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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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.

Keywords: *Ligularia cyathiceps*; *Ligularia lamarum*; *Ligularia subspicata*; Asteraceae; structure elucidation; chemical diversity; eremophilane; subspicatin; subspicatol; internal transcribed spacer (ITS)

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 (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.

-----<Table 1>-----

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.

-----<Figure 1>-----

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 1,807 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 1,715 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-10, between H_3-14 and H-9β, as well as between H_3-15 and H-10 indicated that the decalin ring was *cis*-fused and adopted a non-steroidal conformation (Fig. 2). Because NOE was observed between H-11 and H-6β and H_3-14, both the oxymethylene

group at C-11 and epoxide at C-7 and C-8 were deduced to be α-oriented, which was further supported by consideration of plausible mechanism of epoxy-lactone formation (Saito *et al*., 2012). H-1 resonated at δ 5.16 (td, *J* = 11.2, 4.7 Hz), indicating that it was axial, and NOE was observed between H-1 and H-6α. These observations indicated that H-1 was α-oriented. Although the position of two different acyloxy groups was not established by 2D correlations, angeloyloxy and acetoxy group should be at C-1 and C-13, respectively, when compared the chemical shift of H-1 in **1** (δ 5.16) with that of subspicatin H (δ 5.13) (systematic name: 11β*H*-1β-angeloyloxy-7α,8α-epoxyeremophilan-12,8β-olide) described previously (Saito *et al*., 2011b). If the acetoxy group of **1** was at C-1, H-1 would resonate at higher field than the case of subspicatin H. Compound **1** was a series of eremophilane-type sesquiterpenoids bearing a 1β-acyloxy group, and therefore, named subspicatin M (11β*H*-13-acetoxy-1β-angeloyloxy-7α,8α-epoxyeremophilan-12,8β-olide).

-----<Figure 2>-----

The molecular formula of compound **2** was determined to be $C_{20}H_{28}O_6$ from HRMS data. Compound **2** exhibited spectroscopic features similar to those of compound **1**. The ^{1}H NMR spectrum showed the presence of a singlet methyl (δ 0.74), a doublet methyl (δ 0.57), an oxymethine (δ 4.72), an oxymethylene (δ 3.72 and 3.74), and an angeloyl moiety [δ 1.99 (dq), 1.82 (quintet), 5.70 (qq)] (Table 2). The presence of an epoxy-lactone was suggested by IR absorption (1,800 cm^{-1}). The partial structure of C-11/C-13 was inferred from a typical NMR signal for H-11 at δ 2.54 (dd), coupled with oxymethylene protons at δ 3.72 and 3.74 (each ddd) for H_2-13. The position of the angeloyloxy group was determined to be C-1 because the downfield shifted oximethine proton resonated at δ 4.72 was assigned to H-1 by COSY spectrum [H_2-9 (δ 1.94 and 2.12)/H-10 (δ 1.44–1.51)/H-1 (δ 4.72)] (Fig. 3). The relative configuration was revealed in a NOESY experiment. The decalin ring was deduced to be *cis*-fused because NOE was observed between H_3-14 and H-9β and further supported by the NOE between H_3-15 and H-10 (Fig. 3). The angeloyloxy group was attached to C-1β, as indicated by NOE between H-1α and H-6α. H-11 and epoxide were determined to be α- and β-oriented, respectively, by NOE between H-11 and H-6α. Therefore, compound **2** was established to be 11α*H*-1β-angeloyloxy-7β,8β-epoxy-13-hydroxyeremophilan-12,8α-olide and named subspicatin N.

-----<Figure 3>-----

Compound **3** ($C_{20}H_{28}O_5$) had a characteristic ^{1}H NMR peak at δ 2.73 (q), assignable to H-11 of an epoxy lactone, supported by IR absorption at 1,807 cm^{-1}. The presence of an angeloyloxy group was indicated by the NMR spectra (Tables 2 and 3), although the position was not at C-1, but at C-6, as indicated by HMBC (Fig. 4). The *cis* nature of the decalin ring was established by an NOE between

H_3-14 and H-10 as well as between H_3-15 and H-10. NOEs between H-11 and H_3-14 and between H_3-13 and H-6α suggested that H_3-13 was α-oriented, and hence, the epoxide oxygen atom was α-oriented, similar to the case of **1**. Compound **3** was established to be 11β*H*-6β-angeloyloxy-7α,8α-epoxyeremophilan-12,8β-olide and named eremopetasitenin A_8 (Saito *et al*., 2014).

-----<Figure 4>-----

Compound **4** ($C_{20}H_{28}O_4$) had an angeloyl group (Tables 2 and 3). Two oxymethine protons at δ 2.94 (td, *J* = 10.8 and 4.8 Hz) and 6.60 (br s) were detected as well as a proton assignable to a furan moiety (δ 6.96, br s). The ^{1}H-^{1}H COSY correlations H_2-9/H-10/H-1/H_2-2/H_2-3/H-4/H_3-15 were detected, and the HMBC spectrum showed correlations between H_3-14 and C-4, 5, 6, and 10; between H_3-13 and C-7, 11, and 12; and between H-6 and C-7 and 8 (Fig. 5). From these observations, an eremophilane skeleton was constructed (Fig. 5). An angeloyloxy group was attached to C-6, as indicated by the correlation detected between H-6 and C-1′. The non-steroidal conformation, including the A/B-*cis* ring system (Fig. 5), was supported by the NOE between H_3-14 and H-9β and between H_3-15 and H-10. The configuration of the hydroxy group at C-1 was determined to be β-oriented because NOE was observed between H-1 and H-6. Therefore, compound **4** was established to be 6β-angeloyloxyfuranoeremophilan-1β-ol and named subspicatol A.

-----<Figure 5>-----

Compounds **5** and **6** were inseparable even with HPLC; therefore, structural analysis was performed for the mixture. The mass spectrum showed a quasi-molecular ion peak at *m/z* 403. The ^{1}H NMR spectrum indicated the presence of angeloyl and isobutyroyl moieties (Table 2). The 2D analysis revealed that both compounds had an eremophilane skeleton with angeloyloxy and isobutyroyloxy groups at C-1 and/or C-6, respectively (Fig. 6). Only one quasi-molecular ion peak was detected; one had an angeloyloxy group at C-1 and an isobutyroyloxy group at C-6, and the other had the angeloyloxy group at C-6 and the isobutyroyloxy group at C-1, which could be distinguished from the chemical shifts in H-1 (δ 5.00 for **5** and 4.92 for **6**) and H-6 (δ 6.62 for **5** and 6.72 for **6**). Compound **6** was slightly major (**5**:**6**=5:6), as judged from the integration of the ^{1}H NMR peaks. Compound **6** was considered to be an isobutyroyl derivative of compound **4**. Compounds **5** and **6** were named subspicatins O_1 and O_2, respectively.

-----<Figure 6>-----

-----<Table 2>-----

-----<Table 3>-----

-----<Figure 7>-----

The known compounds were furanoeremophilane (**7**) (Ishii *et al*., 1966), furanoeremophilan-6β-ol (**8** = ligularol) (Ishii *et al*., 1965), 6β-isobutyroyloxyfuranoeremophilane (**9**) (Saito *et al*., 2011a), 6β-angeloyloxyfuranoeremophilane (**10**) (Bohlmann *et al*., 1979), 1β-angeloyloxyfuranoeremophilan-13-ol (**11** = subspicatin A) (Tori *et al*., 2008b), 1β-angeloyloxyfuranoeremophilan-6β-ol (**12** = subspicatin B) (Tori *et al*., 2008b), 6β-(2′-methylbutyroyloxy)furanoeremophilan-10β-ol (**13**) (Tori *et al*., 2008a), 6β-angeloyloxyfuranoeremophilan-10β-ol (**14**) (Bohlmann *et al*., 1974), 6β-acetoxyfuranoeremophilan-10β-ol (**15**) (Tada *et al*., 1974), 6β-isobutyroyloxyfuranoeremophilan-10β-ol (**16**) (Jennings *et al*., 1976), 6β-angeloyloxyfuranoeremophilan-9-one (**17**) (Bohlmann *et al*., 1986), 6β-isobutyroyloxyfuranoeremophilan-9-one (**18**) (Bohlmann and Zdero, 1978), 6β-angeloyloxyfuranoeremophilan-9β-ol (**19**) (Saito *et al*., 2016), 6β-hydroxyeremophil-7(11)-en-12,8α-olide (**20**) (Ishii *et al*., 1966), 11β*H*-1β-angeloyloxy-6β-hydroxyeremophil-7-en-12,8-olide (**21** = subspicatin F) (Saito *et al*., 2011a), norsubspicatin A (**22**) (Saito *et al*., 2011b), fukinone (**23**) (Naya *et al*., 1968), 7α*H*-eremophil-11-en-8-one (**24**) (Bohlmann *et al*. 1986), bakkenolide A (**25**) (Abe *et al*., 1968), (2*R*,3*S*)-5-acetyl-6-hydroxy-2-(prop-1-en-2-yl)-2,3-dihydrobenzofuran-3-yl (*Z*)-2-methylbut-2-enoate (**26**) (Bohlmann *et al*., 1977) (2*R*,3*S*)-5-acetyl-6-hydroxy-2-(prop-1-en-2-yl)-2,3-dihydrobenzofuran-3-yl (*Z*)-2-(acetoxymethyl)but-2-enoate (**27**) (Bohlmann *et al*., 1977), 5,6-dimethoxy-2-(prop-1-en-2-yl)benzofuran (**28**) (Murae *et al*., 1968), and 5-acetyl-6-hydroxy-2-(prop-1-en-2-yl)benzofuran (**29** = euparin) (Kamthong and Robertson, 1939) (Fig. 7). The composition in each sample is shown in Table 4.

-----<Table 4>-----

2.3. LC-MS analysis

Chemical compositions of the three hybrid samples between *L. cyathiceps* and the L/S complex collected in Tianchi (samples 1–3) were compared by LC-MS analyses (reverse-phase) of the ethanol extracts. The total ion chromatograms (TICs) are shown in Fig. 8. Various furanoeremophilanes were detected in sample 1. A peak at t_R = 16.9 min consisted of both subspicatin A (**11**) and 9-oxofuranoeremophilane **17**; the former of which is a characteristic compound of L/S complex. The peak at 17.5 min was 6-ethoxyfuranoeremophilan-10β-ol (**32**), which is probably an artifact generated from furanoeremophilan-6,10-diol (**30**, 11.5 min) during ethanol extraction. Compound **15**, isolated from dried roots, may also be an artifact generated from **30** due to ethyl acetate extraction. The TICs of samples 2 and 3 appeared similar; however, the major peaks in sample 2 were ligularol (**8**, 15.4 min) and subspicatin A (**11**, 16.8 min), whereas the two major peaks in sample 3 were the 9-

oxofuranoeremophilanes **18** (15.3 min) and **17** (16.9 min).

-----<Figure 8>-----

2.4. Chemical outcome of hybridization

2.4.1 Major components of hybrids of L. cyathiceps and L/S complex

The DNA analysis indicated that samples 1–3 were hybrids of *L. cyathiceps* and L/S complex. The chemical outcome of the samples was almost parallel with previously analyzed ones (Shimizu *et al*., 2014; Saito *et al*., 2016). All analyzed hybrid samples between *L. cyathiceps* and the L/S complex at Tianchi described in this and the previous reports are summarized in Table 5. Among the major components detected in the LCMS, subspicatin A (**11**) and ligularol/tetradymol derivatives (**8**, **30**, **32**) are originated from L/S complex (Saito *et al*., 2011a), and 9-oxofuranoeremophilanes (**17** and **18**) must have originated from *L. cyathiceps*; however, their substituents are not exactly the same as those in pure *L. cyathiceps* (see 2.4.2) (Nagano *et al*., 2009).

The hybrid samples were classified into three groups according to their chemical composition detected by LCMS [See previous report for TICs of samples 6–8 (Shimizu *et al*., 2014). Although TIC of sample 5 was not shown in the previous report (Saito *et al*., 2016), it was very similar to that of sample 1]. One group consisted of samples 1, 5, and 7 (mixed-type). In these samples, both 9-oxofuranoeremophilanes and ligularol/tetradymol derivatives were detected. A second group consisted of samples 2 and 8 (L/S-type), the TICs of which were almost identical to that of *L. subspicata* collected sympatrically [sample C of the previous report (Shimizu *et al*., 2014)]. A third group consisted of samples 3 and 6 (*cyathiceps*-type). The major components of these samples were the 9-oxofuranoeremophilanes. Each group (chemotype) consists of at least two samples, and their total outcome (morphology, ITS sequence, and chemotype) are complex (Table 5). Sample 1 was *L. subspicata* in morphology but the other mixed-type samples (samples 5 and 7) were *L. cyathiceps* in the ITS sequences. Samples 2 and 8 (L/S-type) were intermediate and *L. lamarum*, respectively, in morphology. This complex heterogeneity is likely to have resulted from backcrossing, as discussed earlier for sample 1 and previously for other samples (Shimizu *et al*., 2014; Saito *et al*., 2016).

-----<Table 5>-----

2.4.2 Difference in chemical composition between hybrids and parent species

The major 9-oxofuranoeremophilanes isolated from *L. cyathiceps* had either a 1,10-epoxy or 1(10)-ene moiety (Nagano *et al*., 2009). However, the major 9-oxofuranoeremophilanes in the hybrid samples were the 1,10-saturated derivatives **17** and **18**, which were also isolated from L/S-type hybrid

(sample 2), although minor components. Tetradymol (**31**) was detected in hybrid samples 1, 5, and 7 (Fig. 8), but not in either parental species. Similarly, tetradymol was detected in a hybrid of *L. subspicata* and *L. nelumbifolia* (Bureau & Franch.) Hand.-Mazz., but not in the parental species collected sympatrically (Hanai *et al*., 2012). These data suggested that hybridization mixes the biochemical pathways to generate new compounds (Bjeldanes and Geissman, 1971).

2.4.3 Origin of benzofurans in L. subspicata

Euparin-type benzofurans were isolated from sample 4. These compounds have been isolated from various *Ligularia* species, such as *L. latihastata* (W. W. Smith) Hand.-Mazz. (Kuroda *et al*., 2007) and *L. stenocephala* (Maximowicz) Matsumura & Koidzumi (Murae *et al*., 1968), but not from L/S complex. Eremophilane sesquiterpenes have been isolated from all samples of L/S complex (Saito *et al*., 2011a; Tori *et al*., 2008b). As euparin-type benzofurans are not terpenoids, sample 4 belonged to a different chemical lineage from other *L. lamarum*/*L. subspicata* samples. Although the morphology and ITS sequence of sample 4 were of *L. subspicata*, its short pappus and production of euparin suggested that it is introgressed. The hybridized species is likely to be *L. latihastata*, which has a short pappus (Liu and Illarionova, 2011), produces benzofurans, and is distributed in the area where sample 4 was collected.

3. Conclusions

Six new eremophilane sesquiterpenes **1**–**6** together with known eremophilanes (**7**–**21**, **23**, and **24**) and related compounds (**22** and **25**) were isolated from hybrids between *L. cyathiceps* and *L. lamarum*/*L. subspicata* (L/S complex), whereas euparin (**29**) and its derivatives (**26**–**28**) were isolated from *L. subspicata* for the first time. Subspicatins and 9-oxofuranoeremophilanes were detected in the hybrids; however, their chemical composition differed. Some of the compounds in the hybrids were not detected in either parent. The production of euparin derivatives by *L. subspicata* was inferred to be a result of introgression. The production of "new compounds" or "imported compounds" through hybridization and introgression, as observed in the present study, may be a diversification step of chemical composition.

4. Experimental

4.1. General experimental procedures

IR spectra were measured using a SHIMADZU FT/IR-8400S (SHIMADZU, Kyoto, Japan); ^{1}H and ^{13}C NMR spectra were obtained using a Varian 400-MR (400 and 100 MHz, respectively) spectrometer (Agilent Technologies, Palo Alto, CA, USA). Mass spectra were recorded using a JEOL JMS-700 MStation (JEOL, Tokyo, Japan). A Chemcopak Nucleosil 50-5 (250 × 4.6 mm, Chemcoplus, Osaka, Japan) with a solvent system of either *n*-hexane–ethyl acetate or a TSK-GEL G1000H_{HR} (300

× 7.8 mm, Tosoh, Tokyo, Japan) with ethyl acetate was used for HPLC (JASCO, Tokyo, Japan). Silica gel BW-127ZH or BW-300 (Fuji Silysia, Aichi, Japan) was used for column chromatography (CC). Silica gel 60 F254 plates (Merck, Darmstadt, Germany) were used for TLC. DNA was purified from the remnant of the extracted roots of samples 1 and 2 using the DNeasy Plant Minikit (QIAGEN, Hilden, Germany). The ITS1-5.8S-ITS2 region was amplified by polymerase chain reaction with the HotStarTaq *plus* Master Mix kit (QIAGEN) and the LC5 and LC6 primers (Hanai *et al*., 2005; Nagano *et al*., 2010). The product was separated using agarose gel electrophoresis, purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Basel, Switzerland), and re-amplified with Q5 Hot Start polymerase (New England Biolabs, Ipswich, MA, USA) and a high-fidelity polymerase during 20 thermal cycles. Sequencing reactions were performed using the LC1–LC4 primers (Hanai *et al*., 2005; Nagano *et al*., 2010) and the BigDye terminator cycle sequencing kit ver. 3.1 (Applied Biosystems, MA, USA) and analyzed on a 3500 Genetic Analyzer (Applied Biosystems). DNA analysis of samples 3 and 4 was performed as previously described (Hanai *et al*., 2005; Nagano *et al*., 2010).

4.2. Plant materials

Three natural hybrids of *Ligularia* (Asteraceae) (samples 1–3) were collected at Tianchi (altitude: 3,900 m) in 2008. Samples 1 (specimen no. 2008-32, Kunming Institute of Botany) and 2 (specimen no. 2008-33) were collected at the same location (in a small hollow) approximately 100 m from the pond (N 27.6240˚, E 99.6436˚). Sample 3 (specimen no. 2008-37) was collected approximately 200 m from the pond (N 27.6247˚, E 99.6440˚). An introgressed individual of *L. subspicata* (Bureau & Franch.) Hand.-Mazz. (sample 4, specimen no. 2007-133) was collected at Qianhushan (altitude: 3,300 m) approximately 30 km south of Tianchi (N 27.4352˚, E 99.8092˚) in 2007. Each sample was identified by X.G. (author).

4.3. Extraction and LC-MS analysis

Parts of the fresh roots (a few grams) of each sample were extracted with ethanol immediately after harvesting, and the extracted ethanol solutions were filtered and subjected to LC-MS analysis using an Agilent 1100 series LC/MSD mass spectrometer [capillary voltage, 3.5 kV; corona current, 4 μA; capillary exit voltage (fragmentor), 90 V; drying temperature, 330°C; drying flow, 9 L/min; and nebulizer pressure, 50 psig; Agilent Technologies] with a 5C18-MS-II (COSMOSIL; 4.6 × 150 mm; 5 μm octadecyl column; Nacalai Tesque, Kyoto, Japan) using a gradient system (methanol/water; 0 min (7:3)－20 min (10:0)－35 min (10:0)－40 min (7:3)－45 min (7:3); 0.5 mL/min) as the eluent.

4.4. Extraction and isolation

The dried roots of sample 1 (18.2 g) were cut into pieces and extracted with ethyl acetate at room temperature. The filtrate was concentrated to give an extract (1.7 g), and a portion (744.2 mg) was separated using silica gel CC (gradient of *n*-hexane and ethyl acetate). Each fraction was further separated using HPLC (*n*-hexane–ethyl acetate) to give **5** and **6** (0.5 mg), **11** (113.9 mg), **12** (7.8 mg), **13** and **14** (16.6 mg), **15** (2.3 mg), **16** (14.1 mg), **17** (10.5 mg), **18** (13.3 mg), **20** (3.5 mg), and **23** (1.0 mg).

The extract of sample 2 (689.4 mg from 8.5 g of roots) was similarly treated to give **1** (0.8 mg), **2** (0.4 mg), **8** (0.6 mg), **11** (68.3 mg), **12** (3.9 mg), **13** and **14** (6.3 mg), **17** (24.6 mg), **18** (10.9 mg), **19** (0.1 mg), **20** (6.9 mg), **21** (1.1 mg), and **22** (0.4 mg).

The extract of sample 3 (727.4 mg from 10.4 g of roots) was similarly treated to give **3** (1.0 mg), **4** (0.9 mg), **7** (2.0 mg), **9** (15.3 mg), **10** (69.8 mg), **14** (5.8 mg), **17** (300.4 mg), **18** (3.3 mg), **19** (6.6 mg), **24** (1.4 mg), and **25** (1.0 mg).

The extract of sample 4 (153.6 mg from 4.0 g of roots) was similarly treated to give **26** (0.7 mg), **27** (6.0 mg), **28** (14.7 mg), and **29** (0.9 mg).

*4.4.1. Subspicatin M (**1**)*

oil; $[\alpha]_D^{21}$ −50.6 (*c* 0.08, EtOH); CD [θ] (EtOH): +600 (255 nm), −1800 (234 nm), +4200 (215 nm); FTIR (KBr): 1807, 1715 cm^{-1}; For ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 2 and 3; MS (CI): *m/z* 407 $[M + H]^+$, 347, 282, 247 (base); HRCIMS: Obs. *m/z* 407.2084 $[M + H]^+$ (calcd for $C_{22}H_{31}O_7$: 407.2070).

*4.4.2. Subspicatin N (**2**)*

oil; $[\alpha]_D^{20}$ −86.0 (*c* 0.01, EtOH); CD [θ] (EtOH): +33000 (244 nm), +17500 (228 nm), +23000 (220 nm); FTIR (KBr): 3393, 1800, 1713 cm^{-1}; For ^1H NMR spectroscopic data, see Table 2; MS (CI): *m/z* 365 $[M + H]^+$, 347, 265, 247 (base); HRCIMS: Obs. *m/z* 365.1974 $[M + H]^+$ (calcd for $C_{20}H_{29}O_6$: 365.1965).

*4.4.3. Eremopetasitenin A_8 (**3**)*

oil; $[\alpha]_D^{23}$ −10.2 (*c* 0.13, EtOH); CD [θ] (EtOH): −280 (300 nm), +250 (254 nm), −5900 (220 nm); FTIR (KBr): 1807, 1722 cm^{-1}; For ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 2 and 3; MS (CI) *m/z* 349 $[M + H]^+$, 249 (base), 83; HRCIMS: Obs. *m/z* 349.2021 $[M + H]^+$ (calcd for $C_{20}H_{29}O_5$: 349.2015).

*4.4.4. Subspicatol A (**4**)*

oil; $[\alpha]_D^{23}$ −42.2 (*c* 0.07, EtOH); FTIR (KBr): 3439, 1713 cm^{-1}; For ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 2 and 3; MS (CI): *m/z* 332 $[M]^+$, 233 (base), 232, 215, 83; HRCIMS:

Obs. *m/z* 332.1982 $[M]^+$ (calcd for $C_{20}H_{28}O_4$: 332.1988).

*4.4.5. Subspicatins O_1 and O_2 (**5** and **6**)*

oil; For ^1H NMR spectroscopic data, see Table 2; CIMS: *m/z* 403 $[M + H]^+$, 315 (base), 215.

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TABLES

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

	ITS1														5.8S	ITS2							
	1	1	4	9	1	1	1	1	2	2	2	2	2	2	1					1	1	1	2
	1	1	4	9	1	2	2	2	0	1	2	3	3	4	3	1	1	3	9	0	0	5	2
	1	3	6	4	3	5	6	7	6	7	3	3	6	0	7	1	5	0	1	7	9	9	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.

Table 2. ^{1}H NMR data of compounds **1**–**6** (400 MHz in benzene-d_6)

Positions	**1**	**2**	**3**	**4**	**5**	**6**
1	5.16 (td, 11.2, 4.7)	4.72 (td. 8.8, 4.5)	1.61 (qd, 13.4, 4.4)	2.94 (td, 10.8, 4.8)	5.00 (td, 10.0, 4.7)	4.92 (td, 10.4, 4.7)
	—	—	0.83-0.90 (m)	—	—	—
2	1.93-2.06 (m)	1.58-1.66 (m)	1.14-1.22 (m)	1.34-1.42 (m)	1.82-1.88 (m)	1.82-1.88 (m)
	1.13-1.27 (m)	1.36-1.42 (m)	1.04-1.14 (m)	1.22-1.27 (m)	1.41-1.50 (m)	1.36-1.46 (m)
3	1.39 (tt, 14.6, 4.7)	1.35-1.40 (m)	1.86 (tt, 12.9, 4.1)	2.23 (tt, 15.4, 4.6)	2.17-2.28 (m)	2.07-2.18 (m)
	0.91-0.99 (m)	1.07-1.14 (m)	1.02-1.09 (m)	1.22-1.25 (m)	1.19-1.30 (m)	1.19-1.30 (m)
4	0.83-0.93 (m)	0.87-0.94 (m)	1.45-1.53 (m)	1.60-1.70 (m)	1.51-1.62 (m)	1.57-1.70 (m)
6	1.99 (d, 16.6)	1.84 (d, 16.5)	5.94 (s)	6.60 (br s)	6.62 (s)	6.71 (s)
	0.99 (d, 16.6)	1.48 (d, 16.5)	—	—	—	—
9	2.55 (d, 15.5)	2.12 (dd, 15.4, 4.8)	1.99 (dd, 15.2, 7.6)	3.17 (d, 17.1)	2.69 (d, 16.6)	2.69 (d, 16.6)
	1.80 (dd, 15.5, 6.7)	1.94 (dd, 15.4, 5.5)	1.70 (br s)	2.52 (ddd, 17.1, 5.4, 2.2)	2.58 (dd, 16.6, 4.7)	2.58 (dd, 16.6, 4.7)
10	1.49 (dd, 11.2, 6.7)	1.44-1.51 (m)	1.18-1.25 (m)	1.58-1.64 (m)	2.06-2.13 (m)	1.99-2.06 (m)
11	2.47 (dd, 8.8, 5.7)	2.54 (dd, 8.8, 5.7)	2.73 (q, 7.1)	—	—	—
12	—	—	—	6.96 (br s)	6.87 (br.s)	6.86 (br.s)
13	3.79 (dd, 10.4, 5.7)	3.74 (ddd, 10.4, 5.7, 4.1)	1.27 (d, 7.1)	1.91 (d, 1.2)	1.82 (br.s)	1.81 (br.s)
	3.71 (dd, 10.4, 8.8)	3.72 (ddd, 10.4, 8.8, 6.6)	—	—	—	—
14	0.35 (s)	0.74 (s)	0.54 (s)	0.93 (s)	0.98 (s)	0.97 (s)
15	0.61 (d, 7.2)	0.57 (d, 7.1)	0.64 (d, 7.3)	0.77 (d, 7.3)	0.78 (d, 7.2)	0.76 (d, 7.2)
1-*O*-Acyl						
2'	—	—	—	—	—	2.32 (sept, 7.0)
3'	5.70 (qq, 7.2, 1.5)	5.70 (qq, 7.2, 1.5)	—	—	5.67 (qq, 7.2, 1.4)	1.04 (d, 7.0)
4'	1.99 (dq, 7.2, 1.5)	1.99 (dq, 7.2, 1.5)	—	—	1.94 (dq, 7.2, 1.4)	1.03 (d, 7.0)
5'	1.83 (quint, 1.5)	1.82 (quint, 1.5)	—	—	1.83 (quint, 1.4)	—
6-*O*-Acyl						
2"	—	—	—	—	2.39 (sept, 7.0)	—
3"	—	—	5.70 (qq, 7.4, 1.4)	5.76 (qq, 7.3, 1.4)	1.07 (d, 7.0)	5.74 (qq, 7.2, 1.4)
4"	—	—	1.90 (dq, 7.4, 1.4)	2.02 (dq, 7.3, 1.4)	1.07 (d, 7.0)	2.00 (dq, 7.2, 1.4)
5"	—	—	1.66 (quint, 1.4)	1.87 (quint, 1.4)	—	1.85 (quint, 1.4)
13-OAc	1.64 (s)	—	—	—	—	—
OH	—	1.05 (dd, 6.6, 4.1)	—	—	—	—

Table 3. ^{13}C NMR data of compounds **1**, **3**, and **4** (100 MHz in benzene-d_6)

Positions	**1**	**3**	**4**
1	71.7	28.9	68.0
2	26.7	20.6	30.7
3	26.7	28.6	27.3
4	36.5	32.8	32.6
5	36.7	39.9	42.1
6	34.1	67.3	69.1
7	62.6	65.5	116.3
8	86.6	86.9	151.2
9	19.6	25.8	20.5
10	38.3	34.6	45.6
11	52.0	41.0	120.0
12	173.2	175.4	138.9
13	60.2	11.0	8.9
14	25.7	20.0	20.5
15	14.3	14.4	20.3
1'	166.8	166.1	167.7
2'	128.0	126.8	128.0
3'	137.8	140.9	139.1
4'	15.9	15.9	16.0
5'	20.9	20.6	21.0
Ac	170.1	—	—
Ac	20.4	—	—

Table 4. Chemical composition of samples 1–4

sample	furanoeremophilanes/eremophilanolides																				others									
no.	subspicatins/subspicatol									9-ol/9-ones				10-ols				others								simple			rearranged			benzofurans			
1					**5**	**6**	**11[a]**	**12**			**17**	**18**			**13**	**14**	**15**	**16**							**20**		**23**								
2	**1**	**2**					**11[a]**	**12**	**21**		**17**	**18**	**19**		**13**	**14**						**8**			**20**				**22**					
3				**4**							**17[a]**	**18**	**19**			**14**			**3**	**7**		**9**	**10**				**24**			**25**				
4																															**26**	**27**	**28[a]**	**29**

[a]Major constituent.

Table 5. Summary of the ITS sequence and the major compounds in *L. cyathiceps* and *L. lamarum*/*L. subspicata* hybrids

Sample No.	Morphology	ITS[a]	Major components[b]	Reference
1	*L. subspicata*	L/S + C	**17, 18, 30, 32**	
2	ambiguous	L/S + C	**8, 11**	
3	ambiguous	L/S + C	**17, 18**	
5	ambiguous	C	**17, 18, 30, 32**	Saito *et al*., 2016
6	ambiguous	L/S + C	**17, 18**	Shimizu *et al*., 2014 (sample B)
7	ambiguous	C	**17, 18, 30, 32**	Shimizu *et al*., 2014 (sample A)
8	*L. lamarum*	L/S + C	**8, 11**	Shimizu *et al*., 2014 (sample D)

[a]L/S and C indicate sequences typical of the *L. lamarum*/*L. subspicata* complex and *L. cyathiceps*, respectively. See Table 1.

[b]Detected by LC-MS. Compounds **8**, **11**, **30**, and **32** are L/S complex origin, and **17** and **18** are *L. cyathiceps* origin (see text).

Figure legends

Figure 1. Previously undescribed compounds isolated in this study.

Figure 2. Selected two-dimensional correlation of compound **1**.

Figure 3. Selected two-dimensional correlation of compound **2**.

Figure 4. Selected two-dimensional correlation of compound **3**.

Figure 5. Selected two-dimensional correlation of compound **4**.

Figure 6. Selected two-dimensional correlation of compounds **5** and **6**.

Figure 7. Known compounds **7**–**29** isolated in this study.

Figure 8. LC-MS profile (total ion chromatogram) of samples 1–3. For not isolated compounds; **30** = furanoeremophilane-6,10-diol, **31** = tetradymol, **32** = 6-ethoxyfuranoeremophilan-10-ol.

Figure 1.

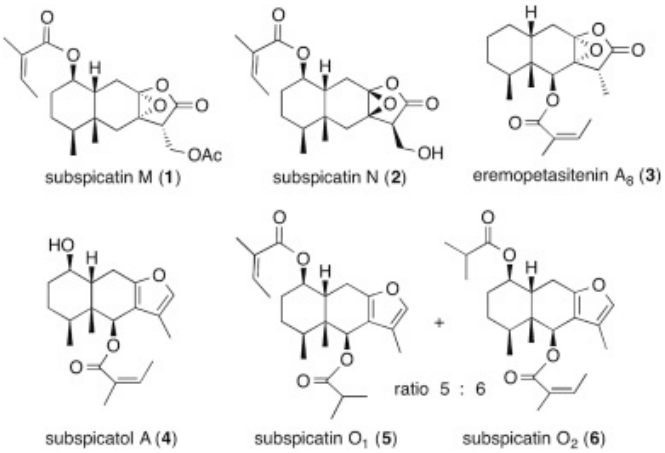


Figure 2.

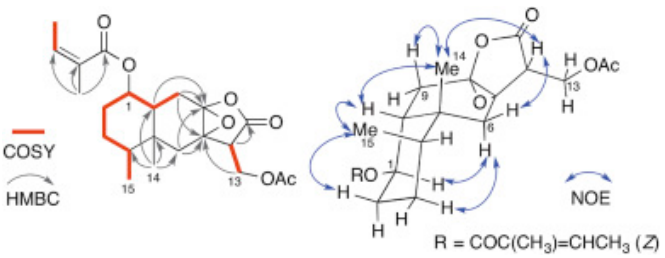


Figure 3.

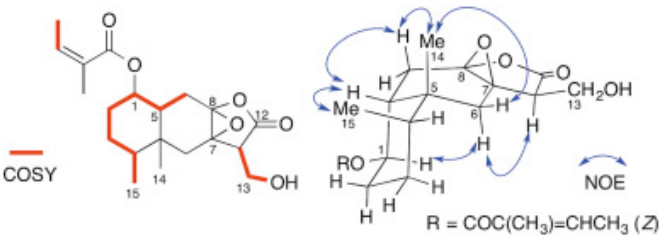


Figure 4.

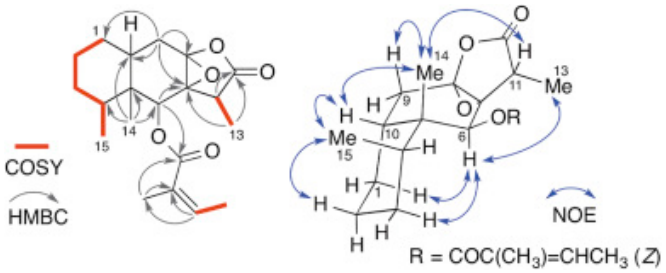


Figure 5.

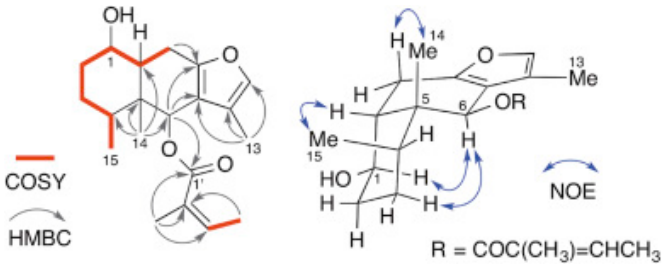


Figure 6.

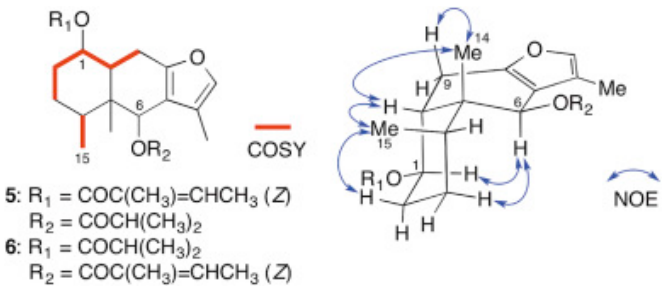


Figure 7.

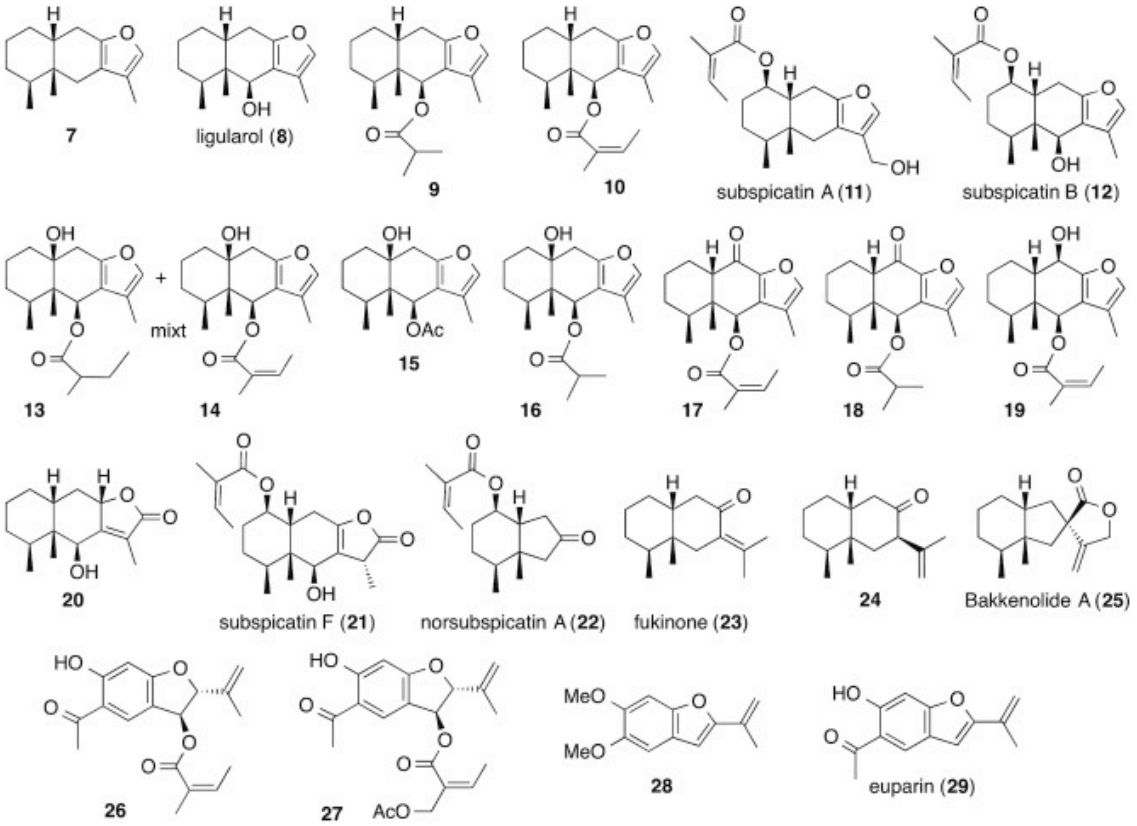
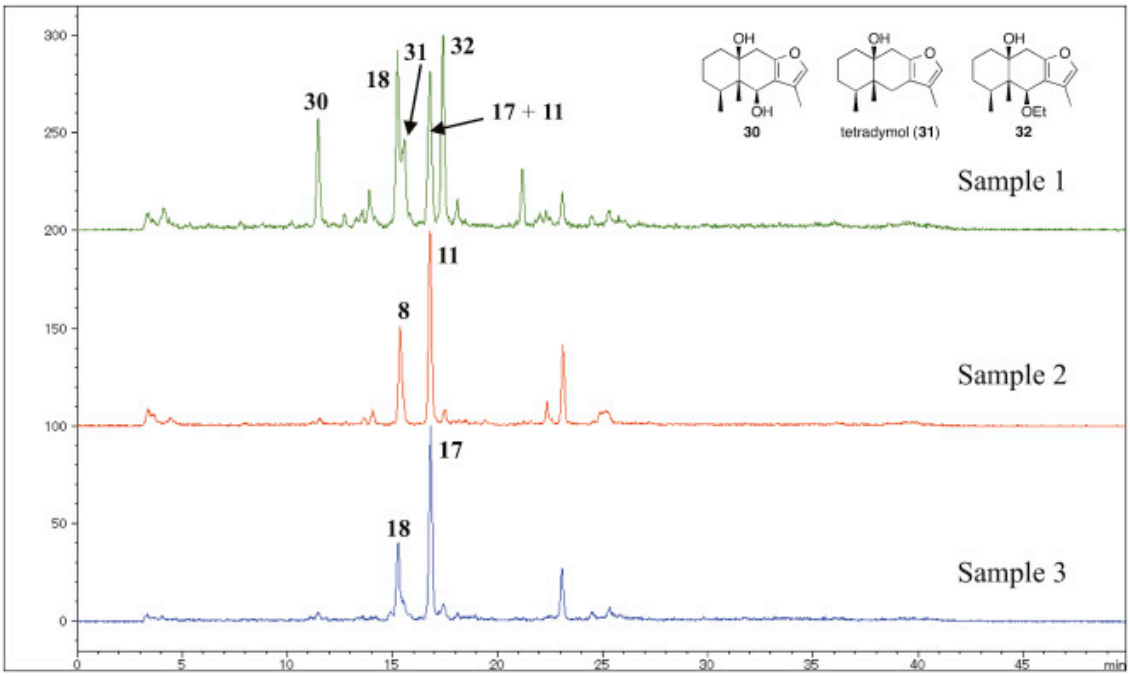

7
ligularol (8)
9
10
subspicatin A (11)
subspicatin B (12)
mixt
13
14
15
16
17
18
19
20
subspicatin F (21)
norsubspicatin A (22)
fukinone (23)
24
Bakkenolide A (25)
26
27
28
euparin (29)

Figure 8.


300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
45
min
30
18
31
32
17 + 11
11
8
17
18
Sample 1
Sample 2
Sample 3
OH
OH
30
OH
O
tetradymol (31)
OH
O
OEt
32