Terpenoids from the *in vitro* Cultured Liverwort *Riella helicophylla*§

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Z. Naturforsch. 54c, 997–1004 (1999); received September 2, 1999

* Riella, Liverwort, Menthan, Monoterpen Peroxide, Kaurane, Labdane, Phytane

The liverwort *Riella helicophylla* was cultivated *in vitro* under aseptic conditions. The lipophilic extract of the plant material yielded seventeen monoterpenes and eleven diterpenes. Seven monoterpenes were hydroperoxides. From the diterpenes six belonged to the labdane type skeleton and one to the kaurane type, the other diterpenes were phytane derivatives.

**Introduction**

The liverwort *Riella helicophylla* (Borg et Montagne) Montagne is native to the western Mediterranean area (Spain, Algeria, Tunisia). It grows in saline lakes (pH 7.8–8) in a depth of about 70 cm (Müller, 1954). The propagation of the plant is either sexually by spores or vegetatively by gametophytes. Because of its morphology and good regenerative power *Riella helicophylla* has been the subject of many ontogenetic and physiological studies (e.g. Stange, 1977; Witt, 1992).

Little is known about the chemistry of this tiny plant. In earlier studies lunularic acid (Grotha and Schmidt, 1976) have been detected and the GC analysis of the essential oil led to the identification of *p*-mentha-1,3,5-triene-7-ol, first isolated from *Citrus reticulata* (Kugler and Kovats, 1963) is new for liverworts. The spectroscopic data from *Riella helicophylla* could be proven by comparison of its optical rotation ([α]20°D = +20°) with those of the *4R*- form, which could be proven by comparison of its optical rotation ([α]20°D = +20°) with those of the *4R*- and *4S*- enantiomers synthesised by Delay and Ohloff (1979). The hydroperoxides 2, 4-hydroperoxy-*p*-mentha-1,8-dien, and 4, 8-hydroperoxy-*p*-mentha-1,3,5-trien have been recently described by Buchanan et al. (1998) from the liverwort *Jungermannia obovata*. Compound 3, *p*-mentha-1,3,5-triene-8-ol, first isolated from *Citrus reticulata* (Kugler and Kovats, 1963) is new for liverworts. The spectroscopic data from 6 were in agreement with *p*-mentha-1,3,5-triene-2,8-diol, first isolated from *Lavandula gibsonii* (Patwardhan and Gupta, 1983). 13, *p*-menth-2-en-1α, 4β, 8- triol, was known from *Asiasari radix* (Yahara et al., 1990) and the tris nor monoterpensoid 16, 4-hydroxy-4-methyl-cyclohex-2-en-1-one, a degradation product of the hydroperoxide ascaridole had been isolated by Connolly (1990) for the first time.

Among the diterpenes 16-kaurene (18) was identified by GC-MS and compared with literature data (Stenhagen et al., 1974). It has been previously described in various liverwort species (Asakawa, 1995). A second diterpene hydrocar-

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§ Publication No. 140 in the series “Arbeitskreis Chemie und Biologie der Moose”.

0939–5075/99/1200–0997 $ 06.00 © 1999 Verlag der Zeitschrift für Naturforschung, Tübingen · www.znaturforsch.com · D
bon, labda-8(17),13(16),14-triene (19) was identified through its $^1$H-NMR spectrum in comparison with the respective 19-carboxy acid (Carman and Deeth, 1971. 8a,15-dihydroxy-13-labdene (20) and 3β, 8α,15-trihydroxy-13-labdene 21 were characterised through their $^1$H NMR and $^{13}$C NMR data (Forster $et$ $al.$, 1985). The 3β-acetoxy derivative (22) and the 3β,8α,15-trihydroxy-13-labdene 21 were characterised through their $^1$H NMR and $^{13}$C NMR data (Forster $et$ $al.$, 1985). The 3β-acetoxy derivative (22) and the 3β,15-diacetoxy derivative (23) of 21 were found to be new natural products.

The $^1$H NMR and $^{13}$C NMR data of 24 were identical with 19-acetoxy-8a,15-dihydroxy-13-labdene previously isolated from Juniperus sabina (Feliciano $et$ $al.$, 1991). The following phytane derivatives were detected and characterised as 2-phyten-1-ol (25), (2E, 2′E)-phyt-2′-eny phyt-2-enoate 26 (Spörle $et$ $al.$, 1991), (2E)-phyt-2-enyl phytanoate 27 (Buchanan $et$ $al.$, 1995) and (2E)-phyt-2-enyl hexadecanoate 28 (Rasool $et$ $al.$, 1991).

Compound 5 showed on TLC a positive reaction with each of three hydroperoxide specific spray reagents (Rieche and Schulz, 1958; Abraham $et$ $al.$, 1957; Huber and Fröhlike, 1972). Its $^1$H NMR revealed the signals of a 2,5 disubstituted cyclohexa-1,4-diene (δH 5.77, H-2, and δH 5.45, H-5, both br s; δH 2.68, m, 4H, 2H-3 and 2H-6 ), three singlet methyls of which one (δH = 1.66, 3H-7) is linked to a double bond and two (δH 1.33, 6H, 3H-9 and 3H-10) belong to a dimethyl carbinol and one proton at δH 7.48 (s), exchangeable with D2O. On the basis of the above evidence compound 5 is 8-hydroperoxy-β-mentha-1,4-diene. 5 is not very stable. After a few days in solution, its $^1$H NMR showed the presence of 8-hydroperoxy-β-mentha-1,3,5-trien (4), due to the aromatisation of the cyclohexa-1,4-diene ring. Its EIMS spectrum only showed the molecular ion peak of the dehydrated product at $m/z = 166$ (4%) with a base peak at $m/z = 133$ which is indicative for a loss of an OOH group.

Compound 7, 8-hydroperoxy-p-mentha-1,3,5-trien-2-ol, also gave a positive reaction with the hydroperoxide spray reagents. Its $^1$H NMR spectrum was nearly identical with that of p-mentha-1,3,5-triene-2,8-diol (6), however an additional proton signal appeared at δH 7.27, indicating a hydroperoxide group. The position of the hydroperoxide at C-8 could easily be concluded from its $^{13}$C NMR spectrum, in which C-8 appears at δC 83.8, in good accordance to the chemical shift of the hydroperoxyl substituted C-8 in 5 (see Table I).

Compound 8 was obtained as a colourless oil. The molecular formula, C10H16O3, was determined by DCI mass spectrometry ([M+H]+ $m/z$ 185). Its $^1$H and $^{13}$C NMR spectra (Table I) revealed a derivative of the known monoterpene peroxide ascarirole (Nitz $et$ $al.$, 1989; Bohlmann and Zeisberg, 1974) with an additional hydroxyl group at C-8 (δC 72.4, 5, C-8; δH 1.28, 6H, 5, 3H-9 and 3H-10). Therefore 8 could be deduced as 8-hydroxyascaridole.

The structure of compound 9 followed immediately from the comparison of its $^1$H and $^{13}$C NMR spectra with those of 8. The downfield shift of C-8 (δC 83.9, ΔδC 8 11.8) in the $^{13}$C NMR spectrum and the appearance of a singlet at δH 8.18, exchangeable with D2O, in the $^1$H NMR spectrum proved 9 to be 8-hydroperoxyascaridole. The DCI mass spectrum with ions at $m/z$ 201 ([M+H]+) and $m/z$ 168 ([M+H]+-OOH) supported the proposed structure.

Table I. $^{13}$C NMR spectral data for compounds 5, 7–11, 14, (CDCl3).

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<td>24.4 q</td>
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All assignments were confirmed by DEPT measurements.  
$a, b$ Values may be interchanged within the same column.
EIMS of 10 ([M]+ at m/z 168) led to the molecular formula C_{10}H_{16}O_{2}. The $^{13}$C NMR spectrum displayed resonances for ten carbons, including a trisubstituted double bond ($\delta_C$ 140.5, s, C-1; $\delta_C$ 122.3, d, C-2) two oxygenated quaternary ($\delta_C$ 67.6 and 64.0, C-4 and C-8) and one oxygenated secondary carbon ($\delta_C$ 65.2, C-3), two methylenes and three methyl groups. The $^1$H NMR spectrum, recorded in CDCl$_3$, revealed the presence of a vinyl methyl group ($\delta_H$ 1.71, 3H-7) and two singlet methyls ($\delta_H$ 1.35, 6H, 3H-9 and 3H-10) of a geminal dimethyl carbinol. The vinyl proton ($\delta_H$ 5.52, H-2) shows a coupling ($J$ 4.9 Hz) to the oxygen bearing methine at $\delta_H$ 3.90 (H-3). It is evident from these spectral data that 10 is a trioxygenated $p$-menth-1-ene with one hydroxyl and one ether or epoxide function located at C-3, C-4 or C-8. The position of the hydroxyl group could be taken

![Chemical structures](image-url)
from the $^1$H spectrum recorded in acetone-$d_6$, where the OH proton was visible ($\delta_{\text{H}} = 3.40$, br s) and showed a weak coupling to H-3 ($\delta_{\text{H}} = 3.82$, br d) in the H-H-COSY. Consequently C-4 and C-8 belong to an epoxide and 10 therefore represents 4,8-epoxy-3-hydroxy-$\beta$-menth-1-ene. Because of its instability the relative configuration of 10 remains undetermined.

The molecular formula of 11 ([M]$^+$ at m/z = 168) was determined by EIMS. Its $^1$H and $^{13}$C NMR spectra displayed one exomethylene, one trisubstituted double bond, a secondary and a tertiary alcohol, two methylenes together with a vinyl methyl and a methyl carbinol. This assumption led to a derivative of $\beta$-mentha-3,8-dien-1-ol with an additional hydroxyl group. Its position at C-2 followed immediately from its multiplicy and that of the vinyl proton H-3 which both appeared as broad singlets in the $^1$H NMR spectrum indicating that they are vicinal neighbours with a dihedral angle of ca 90°. Due to the instability of 11 the relative configuration of $\beta$-mentha-3,8-dien-1,2-diol could not be determined.

Compound 12 gave positive reactions with the peroxide reagents. The compound was unstable, however the structure could be determined from its $^1$H NMR spectrum, which gave rise to a monoterpenic skeleton with a $\beta$-menthene skeleton. At $\delta_{\text{H}} = 7.38$ and $\delta_{\text{H}} = 7.34$ two peroxide protons appear as singlets. The chemical shift of the methyl group H-7 ($\delta_{\text{H}} = 1.33$, 3H, s) places one peroxide at position C-1. The signals at $\delta_{\text{H}} = 5.04$ and $\delta_{\text{H}} = 5.07$ (H-9a and H-9b, both s) belong to an exomethylene which is part of an isopropylidenic group. The corresponding methyl group appears at 1.83 ppm (H-10, 3H, s). The remaining signals reveal two methylene groups ($\delta_{\text{H}} = 2.01$ and 1.80, both 2H, both m, 2H-5 and 2H-6) and a cis configured double bond ($\delta_{\text{H}} = 6.03$ and 5.89, both d, H-2 and H-3, $J_{2,3} = 10.3$ Hz). These data can only be correlated with 1,4-dihyroperoxy-$\beta$-mentha-2,8-diene.

MS and $^1$H NMR spectra of 14 are nearly identical to those of $\beta$-menth-2-en-1$\alpha$, 4$\beta$, 8-triol (13), however the optical rotation of both compounds are quite different (14: $[\alpha]_D^{20} = +$ 50°; 13: $[\alpha]_D^{20} = +$ 2.3°) and the $^{13}$C NMR shifts of C-2, C-3, C-6, C-7 and C-8 differ about 2–3 ppm. These facts indicate that 14 is a diastereomer of 13. The hydroxyl groups at C-1 and C-4 in 14 should be cis orientated in contrast to 13 were they are trans.

The $^1$H NMR data of 15 led to $\beta$-meth-2-en-1$\alpha$, 2$\alpha$, 8-triol. Its $\beta$-acetyl derivative had already been described from Asiasari radix (Yahara et al. 1990). The cis orientation of the hydroxyls at C-1 and C-2, based on the correlation between methyl H-7 and H-2 in the NOESY spectrum, is in agreement with the published structure.

Apart from the known 4-hydroxy-$\beta$-menth-2-en-1-ol 16 (Connolly, 1990) we found a similar compound 17 as a minor component. In contrast to 16, compound 17 gave a positive reaction in our peroxide tests and its $^1$H NMR showed an additional hydroperoxy proton at $\delta_{\text{H}} = 7.68$ (s). Therefore 17 is 4-hydroperoxy-4-methyl-cyclohex-2-en-1-ol.

It is known that terpene hydroperoxides are formed from unsaturated terpenes in the presence of light, oxygen and chlorophyll. Therefore the question arose if the isolated products were genuine or artefacts. To test this fresh plant material was extracted with dichloromethane in the dark and chlorophyll omitted by gelfiltration with Sephadex LH 20. Fractions containing monoterpenes were chromatographed on TLC silica plates with n-hexane/ethylacetate (80:20 v/v) together with the isolated compounds. The plates were sprayed with the peroxide reagents mentioned above. The test proved that the peroxides were present in the extract. A further evidence that the peroxides are genuine is the amount isolated in relation to the respective alcohol. E.g. 4-hydroperoxy-$\beta$-mentha-1,8-dien (2) was isolated in an amount of 80 mg compared to 3 mg of $\beta$-mentha-1,8-dien-4-ol (1).

**Experimental**

**Plant material**

Gemmae of female plants from *Riella helicophylla* (Borg et Montagne) Montagne were kindly provided by Prof. Stange, Kassel, Germany and cultivated aseptically in 1 l glass cylinders covered with a glass lid. The medium was according to Viell (1983). The plants were kept for 4 to 5 weeks in 2000 lux 12 h/12 h dark at 20 °C. The plants were harvested separately from the gemmae and dried at room temperature with a fan. The gemmae were used as seed material for new cultures. The plant material was stored at -15 °C before extraction. A voucher specimen is retained in the department of Pharmacognosy and Analytical Phytochemistry, University of Saarland, Saarbrücken, Germany.
Extraction and isolation

General: All HPLC separations were performed isocratic. The composition of the mobile phase is given as v/v (in parenthesis).

260 g of *R. helicophylla* were pulverised and successively extracted with diethyl ether and CH$_2$Cl$_2$. Since the diethyl ether and the CH$_2$Cl$_2$ extracts exhibited identical TLC and HPLC chromatograms, they were combined to yield 15.8 g crude lipophilic extract. This extract was subjected to SEC on Sephadex LH 20 using CH$_2$Cl$_2$/MeOH (1:1) as mobile phase to give five main fractions (I-V). Fraction I mainly consisted of chlorophyll, carotenoids and fat. Fraction II was rechromatographed under the same conditions to yield a chlorophyll free fraction. This fraction was subjected to VLC on silica gel in a n-hexane/ethyl acetate gradient (0–100%) to yield 10 subfractions II.2.1–10. Fraction II.2.1, containing the hydrocarbons,
was separated by HPLC on silica gel (100% n-hexane) to yield 19 (2 mg). GC-MS of the same fraction led to 18. HPLC of fraction II.2.2 (silica gel, n-hexane/EtOAc 99.5/0.5) resulted in 26 (10 mg), 27 (3.3 mg) and 28 (2.4 mg). Fraction II.2.4 was found to be pure 25 (252 mg). HPLC of fraction II.2.6 (silica gel, n-hexane/EtOAc 98/2) gave 10 (18.5 mg) and 23 (3.5 mg). Fraction II.2.7 was separated on DIOIL modified silica gel via HPLC (n-hexane/EtOAc 75/25) to give 20 (123.5 mg). HPLC on CN modified silica gel led for fraction II.2.8 (n-hexane/EtOAc 75/25) to 22 (126.5 mg) and 24 (62 mg) and for fraction II.2.9 (n-hexane/EtOAc 70/30) to 21 (46.5 mg). Fraction III was further separated by VLC (silica gel, n-hexane/ethyl acetate gradient, 0–100% EtOAc) to give 3 (44.5 mg) and 7 subfractions III.1-7. Fraction III.1 upon HPLC (silica gel, n-hexane/EtOAc 95/5) gave 1 (3 mg), 2 (80 mg) 4 (20.5 mg) and 5 (4 mg). Fraction III.3 was almost pure and gave 9 (23 mg) after HPLC on silica gel (n-hexane/EtOAc 80/20). Fraction III.4 was purified by HPLC (Si, n-hexane/EtOAc 75/25) and gave rise to 7 (2 mg). 8 (6 mg), 12 (3 mg) and 17 (1 mg). Fraction III.5 on HPLC (Si, n-hexane/EtOAc 70/30) gave 6 (2 mg). Fraction III.6 was separated with HPLC on CN modified silica gel (n-hexane/EtOAc 85/15) and gave rise to 11 (3 mg) and 16 (7.5 mg). HPLC on DIOIL modified silica gel led for fraction III.7 (n-hexane/EtOAc 50/50) to 14 (3 mg) and for fraction III.8 (n-hexane/EtOAc 50/50) to 13 (4 mg) and 15 (2.5 mg).

Spectroscopic methods

NMR-spectroscopy: BRUKER AM 400, CDCl3, ambient temperature, 400 MHz (1H), 100 MHz (13C); chemical shifts are given in δ values (ppm) relative to CHCl3 at δH 7.24 or CDCl3 at δC 77.0 and acetone-d6 at δH 2.04. mass spectrometry: VARIAN MAT 311 (DCI); GC-MS (EIMS) was performed on a HP-1 capillary column with a G 1800A GCD (HP).

Spectroscopic data

8-Hydroxyascaridole (8): colourless oil, [δ]230° = +3.2° (c = 0.5); 1H NMR (CDCl3): δH 6.66 (d, J 8.6 Hz, H-3), 6.42 (d, J 8.6 Hz, H-2), 2.3-1.4 (m, 2H-5 and 2H-6), 1.38 (s, 3H-7), 1.28 (s, 3H-9 and 3H-10); 13C NMR: see Table I; DCIMS, m/z (rel. int.) = 185 (11) [M+H]+, 167 (43), 151 (100), 151 (87), 137 (85), 133 (54), 111 (43), 110 (84), 109 (100), 59 (65).

8-Hydroxyascaridole (9): colourless oil, 1H NMR (CDCl3): δH 6.65 (d, J 8.6 Hz, H-3), 6.42 (d, J 8.6 Hz, H-2), 2.3-1.4 (m, 2H-5 and 2H-6), 1.36, 1.35 and 1.31 (all s, 3H-7, 3H-9 and 3H-10); 13C NMR: see Table I; DCIMS, m/z (rel. int.) = 201 (9) [M+H]+, 183 (63), 168 (86), 167 (70), 152 (84), 151 (98), 149 (77), 136 (82), 135 (100), 133 (88), 109 (74).

8-Hydroxypimara-8,14-diene (10): colourless oil, [δ]230° = -0.5° (c = 1.5); 1H NMR (CDCl3): δH 6.52 (d, J 4.9 Hz, H-2), 2.27 (m, H-6a), 2.08 (m, H-6b), 1.71 (s, 3H-7), 1.48 (m, 2H-5), 1.35 (s, 3H-9 and 3H-10); 13C NMR: see Table I; DCIMS, m/z (rel. int.) = 168 (57) [M]+, 107 (45), 97 (60), 84 (69), 83 (57), 80 (100), 70 (52), 69 (38), 67 (67), 55 (40).

1,4-Dihydroxy-pimara-8,14-diene (11): colourless oil, 1H NMR (CDCl3): δH 5.72 (br s, H-3), 5.05 (s, H-9a), 4.96 (s, H-9b), 4.19 (br s, H-2), 2.40 (d-dd, J 17.3, 2.3 Hz, H-5), 2.29 (d-dd, J 17.3, 8.9, 2.3 Hz, H-5b), 1.91 (s, 3H-7), 1.20 (s, 3H-10); 13C NMR: see Table I; DCIMS, m/z (rel. int.) = 168 (7) [M]+, 126 (1), 125 (100), 111 (12), 110 (42), 97 (25), 83 (15), 67 (62), 68 (9), 55 (8).

1,4-Dihydroxy-pimara-8,14-diene (12): colourless oil, 1H NMR (CDCl3): δH 7.38 and 7.34 (both s, both OOH), 6.03 and 5.89 (both d, J 10.3 Hz, H-2 and H-3), 5.07 and 5.04 (both s, H-9a and H-9b), 2.01 and 1.80 (both m, 2H-5 and 2H-6), 1.83 (s, 3H-10), 1.33 (s, 3H-7).

p-Mentha-3,8-dien-1,2-diol (13): colourless oil, 1H NMR (CDCl3): δH 7.24 (s, H-3), 7.24 (s, H-2), 5.05 (s, H-9a), 4.96 (s, H-9b), 4.19 (br s, H-2), 2.40 (d-dd, J 17.3, 2.3 Hz, H-5), 2.29 (d-dd, J 17.3, 8.9, 2.3 Hz, H-5b), 1.91 (s, 3H-7), 1.20 (s, 3H-10); 13C NMR: see Table I; DCIMS, m/z (rel. int.) = 168 (7) [M]+, 126 (1), 125 (100), 111 (12), 110 (42), 97 (25), 83 (15), 67 (62), 68 (9), 55 (8).

5:8-Epoxy-pimara-8,14-diene (14): colourless oil, 1H NMR (CDCl3): δH 6.71 (7H, 3H-7, 3H-9 and 3H-10); 13C NMR: see Table I; DCIMS, m/z (rel. int.) = 168 (7) [M]+, 126 (1), 125 (100), 111 (12), 110 (42), 97 (25), 83 (15), 67 (62), 68 (9), 55 (8).
p-Methyl-2-en-1a, 2a, 8-triol (15): colourless oil, $[\alpha]_D^{20} = +8.3^\circ$ (c = 0.25); $^1$H NMR (CDCl$_3$): $\delta_H$ 5.71 (d, $J$ 1.7 Hz, H-3), 3.86 (d, $J$ 1.7 Hz, H-2), 2.28, 2.05, 1.82, 1.58 (m, H-5a, H-5b, H-6a, H-6b) 1.32 (s, 3H-9 and 3H-10), 1.19 (s, 3H-7); DCIMS, $m/z$ (rel. int.) = 185 (5) [M+H]$^+$, 169 (30), 151 (100), 133 (29), 126 (35), 123 (30), 110 (47), 109 (37), 107 (51), 95 (44).

4-Hydroperoxy-4-methyl-cyclohex-2-en-1-one (17): colourless oil, $[\alpha]_D^{20} = -100^\circ$ (c = 0.05); $^1$H NMR (CDCl$_3$): $\delta_H$ 7.68 (s, OOH), 6.83 (d, $J$ 10.3 Hz, H-3), 6.00 (d, $J$ 10.3 Hz, H-2), 2.67 (m, H-6a), 2.37 (m, H-6b), 2.00 (m, 2H-5), 1.44 (s, 3H-7); DCIMS, $m/z$ (rel. int.) = 143 (66) [M+H]$^+$, 127 (100), 109 (92), 98 (53), 81 (80).

Acknowledgement
We thank Prof. Stange, Kassel, Germany, for providing gemmae of R. helicophylla and Dr. J. Zapp, Saarbrücken, Germany, for running NMR-spectra. Financial support of BMBF is acknowledged.


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