Phytochemistry 140 (2017) 69–76

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Chemical constituents of hybrids of *Ligularia cyathiceps and L. lamarum/L. subspicata* collected in China: Structures of subspicatins M, N, O_1, and O_2, and related compounds

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ARTICLE INFO

Article history:
Received 17 January 2017
Received in revised form
13 April 2017
Accepted 18 April 2017

Keywords:
Ligularia cyathiceps
Ligularia lamarum
Ligularia subspicata
Asteraceae
Structure elucidation
Chemical diversity
Eremophilane
Subspicatin
Subspicatol
Internal transcribed spacer (ITS)

ABSTRACT

Three natural hybrids and an introgressed individual of *Ligularia* were evaluated based on a combination of morphology, root chemicals, and nucleotide sequences of evolutionally neutral regions to understand the chemical outcomes of hybridization and introgression. Six previously undescribed eremophilane sesquiterpenes were isolated from hybrids between *L. cyathiceps* and *L. lamarum*/*L. subspicata*, and benzofurans were isolated from *L. subspicata* for the first time. Their structures were elucidated based on spectroscopic analyses. Some compounds produced by hybrids have not been detected in either parental species, indicating that the metabolic profile was altered by hybridization and introgression.

1. Introduction

The genus *Ligularia* (Asteraceae) in the Hengduan Mountains area of China is highly diverse and its evolution is considered to be continuing (Liu et al., 1994). We have been studying diversity in this genus using two indices, root chemicals and evolutionally neutral DNA sequences, such as the internal transcribed spacers (ITSs) of the nuclear ribosomal RNA (rRNA) gene. To date, we have found that many *Ligularia* species are intra-specifically diverse and that furanoeremophilanes and related sesquiterpenoids are the major compounds in most of the major species (Kuroda et al., 2012, 2014).

L. lamarum (Diels) C. C. Chang and L. *subspicata* (Bureau & Franch.) Hand.-Mazz. are widely distributed in the Hengduan Mountains area. These two species are morphologically very similar and only differ in the presence (*L. lamarum*) or absence (*L. subspicata*) of ray florets (Liu and Illarionova, 2011). We previously reported that these two species are indistinguishable based on their root chemicals and ITS sequences and presumably formed a complex (Saito et al., 2011a) (hereafter we call this L/S complex). Subspicatins (1β-acyloxy-furanoeremophilanes and eremophilanolides) are characteristic chemicals of these species. On the other hand, *L. cyathiceps* Hand.-Mazz. grows in northwestern Yunnan Province (Liu and Illarionova, 2011). We showed that this species was almost uniform in our two indices (Nagano et al., 2009). From this species, 9-oxofuranoeremophilanes were isolated as the major sesquiterpenoids.

Hybridization is an important pathway in plant evolution

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http://dx.doi.org/10.1016/j.phytochem.2017.04.015
0031-9422/

(Riesberg and Carney, 1998). We found natural hybrids during the course of our study on *Ligularia* diversity (Pan et al., 2008; Zhou et al., 2008; Yu et al., 2011, 2014), and the root chemical composition of some has been studied (Hanai et al., 2012, 2016; Shimizu et al., 2016). A variety of hybrids and introgressed individuals have arisen from *L. cyathiceps* and L/S complex near Tianchi pond, Shangrila County, Yunnan Province, China (Shimizu et al., 2014; Saito et al., 2016). For example, one sample contained the ITS sequence of *L. cyathiceps* alone; however, its root chemicals originated from both the *L. cyathiceps* and L/S complex. Another sample was typical of *L. lamarum* with regard to morphology and root chemicals; however, it contained the ITS sequences of both *L. cyathiceps* and L/S complex.

Here we describe the root chemical composition of three additional putative hybrids of *L. cyathiceps* and L/S complex collected at Tianchi (samples 1–3). A morphologically ambiguous sample, collected at Qianhushan (sample 4), approximately 30 km south of Tianchi, was also analyzed. Four new 1β-acyloxyfuranoeremophilanes (or eremophilanolides) were isolated from the hybrid samples and named subspicatins M, N, O_1, and O_2. Related new compounds, 1β-hydroxyfuranoeremophilane (subspicatol A) and eremophilanolide (eremopetasitenin A_8), were also isolated along with 23 known compounds. The chemical outcomes of hybridization are discussed.

2. Results and discussion

2.1. Morphology and DNA analysis

Sample 1 had no ray florets and other morphological characteristics were also in accord with those of *L. subspicata*. Sample 2 had flowers and leaves with morphologies that were intermediate between *L. cyathiceps* and *L. lamarum*. Sample 3 was similar to *L. lamarum* but had flowers morphologically intermediate between *L. cyathiceps* and *L. lamarum*. Sample 4 had no ray florets and was tentatively identified as *L. subspicata*; however, its pappus was shorter than that of typical *L. subspicata*. To assess the genetic constitution of the samples, DNA sequence was determined for the ITS1-5.8S-ITS2 region of the nuclear rRNA gene cluster. The results are summarized in Table 1. The sequence of sample 4 was typical of L/S complex (Saito et al., 2011a; Tori et al., 2008b) and thus the sample was identified as *L. subspicata*. However, the other three samples contained sequences of *L. cyathiceps* and L/S complex, indicating hybridization. An F1 individual of *L. subspicata* and *L. cyathiceps* would have ray florets; thus, the lack thereof indicated backcrossing in sample 1.

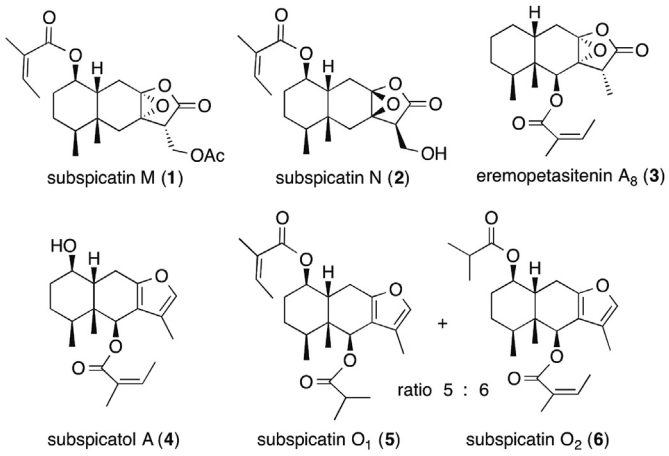

Fig. 1. Previously undescribed compounds isolated in this study.

2.2. Chemical analysis—Isolation of root chemicals

The chemical constituents in each sample were isolated using standard methods, such as silica-gel column chromatography and HPLC, and the structures were determined using spectroscopic methods. Compounds **1**–**29** were isolated, of which **1**–**6** were new (Fig. 1). The structures of the six new compounds were determined as follows.

Compound **1** showed a quasi-molecular ion peak at *m/z* 407, and its molecular formula was determined to be $C_{22}H_{30}O_7$ from HRMS and ^{13}C NMR data. The IR spectrum exhibited absorption at 1807 cm^{-1}, indicating the presence of epoxy- or enol-lactone (Nagano et al., 2009; Saito et al., 2011a, 2011b; Tori et al., 2008a), and at 1715 cm^{-1} (ester). The ^1H NMR spectrum showed the presence of a singlet methyl (δ 0.35), a doublet methyl (δ 0.61), an oxymethine (δ 5.16), an oxymethylene (δ 3.71 and 3.79), and an angelate moiety [δ 1.99 (3H, dq), 1.83 (3H, quintet), 5.70 (1H, qq)] (Table 2). These observations along with an analysis of the two-dimensional (2D) NMR spectra established that the compound was an eremophilanolide with angeloyloxy and acetoxy groups. The 2D correlation indicated that C-13 was oxidized to an oxymethylene (δ 3.71 and 3.79). The lactone had an epoxide ring at C-7 (δ_C 62.6) and C-8 (δ_C 86.6), as suggested by HMBC (Fig. 2). Stereochemistry was revealed by NOESY. The NOEs between H_3-14 and H-

Table 1
Sequences of the ITS1-5.8S-ITS2 region.[a]

	ITS1														5.8S	ITS2								
	1 1	1 3	4 6	9 4	1 1 3	1 2 5	1 2 6	1 2 7	2 0 6	2 1 7	2 2 3	2 3 3	2 3 6	2 4 0	1 3 7	1 1	1 5	3 0	9 1	1 0 7	1 0 9	1 5 9	2 2 0
L. subspicata	C	A	T	T	G	T	C	C	C	C	T	A	G	G	C	T	A	T	C	C	T	C	C
sample 1	C	R	Y	C	R	Y	Y	M	Y	[b]	K	A	R	G	C	[c]	[d]	Y	C	Y	G	M	Y
sample 2[e]	C	A	Y	C	G	Y	Y	M	C	[b]	S	A	G	G	C	[c]	[d]	T	C	C	G	C	Y
sample 3	Y	G	Y	Y	R	Y	Y	M	Y	[b]	Y	A	R	G	C	[c]	[d]	T	C	Y	G	M	Y
sample 4[e,f]	C	W	Y	Y	G	T	C	C	C	[b]	Y	R	G	R	Y	T	A	T	Y	C	G	C	C
L. cyathiceps	C	G	C	C	A	C	T	A	T	–	C	A	A	G	C	C	–	T	C	T	G	A	C

[a] Only differences among samples are shown. Base numbering is according to the *L. subspicata* sequence. The database ID of the *L. subspicata* sequence was DQ272338; *L. cyathiceps*, DQ272328. K = G + T; M = A + C; R = A + G; S = C + G; W = A + T; Y = C + T.
[b] Two sequences with or without C were present.
[c] Two sequences with T or C were present.
[d] Two sequences with or without A were present.
[e] A sequence with AAA in place of AAAA at 179–182 of ITS1 was also present.
[f] A sequence with CCC in place of CC at 69–70 of ITS2 was also present.