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# Overlapping chemical and genetic diversity in *Ligularia lamarum* and *Ligularia subspicata*. Isolation of ten new eremophilanes and a new seco-bakkane compound

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ABSTRACT

Root chemicals and DNA sequences were analyzed for *Ligularia lamarum* samples collected in Yunnan and Sichuan Provinces of China and the relationship between the plant and *Ligularia subspicata*, its taxonomically closest species, was examined. Both species produce furanoeremophilanes and eremophilan-8-one derivatives as the major components. Eleven new compounds, including a new seco-bakkane compound, were isolated. The two species were found to harbor overlapping intra-specific diversities with respect to the chemicals and the DNA sequences.



## 1. Introduction

Diversification and evolution of higher plants is a subject of fundamental importance in natural science. *Ligularia* Cass. (Asteraceae) in the Hengduan Mountainous area is a genus suitable for the investigation of speciation, diversification, and adaptability to alpine environments. We have been studying the diversity in *Ligularia* by the combination of two independent approaches: analysis of chemical components in roots and determination of base sequences of neutral DNA regions. To date, intra-specific diversity has been revealed in many species and in several different modes. For example, each of *Ligularia pleurocaulis* (Franch.) Hand.-Mazz.,[1] *Ligularia tsangchanensis* (Franch.) Hand.-Mazz.,[2] and *Ligularia virgaurea* var. *virgaurea* (Maxim.) Mattf.[3] contained chemically distinct populations. The chemical spectrum was continuous in *Ligularia dictyoneura* (Franch.) Hand.-Mazz.[4] and *Ligularia kanaitzensis* (Franch.) Hand.-Mazz.[5] *Ligularia cymbulifera* (W.W. Smith) Hand.-Mazz.[6] and *Ligularia cyathiceps* Hand.-Mazz.[7] were chemically almost uniform. These results implied that a number of mechanisms might be involved in the generation of chemical diversity in *Ligularia*.

Recently we reported that *Ligularia subspicata* (Bureau and Franch.) Hand.-Mazz., collected in northwestern Yunnan and southwestern Sichuan Provinces, China, was diverse both in the chemical composition and the nucleotide sequences and that the difference among the samples was continuous.[8] Furanoeremophilanes, such as ligularol were obtained from all the collected samples. Furano- and nonfurano-eremophilane sesquiterpenes oxidized at C-1 position (subspicatins A–D) were isolated as characteristic compounds of the species.

Further search in the field has provided us with additional samples of *L. subspicata*, as well as *Ligularia lamarum* (Diels) C.C. Chang. *L. lamarum* is distributed more widely than *L. subspicata*,[9]

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

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and is a species taxonomically closest to *L. subspicata*, both belonging to the section *Ligularia*, series *Ligularia*.[9] The difference between the two species is only in the presence (*L. lamarum*) or the absence (*L. subspicata*) of ligulate florets and in the color of pappi.[9,10] The two species occupy similar habitats, such as swamps, scrub, and forest understories of around 3000–5000 m in altitude, and we have found them intermingled at some locations (vide infra). To the best of our knowledge, the chemical constituents of *L. lamarum* have not been reported. In the present study, we focused on the similarity and difference of the two species in chemical composition and DNA sequence of evolutionally neutral regions, aiming to clarify the level of diversification within each *L. lamarum* and *L. subspicata* and between the two species. The answers will have important implications for investigating speciation, diversification, and adaptability to alpine environments of *Ligularia* species in Hengduan Mountains. Here we report that the two species are indistinguishable in our two indices in spite of distinct morphological difference.

## 2. Results

Samples of *L. lamarum* and *L. subspicata* were collected in Yunnan and Sichuan Provinces of China (Tables 1 and 2 and Fig. 1). Some samples of the two species were sympatric at six locations: at locations 1, 2, and 5, they were growing intermingled; at locations 3 and 4, they were within 20 m.

**Table 1**
Collection locality and Ehrlich's test of *L. lamarum* and *L. subspicata* samples collected at the same field

| Location | Sample number | Species | Locality | Elevation | Ehrlich's test |
|---|---|---|---|---|---|
| 1 | 1A | *L. lamarum* | Daxueshan | 4100 | Negative |
| | 1B | *L. subspicata* | | | Negative |
| | 1C | *L. lamarum* | | | Negative |
| 2 | 2A | *L. lamarum* | Daxueshan | 4100 | Positive |
| | 2B | *L. subspicata* | | | Positive |
| | 2C | *L. lamarum* | | | Positive |
| 3 | 3A | *L. lamarum* | Tianchi | 3500 | Positive |
| | 3B | *L. lamarum* | | | Positive |
| | 3C | *L. subspicata* | | | Positive |
| 4 | 4A | *L. lamarum* | Nixi | 3600 | Positive |
| | 4B[a] | *L. subspicata* | | | Positive |
| 5 | 5A | *L. lamarum* | Baima-xueshan | 4100 | Positive |
| | 5B[a] | *L. subspicata* | | | Positive |

[a] Published data; see Ref. 8.

**Table 2**
Collection locality and Ehrlich's test of *L. lamarum* and *L. subspicata* samples collected independently

| Sample number | Species | Locality | Elevation | Ehrlich's test |
|---|---|---|---|---|
| 6 | *L. lamarum* | Laojunshan | 4000 | Positive |
| 7 | *L. lamarum* | Laojunshan | 4000 | Positive |
| 8 | *L. lamarum* | Gonggashan | 3500 | Negative |
| 9 | *L. lamarum* | Maerkang/Xiaojin | 3900 | Positive |
| 10 | *L. subspicata* | Changhaizi | 3700 | Negative |
| 11 | *L. subspicata* | Jiulong | 3600 | Negative |
| 12 | *L. subspicata* | Jiulong/Kangding | 4100 | Positive |
| 13 | *L. subspicata* | Gaoersishan | 3700 | Negative |
| 14 | *L. subspicata* | Yajiang | 4000 | Positive |
| 15 | *L. subspicata* | Haizishan(Batang County) | 4200 | Negative |

Samples 1C and 2C had ligulate florets and thus were identified as *L. lamarum*, however, ligules were shorter than typically observed. The individuals may be natural hybrid between *L. lamarum* and *L. subspicata*. Two previously reported *L. subspicata* samples[8] 4B and 5B are included in Table 1 for comparison with the *L. lamarum* samples collected sympatrically.

Roots were extracted with EtOH and the extracts were subjected to Ehrlich's test on TLC plates as previously described.[1–8] Although all the *L. subspicata* samples reported in the previous paper contained furanoeremophilanes,[8] some of the newly collected *L. subspicata* samples were negative to the test, suggesting the absence of furanoeremophilanes (Tables 1 and 2). The TLC pattern was quite similar within each of four sets of samples collected sympatrically (samples 1A–C, 2A–C, 4A and 4B, and 5A and 5B); the pattern in 3A was slightly different from that in 3B and 3C.

Chemical constituents were isolated and their structures were determined. As summarized in Table 3, the compounds were of six categories and mostly eremophilanes: (1) subspicatins (1-acyloxyfuranoeremophilane derivatives): subspicatin A (**1**),[8] subspicatin B (**2**),[8] subspicatin E (**3**), subspicatin F (**4**), and subspicatin G (**5**); (2) ligularol (**6**),[11] and its derivatives **7**,[12] **8**,[13] **9**,[14] **10**,[15] **11**, **12**, **13**,[16] **14**,[17] **15**,[18] eremofarfugin C (**16**),[5] and eremopetasitenin A4 (**17**); (3) tetradymol (**18**),[19] and its derivatives **19**,[5] **20**,[20] **21**,[5] **22**,[21] **23**,[22] and **24**[23]; (4) fukinone (**25**),[24] and its derivatives **26**,[25] **27**,[26] isopetasin (**28**),[27] **29**,[27] **30**,[28] **31**,[29] **32**,[30,31] **33**,[32] petasin (**34**),[31] **35**,[5] and **36**[33]; (5) oxidized fukinone derivatives: **37**,[34] **38**,[5] **39**,[34] **40**,[35] **41**, **42**, **43**, **44**,[36] **45**,[8] **46**, and **47**[18]; (6) compounds having other carbon skeletons: liguhodgsonal (**48**),[37] **49**,[3] **50**,[38] and **51**. Eremophilan-8-one derivatives (fukinones in Table 3) were the major constituents in the samples negative to Ehrlich's test, regardless of the species.

The structures of 11 new compounds, **3**, **4**, **5**, **11**, **12**, **17**, **41**, **42**, **43**, **46**, and **51**, were determined as follows.

The molecular formula of compound **3** was determined to be $C_{20}H_{28}O_6$ by HRCIMS. The $^1$H NMR spectrum showed the presence of an angeloyl group [$\delta$ 5.69 (qq, $J$=7.2, 1.5 Hz, H-3′), 1.81 (quint, $J$=1.5 Hz, H-5′), and 1.97 (dq, $J$=7.2, 1.5 Hz, H-4′)], one singlet [$\delta$ 0.44 (s, H-14)] and two doublet [$\delta$ 0.62 (d, $J$=7.2 Hz, H-15) and 1.33 (d, $J$=7.3 Hz, H-13)] methyl groups, and two methine protons bearing oxygen functions [$\delta$ 3.64 (d, $J$=6.0 Hz, H-6) and 5.08 (td, $J$=11.4, 4.7 Hz, H-1)] (Table 4). The $^{13}$C NMR spectrum indicated the presence of two carbonyl [$\delta$ 166.8 (C-1′) and 175.8 (C-12)], two olefinic [$\delta$ 128.2 (C-2′) and 138.1 (C-3′)], and four carbons bearing oxygen functions [$\delta$ 66.5 (C-7), 66.6 (C-6), 71.1 (C-1), and 86.7 (C-8)] (Table 5). The IR spectrum showed the presence of an epoxy- or enol-lactone[5,8,39,40] at 1805 cm$^{-1}$ and carbonyls at 1715 cm$^{-1}$ as well as a hydroxy group at 3500 cm$^{-1}$. The COSY spectrum indicated proton connectivities for H10–H1–H2, H3–H4–H15, and H11–H13 (Fig. 2). The HMBC spectrum showed correlations between H-15 and C-3, between H-14 and C-4, 5, 6, and 10, between H-13 and C-7, 11, and 12, between H-9 and C-5, 8, and 10, and between H-6 and C-8 (Fig. 2). These observations indicated that this compound had the eremophilane skeleton with an epoxy lactone at C-7 and C-8 positions. An angeloyl group was deduced to be attached at C-1 position ($\delta_H$ 5.09, $\delta_C$ 71.1) and the hydroxy group, at C-6 position ($\delta_H$ 3.64, $\delta_C$ 66.6), respectively, because H-1 was correlated with C-1′. Therefore, the planar structure of **3** was determined as shown in the formula. Its relative configuration was determined by NOEs between H-13 and H-6$\alpha$, between H-11 and H-14, between H-14 and H-10, and between H-6$\alpha$ and H-3$\alpha$ (Fig. 3). The compound was named subspicatin E.

The molecular formula of **4** was determined to be $C_{20}H_{28}O_5$ by HRCIMS. The IR spectrum showed absorptions at 3500, 1800, and 1710 cm$^{-1}$, indicating the presence of an epoxy- or an enol-lactone as well as hydroxy and carbonyl groups. The $^1$H and $^{13}$C NMR spectra showed the presence of a singlet and two doublet methyl groups, two oxymethine protons, and an angelate moiety, although they appeared as broad peaks (Tables 4 and 5). The COSY and HMBC spectra indicated proton connectivities for H9–H10–H1–H2–H3–H4–H15 and correlations between H-15 and C3, 4, and 5, between H-13 and C-7, 11, and 12, between H-4′ and C-2′ and 3′, and between H-5′ and C-2′ and 3′ (Fig. 4). Thus, the resonance at $\delta$ 116.6 was assigned to C-7, and that at $\delta$ 148.0 to

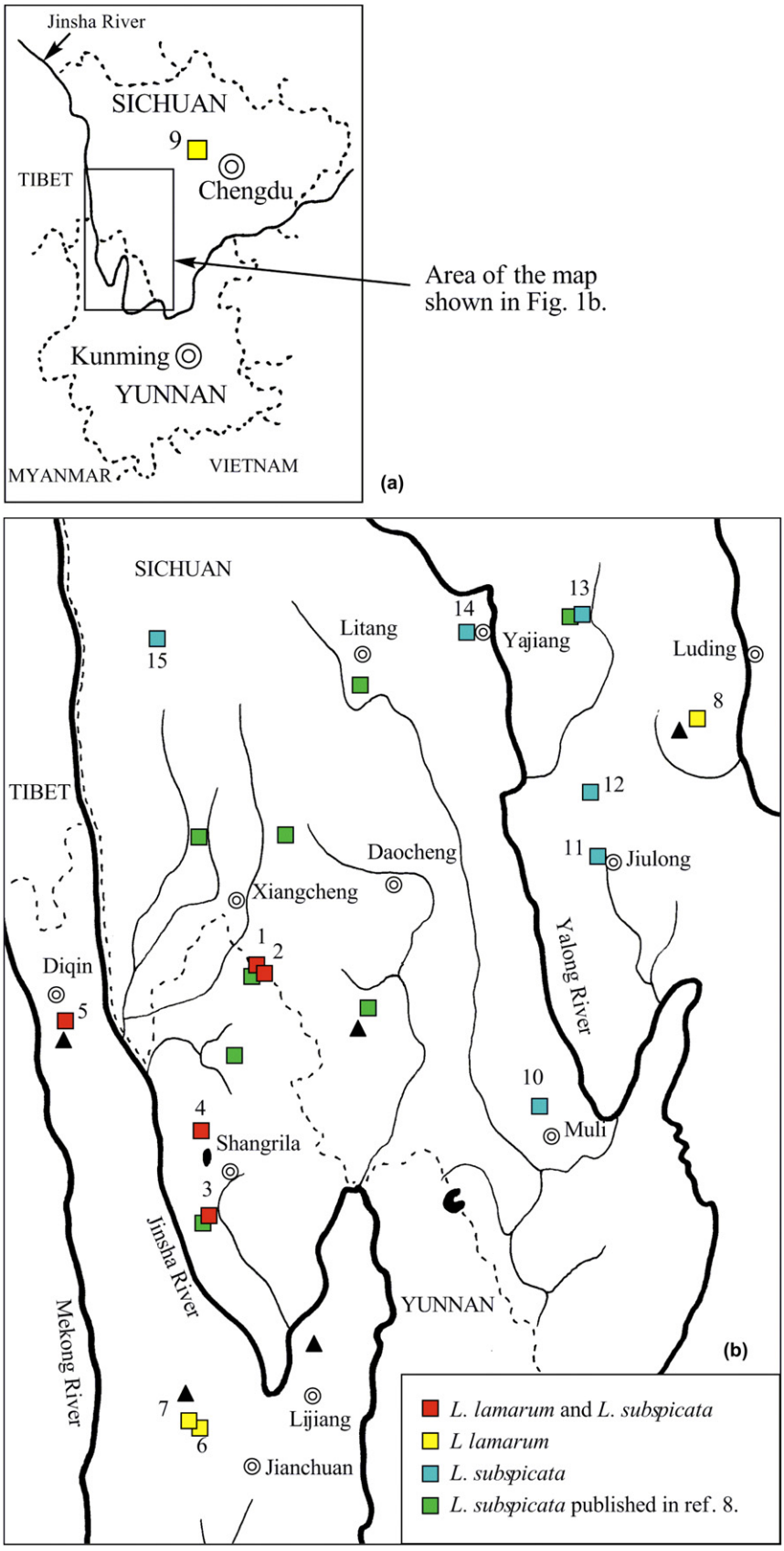



**Fig. 1.** a and b. Locations where samples of *L. lamarum* and *L. subspicata* species (squares) were collected. Filled triangles and double circles indicate major peaks and major cities, respectively.

**Table 3**
Chemical composition of *L. lamarum* and *L. subspicata* samples

| Sample number[a] | Furanoeremophilanes | | | Other eremophilanes | | Other carbon skeleton |
|---|---|---|---|---|---|---|
| | Subspicatins | Ligularols | Tetradymols | Fukinones | Oxidized fukinones | |
| 1A | | | | **25**, **36** | | |
| 1B | | | | **25** | **39**–**41**, **43**, **44** | |
| 1C | | | | **25** | **39**–**44** | **48** |
| 2A | **1** | | | **36** | | |
| 2B | | **14**, **15** | | | **38**, **45**, **47** | |
| 2C | | **14**, **15**, **17** | | | **37**, **38**, **45**, **46** | **51** |
| 3A | **1**–**4** | **6**, **10**–**12**, **14**, **16** | **20**, **21** | | **38** | **49**, **50** |
| 3B | **1** | **6**, **8**, **9** | **18**, **19** | | | |
| 3C | **1** | **15** | **20**–**22** | | | |
| 4A | | **6**, **13** | **21** | **31**, **33**–**35** | | |
| 4B[b] | **1** | **6** | | **31**–**35** | | |
| 5A | **2**, **4** | **6**, **7**, **14**, **15** | | | **38** | |
| 5B[b] | **2**, **52** | **6** | | | **45** | |
| 6 | **1**, **2** | **6**, **15** | **21** | | **38** | **49** |
| 7 | **1**, **2** | **6**, **15** | | | | |
| 8 | | | | **25** | | |
| 9 | | **6** | **22**–**24** | | | |
| 10 | | | | **31**–**36** | | |
| 11 | | | | **27**, **28**, **29**, **31**, **33**, **34**, **35** | | |
| 13 | | | | **27**–**35** | | |
| 15 | | | | **25**–**27** | **39**–**41**, **44** | **48** |

[a] Chemical constituents of samples 12 and 14 were not examined because of decomposition during storing.
[b] Published in Ref. 8. Samples 4B and 5B are samples 3 and 5 in Ref. 8, respectively.

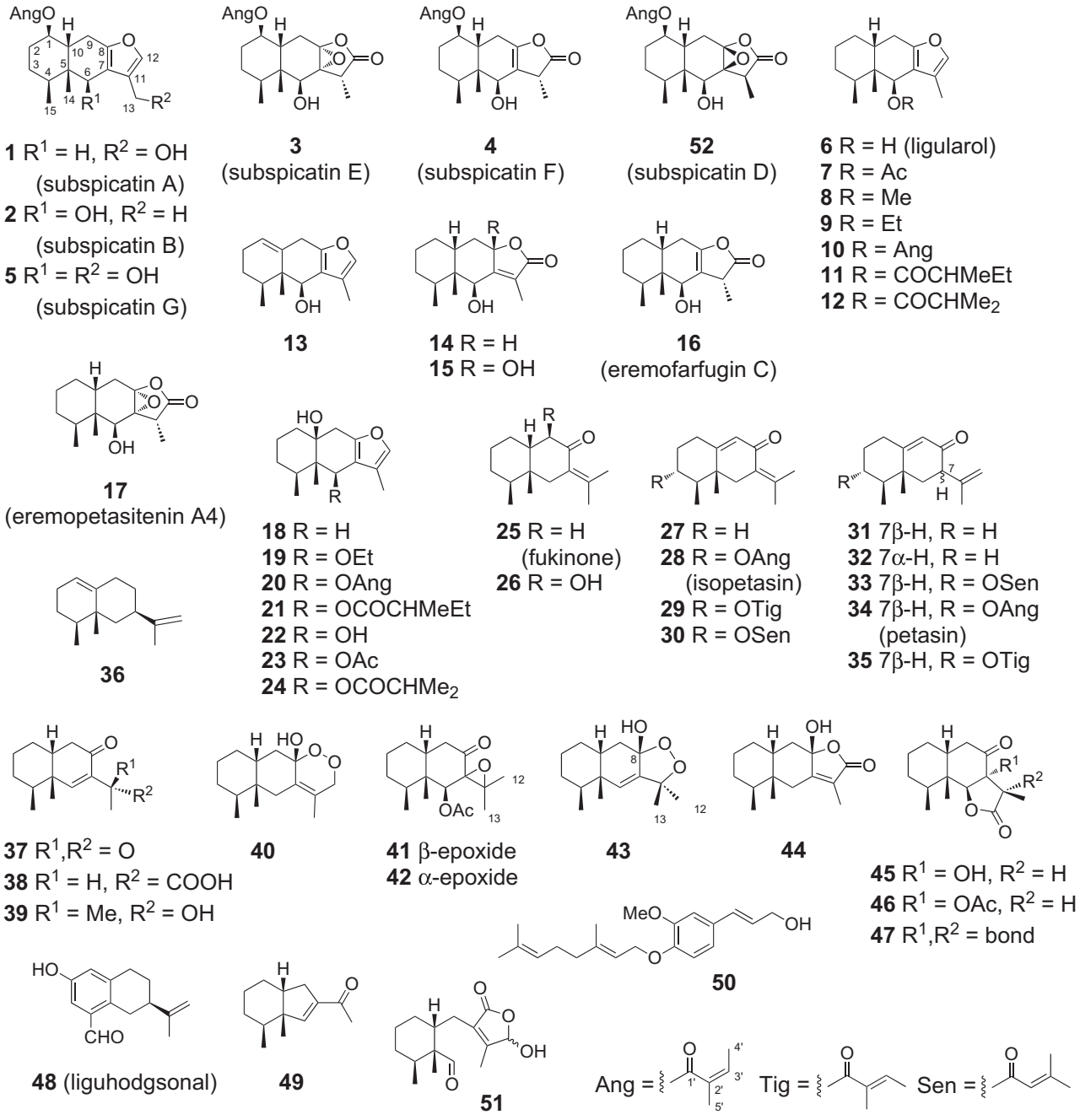
C-8, forming an enol-lactone. Although the position of an angeloyl group ($\delta_H$ 5.68) could not be determined by the HMBC spectrum, chemical shift values of H-1 and C-1 ($\delta_H$ 4.79, $\delta_C$ 71.0) indicated that the angeloyloxy group was attached to C-1. Therefore, those of H-6 and C-6 ($\delta_H$ 4.19, $\delta_C$ 66.1) indicated that the hydroxy group was attached to C-6. The relative configuration of **4** was determined by the NOESY spectrum (Fig. 5). The NOEs between H-10 and H-14 and between H-6 and H-3α indicated that the rings A and B were cis-fused. The relative configuration of the angeloyloxy group was determined to be β by the coupling pattern of H-1 and also by the NOE between H-6α and H-1α (Fig. 5). Since an NOE was detected between H-6 and H-13, the methyl group at C-11 was assigned as α. Thus, the structure of **4** was established as depicted in the formula and the compound was named subspicatin F.

The molecular formula of compound **5** was determined to be $C_{20}H_{28}O_5$ by HRCIMS. The $^1$H NMR spectrum showed the presence of an angeloyl moiety [$\delta$ 5.66 (qq, $J$=7.4, 1.5 Hz, H-3′), 1.82 (3H, quint, $J$=1.5 Hz, H-5′), and 1.92 (3H, dq, $J$=7.4, 1.5 Hz, H-4′)], an oxymethine proton [$\delta$ 4.88 (br s, H-6)], and an oxymethylene unit [$\delta$ 4.24 (d, $J$=12.7 Hz) and 4.17 (d, $J$=12.7 Hz)] (Table 4). The presence of hydroxy groups was indicated by an absorption at 3350 cm$^{-1}$ in its IR spectrum. The HMBC spectrum showed correlations between H-15 and C-3, 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-7 and 8 (Fig. 6), Therefore, **5** was determined to be an eremophilane derivative with an angeloyloxy and hydroxy groups. Although the position of the angeloyloxy group could not be determined by the HMBC spectrum, it was deduced by the chemical shift values of H-1 ($\delta$ 4.90) and C-1 ($\delta$ 71.6) (Tables 4 and 5), as discussed for compound **4**. Therefore, the two hydroxy groups were determined to be attached to the C-6 and C-13 positions. The relative configuration of **5** was determined by the NOESY spectrum. NOEs between H-10 and H-14, between H-10 and H-15, and between H-9β and H-14 indicated that the rings A and B were cis-fused. Because H-1α was axially oriented [$\delta$ 4.90 (td, $J$=11.4, 4.9 Hz)], the whole structure of **5** was established as in Fig. 7 and the compound was named subspicatin G.

$^1$H and $^{13}$C NMR spectra indicated that compounds **11** and **12** were 6β-acyloxyderivatives of ligularol (Tables 4 and 5). The proton at C-6 of **11** and **12** appeared at $\delta$ 6.38 and 6.42 as a singlet, respectively. The proton at C-12 was observed at $\delta$ 6.96 for both compounds. In addition to the singlet and doublet methyl groups of the eremophilane skeleton, a doublet and a triplet methyl groups were observed at $\delta$ 1.11 and 0.83 for compound **11**, while two doublet methyl groups were detected at $\delta$ 1.11 and 1.09 for compound **12**. Therefore, the acyl groups of **11** and **12** were judged to be 2-methylbutyrate and 2-methylpropionate, respectively. Thus, the structures of **11** and **12** were established to be 6β-(2′-methylbutyryloxy)furanoeremophilane and 6β-(2′-methylpropionyloxy) furanoeremophilane, respectively, which were supported by the HMBC and NOESY spectra. The chirality at C-2′ of **11** was not determined.

The molecular formula of **17** was determined to be $C_{15}H_{22}O_4$ by HRCIMS. The IR spectrum showed absorptions at 3500 and 1800 cm$^{-1}$. The $^1$H NMR spectrum of **17** indicated that no angeloyl group was present in this molecule (Table 4). 2D NMR experiments indicated that **17** was a derivative of **3** without the angeloyloxy group at the C-1 position (Fig. 8). The relative configuration of **17** was determined by the NOESY spectrum. NOEs were observed between H-11 and H-14, between H-14 and H-10, between H-6 and H-1α, and between H-6 and H-3α (Fig. 9). Therefore, the structure of **17** was established as de-angeloyloxy subspicatin E and the compound was named eremopetasitenin A4.

Compound **41** showed a quasi-molecular ion peak at $m/z$ 295 by HRCIMS and the molecular formula was determined to be $C_{17}H_{26}O_4$. The $^1$H NMR spectrum showed the presence of an acetyl group ($\delta$ 1.62), three singlet methyl groups ($\delta$ 0.76, 1.02, and 1.23), and a doublet methyl group ($\delta$ 0.69) (Table 4). The IR spectrum indicated the presence of two carbonyl groups (1730, 1745 cm$^{-1}$). Connectivities were shown for H9–H10–H1–H2 and H3–H4–H15 in the COSY spectrum (Fig. 10). The HMBC spectrum showed correlations between H-14 and C-4, 5, 6, and 10, between H-13 and C-7, 11, and 12, between H-9 and C-7 and 8, and between H-6 and C-8. These data indicated that an epoxide was present between C-7 and C-11. The position of an acetoxy group ($\delta_H$ 1.62) was determined to be at C-6 by a correlation between H-6 and C-1′. The relative configuration of **41** was deduced by the NOESY spectrum (Fig. 11). The cis fusion of A/B rings was shown by an NOE between H-10 and H-14, which was supported by NOEs between H-4 and H-9α and between H-2α and H-9α. The acetoxy group was determined to be in the β-orientation, because H-6 showed an NOE to H-15. The




β orientation of the epoxide oxygen atom was inferred from the NOE between H-6 and H-13. This compound was crystallized and the structure was unambiguously determined by the X-ray crystallographic analysis (Fig. 12).

Compound **42** was spectroscopically very similar to **41**. The differences were in the chemical shift values for H-4, H-12, and H-13 (Table 4) and for C-7, 8, 11, 12, and 13 (Table 5). 2D NMR experiments indicated that this was a diastereoisomer of compound **41** with respect to the orientation of the epoxide oxygen atom (Figs. 13 and 14); the oxygen atom of the epoxide ring was in the α-configuration.

Compound **43** showed a quasi-molecular ion peak at *m*/*z* 275 $[M+Na]^+$ in HRFABMS and its molecular formula was determined to be $C_{15}H_{24}O_3$ with 4° of unsaturation. The IR spectrum indicated the presence of a hydroxy group (3400 $cm^{-1}$). Three singlet methyl groups at $\delta$ 0.87, 1.25, and 1.30, a doublet methyl group at $\delta$ 0.66, and an olefinic proton at $\delta$ 5.24 were detected in its $^1H$ NMR spectrum (Table 4). The $^{13}C$ NMR spectrum indicated the presence of a carbon bearing two oxygen functions at $\delta$ 102.8, a double bond at $\delta$ 129.0 and 149.2, and a quaternary carbon bearing an oxygen function at $\delta$ 81.6 (Table 5). The COSY spectrum showed proton connectivities for H9–H10–H1–H2 and H4–H15 (Fig. 15). The HMBC spectrum showed correlations between H-14 and C-4, 5, 6, and 10, between H-15 and C-3, between H-12 and C-7, 11, and 13, between H-9 and C-7 and 8, and between H-6 and C-8 (Fig. 15). These observations indicated that there were three rings in this molecule. Since this molecule contained three oxygen atoms, a peroxy bridge was deduced to be present between C-8 and 11. Therefore, the planar structure of **43** was determined as shown in Fig. 15. Its relative configuration was determined by the NOESY spectrum (Fig. 16). The

**Table 4**
$^1$H NMR data of new compounds (in $C_6D_6$; $\delta$, $J$ in Hz)

| C | 3 | 4[a] | 5 | 11[b] | 12[c] | 17[d] | 41 | 42 | 43 | 46 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 5.08 (td, 11.4, 4.7) | 4.79 (td, 10.4, 4.4) | 4.90 (td, 11.4, 4.9) | 1.37–1.43 (m) | 1.31–1.39 (m) | 1.45 (qd, 13.4, 4.2) | 1.30–1.40 (m) | 1.28–1.37 (m) | 1.58–1.69 (m) | 1.19 (tt, 13.4, 3.9) |
| | — | — | — | 1.10–1.19 (m) | 1.09–1.17 (m) | 0.81–0.88 (m) | 0.88–0.96 (m) | 0.94–1.03 (m) | 1.23–1.30 (m) | 0.80 (br d, 13.4) |
| 2 | 2.05 (dq, 11.7, 4.7) | 1.84 (m) | 1.47 (qd, 11.4, 4.4) | 1.26–1.36 (m) | 1.25–1.35 (m) | 1.19–1.25 (m) | 1.11–1.21 (m) | 1.16–1.23 (m) | 1.23–1.30 (m) | 1.04–1.14 (m) |
| | 1.16–1.26 (m) | 1.39 (m) | 1.92–1.99 (m) | 1.26–1.36 (m) | 1.25–1.35 (m) | 1.12 (qt, 13.4, 4.0) | 1.11–1.21 (m) | 1.16–1.23 (m) | 0.95–1.07 (m) | 1.04–1.14 (m) |
| 3 | 1.30–1.40 (m) | 1.54 (m) | 1.18 (br d, 14.5) | 1.46–1.53 (m) | 1.52–1.62 (m) | 1.30 (tt, 13.4, 4.4) | 1.11–1.21 (m) | 1.19–1.28 (m) | 1.09–1.15 (m) | 1.02–1.08 (m) |
| | 0.94–1.00 (m) | 1.08 (m) | 1.81–1.88 (m) | 1.16–1.24 (m) | 1.15–1.24 (m) | 0.97–1.03 (m) | 1.11–1.21 (m) | 0.95–1.07 (m) | 0.97 (qd, 12.1, 2.6) | 0.84–0.95 (m) |
| 4 | 1.35–1.44 (m) | 1.34 (m) | 1.94–2.02 (m) | 1.36–1.45 (m) | 1.39–1.48 (m) | 1.44–1.51 (m) | 1.54 (dqd, 12.1, 6.4, 2.0) | 2.17–2.26 (m) | 1.12–1.21 (m) | 1.45–1.54 (m) |
| 6 | 3.64 (d, 6.0) | 4.19 (br s) | 4.88 (br s) | 6.38 (s) | 6.42 (s) | 3.80 (d, 6.3) | 5.63 (s) | 5.54 (br s) | 5.24 (s) | 4.25 (s) |
| 9 | 2.51 (d, 15.5) | 2.20 (m) | 2.53 (ddd, 17.1, 5.6 2.0) | 2.54 (dd, 17.3, 6.8) | 2.55 (dd, 17.2, 6.7) | 1.93 (dd, 15.2, 7.3) | 2.40 (t, 13.9) | 2.70–2.82 (m) | 1.87 (dd, 13.5, 12.3) | 2.13 (dd, 13.4, 3.9) |
| | 1.80 (dd, 15.5, 7.0) | 2.00 (m) | 2.74 (d, 17.1) | 2.40 (dd, 17.3, 7.4) | 2.35 (dd, 17.2, 6.7) | 1.62 (d, 15.2) | 2.14 (dd, 13.9, 4.4) | 2.22 (dd, 14.1, 6.9) | 1.59 (dd, 12.3, 2.6) | 1.86 (dd, 13.4, 10.3) |
| 10 | 1.61 (m) | 1.78 (m) | 1.94–2.00 (m) | 1.96–2.03 (m) | 1.90–1.98 (m) | 1.11–1.17 (m) | 2.09 (dq, 13.9, 4.4) | 2.00–2.09 (m) | 2.05–2.11 (m) | 1.87–1.92 (m) |
| 11 | 2.81 (q, 7.3) | 2.93 (m) | — | — | — | 2.79 (q, 7.3) | — | — | — | 3.04 (q, 7.4) |
| 12 | — | — | 6.87 (s) | 6.96 (br s) | 6.96 (q, 1.1) | — | 1.02 (s) | 1.40 (s) | 1.25 (s) | — |
| 13 | 1.33 (d, 7.3) | 1.13 (d, 7.7) | 4.17 (d, 12.7) | 1.99 (d, 1.0) | 1.96 (d, 1.1) | 1.32 (d, 7.3) | 1.23 (s) | 1.35 (s) | 1.30 (s) | 1.42 (d, 7.4) |
| | — | — | 4.24 (d, 12.7) | — | 0.95 (s) | 0.39 (s) | 0.76 (s) | 0.79 (s) | 0.87 (s) | 0.84 (s) |
| 14 | 0.44 (s) | 0.62 (br s) | 0.86 (s) | 0.97 (s) | 0.78 (d, 6.8) | 0.65 (d, 7.4) | 0.69 (d, 6.4) | 0.85 (d, 6.6) | 0.66 (d, 6.5) | 0.58 (d, 6.6) |
| 15 | 0.62 (d, 7.2) | 0.70 (d, 7.7) | 0.82 (d, 7.3) | 0.78 (d, 6.8) | — | — | 1.62 (s) | 1.63 (s) | — | 1.60 (s) |
| Ac | — | — | — | — | 2.43 (sept, 7.1) | — | — | — | — | — |
| 2′ | — | — | — | 2.29 (sext, 7.1) | 1.11 (d, 7.1) | — | — | — | — | — |
| 3′ | 5.69 (qq, 7.2, 1.5) | 5.68 (qq, 7.7, 1.6) | 5.66 (qq, 7.4, 1.5) | 1.73 (dquint, 13.7, 7.1) | — | — | — | — | — | — |
| | — | — | — | 1.37 (dquint, 13.7, 7.1) | 1.09 (d, 7.1) | — | — | — | — | — |
| 4′ | 1.97 (dq, 7.2, 1.5) | 1.94 (dq, 7.7, 1.6) | 1.92 (dq, 7.4, 1.5) | 0.83 (t, 7.1) | — | — | — | — | — | — |
| 5′ | 1.81 (quint, 1.5) | 1.82 (quint, 1.6) | 1.82 (quint, 1.5) | 1.11 (d, 7.1) | | | | | | |

[a] 400 MHz.
[b] Measured at 60 °C.
[c] Measured at 50 °C.
[d] $\delta$ 0.72 (d, 6.3, OH).

**Table 5**
$^{13}$C NMR data of new compounds (in $C_6D_6$)

| C | 3 | 4 | 5 | 11 | 12[a] | 17 | 41 | 42 | 43 | 46 | 51[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 71.1 | 71.0 | 71.6 | 26.9 | 27.1 | 28.9 | 25.8 | 27.7 | 28.2 | 28.0 | 25.8, 25.7 |
| 2 | 26.7 | 26.6 | 26.9 | 20.4 | 20.4 | 20.9 | 19.4 | 20.6 | 21.8 | 20.2 | 20.2 |
| 3 | 26.4 | 26.8 | 27.0 | 29.6 | 29.7 | 28.7 | 30.9 | 30.1 | 31.3 | 30.0 | 30.1 |
| 4 | 31.4 | 30.1 | 31.6 | 31.5 | 31.7 | 32.2 | 29.2 | 30.6 | 35.5 | 30.6 | 31.4, 31.3 |
| 5 | 42.1 | 31.5 | 42.8 | 40.6 | 40.8 | 40.3 | 41.6 | 41.8 | 38.3 | 39.2 | 51.2 |
| 6 | 66.6 | 66.1 | 66.9 | 69.4 | 69.5 | 66.6 | 79.2 | 78.1 | 129.0 | 87.3 | 206.0, 205.9 |
| 7 | 66.5 | 116.6 | 118.8 | 116.4 | 116.4 | 66.8 | 69.9 | 67.8 | 149.2 | 87.5 | 129.5, 129.4 |
| 8 | 86.7 | 148.0 | 149.7 | 152.6 | 152.4 | 87.1 | 203.6 | 205.9 | 102.8 | 203.6 | 171.3 |
| 9 | 19.3 | 19.9 | 21.2 | 25.8 | 26.0 | 25.9 | 43.4 | 43.4 | 33.9 | 40.2 | 23.7, 23.6 |
| 10 | 39.1 | 41.0 | 42.3 | 36.2 | 36.5 | 34.4 | 38.6 | 39.0 | 38.3 | 37.0 | 38.6, 38.3 |
| 11 | 40.5 | 39.1 | 125.1 | 119.9 | 119.9 | 40.6 | 66.0 | 64.2 | 81.6 | 43.7 | 156.2 |
| 12 | 175.8 | 178.7 | 138.9 | 138.5 | 138.5 | 176.0 | 19.3 | 21.7 | 25.7 | 174.1 | 97.7, 97.6 |
| 13 | 11.6 | 14.3 | 56.0 | 8.7 | 8.5 | 11.6 | 22.4 | 21.4 | 28.9 | 12.1 | 11.1, 11.0 |
| 14 | 18.6 | 18.7 | 19.0 | 17.3 | 17.7 | 18.7 | 16.4 | 16.8 | 18.9 | 16.8 | 17.1, 17.0 |
| 15 | 14.2 | 14.4 | 14.6 | 14.2 | 15.4 | 14.6 | 17.9 | 16.3 | 16.3 | 16.2 | 15.3, 15.2 |
| 1′ | 166.8 | 166.9 | 167.0 | 175.9 | 176.2 | 167.0 | 169.7 | 169.0 | — | 168.5 | — |
| 2′ | 128.2 | 128.2 | 128.2 | 41.7 | 34.6 | 127.6 | 20.3 | 20.3 | — | 20.8 | — |
| 3′ | 138.1 | 138.3 | 137.8 | 26.9 | 19.4 | 138.3 | — | — | — | — | — |
| 4′ | 15.9 | 15.9 | 15.9 | 11.9 | 19.0 | 15.9 | — | — | — | — | — |
| 5′ | 20.8 | 20.8 | 20.8 | 15.5 | | 20.8 | — | — | — | — | — |

[a] Measured at 50 °C.
[b] Some peaks doubled due to the isomer.

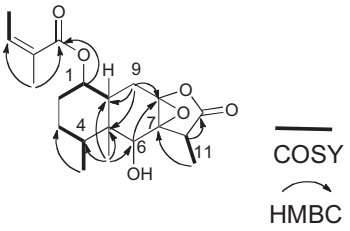



**Fig. 2.** Major HMBC correlations for **3**.

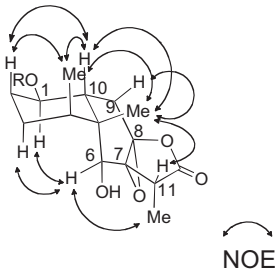



**Fig. 3.** NOEs detected for compound **3**.

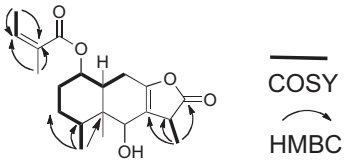



**Fig. 4.** Major HMBC correlations for **4**.

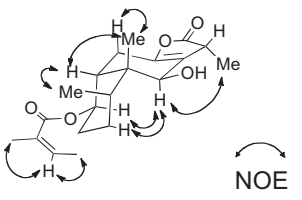



**Fig. 5.** NOEs detected for compound **4**.

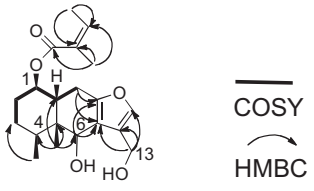



**Fig. 6.** Major HMBC correlations for **5**.

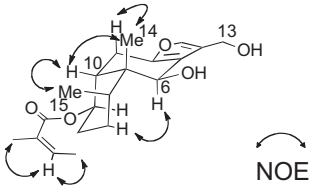



**Fig. 7.** NOEs detected for compound **5**.

NOE between H-10 and 14 indicated that the A and B rings were cis fused. The configuration of the hydroxy group at C-8 was determined to be β, because an NOE was detected between H-1β and H-14 and indicated the steroidal conformation as shown in Fig. 16.

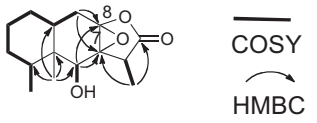



**Fig. 8.** Major HMBC correlations for **17**.

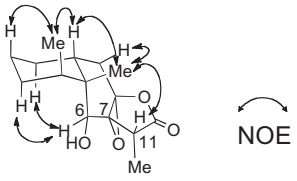



**Fig. 9.** NOEs detected for compound **17**.

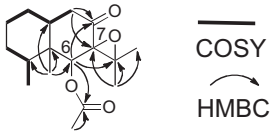



**Fig. 10.** Major HMBC correlations for **41**.

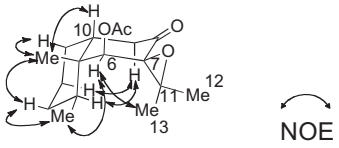



**Fig. 11.** NOEs detected for compound **41**.

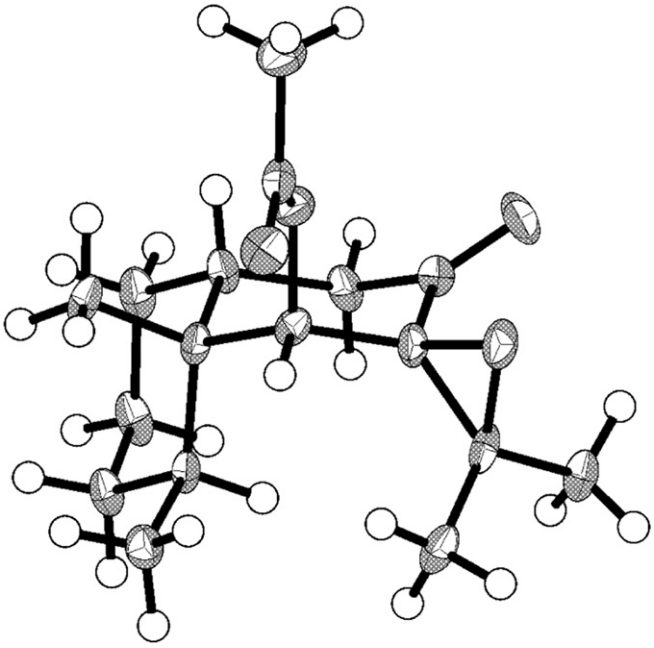


**Fig. 12.** The stereostructure of **41**.

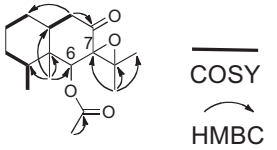



**Fig. 13.** Major HMBC correlations for **42**.

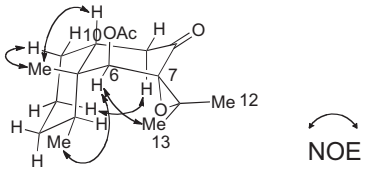



**Fig. 14.** NOEs detected for compound **42**.

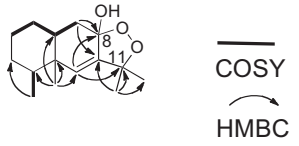



**Fig. 15.** Major HMBC correlations for **43**.

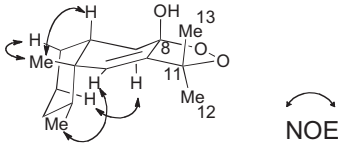



**Fig. 16.** NOEs detected for compound **43**.

With the α-configuration for the hydroxy group at C-8, the conformation would be non-steroidal.[39] Therefore, the structure of **43** was established as depicted in the formula in Fig. 16.

Compound **46** showed a quasi-molecular ion peak at *m*/*z* 309 and its molecular formula was determined to be $C_{17}H_{24}O_5$ by HRMS. The IR spectrum showed absorptions at 1767, 1745, and 1726 $cm^{-1}$, indicating the presence of a lactone (δ 174.1), an ester (δ 168.5), and a ketone (δ 203.6) (Table 5). The presence of a singlet and two doublet methyl groups as well as an acetyl group (δ 1.60) was shown by the $^1H$ NMR spectrum (Table 4). The HMBC spectrum revealed the eremophilane skeleton with a lactone between C-6 and C-12 (Fig. 17). The position of the acetoxy group was determined by the chemical shift value of C-7 (δ 87.5). An NOE between H-10 and H-14 showed that the A and B rings were cis fused (Fig. 18). From NOEs between H-1β and H-14, and between H-2α and H-9α, this compound was shown to adopt the steroidal conformation. Because an NOE was observed between H-6 and H-15, H-6 was determined to be α-equatorial. Therefore, the configuration of the acetoxy group was determined to be α-axial. The configuration of the methyl group at C-11 was shown to be β by an NOE between H-11 and H-6. Based on these data, the structure of **46** was established as depicted in the formula, the acetate of subspicatolide. Subspicatolide was previously isolated from *L. subspicata*.[8] A positive Cotton effect (+3800) at 299 nm was shown in the CD spectrum of **46**, which was very similar to that of subspicatolide. Therefore, the absolute configuration was the same as that of subspicatolide as depicted in the formula.

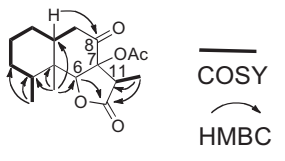



**Fig. 17.** Major HMBC correlations for **46**.

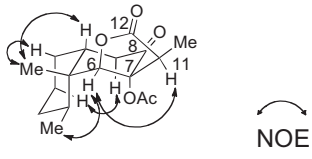



**Fig. 18.** NOEs detected for compound **46**.

Compound **51** was labile and obtained as a mixture of two isomers. The spectrum consisted of two sets of signals. The molecular formula was determined to be $C_{15}H_{22}O_4$ with 5° of unsaturation by HRCIMS. The $^1H$ NMR spectrum showed the presence of a doublet methyl, two singlet methyl groups, an aldehyde (δ 9.32, 9.35), and methine protons at δ 5.00 and 5.07. The $^{13}C$ NMR spectrum showed the presence of two carbonyl groups and a double bond (Table 5). The IR spectrum indicated the presence of a hydroxy (3340 $cm^{-1}$), a lactone (1759, 1745 $cm^{-1}$), and a formyl group (1726 $cm^{-1}$). These observations indicated that this compound was bicyclic. Connectivities were detected for H9–H10–H1 and H3–H4–H15 in the COSY spectrum (Fig. 19). The HMBC spectrum showed correlations between H-14 and C-4, 5, 6, and 10, between H-13 and C-7, 11, and 12, and between H-9 and C-7, 8, and 11 (Fig. 19). Therefore, the planar structure of **51** was established as shown in Fig. 19. Its relative configuration was determined by the NOESY spectrum, in which NOEs were observed between H-15 and H-10, H-10 and H-14, H-4 and H-6, H-6 and H-9, and H-9 and H-13 (Fig. 20). These data indicated that the formyl group adopted the α-axial orientation, and H-10, the β-axial. Compound **51** was a 1:1 mixture of the epimers at C-12.

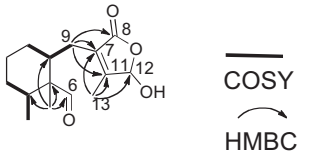



**Fig. 19.** Major HMBC correlations for **51**.

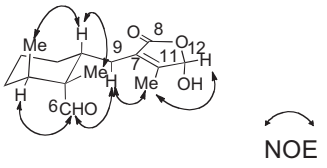



**Fig. 20.** NOEs detected for compound **51**.

Compounds **26**,[25] **40**,[35] and **47**[18] have been reported as reaction products. In the present study, they were isolated as natural products for the first time, and their spectroscopic data are recorded in the Experimental.

DNA sequences of the ITS1-5.8S-ITS2 region of the ribosomal RNA gene in the nuclear genome and the *atp*B-*rbc*L intergenic region in the plastid genome were determined. The results are shown in Table 6. When the DNA sequences of the ITS1-5.8S-ITS2 region of the present *L. lamarum* and *L. subspicata* samples and the previous *L. subspicata* samples were subjected to standard phylogenetic analyses using PAUP*,[41] they were not separated into clades. Gene introgression between the species can be inferred from the presence of many heterozygous sites in the ITS1-5.8S-ITS2 region of almost all samples and diversity in the chemicals.

## 3. Discussion

Fifty-one compounds were isolated from 12 *L. lamarum* and 7 *L. subspicata* samples. Among the 11 new compounds, **51** has an unusual carbon skeleton, the 6,7-seco-bakkane. The compound is presumably derived from a bakkane derivative, which is considered to be generated from an eremophilane derivative.[42] A plausible biosynthetic pathway is described in Fig. 21. Seco-type eremophilane compounds known so far have structures with bond fission between C-5 and C-6[43,44] or C-8 and C-9.[45–47] The compound is the first example of a C6-C7 seco-type skeleton.

**Table 6**
atpB-rbcL and ITS1-5.8S-ITS2 base sequences of *L. lamarum* and *L. subspicata* samples[a,b]

| no. | atpB-rbcL | ITS1 | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 3 | | 4 | | | | | | | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | | 2 | 2 | 2 |
| | 2 | 4 | | 0 | | 1 | 4 | 5 | 6 | 8 | 9 | 9 | 0 | 0 | 2 | 2 | 2 | 3 | 6 | 9 | 0 | 1 | 1 | | 2 | 2 | 2 |
| | 8 | 4 | T[c] | 9 | A[d] | 3 | 6 | 8 | 8 | 2 | 4 | 6 | 1 | 8 | 5 | 6 | 7 | 2 | 6 | 7 | 0 | 3 | 5 | [e] | 3 | 4 | 5 |
| 1A[f] | G | T | 9 | T | 9 | A | T | G | C | T | C | A | C | G | T | C | C | C | R | G | T | Y | C | — | T | G | G |
| 1B[g] | G | T | 9 | T | 9 | A | T | G | C | T | C | A | C | G | T | C | C | C | G | G | T | C | C | — | T | G | G |
| 1C[f] | G | T | 9 | T | 9 | A | T | G | C | T | C | A | C | G | T | C | C | C | R | G | T | Y | C | — | T | G | G |
| 2A | A | G | 9 | A | 9 | A | T | G | C | T | C | A | C | G | T | C | C | C | G | G | T | Y | C | — | T | G | K |
| 2B | A | G | 9 | A | 9 | A | T | G | C | T | C | A | C | G | T | C | C | C | G | G | T | Y | C | — | T | G | G |
| 2C | A | G | 9 | A | 9 | A | T | G | Y | T | C | A | C | G | T | C | C | C | G | G | T | Y | C | — | T | G | K |
| 3A | G | T | 9 | T | 9 | A | T | G | Y | T | Y | A | Y | G | T | C | C | C | G | G | T | C | C | — | T | G | G |
| 3B | G | T | 9 | T | 9 | A | T | G | Y | T | C | A | C | G | T | C | C | C | G | G | T | C | C | — | T | K | G |
| 3C | G | T | 9 | T | 9 | A | T | G | Y | T | Y | A | C | G | T | C | C | C | G | G | T | C | C | — | T | G | G |
| 4A | G | T | 8 | A | 10 | A | T | G | Y | T | Y | A | C | G | T | C | C | C | G | G | T | C | C | — | T | G | G |
| 4B | G | T | 8 | A | 10 | A | T | G | Y | T | Y | A | C | G | T | C | C | C | G | G | T | C | C | — | T | K | G |
| 5A | G | T | 9 | A | 10 | A | T | G | C | Y | C | A | C | G | T | C | C | C | G | G | T | C | C | — | T | G | G |
| 5B | G | T | 9 | A | 10 | A | T | G | C | Y | C | A | C | G | T | C | C | C | G | G | T | C | C | — | T | G | G |
| 6 | G | T | 9 | A | 10 | A | T | K | Y | T | C | R | C | G | T | C | C | C | G | G | T | C | C | — | T | G | G |
| 7[f] | G | T | 10 | T | 9 | A | T | G | Y | T | C | A | C | G | T | C | C | Y | G | G | T | C | C | — | T | G | G |
| 8 | G | T | 9 | A | 8 | A | T | G | C | T | C | A | C | R | T | C | C | C | G | G | A | C | Y | C | T | G | G |
| 9 | G | T | 9 | A | 10 | A | T | G | C | T | C | A | C | G | T | C | C | C | G | R | A | C | C | C | T | G | G |
| 10[f] | G | T | 8 | A | 10 | A | T | G | C | T | C | A | C | G | T | C | C | C | G | G | T | C | C | — | T | G | G |
| 11[h] | G | T | 9 | T | 9 | A | T | G | C | T | C | A | C | G | T | C | C | C | G | G | T | Y | C | — | T | G | G |
| 12[f] | G | T | 9 | A | 10 | A | T | G | C | T | C | A | C | G | T | C | C | C | G | G | T | Y | C | — | T | G | G |
| 13 | G | T | 8 | A | 10 | A | T | G | C | T | C | A | C | G | T | C | C | C | G | G | T | Y | C | — | T | G | K |
| 14[i] | G | T | 9 | T | 9 | A | T | G | C | T | C | A | C | G | T | C | C | C | G | G | T | Y | C | — | T | G | K |
| 15[j] | G | T | 9 | T | 9 | W | Y | G | C | T | C | A | C | G | Y | Y | M | C | G | G | T | Y | C | — | Y | G | K |

| | 5.8S | ITS2 | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | | | | | | | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| | 5 | | 1 | 1 | 1 | 2 | 3 | 6 | 9 | 9 | 0 | 0 | 1 | 4 | 6 | 8 | 9 | 1 |
| | 2 | 5 | 1 | 3 | 9 | 7 | 0 | 3 | 8 | 9 | 4 | 9 | 8 | 6 | 7 | 2 | 6 | 7 |
| 1A | C | C | T | C | C | Y | Y | C | C | C | C | G | T | C | T | C | C | C |
| 1B | C | C | T | C | C | Y | Y | C | C | C | C | G | T | C | T | C | C | C |
| 1C | C | C | T | C | C | Y | Y | C | C | C | C | G | T | C | T | C | C | C |
| 2A | C | C | T | C | C | Y | T | C | C | C | C | G | T | C | T | C | C | C |
| 2B | C | C | T | C | C | Y | Y | C | C | C | C | G | T | C | T | C | C | C |
| 2C | C | C | T | C | C | Y | T | C | C | C | C | G | T | C | T | C | C | C |
| 3A | C | C | T | C | C | C | Y | C | C | C | C | K | T | C | T | C | C | C |
| 3B | C | C | T | Y | C | C | Y | C | C | C | Y | G | T | C | T | C | C | C |
| 3C | C | Y | T | C | C | Y | T | C | C | C | C | G | T | C | T | C | C | C |
| 4A | C | Y | T | C | C | Y | T | C | C | C | C | G | T | C | T | C | C | C |
| 4B | C | C | T | Y | C | Y | T | C | C | C | C | G | Y | C | T | C | C | C |
| 5A | C | Y | T | C | C | Y | T | C | C | C | C | G | T | C | T | C | C | Y |
| 5B | C | T | T | C | C | Y | T | C | C | C | C | G | T | C | T | C | C | Y |
| 6 | C | Y | T | C | C | C | T | C | C | C | C | G | T | C | T | C | C | C |
| 7 | C | C | T | C | C | Y | T | Y | C | C | C | G | T | C | T | C | C | C |
| 8 | C | T | T | C | C | C | T | C | C | C | C | G | T | C | T | C | C | C |
| 9 | C | Y | T | C | C | C | T | C | C | C | C | G | T | C | Y | C | Y | C |
| 10 | C | C | T | C | C | C | Y | C | C | C | C | G | T | C | T | C | C | C |
| 11 | C | C | T | C | C | Y | T | C | C | C | C | G | T | C | T | Y | C | C |
| 12 | C | C | T | C | C | Y | T | C | C | C | C | G | T | C | T | Y | C | C |
| 13 | C | C | T | C | C | Y | T | C | C | C | C | G | T | C | T | C | C | C |
| 14 | C | C | T | C | C | Y | T | C | C | C | C | G | T | C | T | Y | C | C |
| 15 | Y | C | Y | C | Y | Y | T | C | Y | Y | C | G | T | Y | Y | C | C | C |

[a] Only the differences among the samples are shown. Numbering of the bases is based on the published atpB-rbcL sequence of L. tongolensis (Ref. 6) and on the ITS1-5.8S-ITS2 sequence of sample 7, which was deposited in the database (accession AB426703).
[b] K=G+T; M=A+C; R=A+G; S=C+G; W=A+T; Y=C+T.
[c] The number of thymines in a stretch around the 390th base.
[d] The number of adenines in a stretch around the 510th base.
[e] Base insertion between 217 and 218 of ITS1.
[f] A variant with deletion of AC at 18–19 in ITS2 was superimposed with a weaker intensity.
[g] A variant with AAA in place of AA at 172–173 in ITS2 was superimposed with a weaker intensity.
[h] A variant with TT in place of TTT at 106–108 in ITS1 and a variant with an insertion of C between 217 and 218 of ITS1 were superimposed with weaker intensities. A variant with GCG in place of ACGCC at 18–22 in ITS2 was superimposed with an equal intensity.
[i] A variant with an insertion of C between 217 and 218 of ITS1 and a variant with GCG in place of ACGCC at 18–22 in ITS2 were superimposed with weaker intensities.
[j] A variant with TTCCCC in place of TTYCCCC at 211–217 in ITS1 was superimposed with weaker intensities.

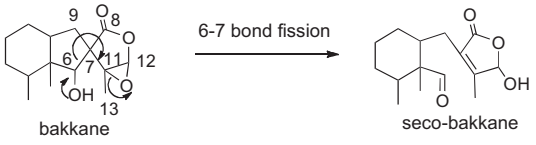



**Fig. 21.** Plausible biosynthetic route of compound **51**.

Although all the *L. subspicata* samples that we previously reported on were positive to Ehrlich's test,[8] some of the *L. subspicata* samples obtained afterwards were negative to the test and eremophilan-8-one derivatives were obtained from them as the major components (samples 1B, 10, 11, 13, and 15). The chemical composition of *L. lamarum* was very similar to that of *L. subspicata*:

eremophilan-8-one derivatives were isolated from Ehrlich-negative samples (samples 1A, 1C, and 8); subspicatins (**1**–**5**) and/or ligularol derivatives (**6**–**15**) were isolated from Ehrlich-positive samples (Table 3). This similarity is especially clear among samples collected at the same locations, when analyzed by TLC. Although some Ehrlich-positive components could not be isolated due to instability during isolation procedure, subspicatin A (**1**) was isolated commonly from the three samples collected at location 3, and fukinone (**25**) was obtained from the three samples at location 1. The similarity was observed not only in the chemical components but also in the neutral DNA sequences. The sequences of *atp*B-*rbc*L region were completely the same within each set of samples collected sympatrically (Table 6).

The chemical spectrum of the samples listed in Table 3 is continuous, e.g., both furano- and non-furanoeremophilanes are found in samples 2A-C, 3A, 4A, 4B, 5A, 5B, and 6. Our previous *L. subspicata* samples contained compounds **1**, **2**, **6**, and **22** as major components in different compositions.[8] Thus, the chemical spectrum in the samples of the two species as a whole was also continuous, which conclusion was also reached by sequencing of the ITS1-5.8S-ITS2 region.

We previously proposed a working hypothesis that plants producing furanoeremophilane are ecologically more advantageous than those producing only eremophilan-8-one derivatives.[4,5] Since furanoeremophilanes are considered to be generated from eremophilan-8-one derivatives,[48] plants producing only the latter compounds are likely to be evolutionarily older.[5] Therefore, we think that stages of evolution in root chemicals from eremophilan-8-ones to furanoeremophilanes are currently seen in *L. lamarum* and *L. subspicata*.

## 4. Conclusion

Furanoeremophilane and eremophilan-8-one derivatives were isolated as the major components from *L. lamarum* and *L. subspicata*. Eleven new compounds were obtained. One of the new compounds, **51**, had a unique seco-bakkane carbon skeleton. Each species was found to harbor chemical diversity, and the chemical spectra in the two species were overlapping. The two species were indistinguishable by DNA sequencing, and in addition, we found *L. lamarum* individuals with short ligules (samples 1C and 2C). It has been known that the formation of ligulate florets can be controlled by a single genetic locus.[49] The fact that the ribosomal RNA gene has copy variants, shown by multiple-base sites and length variations (Table 6), indicates hybridization between these plants and/or with other plants. These results indicate that *L. lamarum* and *L. subspicata* may constitute a complex with genetic exchange, resulting in their chemical similarities.

## 5. Experimental

### 5.1. General

See our previous report[3,5,6] for CD, IR, NMR, mass spectra, HPLC, column chromatography, and TLC. X-ray analysis was performed using a Bruker AXS SMART APEX II analyzer.

### 5.2. Plant materials

Samples of *L. lamarum* and *L. subspicata* were collected in August, 2004–2008 at the locations shown in Table 1 and Fig. 1. Sample 3B was collected in 2004; samples 4A and 8 were collected in 2005; sample 5A was collected in 2006; samples 1A-C, 2A-C, and 10–15 were collected in 2007; samples 3A, 3C, 6, and 7 were collected in 2008; sample 9 was collected in 2009. Each plant was identified by Xun Gong, one of the authors.

### 5.3. Extraction for Ehrlich's test

Roots (2–10 g) were extracted with ethanol immediately after harvest without drying. Solid plant materials were removed after several days, and the extracts were subjected to Ehrlich's test without concentration. See our previous report for the test on TLC.[6]

### 5.4. DNA sequencing

DNA was purified from dried leaves by use of DNeasy Plant Mini Kit (QIAGEN) and GLASSMILK (Qbiogene). DNA fragments were amplified by polymerase chain reaction (PCR) with HotStarTaq or HotStarTaq plus DNA polymerase (QIAGEN) and purified by agarose gel electrophoresis and with High Pure PCR Product Purification Kit (Roche Diagnostics). DNA sequencing was carried out with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and on a 3130xl Genetic Analyzer (Applied Biosystems). The primers used for PCR were: for the ITS1-5.8S-ITS2 region, a pair of its5m and its4[50] or a pair of LC5 and LC6;[51] for the *aptB-rbcL* intergenic region, a pair of ast-atpB and ast-rbcL[6] or a pair of La and ar.[51] The annealing temperature was 56 °C and the number of cycles was 40. Using a *L. lamarum* sample, it was confirmed that the sequencing results were the same, whether the number of cycles was 30 or 40, or whether either pair of primers was used. The primers used for DNA sequencing were: for the ITS1-5.8S-ITS2 region amplified with its5m and its4, its5m, its2B, its3, and its4;[1,50] for the ITS1-5.8S-ITS2 region amplified with LC5 and LC6, LC1, LC2, LC3, and LC4;[51] for the *aptB-rbcL* intergenic region amplified with ast-atpB and ast-rbcL, ast-atpB and ast-rbcL; for the *aptB-rbcL* intergenic region amplified with La and ar, La and ar.

### 5.5. Extraction and purification for structure determination

The roots of each sample (2–10 g) were dried and extracted with AcOEt at room temperature. Oily extracts were obtained by the standard method. Nucleosil 50-5 (4.6×250 mm) and/or TSK-GEL G1000$H_{HR}$ (7.8×300 mm) was used for HPLC.

The AcOEt extract (71.4 mg) of sample 1A was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **25** (14.4 mg) and **36** (3.1 mg).

The AcOEt extract (29.0 mg) of sample 1B was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **25** (4.8 mg), **39** (0.4 mg), **40** (0.9 mg), **41** (0.3 mg), **43** (0.4 mg), and **44** (0.4 mg).

The AcOEt extract (135.2 mg) of sample 1C was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **25** (31.1 mg), **39** (1.9 mg), **40** (0.9 mg), **41** (4.3 mg), **42** (2.1 mg), **43** (2.0 mg), **48** (0.3 mg), and **44** (0.6 mg).

The AcOEt extract (86.8 mg) of sample 2A was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **1** (2.0 mg), and **36** (1.9 mg).

The AcOEt extract (72.6 mg) of sample 2B was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **14** (0.4 mg), **15** (2.8 mg), **38** (0.8 mg), **45** (1.8 mg), and **47** (0.7 mg).

The AcOEt extract (32.5 mg) of sample 2C was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **14** (0.3 mg), **15** (0.9 mg), **17** (0.4 mg), **37** (0.3 mg), **38** (0.1 mg), **45** (0.6 mg), **46** (0.4 mg), and **51** (0.3 mg).

The AcOEt extract (209.2 mg) of sample 3A was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **1** (2.0 mg), **2** (9.8 mg), **4** (4.1 mg), **3** (1.0 mg), **6** (7.5 mg), **14** (1.8 mg), **16** (2.9 mg) **10** (0.7 mg), **11** (1.5 mg), **12** (0.2 mg), **20** and **21** (mixture 0.9 mg), **38** (9.6 mg), **49** (1.4 mg), and **50** (1.8 mg).

The AcOEt extract (672.2 mg) of sample 3B was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **1** (6.5 mg), **6** (1.9 mg), **8** (8.9 mg), **9** (13.9 mg), **18** (1.8 mg), and **19** (2.4 mg).

The AcOEt extract (218.1 mg) of sample 3C was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **1** (1.7 mg), **15** (2.6 mg), **20** and **21** (mixture 1.0 mg), and **22** (0.5 mg).

The AcOEt extract (530.0 mg) of sample 4A was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **6** (2.6 mg), **13** (3.2 mg), **31** (7.0 mg), **33** (3.4 mg), **34** (4.7 mg), **35** (3.8 mg), and **21** (4.2 mg).

The AcOEt extract (441.5 mg) of sample 5A was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **2** (12.8 mg), **4** (1.7 mg), **6** (61.9 mg), **7** (40.3 mg), **14** (1.2 mg), **15** (13.8 mg), and **38** (0.6 mg).

The AcOEt extract (994.7 mg) of sample 6 was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **1** (25.3 mg), **2** (47.0 mg), **4** (14.1 mg), **5** (9.1 mg), **6** (17.8 mg), **15** (25.7 mg), **21** (13.9 mg), **38** (11.6 mg), and **49** (2.1 mg).

The AcOEt extract (88.8 mg) of sample 7 was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **1** (20.0 mg), **2** (1.1 mg), **6** (11.0 mg), and **15** (1.8 mg).

The AcOEt extract (530.0 mg) of sample 8 was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **25** (34.4 mg).

The AcOEt extract (521.1 mg) of sample 9 was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **24** (3.2 mg), **6** (3.0 mg), **23** (4.8 mg), and **22** (22.4 mg).

The AcOEt extract (156.2 mg) of sample 10 was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) to obtain a mixture of **36** (2.4 mg), a mixture of **31** and **32** (9.2 mg, ratio 2:1), and a mixture of **34**, **35**, and **33** (64.3 mg, ratio 1:4:1).

The AcOEt extract (83.6 mg) of sample 11 was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) to obtain **28** (0.9 mg), **35** (11.3 mg), **33** (0.3 mg), a mixture of **31** and **27** (3.0 mg, ratio 2:1), and a mixture of **34** and **29** (4.5 mg, ratio 1:1).

The AcOEt extract (92.1 mg) of sample 13 was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) to obtain a mixture of **28**, **29**, and **30** (9.7 mg, ratio 5:4:2), a mixture of **34**, **35**, **33**, and **29** (8.9 mg, ratio 7:6:4:5), **35** (6.1 mg), and a mixture of **32**, **31**, and **27** (3.6 mg, ratio 1:4:4).

The AcOEt extract (379.4 mg) of sample 15 was separated by silica-gel column chromatography (Hexane–AcOEt, in gradient) followed by HPLC to isolate **25** (67.5 mg), **27** (1.7 mg), **26** (0.3 mg), **39** (2.0 mg), **40** (6.6 mg), **41** (2.7 mg), **44** (1.3 mg), and **48** (0.8 mg).

Samples 12 and 14 showed clear Ehrlich-positive TLC patterns when they were extracted and checked immediately. However, the extracts after evaporation of the solvent were deteriorated during storing.

### 5.6. Spectral data of the new compounds

$^1$H and $^{13}$C NMR spectra except for **51** are given in Tables 4 and 5, respectively. Other spectral data are as follows.

10βH,11βH-1β-angeloyloxy-7α,8α-epoxy-6β-hydroxyeremophilan-12,8β-olide (subspicatin E) (**3**): $[\alpha]_D^{23}$ −49.6 (*c* 0.09, EtOH); FTIR 3500, 1805, 1715 cm$^{-1}$; MS (CI) *m*/*z* 365 $[M+H]^+$, 347, 265, 247 (base), 83; HRMS (CI) Obs *m*/*z* 365.1966 $[M+H]^+$ (calcd for $C_{20}H_{29}O_6$ 365.1964); CD [$\theta$] (EtOH) −7620 (221 nm).

10βH,11βH-1β-angeloyloxy-6β-hydroxyeremophil-7-en-12,8-olide (subspicatin F) (**4**): $[\alpha]_D^{21}$ −50.1 (*c* 0.17, EtOH); FTIR 3500, 1800, 1760, 1710 cm$^{-1}$; MS (CI) *m*/*z* 349 $[M+H]^+$, 331, 249 (base), 231, 109; HRMS (CI) Obs *m*/*z* 349.2025 $[M+H]^+$ (calcd for $C_{20}H_{29}O_5$ 349.2015).

10βH-1β-angeloyloxyfuranoeremophilane-6β,13-diol (subspicat in *G*) (**5**): $[\alpha]_D^{22}$ −43.5 (*c* 0.12, EtOH); FTIR 3350, 1710 cm$^{-1}$; MS (CI) *m*/*z* 349 $[M+H]^+$, 331, 231, 140, 109 (base); HRMS (CI) Obs *m*/*z* 349.2023 $[M+H]^+$ (calcd for $C_{20}H_{29}O_5$ 349.2015).

10βH-6β-(2′-methylbutyryloxy)furanoeremophilane (**11**): $[\alpha]_D^{23}$ −58.2 (*c* 0.072, EtOH); FTIR 1730 cm$^{-1}$; MS (CI) *m*/*z* 318 $[M]^+$, 217 (base), 216; HRMS (CI) Obs *m*/*z* 318.2191 $[M]^+$ (calcd for $C_{20}H_{30}O_3$ 318.2194).

10βH-6β-(2′-methylpropionyloxy)furanoeremophilane (**12**): $[\alpha]_D^{23}$ −30.1 (*c* 1.14, EtOH); FTIR 1730 cm$^{-1}$; MS (CI) *m*/*z* 304 $[M]^+$, 217 (base), 216; HRMS (CI) Obs *m*/*z* 304.2048 $[M]^+$ (calcd for $C_{19}H_{28}O_3$ 304.2039).

10βH,11βH-7α,8α-epoxy-6β-hydroxyeremophilan-12,8β-olide (eremopetasitenin A4) (**17**): $[\alpha]_D^{23}$ −0.5 (*c* 0.04, EtOH); FTIR 3500, 1800 cm$^{-1}$; MS (CI) *m*/*z* 267 $[M+H]^+$, 249 (base), 221, 109; HRMS (CI) Obs *m*/*z* 267.1601 $[M+H]^+$ (calcd for $C_{15}H_{23}O_4$ 267.1596); CD [$\theta$] (EtOH) +1400 (290 nm), −5060 (220 nm).

10βH-9β-hydroxyeremophil-7(11)-en-8-one (**26**): $[\alpha]_D^{24}$ +83.3 (*c* 0.03, EtOH); MS (CI) *m*/*z* 237 $[M+H]^+$, 219 (base), 201; HRMS (CI) Obs *m*/*z* 237.1862 $[M+H]^+$ (calcd for $C_{15}H_{25}O_2$ 237.1855); $^1$H NMR (500 MHz, $C_6D_6$) $\delta$ 0.59 (3H, d, *J*=6.6 Hz, H-15), 0.65 (3H, s, H-14), 1.07 (1H, qd, *J*=12.8, 4.7 Hz, H-3), 1.21–1.27 (1H, m, H-3), 1.37–1.42 (1H, m, H-1), 1.42 (3H, d, *J*=1.5 Hz, H-13), 1.39–1.48 (1H, m, H-4), 1.50–1.56 (1H, m, H-2), 1.51–1.56 (1H, m, H-1), 1.52–1.59 (1H, m, H-10), 1.55–1.61 (1H, m, H-6), 1.84 (3H, d, *J*=2.4 Hz, H-12), 2.15–2.22 (1H, m, H-2), 2.52 (1H, d, *J*=14.7 Hz, H-6), 4.04 (1H, d, *J*=2.4 Hz, OH), 4.19 (1H, dd, *J*=11.1, 2.4 Hz, H-9); $^{13}$C NMR (125 MHz, $C_6D_6$) $\delta$ 16.5 (C-15), 20.4 (C-1), 20.7 (C-13), 20.8 (C-14), 22.2 (C-12), 22.9 (C-2), 30.9 (C-3), 32.0 (C-4), 37.7 (C-5), 41.6 (C-6), 51.3 (C-10), 73.9 (C-9), 129.8 (C-7), 139.1 (C-11), 206.0 (C-8).

10βH-8α,12-epidioxyeremophil-7(11)-en-8β-ol (**40**): $[\alpha]_D^{24}$ −54.6 (*c* 0.42, EtOH); FTIR 3480 cm$^{-1}$; MS (FAB) *m*/*z* 259 $[M+Na]^+$, 235 (base), 219, 109; HRMS (FAB) Obs *m*/*z* 259.1652 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_2Na$ 259.1674); $^1$H NMR (500 MHz, $C_6D_6$) $\delta$ 0.65 (3H, d, *J*=6.6 Hz, H-14), 0.81 (3H, s, H-14), 0.97–1.08 (1H, m, H-3), 1.02–1.09 (1H, m, H-1), 1.14–1.19 (1H, m, H-2), 1.15–1.21 (1H, m, H-3), 1.18–1.23 (1H, m, H-2), 1.24 (3H, br s, H-13), 1.35–1.44 (1H, m, H-4), 1.60 (1H, dd, *J*=13.0, 4.0 Hz, H-9), 1.57–1.68 (1H, m, H-1), 1.76 (1H, br d, *J*=14.3 Hz, H-6), 1.85 (1H, dd, *J*=13.0, 12.7 Hz, H-9), 1.90–1.97 (1H, m, H-10), 2.38 (1H, d, *J*=14.3 Hz, H-6), 3.59 (1H, dd, *J*=15.9, 2.9 Hz, H-12), 4.54 (1H, ddd, *J*=15.9, 2.9, 1.2 Hz, H-12); $^{13}$C NMR (125 MHz, $C_6D_6$) $\delta$ 13.6 (C-13), 16.6 (C-15), 20.8 (C-2), 21.2 (C-14), 26.5 (C-1), 29.2 (C-4), 30.9 (C-3), 34.6 (C-9), 36.2 (C-6), 38.0 (C-5), 39.4 (C-10), 72.9 (C-12), 97.7 (C-8), 125.1 (C-11), 128.8 (C-7).

10βH-6β-acetoxy-7β,11β-epoxyeremophilan-8-one (**41**): mp 144–147 (hexane); $[\alpha]_D^{23}$ +32.3 (*c* 0.43, EtOH); FTIR 1745, 1730, 1229 cm$^{-1}$; MS (CI) *m*/*z* 295 $[M+H]^+$, 236, 235 (base), 194; HRMS (CI) Obs *m*/*z* 295.1906 $[M+H]^+$ (calcd for $C_{17}H_{27}O_4$ 295.1910); CD [$\theta$] (EtOH) −3300 (299 nm), +1890 (243 nm), +1120 (226 nm).

10βH-6β-acetoxy-7α,11α-epoxyeremophilan-8-one (**42**): $[\alpha]_D^{24}$ +42.0 (*c* 0.21, EtOH); FTIR 1745, 1728, 1227 cm$^{-1}$; MS (CI) *m*/*z* 295 $[M+H]^+$, 236, 235 (base), 194; HRMS (CI) Obs *m*/*z* 295.1909 $[M+H]^+$ (calcd for $C_{17}H_{27}O_4$ 295.1910); CD [$\theta$] (EtOH) +1240 (305 nm), +4060 (206 nm).

10βH-8α,11-epidioxyeremophil-6-en-8β-ol (**43**): $[\alpha]_D^{23}$ +41.9 (*c* 0.20, EtOH); FTIR 3400 cm$^{-1}$; MS (FAB) *m*/*z* 275 $[M+Na]^+$, 235, 219, 154 (base), 136; HRMS (FAB) Obs *m*/*z* 275.1633 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_3Na$ 275.1623); CD [$\theta$] (EtOH) +2870 (221 nm).

10βH,11αH-7α-acetoxy-8-oxoeremophilan-12,6β-olide (subspicatolide acetate) (**46**): $[\alpha]_D^{24}$ +20.8 (*c* 0.04, EtOH); FTIR 1767, 1745, 1726 cm$^{-1}$; MS (CI) *m*/*z* 309 $[M+H]^+$, 249 (base), 231; HRMS (CI) Obs *m*/*z* 309.1699 $[M+H]^+$ (calcd for $C_{17}H_{25}O_5$ 309.1702); CD [$\theta$] (EtOH) +3800 (299 nm).

10βH-8-oxoeremophil-7(11)-en-12,6β-olide (**47**): $[\alpha]_D^{20}$ +15.9 (*c* 0.12, EtOH); FTIR 1760, 1690 cm$^{-1}$; MS (CI) *m*/*z* 249 $[M+H]^+$ (base), 231, 109; HRMS (CI) Obs *m*/*z* 249.1493 $[M+H]^+$ (calcd for $C_{15}H_{21}O_3$

249.1491); CD [$\theta$] (EtOH) +3490 (344 nm), −1130 (273 nm), −9360 (246 nm); $^1$H NMR (500 MHz, $C_6D_6$) $\delta$ 0.34 (3H, s, H-14), 0.66 (3H, d, $J$=7.1 Hz, H-15), 0.59–0.69 (1H, m, H-1), 0.86 (1H, dq, $J$=13.4, 3.2 Hz, H-1), 1.04–1.11 (1H, m, H-3), 1.07–1.15 (2H, m, H-2), 1.11–1.17 (1H, m, H-10), 1.46–1.53 (1H, m, H-3), 1.82 (1H, dd, $J$=17.4, 2.4 Hz, H-9), 1.84–1.90 (1H, m, H-4), 1.95 (1H, dd, $J$=17.4, 6.1 Hz, H-9), 1.99 (3H, d, $J$=2.4 Hz, H-13), 4.69 (1H, q, $J$=2.4 Hz, H-6); $^{13}$C NMR (125 MHz, $C_6D_6$) $\delta$ 9.6 (C-13), 14.5 (C-15), 16.5 (C-14), 20.3 (C-2), 27.8 (C-3), 29.2 (C-1), 33.7 (C-4), 35.8 (C-10), 40.5 (C-5), 45.0 (C-9), 81.8 (C-6), 130.9 (C-11), 151.4 (C-7), 172.4 (C-12), 196.1 (C-8).

12-Hydroxy-6-oxo-6,7-secobakk-7(11)-en-8,12-olide (**51**): $[\alpha]_D^{25}$ +13.9 ($c$ 0.09, EtOH); FTIR 3340, 1759, 1745, 1726 cm$^{-1}$; MS (CI) $m/z$ 267 $[M+H]^+$, 249 (base), 221; HRMS (CI) Obs $m/z$ 267.1597 $[M+H]^+$ (calcd for $C_{15}H_{23}O_4$ 267.1596); $^1$H NMR (400 MHz, $C_6D_6$) $\delta$ 0.71 (3H, d, 6.7, H-15) and 0.72 (3H, d, 6.8, H-15), 0.84, and 0.85 (3H, s, H-14), 0.98–1.03 (m, H-3a), 1.15–1.24 (m, H-2a), 1.23–1.34 (4H, m, H-1, 2b, 3b), 1.37, and 1.38 (3H, s, H-13), 1.76–1.83 (m, H-4), 1.84–1.93 (m, H-10), 2.16–2.20 (m, H-9a), 2.26 (dd, 13.7, 10.5, H-9b), and 2.27 (dd, 13.7, 10.8, H-9b), 2.44 (br s, OH), 5.00, and 5.07 (s, H-12), 9.35 and 9.32 (s, H-6).

### 5.7. X-ray crystallographic analysis of compound 41

All diagrams and calculations were performed using maXus (Bruker Nonius, Delft and MacScience, Japan). Mo K$\alpha$ radiation, $k$=0.71073 Å, Data collection: DIP Image plate, Program(s) used to refine structure: SHELXL-97 (Sheldrick, 1997); refinement on F2, full matrix least squares refinement. Crystal data: monoclinic, p21, $a$=7.697(3) Å, $b$=10.222(3) Å, $c$=10.402(3) Å, $\alpha$=90°, $\beta$=99.627(3)°, $\gamma$=90°, $V$=1155(2) Å$^3$, $R$=0.1126. Crystallographic data for compound **41** have been deposited at the Cambridge Crystallographic Data Center as supplementary publication number CCDC 803640. Copies of the data can be obtained, free of charge, via www.ccdc.cam.ac.uk/data_request/cif, or by mailing to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336033 or e-mail: data_request@ccdc.cam.ac.uk).

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