	A	W	K	R	Y	K	T	C	G	T	C	C	G	R	C	G	C	c	c	c	g	c	c	c	c	c	c	t	c	g	c	c	t	a	t	a	a	c	c	t	C	c	t	t	c	t	c	t	c	c
12[d]	C	A	C	A	W	G	R	T	G	T	C	G	T	C	C	G	R	C	G	C	c	c	c	g	c	c	c	c	c	c	t	c	g	c	c	t	a	t	a	a	c	c	t	C	c	t	t	y	t	c	t	c	c
15	C	A	C	A	W	G	R	T	G	T	C	G	T	C	C	G	G	C	G	C	C	C	C	G	C	C	C	C	C	C	T	C	G	C	C	T	A	T	A	A	C	C	T	C	C	T	T	Y	T	C	T	C	C
14[e]	c	a	c	a	t	g	a	t	g	t	c	k	t	c	S	g	g	c	g	c	c	C	C	G	C	C	C	C	C	C	T	C	G	C	C	T	A	T	A	A	C	C	T	C	C	T	T	Y	T	C	T	C	C
7[f]	y	t	c	r	t	g	a	t	g	y	c	g	c	t	A	g	g	y	—	y	c	C	C	G	Y	C	Y	C	Y	Y	C	C	A	C	—	A	A	Y	W	Y	T	Y	C	C	C	W	Y	T	T	C	C	C	C
5[f]	y	t	c	r	t	g	a	t	g	y	c	g	c	t	A	g	g	y	—	y	c	C	C	G	Y	C	Y	C	Y	Y	C	C	A	C	—	A	A	Y	W	Y	T	Y	C	C	C	W	Y	T	T	C	C	C	C
6[f]	y	t	c	r	t	g	a	t	g	y	c	g	c	t	A	g	g	y	—	y	c	C	C	G	Y	C	Y	C	Y	Y	C	C	A	C	—	A	A	Y	W	Y	T	Y	C	C	C	W	Y	T	T	C	C	C	C
8[f]	y	t	c	r	t	g	a	t	g	y	c	g	c	t	A	g	g	y	—	y	c	C	C	G	Y	C	Y	C	Y	Y	C	C	A	C	—	A	A	Y	W	Y	T	Y	C	C	C	W	Y	T	T	C	C	C	C
3[g]	y	t	c	r	t	g	a	t	g	y	y	g	c	t	A	g	g	y	—	y	c	Y	Y	K	C	C	C	C	C	C	C	C	A	Y	—	A	R	K	A	Y	T	Y	C	C	C	W	C	T	T	C	C	C	Y
Ref[h]	C	A	C	A	T	G	A	T	G	T	C	G	T	C	C	G	G	C	G	C	C	C	C	G	C	C	C	C	C	C	T	C	G	C	C	T	A	T	A	A	C	C	T	C	C	T	T	C	T	C	T	C	C

[a] Only the differences from the reference sequence are listed. '—' indicates deletion; K=G+T; M=A+C; R=A+G; S=C+G; W=A+T; Y=C+T. Bases in lower-case letters were determined from data on only one strand because of the presence of two sequences of different lengths as noted below, making the base calling less accurate.
[b] An additional sequence with six Cs in place of five Cs at the positions 155−159 in ITS1 was present.
[c] An additional sequence with two Ts in place of three Ts at the positions 117−119 in ITS1 was present. Besides, an additional sequence with one T in place of two Ts at the positions 67 and 78 of ITS2 was present.
[d] An additional sequence with two As in place of one A at the 118th position of ITS2 was present.
[e] An additional sequence with two Ts in place of three Ts at the positions 117−119 in ITS1 was present.
[f] An additional sequence with six Cs in place of five Cs at sites 155−159 in ITS1 was present.
[g] Sequence with six and seven Cs in place of five Cs at sites 155−159 in ITS1 was present.
[h] Voucher 02269 (Pan and Gong; KIB); accession DQ272335.

4. Conclusion

Six new compounds were isolated from root extracts of *L. kanaitzensis*. Kanaitzensol (**1**) is an isomer of an intermediate from eremophil-7(11)-en-8-one to furanoeremophilanes with respect to the 7(11)-double bond.

The chemical composition was found to be diverse. The plant produces furanoeremophilanes in the Baishuitai and the Lidiping areas, where the plant was abundant. The results of sequencing of the ITSs of the rRNA gene indicated that the populations in Baishuitai and those in Lidiping have undergone genetic differentiation. The chemical results and observation in the field seem to support our previous premise that furanoeremophilanes, which are likely to be produced via a 12-hydroxy derivative(s) of eremophil-7(11)-en-8-ones, are evolutionarily more advanced among eremophilanes and confer ecological advantage on plants.

5. Experimental

5.1. General

Specific rotations and CD spectra were measured on a JASCO DIP-1000 and a JASCO J-725 auto recording polarimeters; IR spectra, on a JASCO FT/IR-5300 spectrophotometer; ^{1}H and ^{13}C NMR spectra, on a Varian Unity 600 (600 MHz and 150 MHz, respectively) and a JEOL ECP 400 (400 MHz and 100 MHz, respectively) spectrometers. Mass spectra, including high-resolution ones, were recorded on a JEOL JMS-700 MStation. X-ray crystallographic analysis was carried out on a Mac Science MXC 18 diffractometer using a DIP image plate. Chemcopak Nucleosil 50-5 (4.6×250 mm) with a solvent system of hexane–ethyl acetate was used for HPLC (a JASCO pump system). Silica gel 60 (70–230 mesh, Fuji Sylisia) was used for column chromatography. Silica gel 60 F_{254} plates (Merck) were used for TLC. Ehrlich's test on TLC was carried out as previously described.[8] Polymerase chain reaction and purification of the products were carried out as described previously.[8–10] DNA sequencing reactions were carried out with the BigDye Terminator Ver 3.0 kit (Applied Biosystems) and the primers described previously[9,10] and analyzed on a 3130xl sequencer (Applied Biosystems).

5.2. Plant materials

Samples of *L. kanaitzensis* were collected in August, 2002–2006 at 15 locations (Table 1 and Fig. 1). Each plant was identified by Xun Gong, one of the authors.

5.3. Extraction, purification, and structural determination

5.3.1. General procedure

For the samples collected in 2002 and 2003, roots were cut into small pieces without drying, and immediately extracted with EtOH at rt. The extract was filtered and concentrated to afford an oily residue with an aqueous phase. EtOAc was added to this oil/water mixture and the organic layer was recovered. Evaporation of the solvent afforded an oily residue, to which water-soluble starch was added for handling purposes. For the samples collected in 2004–2006, roots were dried and extracted with EtOAc at rt. Oily extracts were obtained by the standard method.

Sample 1: the ethanol extract obtained from 80 g of fresh root was separated by silica-gel column chromatography along with HPLC to give **17**[37] (239.4 mg) and **27**[22] (3.3 mg).

Sample 2: the ethanol extract (1.3 g) was separated similarly to give **4** (1.8 mg), **17**[37] (35.0 mg), **18**[38,39] (64.0 mg), and **19**[40] (2.5 mg).

Sample 3: the ethanol extract (648.8 mg) was separated similarly to give **1** (3.0 mg), **17**[37] (438.8 mg), **18**[38,39] (5.8 mg), and **22**[42] (6.6 mg).

Sample 4: the EtOAc extract (1.68 g) was separated similarly to give **13**[33] (4.2 mg), **17**[37] (58.8 mg), and **29**[25,26] (362.2 mg).

Sample 5: the EtOAc extract (721.7 mg) was separated similarly to give **6** (25.2 mg), (2′*S*)-**7**[5] (7.2 mg), **8**[4] (9.5 mg), **11**[32] (29.9 mg), **12**[33] (4.5 mg), **14**[1,34] (271.1 mg), **26**[7] (1.1 mg), and **30**[27–29] (0.7 mg).

Sample 6: the EtOAc extract (612.7 mg) was separated similarly to give **6** (17.6 mg), (2′*S*)-**7**[5] (15.3 mg), **11**[32] (27.5 mg), **12**[33] (7.6 mg), **14**[1,34] (100.9 mg), **16**[36] (3.2 mg), and **20**[40] (5.9 mg).

Sample 7: the ethanol extract obtained from 110 g of fresh root was separated similarly to give **8**[4] (3.1 mg), **9**[5] (12.9 mg), **10** (10.4 mg), and **15**[35] (2.9 mg).

Sample 8: the EtOAc extract (2.69 g) was separated similarly to give **2** (21.3 mg), **3** (4.4 mg), **6** (10.9 mg), (2′*S*)-**7**[5] (103.6 mg), **8**[4] (22.9 mg), **11**[32] (200.8 mg), **14**[1,34] (51.9 mg), **16**[36] (44.9 mg), **20**[41] (4.9 mg), **21**[41] (17.8 mg), **25**[10,46,47] (1 mg), **26**[7] (2.5 mg), **28**[23,24] (3.5 mg), and **30**[27–29] (1.8 mg).

Sample 9: the ethanol extract (712.6 mg) was separated similarly to give (2′*S*)-**7**[5] (15.9 mg), **8**[4] (119.2 mg), **10** (182.2 mg), **11**[32] (42.9 mg), **14**[1,34] (300.2 mg), and **24**[44,45] (21.3 mg).

Sample 10: the ethanol extract (424.2 mg) was separated similarly to give **4** (15.9 mg), **5** (1.0 mg), (2′*S*)-**7**[5] (4.1 mg), **8**[4] (2.9 mg), **11**[32] (5.1 mg) and **18**[38,39] (18.4 mg), **19**[40] (15.2 mg), **23**[39,43] (2.5 mg), and **31**[30,31] (1.5 mg).

Sample 13: the ethanol extract (445.6 mg) was separated similarly to give **6** (14.8 mg), (2′*S*)-**7**[5] (24.6 mg), **8**[4] (8.2 mg), **11**[32] (15.7 mg), **13**[33] (8.5 mg), and **31**[30,31] (1.0 mg).

Sample 14: the EtOAc extract (531.9 mg) was separated similarly to give **17**[37] (56.5 mg) and **23**[39,43] (5.6 mg).

5.3.2. Kanaitzensol A (*1*)

$[\alpha]_D^{16}$ −3.2 (*c* 0.3, EtOH); FTIR (KBr) 3400, 1700, 1630 cm^{-1}; MS (CI) *m/z* 237 $[M+H]^+$ (base), 236, 219; HRMS (CI) obsd *m/z* 237.1833 $[M+H]^+$ (calcd for $C_{15}H_{25}O_2$ 237.1855); ^{13}C NMR (600 MHz, C_6D_6) δ 15.8 (C-15), 17.7 (C-13), 20.5 (C-2), 21.4 (C-14), 27.3 (C-1), 30.1 (C-4), 30.3 (C-3), 36.9 (C-5), 40.3 (C-6), 41.5 (C-10), 44.4 (C-9), 62.8 (C-12), 133.5 (C-7), 139.5 (C-11), 204.8 (C-8); ^{1}H NMR (600 MHz, C_6D_6) δ 0.65 (3H, d, *J*=6.9 Hz, H-15), 0.68 (3H, s, H-14), 0.97 (1H, m, H-1), 1.02 (1H, m, H-3), 1.15 (1H, m, H-2), 1.18 (1H, m, H-2), 1.38 (2H, m, H-3, 4), 1.41 (1H, m, H-1), 1.47 (1H, dq, *J*=11.0, 5.5 Hz, H-10), 1.68 (1H, d, *J*=15.1 Hz, H-6β), 2.10 (3H, d, *J*=1.4 Hz, H-13), 2.17 (1H,

dd, J=15.9, 5.5 Hz, H-9β), 2.38 (1H, dd, J=15.9, 11.0 Hz, H-9α), 2.50 (1H, d, J=15.1 Hz, H-6α), 3.78 (1H, d, J=12.4 Hz, H-12), 3.83 (1H, d, J=12.4 Hz, H-12).

*5.3.3. Eremofarfugin C (**2**)*

$[\alpha]_D^{19}$ +6.8 (*c* 1.07, EtOH); FTIR (KBr) 3460, 1800 cm^{-1}; MS (CI) *m/z* 251 $[M+H]^+$ (base), 233; HRMS (CI) obsd *m/z* 251.1648 $[M+H]^+$ (calcd for $C_{15}H_{23}O_3$ 251.1647); ^{13}C NMR (100 MHz, C_6D_6, 60 °C) δ 14.2 (C-13), 15.0 (C-15), 17.3 (C-14), 20.5 (C-2), 25.3 (C-9), 27.3 (C-1), 29.7 (C-3), 31.4 (C-4), 35.1 (C-10), 39.0 (C-11), 40.4 (C-5), 66.7 (C-6), 116.4 (C-7), 149.6 (C-8), 180.0 (C-12); ^1H NMR (400 MHz, C_6D_6, 60 °C) δ 0.66 (3H, d, J=7.0 Hz, H-15), 0.76 (3H, s, H-14), 1.03 (1H, m, H-1), 1.13 (3H, d, J=7.3 Hz, H-13), 1.15−1.19 (2H, m, H-2, 3), 1.24−1.32 (2H, m, H-1, 2), 1.35 (1H, m, H-3), 1.45 (1H, m, H-4), 1.60 (1H, m, H-10), 1.80 (1H, br d, J=15.4 Hz, H-9b), 2.02 (1H, dd, J=15.4, 3.6 Hz, H-9a), 3.02 (1H, m, H-11), 4.04 (1H, s, H-6).

*5.3.4. 8β,10β-Dihydroxy-6β-isobutyryloxyeremophil-7(11)-en-(12,8)-olide (**3**)*

$[\alpha]_D^{19}$ +104.7 (*c* 0.44, EtOH); FTIR (KBr) 3560, 3350, 1760, 1730 cm^{-1}; MS (CI) *m/z* 353 $[M+H]^+$, 335 (base), 317, 265, 247; HRMS (CI) obsd *m/z* 353.1961 $[M+H]^+$ (calcd for $C_{19}H_{29}O_6$ 353.1964); ^{13}C NMR (100 MHz, C_6D_6) δ 8.7 (C-13), 10.6 (C-14), 16.3 (C-15), 18.7 (C-4′), 18.9 (C-3′), 21.7 (C-2), 29.8 (C-3), 33.2 (C-4), 34.3 (C-2′), 34.8 (C-1), 43.8 (C-9), 47.4 (C-5), 71.8 (C-10), 74.2 (C-6), 103.5 (C-8), 130.0 (C-11), 152.0 (C-7), 171.4 (C-12), 175.7 (C-1′); ^1H NMR (400 MHz, C_6D_6) δ 0.46 (3H, d, J=6.6 Hz, H-15), 0.64 (1H, m, H-4), 0.86−0.93 (3H, m, H-2, 3, 3), 1.04 (3H, s, H-14), 1.05 (3H, d, J=7.0 Hz, H-4′), 1.06 (3H, d, J=7.0 Hz, H-3′), 1.18 (1H, m, H-2), 1.32 (1H, br d, J=12.5 Hz, H-1α), 1.65 (1H, td, J=12.5, 3.6 Hz, H-1β), 1.91 (3H, s, H-13), 2.07 (1H, d, J=14.3 Hz, H-9α), 2.18 (1H, dd, J=14.3, 6.2 Hz, H-9β), 2.33 (1H, m, H-2′), 3.87 (1H, br s, OH), 4.47 (1H, br, OH), 5.66 (1H, s, H-6).

*5.3.5. 3α-Tigloyloxyeremophila-9,11-dien-8-one (**4**)*

$[\alpha]_D^{18}$ +21.7 (*c* 0.18, EtOH); FTIR (KBr) 1700, 1670, 1650 cm^{-1}; MS (CI) *m/z* 317 (base), 316 $[M]^+$, 305, 148; HRMS (CI) obsd *m/z* 317.2105 $[M+H]^+$ (calcd for $C_{20}H_{29}O_3$ 317.2116); ^{13}C NMR (150 MHz, C_6D_6) δ 10.4 (C-15), 12.2 (C-5′), 14.2 (C-4′), 16.5 (C-14), 20.7 (C-13), 30.2 (C-1), 31.8 (C-2), 39.8 (C-5), 41.9 (C-6), 47.6 (C-4), 50.4 (C-7), 73.2 (C-3), 113.7 (C-12), 125.0 (C-9), 129.3 (C-2′), 136.9 (C-3′), 144.3 (C-11), 164.8 (C-10), 167.1 (C-1′), 196.6 (C-8); ^1H NMR (600 MHz, C_6D_6) δ 0.63 (3H, s, H-14), 0.70 (3H, d, J=6.9 Hz, H-15), 1.20 (1H, m, H-2α), 1.28 (1H, dq, J=11.3, 6.9 Hz, H-4), 1.42 (3H, dq, J=7.1, 1.1 Hz, H-4′), 1.64 (1H, dd, J=14.3, 12.9 Hz, H-6α), 1.69 (1H, m, H-1α), 1.71 (1H, dd, J=12.9, 4.4 Hz, H-6β), 1.82 (3H, quint, J=1.4 Hz, H-5′), 1.85 (3H, td, J=1.4, 0.5 Hz, H-13), 1.87 (1H, dd, J=3.4, 1.9 Hz, H-1β), 2.02 (1H, m, H-2β), 2.89 (1H, dd, J=14.3, 4.4 Hz, H-7), 4.83 (1H, s, H-12), 4.93 (1H, td, J=11.3, 4.4 Hz, H-3β), 5.00 (1H, t-like, J=1.6 Hz, H-12), 5.64 (1H, d, J=1.9 Hz, H-9), 6.95 (1H, qq, J=7.1, 1.4 Hz, H-3′); CD (EtOH) $[\theta]_{217\ nm}$ +32,800, $[\theta]_{327\ nm}$ −2480.

*5.3.6. 3α-Propionyloxy-7βH-eremophila-9,11-dien-8-one (**5**)*

$[\alpha]_D^{20}$ +15.5 (*c* 0.104, EtOH); FTIR (KBr) 1730, 1670, 1620 cm^{-1}; MS (CI) *m/z* 291 $[M+H]^+$ (base), 217, 148, 89; HRMS (CI) obsd *m/z* 291.1962 $[M]^+$ (calcd for $C_{18}H_{27}O_3$ 291.1960); ^{13}C NMR (150 MHz, C_6D_6) δ 9.3 (C-3′), 10.3 (C-15), 16.5 (C-14), 20.6 (C-13), 27.8 (C-2′), 30.2 (C-1), 31.8 (C-2), 39.8 (C-5), 41.8 (C-6), 47.4 (C-4), 50.4 (C-7), 72.9 (C-3), 113.7 (C-12), 125.0 (C-9), 144.2 (C-11), 164.6 (C-10), 173.2 (C-1′), 196.5 (C-8); ^1H NMR (600 MHz, C_6D_6) δ 0.61 (3H, s, H-14), 0.66 (3H, d, J=6.6 Hz, H-15), 1.00 (3H, t, J=7.4 Hz, H-3′), 1.16 (1H, m, H-2α), 1.22 (1H, dq, J=11.0, 6.6 Hz, H-4α), 1.32 (1H, m, H-1α), 1.62 (1H, t, J=13.0 Hz, H-6α), 1.67 (1H, m, H-1β), 1.71 (1H, dd, J=13.0, 4.6 Hz, H-6β), 1.84 (3H, q, J=0.5 Hz, H-13), 1.92 (1H, m, H-2β), 2.07 (2H, q, J=7.4 Hz, H-2′), 2.88 (1H, dd, J=13.0, 4.6 Hz, H-7), 4.82 (1H, m, H-12), 4.83 (1H, td, J=11.0, 4.4 Hz, H-3β), 5.01 (1H, t-like, J=1.6 Hz, H-12), 5.64 (1H, d, J=1.6 Hz, H-9); CD (EtOH) $[\theta]_{241\ nm}$ +16,300, $[\theta]_{324\ nm}$ −1600.

*5.3.7. 6β-Acetoxyfuranoeremophilan-10β-ol (**6**)*

$[\alpha]_D^{18}$ −39.4 (*c* 1.09, EtOH); FTIR (KBr) 1730, 3580 cm^{-1}; MS (CI) *m/z* 293 $[M+H]^+$, 275, 233 (base), 215; HRMS (CI) obsd *m/z* 293.1743 $[M+H]^+$ (calcd for $C_{17}H_{25}O_4$ 293.1753); ^{13}C NMR (150 MHz, C_6D_6) δ 8.2 (C-13), 10.0 (C-14), 16.1 (C-15), 20.2 (C-2′), 22.3 (C-2), 29.2 (C-3), 33.5 (C-1), 33.8 (C-4), 34.1 (C-9), 45.5 (C-5), 70.5 (C-6), 75.0 (C-10), 115.6 (C-7), 119.6 (C-11), 139.1 (C-12), 151.9 (C-8), 169.5 (C-1′); ^1H NMR (600 MHz, C_6D_6) δ 0.72 (3H, d, J=6.0 Hz, H-15), 0.94 (1H, m, H-3), 1.00 (1H, m, H-4), 1.05 (3H, s, H-14), 1.07 (1H, m, H-3), 1.09 (1H, m, H-2), 1.36 (1H, m, H-2), 1.45 (1H, dd, J=14.1, 3.0 Hz, H-1), 1.50 (3H, s, Ac), 1.72 (1H, m, H-1), 1.92 (3H, d, J=1.1 Hz, H-13), 2.71 (1H, d, J=17.4 Hz, H-9α), 2.99 (1H, d, J=17.4 Hz, H-9β), 6.37 (1H, s, H-6), 6.94 (1H, q, J=1.1 Hz, H-12).

*5.3.8. 6β-Ethoxyfranoeremophilan-10β-ol (**10**)*

MS (CI) *m/z* 279 $[M+H]^+$, 233 (base), 232, 217, 152; ^{13}C NMR (150 MHz, C_6D_6) δ 8.6 (C-13), 11.3 (C-14), 15.4 (C-2′), 15.7 (C-15), 22.8 (C-2), 29.1 (C-3), 33.8 (C-4), 34.3 (C-1), 35.3 (C-9), 45.6 (C-5), 65.2 (C-1′), 74.9 (C-10), 77.8 (C-6), 117.0 (C-7), 119.5 (C-11), 138.4 (C-12), 151.9 (C-8); ^1H NMR (600 MHz, C_6D_6) δ 0.56 (3H, J=6.6 Hz, H-15), 0.87 (3H, t, J=7.1 Hz, H-2′), 0.92 (1H, m, H-4), 0.94 (1H, m, H-3), 1.12 (1H, m, H-2), 1.16 (1H, m, H-3), 1.29 (3H, s, H-14), 1.42 (1H, m, H-2), 1.56 (1H, br d, J=12.9 Hz, H-1α), 1.78 (3H, J=1.2 Hz, H-13), 1.86 (1H, td, J=12.9, 4.9 Hz, H-1β), 2.93 (1H, d, J=18.0 Hz, H-9β), 3.03 (1H, dq, J=8.5, 7.0 Hz, H-1′), 3.08 (1H, d, J=18.0 Hz, H-9α), 3.36 (1H, dq, J=8.5, 7.0 Hz, H-1′), 6.99 (1H, q, J=1.2 Hz, H-12).

*5.3.9. 6β-((2′S)-2-Methylbutyryloxy)furanoeremophilan-10β-ol ((2′S)-**7**)*

$[\alpha]_D^{19}$ −33.0 (*c* 1.02, EtOH); FTIR (KBr) 3570, 3450, 1730 cm^{-1}; MS (CI) *m/z* 335 $[M+H]^+$, 334 $[M]^+$, 317, 249, 234, 233 (base), 232; HRMS (CI) obsd *m/z* 334.2144 $[M]^+$ (calcd for $C_{20}H_{30}O_4$ 334.2145); ^{13}C NMR (100 MHz, C_6D_6)

δ 8.2, 10.2, 11.7, 16.0, 16.9, 22.3, 26.9, 29.2, 33.5, 33.9, 34.1, 41.5, 45.6, 70.3, 75.1, 115.8, 119.5, 139.2, 151.9, 175.2; ^{1}H NMR (600 MHz, C_6D_6) δ 0.72 (3H, d, *J*=7.4 Hz, H-15), 0.73 (3H, t, *J*=7.4 Hz, H-4′), 0.94 (1H, m, H-3), 0.98 (3H, d, *J*=6.8 Hz, H-5′), 1.02 (1H, m, H-4), 1.06 (1H, m, H-3), 1.09 (1H, m, H-2), 1.10 (3H, s, H-15), 1.21 (1H, dq, *J*=13.7, 6.6 Hz, H-3′), 1.35 (1H, m, H-2), 1.48 (1H, br d, *J*=13.5 Hz, H-1), 1.59 (1H, dq, *J*=13.7, 6.6 Hz, H-3′), 1.74 (1H, m, H-1), 1.95 (3H, d, *J*=1.2 Hz, H-13), 2.03 (1H, sextet, *J*=6.6 Hz, H-2′), 2.74 (1H, d, *J*=17.9 Hz, H-9β), 3.00 (1H, d, *J*=17.9 Hz, H-9α), 6.44 (1H, s, H-6), 6.94 (1H, q, *J*=1.2 Hz, H-12).

5.3.10. 8-Oxo-11S-eremophil-6-en-12-oic acid (**20**)

$[\alpha]_D^{19}$ +18.7 (*c* 0.35, EtOH); FTIR (KBr) 3500–2500, 1710, 1680 cm^{-1}; MS (CI) *m/z* 251 (base) $[M+H]^+$, 233, 207; HRMS (CI) obsd 251.1648 $[M+H]^+$ (calcd for $C_{15}H_{23}O_3$ 251.1647); ^{13}C NMR (100 MHz, C_6D_6) δ 15.8 (C-15), 16.3 (C-13), 20.3 (C-14), 20.5 (C-2), 27.0 (C-1), 30.2 (C-3), 35.6 (C-4), 38.8 (C-11), 39.36 (C-5),* 39.42 (C-9, 10),* 136.8 (C-7), 156.0 (C-6), 179.1 (C-12), 197.9 (C-8) [*assignment ambiguous]; ^{1}H NMR (400 MHz, C_6D_6) δ 0.60 (3H, d, *J*=7.0 Hz, H-15), 0.72 (3H, s, H-14), 0.92 (1H, m, H-3), 0.95 (1H, m, H-1), 1.07 (1H, m, H-3), 1.16 (2H, m, H-2), 1.26 (3H, d, *J*=7.0 Hz, H-13), 1.28 (1H, m, H-1), 1.30 (1H, m, H-4), 1.51 (1H, m, H-10), 2.05 (1H, *J*=17.2, 4.4 Hz, H-9β), 2.33 (1H, *J*=17.2, 12.4 Hz, H-9α), 3.59 (1H, q, *J*=7.0 Hz, H-11), 6.36 (1H, s, H-6); CD (EtOH) $[\theta]_{215\ nm}$ +8800, $[\theta]_{253\ nm}$ −800, $[\theta]_{340\ nm}$ +450.

5.3.11. 8-Oxo-11R-eremophil-6-en-12-oic acid (**21**)

$[\alpha]_D^{20}$ +6.49 (*c* 1.34, EtOH); FTIR (KBr) 3500–2500, 1710, 1680 cm^{-1}; MS (CI) *m/z* 251 (base) $[M+H]^+$, 233, 207; HRMS (CI) obsd 251.1646 $[M+H]^+$ (calcd for $C_{15}H_{23}O_3$ 251.1647); ^{13}C NMR (100 MHz, C_6D_6) δ 16.1 (C-15), 16.6 (C-13), 20.7 (C-2, 14), 27.3 (C-1), 30.5 (C-3), 36.0 (C-4), 38.9 (C-11), 39.2 (C-5),* 39.6 (C-9),* 39.7 (C-11),* 136.8 (C-7), 155.8 (C-6), 178.7 (C-12), 197.6 (C-8) [*assignment ambiguous]; ^{1}H NMR (400 MHz, C_6D_6) δ 0.65 (3H, d, *J*=7.0 Hz, H-15), 0.72 (3H, s, H-14), 0.96 (1H, m, H-3), 0.99 (1H, m, H-1), 1.08 (1H, m, H-3), 1.12 (1H, m, H-2), 1.16 (1H, m, H-2), 1.23 (3H, d, *J*=7.0 Hz, H-13), 1.30 (1H, m, H-1), 1.32 (1H, m, H-4), 1.54 (1H, m, H-10), 2.06 (1H, dd, *J*=17.2, 4.4 Hz, H-9), 2.36 (1H, *J*=17.2, 12.1 Hz, H-9), 3.70 (1H, q, *J*=7.0 Hz, H-11), 6.37 (1H, s, H-6); CD (EtOH) $[\theta]_{204\ nm}$ −3200, $[\theta]_{238\ nm}$ +8200, $[\theta]_{239\ nm}$ +900.

5.4. Preparation of 6β-((2′S)-2-methylbutyryl-oxy)furanoeremophilan-10β-ol ((2′S)-**7**)

Diol **8** (6.8 mg) was treated with (*S*)-(+)-2-methylbutyric anhydride (0.1 mL) in pyridine (0.1 mL) in the presence of *N*,*N*-dimethylaminopyridine (DMAP) (5.8 mg) at rt for 2 days. Methanol was added and the mixture was extracted with EtOAc. The organic layer was washed with 1 M HCl, satd $NaHCO_3$ soln, and brine. After drying over $MgSO_4$, the solvents were evaporated in vacuo to give a residue (11.5 mg). The residue was purified by silica-gel column chromatography (hexane–EtOAc, in gradient) to isolate (2′*S*)-**7** (2.8 mg): $[\alpha]_D^{19}$ −28.3 (*c* 0.28, EtOH).

5.5. Preparation of (2′RS)-7

Diol **8** (38.4 mg) was treated with *rac*-2-methylbutyric acid (37.2 mg) and DCC (64.0 mg) in CH_2Cl_2 (4 mL) in the presence of DMAP (13 mg) at rt overnight. Water was added and the mixture was extracted with EtOAc. The organic layer was washed with 1 M HCl, satd $NaHCO_3$ soln, and brine. After drying over $MgSO_4$, the solvents were evaporated in vacuo to give a residue (99.9 mg). The residue was purified by silica-gel column chromatography (hexane–EtOAc, in gradient) to isolate (2′*R*,*S*)-**7** (26.6 mg). Attempts of separation of the 2′*S* and 2′*R* derivative, by normal phase (Nucleosil 50-5, hexane–EtOAc), reversed phase HPLC (Cosmosil ODS, Cosmosil 5C_{18}-Ar-II, Cadenza CD, Cadenza Intakt: in MeOH–H_2O or CH_3CN–H_2O), Chiralcel OB-H, and Chiralcel OD-H (both in hexane–*i*PrOH), were not successful. ^{1}H NMR (200 MHz, C_6D_6) δ 0.95 (d, *J*=6.5 Hz, H-5′*R*), 0.98 (d, *J*=6.8 Hz, H-5′*S*).

5.6. Preparation of 11R-acid phenacyl ester **32**

A solution of 11*R*-acid (**21**, 16.3 mg), 2,4′-dibromoacetophenone (77.8 mg), and Et_3N (0.1 mL) in acetone (2 mL) was stirred at rt for 1 h. Water was added and the mixture was extracted with EtOAc. The organic layer was washed with 1 M HCl, satd $NaHCO_3$ soln, and brine, dried ($MgSO_4$), and evaporated to give a residue. The residue was purified by silica-gel column chromatography (hexane–EtOAc) to give a crystal (**32**, 24.3 mg): colorless plate; mp 134–136 °C (EtOAc); FTIR (KBr) 1740, 1700, 1670 cm^{-1}; MS (CI) *m/z* 449 and 447 $[M+H]^+$, 234, 233 (base), 232; HRMS (CI) obsd *m/z* 447.1175 $[M+H]^+$ (calcd for $C_{23}H_{28}O_4Br$ 447.1171); ^{1}H NMR (200 MHz, C_6D_6) δ 0.78 (3H, d, *J*=6.8 Hz, H-15), 0.82 (3H, s, H-14), 1.48 (3H, d, *J*=7.4 Hz, H-13), 2.17 (1H, dd, *J*=17.0, 4.8 Hz, H-9), 2.50 (1H, dd, *J*=17.0, 12.0 Hz, H-9), 4.03 (1H, q, *J*=7.0 Hz, H-11), 4.67 (1H, d, *J*=16.2 Hz, H-1′), 4.88 (1H, d, *J*=16.2 Hz, H-1′), 6.60 (1H, s, H-6), 7.03 (2H, d, *J*=9 Hz, H-5′, 7′), 7.20 (2H, d, *J*=9 Hz, H-4′, 8′).

All diagrams and calculations were performed using maXus (Bruker Nonius, Delft & MacScience, Japan). Mo Kα radiation, λ=0.71073 Å, 3808 measured reflections, 3805 independent reflections, 3581 observed reflections, data collection: DIP Image plate, Program(s) used to refine structure: *SHELXL*-97 (Sheldrick, 1997). Crystal data: monoclinic, $P2_1$, *a*=10.8030(7) Å, *b*=8.0320(4) Å, *c*=12.0130(9) Å, α=90.00°, β=92.954(2)°, γ=90.00°, *V*=1040.98 (12) Å^3, *R*(all)=0.0438, *R*(gt)=0.0410, *wR*(ref)=0.1137, Flack parameter=−0.018 (9).[57] Crystallographic data (excluding structure factors) for the structure of this compound have been deposited with Cambridge Crystallographic Data Centre as supplementary publication CCDC 669685. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ (fax: +44-(0)1223-336033 or e-mail:deposit@ccdc.cam.ac.uk).

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