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Chemical and genetic diversity of *Cremanthodium lineare*

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ABSTRACT

Chemical constituents and evolutionally neutral DNA sequences of six samples of *Cremanthodium lineare* Maxim., collected in the Sichuan Province of China, were studied. Three samples produced furanoeremophilanes and the other three, eremophilan-8-ones. The chemotypes were found to be correlated with DNA sequence types, suggesting that the chemical diversity observed has a genetic origin. Production of furanoeremophilanes by a *Cremanthodium* species suggests an evolutionary relationship between *Cremanthodium* and *Ligularia* species, and possibly to related genera.

1. Introduction

Plants in the Hengduan Mountains area in China are of interesting to study plant diversity. Both inter- and intra-specific diversities of *Ligularia* species (Asteraceae, tribe Senecioneae) have been investigated by two independent analyses: one on terpenoid composition of the root and the other on DNA sequences of evolutionally neutral regions, such as internal transcribed spaces (ITSs) of the ribosomal RNA gene and the *atpB-rbcL* intergenic region. Thus far, many *Ligularia* species have been found to harbor intra-specific diversity and that furanoeremophilanes are also included in most of the major species (Kuroda et al., 2012). A hypothesis is proposed that furanoeremophilane-producing plants are ecologically more advantageous than those of the same or related species that produce eremophilan-8-ones (Kuroda et al., 2012). For example, in some species such as *Ligularia kanaitzensis* (Tori et al., 2008a; Shimizu et al., 2012) and *Ligularia subspicata* (Saito et al., 2011a), populations producing furanoeremophilanes appear to be ecologically more advantageous than those producing eremophilan-8-ones.

The genus *Cremanthodium* is taxonomically very similar to *Ligularia*, growing in the high mountains (usually more than 4000 m elevation) of Tibet and adjacent areas (Liu and Illarionova, 2011). It has been proposed that *Ligularia* and *Cremanthodium*, together with *Parasenecio*, form a complex (Liu et al., 2006) and therefore studies on chemical diversity in *Cremanthodium* should advance our understanding of the diversity in *Ligularia*, and *vice versa*; however, chemical studies on *Cremanthodium* are limited, because most *Cremanthodium* species are small (Liu et al., 2001; Liu et al., 2006). To date, chemical components of *Cremanthodium ellisii* (Yang et al., 1995; Chen et al., 1996, 1997; Su et al., 2000; Wang et al., 2004), *Cremanthodium discoideum* (Zhu et al., 1999, 2000), *Cremanthodium potaninii* (Yang et al., 2010), and *Cremanthodium pleurocaule* (Tu et al., 2006) have been reported and bisabolane- and oplopane-type sesquiterpenoids, triterpenoids, and other compounds have been isolated. In the course of continuing study on *Ligularia* and *Cremanthodium* species, we isolated eremophilane-type sesquiterpenoids from *Cremanthodium helianthus* (Saito et al., 2012a) and *Cremanthodium stenactinium* (Saito et al., 2011b), and bisabolane-type sesquiterpenoids from *Cremanthodium rhodocephalum* (Saito et al., 2012b), were isolated. Thus suggests that the root phytochemicals in *Cremanthodium* are

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similar to those in *Ligularia*; however, furanoeremophilanes, the major class of compound in *Ligularia*, have not been obtained from *Cremanthodium*.

The subject of the present study is *C. lineare* Maxim., which grows in alpine meadows and scrubs in the Hengduan Mountains. The species is classified into three variants, var. *eligulatum*, var. *lineare*, and var. *roseum*, based on the presence/absence of ray florets and their color (Liu and Illarionova, 2011). Here in results of analyses of the chemical composition and DNA sequences of six samples of *C. lineare* var. *lineare* collected at six different locations in Sichuan Province, China are presented. To the best of our knowledge, the chemical composition of *C. lineare* has not been reported.

2. Results

2.1. Sample collection

Samples were collected at six different locations in Sichuan Province, China in August 2005, 2007, and 2009, respectively, (Table 1 and Fig. 1).

2.2. Chemical study

A small amount of fresh root (ca. 1 g) of each sample was extracted with ethanol immediately after harvesting and the compounds therein were subjected to Ehrlich's test on TLC plates (Kuroda et al., 2004; Kuroda and Nishio, 2007). The test is useful to quickly examine the presence/absence of furanoeremophilanes, and has been used throughout the studies on the diversity of *Ligularia*. Samples 1, 3, and 4 showed pink spot(s), suggesting the presence of furanoeremophilanes, while samples 2, 5, and 6 were negative in the test.

The chemical composition of each sample was analyzed using air-dried roots. Each root sample was extracted with EtOAc and the compounds therein were separated using silica-gel column chromatography and HPLC by standard methods. From the Ehrlich-positive samples (samples 1, 3, and 4), furanoeremophilanes **1**, **2** (Novotný et al., 1968), **3** (Bohlmann et al., 1974), **4** (Bohlmann and Zdero, 1974), **5** (Bohlmann and Zdero, 1976), and **6** (Bohlmann et al., 1972) were isolated. From the other samples (samples 2, 5, and 6), eremophilan-8-ones **7** (Naya et al., 1968), **8** (Bates and Paknikar, 1966), **9** (Ishihara et al., 1993), **10** (Naya et al., 1972), **11** (Neuenschwander et al., 1979;Novotný et al., 1961), and **12** (Aebi et al., 1955; Stoll et al., 1956; Novotný et al., 1961) were isolated as well as bakkanes **13**, **14** (Jakupovic et al., 1989), and **15** (Abe et al., 1968) (Table 1 and Fig. 2). Compounds **1** and **13** were new and their structures were determined as follows.

Compound **1** exhibited a quasi-molecular ion peak at *m*/*z* 347 and its molecular formula was determined to be $C_{20}H_{27}O_5$ (HRMS) with eight degrees of unsaturation. The ^{1}HNMR spectrum showed the presence of three doublet methyls (δ 0.73, 1.18, and 1.48), two singlet methyls (δ 0.47 and 1.42), an oxymethine proton at δ 4.76 (td, *J* = 11.3, 4.6 Hz), and a proton assignable to a furan at δ 6.77. The ^{13}CNMR spectrum indicated the presence of five methyl, three methylene, five methine, and seven quaternary carbon signals, two of which were carbonyls (δ 169.2 and 185.4). The resonances of a furan ring were also observed (δ 120.8, 135.2, 144.1, and 150.0). The ^{1}H-^{1}H COSY spectrum indicated a proton connectivity from H-1 to H-15 through H-4 (Fig. 3), whereas the HMBC spectrum showed correlations between; H-14 and C-4, C-5, C-6, and C-10; H-13 and C-7 and C-12; H-6 and C-5, C-7, and C-8; and H-12 and C-7 and C-8, respectively. These observations indicated a furanoeremophilane skeleton with an acyl group at C-3 (δ_H 4.76 and δ_C 75.3). The acyl group was inferred as a 2-methyl-2,3-epoxybutyryl moiety, as shown in Fig. 3, because H-3′ resonated at δ_H 2.60 (1H, q, *J* = 5.4 Hz) and C-2′ and 3′ at δ_C 59.8 (C-2′), 59.3 (C-3′). The other carbonyl group was deduced to be at C-9, because this position was the only possibility in which to construct a consistent structure (Fig. 3). The carbon signal at C-7 of furanoeremophilanes without the carbonyl group at C-9 normally appears at around δ_C 115–120 (Tori et al., 2008a; Shimizu et al., 2012; Saito et al., 2011a), while the corresponding chemical shift of C-7 of this compound is δ_C 135.2 (Tori et al., 2008b); therefore, its structure was determined to be 3-acylfuranoeremophilan-9-one.

NOEs were observed between H-14 and H-3 and between H-4 and H-10, indicating that they were in 1,3-diaxialrelationships, and hence rings A and B were *trans*-fused and the acyloxy group at C-3 was α-oriented. NOE was also observed between H-3′ and H-5′, indicating the geometry to be 2′*S**,3′*S** (Fig. 3). Thus, this compound was established as 3α-(2′,3′-epoxybutyryloxy)furanoeremophilan-9-one. The absolute configuration of a terpenoid part was determined as shown in the formula from the negative Cotton effect ([θ] −8400) at 292 nmin the CD spectrum; however, the absolute configuration of the epoxide was not determined.

The quasi-molecular ion peak for compound **13** was observed at *m*/*z* 251 and the molecular formula was determined to be $C_{15}H_{22}O_3$ with five degrees of unsaturation. The ^{1}HNMR spectrum showed the presence of two singlet methyls (δ 0.84, 0.89), a doublet methyl (δ 0.63), and an oxymethine (δ 4.64) proton. The ^{13}CNMRand IR spectra indicated the presence of a lactone (δ 180.5 and 1798 cm^{-1}). The COSY spectrum showed connectivity of H-1/H-10/H-9, H-2/H-3, and H-4/H-15 (Fig. 4), whereas the HMBC spectrum had correlations between: H-15 and C-3 and C-4; H-14 and C-4, C-5, C-6, and C-10; H-6 and C-7 and C-8; H-9 and C-11; H-13 and C-7, C-11, and C-12, and H-12 and C-8, respectively. These correlations clearly indicated the bakkane skeleton, as shown in Fig. 4. The chemical shifts at C-11 and C-12 were δ_C 63.6 and 82.6, indicating the presence of an epoxide. These observations established the structure of compound **13**, as shown in Fig 4.

NOE between H-14 and H-10 indicated a *cis*-fused A/B ring junction. The carbonyl group of the lactone (C-8) should be β, because the NOE was observed between H-13 and H-9 (δ 1.61).

Table 1
Collection locality, *atpB-rbcL* sequences, and isolated compounds of *C. lineare* samples.

Sample no[a]	Locality[b]	Altitude	Isolated Compounds		
			Furano-eremophilanes	Eremophilan-8-ones	Others
1	Gaoersishan (Yajiang)	4200	**4**, **5**		**16**
2	Haizishan (Batang)	4200		**7**	
3	Derongma (Shiqi)	4200	**1**, **2**, **3**		
4	Nixia (Shiqi)	4600	**6**		
5	Zhuqing (Shiqi)	4000		**8**, **9**, **10**, **11**, **12**	
6	Border of Luhuo/Seda	3700		**7**, **8**	**13**, **14**, **15**

[a] Samples 1 and 2 were collected in August 2005 and 2007, respectively. Samples 3–6 were collected in 2009.

[b] County in parentheses.