PERGAMON

Phytochemistry 53 (2000) 645–650

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Biosynthesis of the gymnomitrane-type sesquiterpenes in liverworts

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Received 7 September 1999; received in revised form 29 October 1999

Abstract

The biosynthesis of gymnomitranes in the liverworts *Reboulia hemisphaerica* (L.) Raddi and *Bazzania trilobata* (L.) Gray was investigated. Labelling experiments with 1-^{13}C-, 2-^{13}C- and 6,6-D_2 glucose, 2,2-D_2- and 4,4-D_2 mevalolactone and tissue cultures of the liverworts were carried out.

Keywords: *Reboulia hemisphaerica*; Marchantiales; *Bazzania trilobata*; Jungermanniales; Liverworts; Sesquiterpenes; Gymnomitranes; Biosynthesis; Labelling experiments

1. Introduction

We have investigated the biosynthesis of the gymnomitranes in the liverworts *Reboulia hemisphaerica* and *Bazzania trilobata*. *R. hemisphaerica* is a thalloid liverwort (*Hepaticae*) of the order Marchantiales and *B. trilobata* is a leafy liverwort of the order Jungermanniales (Frahm & Frey, 1992).

The gymnomitranes are tricyclic sesquiterpenes, which are known as characteristic constituents of liverworts (Huneck & Andersen, 1977). So far only some vague ideas concerning the biosynthesis of the gymnomitranes were discussed in the literature (Connolly, Harding & Thornton, 1974). Since cuparanes and gymnomitranes were often found as constituents of the same plant, the cuparanes were assumed as precursors of the gymnomitranes.

Our investigation of the biosynthesis of the gymnomitranes consists of two parts: the biosynthesis of isopentenyl diphosphate (IDP) and the cyclisation of farnesyl diphosphate (FDP) or nerolidyl diphosphate (NDP). According to present knowledge IDP can be biosynthesised via two different pathways: the classical acetate–mevalonate pathway or the triose–pyruvate pathway. In the past, the acetate–mevalonate pathway has been accepted as the only route leading to IDP. Recently the new triose–pyruvate pathway has been discovered. In higher plants IDP is biosynthesised alternatively via both pathways (Schwarz, 1994; Lichtenthaler, Rohmer & Schwender, 1997; Lichtenthaler, 1998). Accordingly, sesquiterpenes, triterpenes and steroids are biosynthesised via the classical acetate–mevalonate pathway in the cytoplasma, whereas monoterpenes, diterpenes, carotinoids and plastoquinone-9 are formed via the triose–pyruvate pathway in the plastides. The same compartmentation was found in the red alga *Cyanidium caldarium* (Lichtenthaler, 1998) and in the liverworts *Ricciocarpos natans* and *Conocephalum conicum* (Thiel, Adam, Zapp & Becker, 1997). In the green algae *Chorella*, *Scenedesmus* and *Chlamydomonas*, all terpenoids are biosynthesised via the triose–pyruvate pathway (Schwender, Seemann, Lichtenthaler & Rohmer, 1996).

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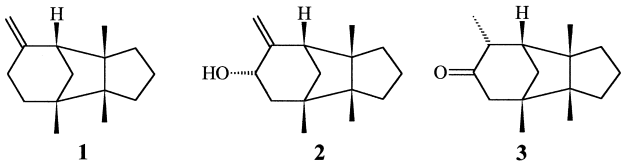
0031-9422/00/$ - see front matter
PII: S0031-9422(99)00587-7

2. Results and discussion

The sesquiterpene constituents of *R. hemisphaerica* (Morais, Harrison & Becker, 1988; Morais, Harrison & Becker, 1991; Morais & Becker, 1991; Warmers & König, 1999a) and *B. trilobata* (Asakawa & Heidelberger, 1982; Asakawa, 1995; Warmers & König, 1999b) have been investigated before. Some of the major constituents were sesquiterpenes with gymnomitrane skeleton: β-barbatene (**1**), gymnomitr-3(15)-en-4α-ol (**2**) and gymnomitran-4-one (**3**) in *R. hemisphaerica* and β-barbatene (**1**) in *B. trilobata* (Scheme 1).

Tissue cultures of both liverworts could be obtained by sterilizing the carefully cleaned plant material and placing it upon a tissue culture medium. The tissue culture of *R. hemisphaerica* was partly self-prepared and partly obtained from Prof. Dr. H. Becker, University of Saarbrücken (Germany). *R. hemisphaerica* was was cultivated on a solid, modified B5 medium with D-glucose (Morais & Becker, 1991). *B. trilobata* was cultivated on a solid, modified Knop medium and as a liquid culture in autoclaved water. Labelled precursors were added to the media of the tissue cultures. These labelled precursors were incorporated by the plant and consumed for the biosynthesis of the sesquiterpenes. Detailed information concerning the biosynthesis of the gymnomitranes could be obtained by inspection of the labelling patterns in the sesquiterpenes. The liverworts were submitted to hydrodistillation and the essential oils were analysed by GC–MS. After isolation of individual compounds by preparative GC the labelled gymnomitranes were investigated by ^{13}C- or ^{2}D NMR. The labelling patterns of the sesquiterpenes were determined by comparison of their NMR spectra with those of the unlabelled compounds, which had been investigated before by several NMR experiments (^{1}H, ^{13}C, DEPT, COSY GS, HMBC, HMQC, NOESY) to fully assign individual signals to the corresponding atoms of the compounds.

As precursors for the labelling experiments 1-^{13}C-, 2-^{13}C- and 6,6-D_2 glucose as well as 2,2-D_2- and 4,4-D_2 mevalolactone (MVL) were used. The labelled glucose was metabolised to pyruvate, glyceraldehyde and acetyl CoA. Using labelled glucose it is possible to distinguish between the two different pathways to IDP. The labelled MVL is only consumed for the biosynthesis of sesquiterpenes in case IDP is biosynthesised via the acetate–mevalonate pathway. The labelled MVL was prepared according to literature reports (Cane & Levin, 1976; Schwarz, 1994).



Scheme 1.

The first labelling experiment was performed with *R. hemisphaerica* and 2,2-D_2- and 4,4-D_2 MVL. The mass spectra of the corresponding sesquiterpenes showed a low labelling rate of only 5%. Two explanations are possible: MVL was not a precursor of the sesquiterpenes and IDP was biosynthesised via the triose–pyruvate pathway or MVL was not incorporated by the plant.

To distinguish between the two different pathways to IDP the labelling experiment was repeated with 1-^{13}C-, 2-^{13}C- and 6,6-D_2 glucose. The labelled gymnomitranes were investigated by NMR. The ^{13}C NMR spectra of the ^{13}C labelled gymnomitranes (**1**, **2**, **3**) clearly showed an increase of the intensities of certain signals compared with those of the unlabelled compounds. The ^{2}D NMR spectrum of the deuterium labelled gymnomitr-3(15)-en-4α-ol (**2**) showed signals for deuterium at the same chemical shifts as the corresponding protons. In Fig. 1 the labelling patterns for the gymnomitranes (**1**, **2**, **3**) as results of the labelling experiments with 1-^{13}C- (A), 2-^{13}C- (B) and 6,6-D_2 glucose (C) are shown.

These labelling patterns could be explained by a biosynthesis of IDP via the classical acetate–mevalonate pathway with mevalonic acid (MVA) as intermediate (Fig. 2).

For the formation of the gymnomitranes several cyclisation- and rearrangement reactions from farnesyl diphosphate (FDP) or nerolidyl diphosphate (NDP) can be anticipated. The initial step was the isomerisation of FDP to NDP, which could adopt the appropriate conformation for further cyclisation reactions. Ionisation of NDP could generate a cation at C-1, which is captured by the 6,7-double bond. The obtained cation at C-7 is attacked by the 10,11-double bond. These cyclisation reactions are followed by a 1,4-hydride shift from C-6 to C-10 and two 1,2-methyl migrations from C-7 to C-6 and from C-11 to C-7. Up to this step the biosynthesis of the gymnomitranes proceeds analogously to the well-known biosynthesis of trichodiene (Cane &

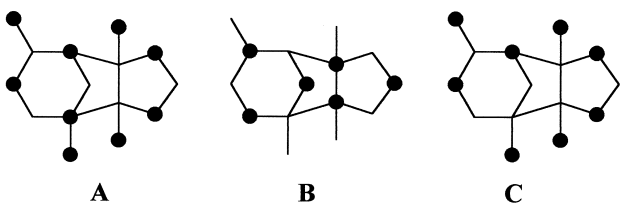


Fig. 1. Labelling patterns of the gymnomitranes resulting by labelling experiments with 1-^{13}C- (A), 2-^{13}C- (B) and 6,6-D_2 glucose (C). ● labelled position.

Ha, 1986, 1988; Cane & Xue, 1996). The cation at C-11 is captured by the 2,3-double bond. The loss of a proton from carbon C-15 yields **1**, further enzymatic oxidation **2** and a rearrangement reaction affords **3** (Fig. 3).

As expected the reactions proceeded stereoselectively. Of special interest was the hydride shift from carbon C-6 to carbon C-10. This shift was proved by the labelling pattern of **2**. The ^{2}D NMR spectrum of the deuterium labelled compound showed that both hydrogen atoms of the methylene group CH_2-10 were labelled. As one hydrogen atom of the methylene group CH_2-10 had nearly the same chemical shift as one of the hydrogen atoms of the methylene group CH_2-9, the assignment of the labelled atom was not clear, but a label in the methylene group CH_2-9 could not be explained by any cyclisation mechanism. The labelling of both hydrogen atoms of the methylene group CH_2-10 was a result of the hydride shift from carbon C-6 to carbon C-10. The spatial relationship between both groups made a chemical reaction highly unlikely and the hydride had to be transferred by an enzyme.

Although the biosynthesis of IDP in *R. hemisphaerica* followed the classical acetate–mevalonate pathway, the labelling rate of the sesquiterpenes was low using MVL as precursor. This could be explained by a low incorporation of MVL by *R. hemisphaerica*. To find out whether or not the low incorporation of MVL is a general problem of liverworts the labelling experiment was repeated with *B. trilobata* and 2,2-D_2- and 4,4-D_2 MVL. The mass spectra of the sesquiterpenes showed a higher labelling rate of 15%. This result confirms that the rate of incorporation of labelled MVL by intact liverworts is generally low. Nevertheless, the occurrence of the acetate–mevalonate pathway can be considered as a fact.

3. Experimental

3.1. NMR spectroscopy

NMR measurements were carried out with a Bruker WM 500-, a Bruker WM 400- or a Varian AM 360 instrument. For the ^{13}C NMR spectra of the labelled compounds the relative intensities referring to the corresponding signals of the unlabelled compounds were reported. The signal intensities were obtained by integration. In some cases the signal intensities of the quaternary carbons were too small.

3.2. Mass spectrometry

GC–MS measurements were run on a HP 5890 gas chromatograph coupled to a VG Analytical 70-250S mass spectrometer.

3.3. Plant material

Fresh plant material of *Reboulia hemisphaerica* was collected at the river Argout near Burlats and Les Salvages (France) by U. Heseler, St. Ingbert. A tissue culture of *R. hemisphaerica* was obtained from Prof. Dr. H. Becker, Universität des Saarlandes, Saarbrücken, Germany. *R. hemisphaerica* was identified by Prof. Dr. R. Mues, Universität des Saarlandes, Saarbrücken, Germany. *Bazzania trilobata* was collected near Gaggenau in the Black Forest (Germany) and identified by Dr. H. Muhle, Universität Ulm, Germany.

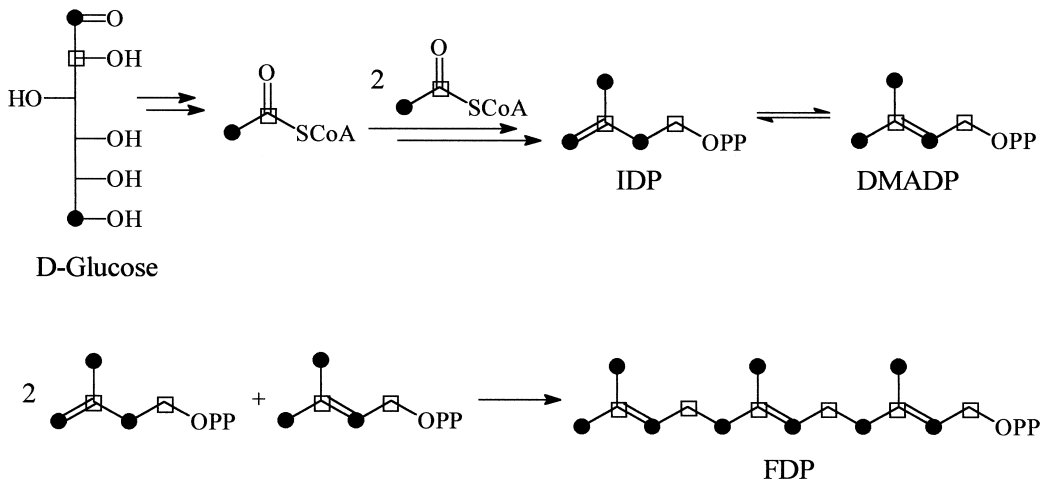


Fig. 2. Biosynthesis of isopentenyl diphosphate (IDP) and farnesyl diphosphate (FDP) via the classical acetate–mevalonate pathway. ● labelling patterns resulting from the labelling experiments with 1-^{13}C- and with 6,6-D_2 glucose, □ labelling patterns resulting from the labelling experiment with 2-^{13}C glucose.

3.4. Preparation of tissue cultures of Reboulia hemisphaerica and Bazzania trilobata

Fresh plant material was cleaned carefully. Single plants were washed 90–120 s with 1% natrium hypochlorite solution. The sterile plants were washed three times with autoclaved water and were placed upon the tissue culture medium.

3.5. Tissue culture of Reboulia hemisphaerica

The tissue culture of *R. hemisphaerica* was partly self-made and partly obtained from Prof. Dr. H. Becker, Universität des Saarlandes, Saarbrücken. *R. hemisphaerica* was cultivated on a solid, modified B5 medium with 5 g D-glucose and 9 g agar per litre medium.

3.6. Tissue culture of Bazzania trilobata

The tissue culture of *B. trilobata* was self-made. *B. trilobata* was cultivated on a solid, modified Knop medium with 9 g agar per litre medium and as a liquid culture in autoclaved water.

3.7. Tissue culture conditions

The tissue cultures were grown at 25°C and under a light–dark change after 12 h. The liquid cultures were placed upon a shaker.

3.8. Labelling experiments with Reboulia hemisphaerica

To the medium of the tissue culture of *R. hemisphaerica* were added 1-^{13}C glucose, 2-^{13}C glucose and 6,6-D_2 glucose. The amount was 5 g glucose per litre

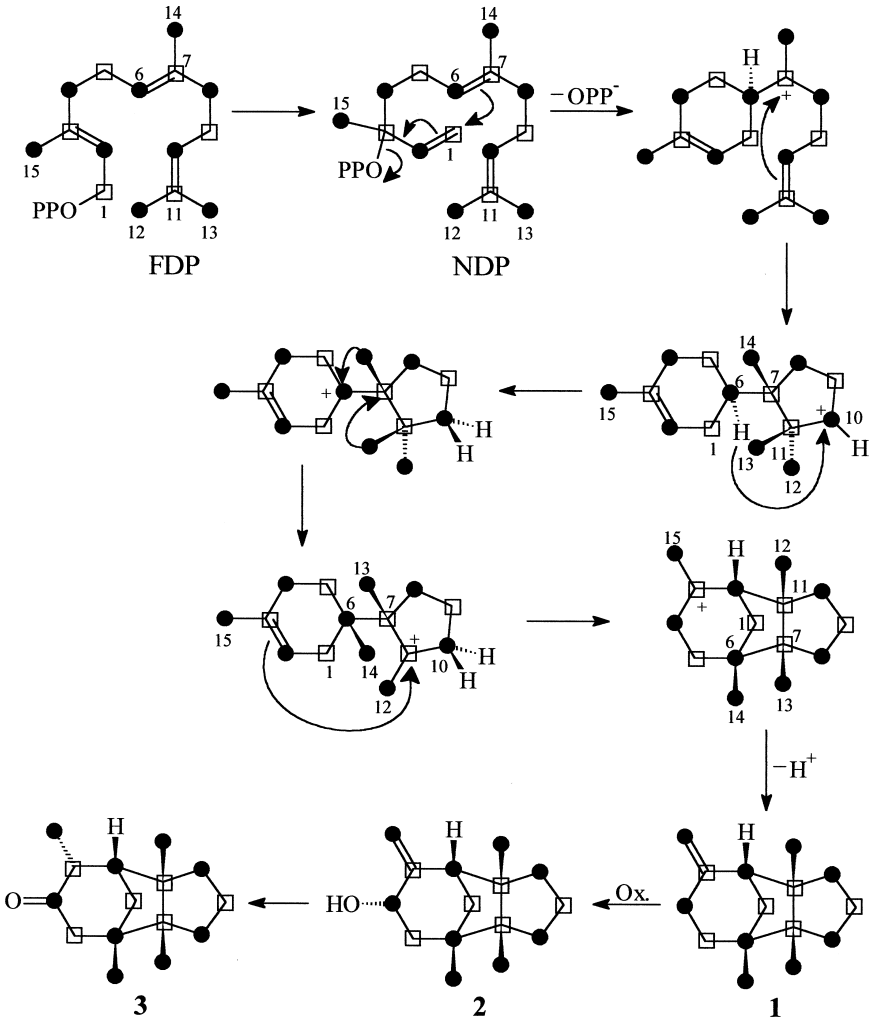


Fig. 3. Biosynthesis of the gymnomitranes (**1**, **2**, **3**) from farnesyl diphosphate (FDP) and nerolidyl diphosphate (NDP). ● labelling patterns resulting from the labelling experiments with 1-^{13}C- and with 6,6-D_2 glucose, □ labelling patterns resulting from the labelling experiment with 2-^{13}C glucose.

medium; in the case of ^{2}D labelled glucose only the labelled glucose was added, in the case of the ^{13}C labelled glucose the labelled glucose was diluted with unlabelled glucose in a ratio 1:4. The liverworts grew 6 weeks upon these media.

4,4-D_2 mevalolactone and 2,2-D_2 mevalolactone were added in amounts of 1 g per litre medium. The liverwort grew 3 months upon these media.

3.9. Labelling experiments with Bazzania trilobata

To the liquid culture of *B. trilobata* were added 1 g per litre medium 4,4-D_2 mevalolactone and 2,2-D_2 mevalolactone. The liverwort grew 3 months upon these media.

3.10. Labelled glucose

The labelled glucose was obtained from the company Isotec.

3.11. Labelled mevalolactone

The labelled mevalolactone was synthesised following a procedure described in the literature (Cane & Levin, 1976; Schwarz, 1994).

3.12. Hydrodistillation

The essential oils were prepared by hydrodistillation (2 h) of aqueous homogenates of fresh and green plants using *n*-hexane as collection solvent.

3.13. Isolation

The isolation was carried out by preparative GC.

3.14. Preparative GC

Modified Varian 1400 instrument, equipped with a stainless steel column (1.85 m × 4.3 mm) with 6.4% polydimethylsiloxane SE 30 on Chromosorb W-HP; flame ionization detection; helium as carrier gas at a flow rate of 240 ml/min (Hardt & König, 1994).

3.15. Unlabelled β-barbatene (**1**)

MS (EI, 70 eV): *m/z* (rel. int.) 204 (2) [M^+], 189 (6), 133 (6), 119 (9), 111 (31), 109 (24), 108 (66), 107 (22), 96 (88), 95 (67), 94 (43), 93 (100), 91 (33), 81 (70), 79 (44), 77 (25), 69 (23), 67 (21), 55 (32), 41 (38); ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.84 (3H, *s*, H-14), 0.90 (3H, *s*, H-13), 1.03 (3H, *s*, H-12), 1.06–1.11 (1H, *m*, H-8β), 1.15–1.19 (1H, *m*, H-10α/β), 1.30 (1H, *d*, *J* = 11.2 Hz, H-1β), 1.38–1.45 (1H, *m*, H-5β), 1.68–1.78 (2H, *m*, H-5α, H-10α/β), 1.76–1.82 (2H, *m*, H-9α, H-9β), 1.88–1.91 (1H, *m*, H-8α), 2.04–2.08 (1H, *m*, H-1α), 2.15 (1H, *d*, *J* = 4.6 Hz, H-2), 2.22 (1H, *dd*, *J* = 8.2, 16.5 Hz, H-4β), 2.40–2.50 (1H, *m*, H-4α), 4.57 (1H, *t* (*dd*), *J* = 2.6 Hz, H-15α), 4.60 (1H, *t* (*dd*), *J* = 2.6 Hz, H-15β); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ (ppm) 23.4 (*q*, C-13), 24.8 (*q*, C-14), 27.5 (*t*, C-9), 27.5 (*q*, C-12), 28.7 (*t*, C-4), 35.6 (*t*, C-8), 37.1 (*t*, C-10), 38.1 (*t*, C-5), 43.1 (*s*, C-6), 46.8 (*t*, C-1), 54.2 (*s*, C-7), 55.4 (*s*, C-11), 56.0 (*d*, C-2), 107.5 (*t*, C-15), 152.0 (*s*, C-3).

3.16. Unlabelled gymnomitr-3(15)-en-4α-ol (**2**)

MS (EI, 70 eV): *m/z* (rel. int.) 220 (2) [M^+], 202 (4), 187 (7), 137 (10), 123 (63), 109 (21), 107 (21), 106 (56), 105 (15), 96 (61), 95 (100), 94 (21), 93 (16), 91 (56), 81 (67), 79 (23), 67 (18), 55 (28), 41 (29); ^{1}H NMR (500 MHz, C_6D_6): δ (ppm) 0.79 (3H, *s*, H-13), 0.85 (3H, *s*, H-14), 0.96 (3H, *s*, H-12), 0.92–1.03 (1H, *m*, H-8β), 1.05–1.20 (2H, *m*, H-9α/β, H-10β), 1.32–1.42 (3H, *m*, H-1α, H-1β, H-9α/β), 1.55–1.65 (2H, *m*, H-5α, H-5β), 1.71–1.75 (1H, *m*, H-8α), 1.92–1.96 (1H, *m*, H-10α), 2.22 (1H, *d*, *J* = 3.8 Hz, H-2), 4.16–4.23 (1H, *m*, H-4), 4.87 (1H, *s*, H-15α), 5.31 (1H, *s*, H-15β); ^{13}C NMR (125 MHz, C_6D_6): δ (ppm) 23.8 (*q*, C-13), 25.3 (*t*, C-9), 26.1 (*q*, C-14), 27.7 (*q*, C-12), 37.2 (*t*, C-1), 38.1 (*t*, C-8), 38.7 (*t*, C-10), 41.8 (*t*, C-5), 45.3 (*s*, C-6), 53.7 (*s*, C-7), 54.0 (*d*, C-2), 55.1 (*s*, C-11), 66.4 (*d*, C-4), 109.3 (*t*, C-15), 151.9 (*s*, C-3).

3.17. Unlabelled gymnomitran-4-one (**3**)

MS (EI, 70 eV): *m/z* (rel. int.) 220 (11) [M^+], 205 (5), 165 (6), 137 (89), 136 (19), 125 (18), 124 (27), 123 (48), 121 (18), 109 (24), 107 (15), 97 (27), 96 (51), 95 (75), 81 (100), 67 (27), 55 (55), 41 (42); ^{1}H NMR (500 MHz, C_6D_6): δ (ppm) 0.71 (3H, *s*, H-14), 0.74 (3H, *s*, H-13), 0.87 (3H, *s*, H-12), 0.92–0.98 (1H, *m*, H-8β), 1.11 (1H, *ddt* (*dddd*), *J* = 1.6, 5.7, 12.5 Hz, H-10β), 1.23 (3H, *d*, *J* = 7.0 Hz, H-15), 1.32 (1H, *d*, *J* = 12.0 Hz, H-1β), 1.43–1.56 (2H, *m*, H-2, H-9β), 1.55–1.66 (1H, *m*, H-9α), 1.63–1.76 (1H, *m*, H-8α), 1.72–1.86 (1H, *m*, H-10α), 1.87 (1H, *dd*, *J* = 1.3, 16.4 Hz, H-5β), 1.96–2.02 (1H, *m*, H-1α), 2.00–2.08 (1H, *s*, H-3), 2.58 (1H, *dd*, *J* = 3.2, 16.4 Hz, H-5α); ^{13}C NMR (125 MHz, C_6D_6): δ (ppm) 14.4 (*q*, C-15), 22.5 (*q*, C-13), 23.3 (*q*, C-14), 28.1 (*t*, C-9), 28.4 (*q*, C-12), 35.8 (*t*, C-8), 38.4 (*t*, C-10), 46.7 (*s*, C-6), 47.6 (*t*, C-1), 50.7 (*d*, C-3), 51.1 (*d*, C-2), 54.7 (*t*, C-5), 55.7 (*s*, C-11), 56.2 (*s*, C-7), 210.5 (*s*, C-4).

3.18. 1-^{13}C Glucose labelled β-barbatene (**1a**) of Reboulia hemisphaerica

^{13}C NMR (100.6 MHz, $CDCl_3$): δ (ppm, relative signal intensity) 23.4 (1.78, C-13), 24.8 (1.65, C-14), 27.5

(1.28, C-9, C-12), 28.7 (1.42, C-4), 35.6 (1.68, C-8), 37.1 (1.98, C-10), 38.1 (1.00, C-5), 43.1 (1.77, C-6), 46.8 (0.94, C-1), 54.2 (C-7), 55.4 (C-11), 56.0 (1.80, C-2), 107.5 (1.71, C-15), 152.0 (C-3).

*3.19. 1-^{13}C Glucose labelled gymnomitr-3(15)-en-4α-ol (**2a**) of Reboulia hemisphaerica*

^{13}C NMR (125 MHz, C_6D_6): δ (ppm, relative signal intensity) 23.8 (1.88, C-13), 25.3 (1.00, C-9), 26.1 (1.90, C-14), 27.7 (1.97, C-12), 37.2 (1.00, C-1), 38.1 (1.90, C-8), 38.7 (1.86, C-10), 41.8 (0.96, C-5), 45.3 (1.69, C-6), 53.7 (0.93, C-7), 54.0 (1.83, C-2), 55.1 (0.88, C-11), 66.4 (1.96, C-4), 109.3 (1.98, C-15), 151.9 (1.18, C-3).

*3.20. 1-^{13}C Glucose labelled gymnomitran-4-one (**3a**) of Reboulia hemisphaerica*

^{13}C NMR (125 MHz, C_6D_6): δ (ppm, relative signal intensity) 14.4 (1.30, C-15), 22.5 (1.64, C-13), 23.3 (3.60, C-14), 28.1 (1.00, C-9), 28.4 (1.86, C-12), 35.8 (1.93, C-8), 38.4 (2.03, C-10), 46.7 (1.18, C-6), 47.6 (1.11, C-1), 50.7 (0.90, C-3), 51.1 (1.69, C-2), 54.7 (0.88, C-5), 55.7 (0.90, C-11), 56.2 (0.92, C-7), 210.5 (1.85, C-4).

*3.21. 2-^{13}C Glucose labelled β-barbatene (**1b**) of Reboulia hemisphaerica*

^{13}C NMR (100.6 MHz, $CDCl_3$): δ (ppm, relative signal intensity) 23.4 (1.00, C-13), 24.8 (1.14, C-14), 27.5 (1.58, C-9, C-12), 28.7 (0.98, C-4), 35.6 (1.03, C-8), 37.1 (1.03, C-10), 38.1 (2.40, C-5), 43.1 (0.87, C-6), 46.8 (2.58, C-1), 54.2 (3.08, C-7), 55.4 (3.33, C-11), 56.0 (0.91, C-2), 107.5 (1.07, C-15), 152.0 (2.46, C-3).

*3.22. 2-^{13}C Glucose labelled gymnomitr-3(15)-en-4α-ol (**2b**) of Reboulia hemisphaerica*

^{13}C NMR (125 MHz, C_6D_6): δ (ppm, relative signal intensity) 23.8 (1.00, C-13), 25.3 (2.21, C-9), 26.1 (1.06, C-14), 27.7 (1.09, C-12), 37.2 (2.12, C-1), 38.1 (1.01, C-8), 38.7 (0.97, C-10), 41.8 (2.21, C-5), 45.3 (0.81, C-6), 53.7 (1.79, C-7), 54.0 (0.99, C-2), 55.1 (2.12, C-11), 66.4 (1.01, C-4), 109.3 (1.01, C-15), 151.9 (2.68, C-3).

*3.23. 2-^{13}C Glucose labelled gymnomitran-4-one (**3b**) of Reboulia hemisphaerica*

^{13}C NMR (125 MHz, C_6D_6): δ (ppm, relative signal intensity) 14.4 (1.00, C-15), 22.5 (1.17, C-13), 23.3 (1.53, C-14), 28.1 (2.78, C-9), 28.4 (1.49, C-12), 35.8 (1.40, C-8), 38.4 (1.30, C-10), 46.7 (1.16, C-6), 47.6 (1.99, C-1), 50.7 (2.05, C-3), 51.1 (1.14, C-2), 54.7 (2.22, C-5), 55.7 (1.98, C-11), 56.2 (2.65, C-7), 210.5 (C-4).

*3.24. 6,6-D_2 Glucose labelled gymnomitr-3(15)-en-4α-ol (**2c**) of Reboulia hemisphaerica*

^{2}D NMR (30.7 MHz, C_6H_6): δ (ppm) 0.71 (D-13), 0.80 (D-14), 0.85 (D-12), 0.85 (D-8β, covered), 1.12 (D-10α/β), 1.69 (D-8α), 1.92 (D-10α/β), 2.17 (D-2), 4.21 (D-4), 4.87 (D-15α), 5.38 (D-15β).

Acknowledgements

We thank Prof. Dr. H. Becker, Prof. Dr. R. Mues, U. Heseler and Dr. H. Muhle for the plant material and Dr. V. Sinnwell and Dr. E. Haupt, Universität Hamburg, and their teams for the NMR measurements and Prof. Dr. H.-P. Mühlbach and Prof. Dr. E. Pratje, Universität Hamburg, for the possibility of using their equipment for tissue cultures.

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