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Biosynthesis of the sesquiterpene hodgsonox from the New Zealand liverwort *Lepidolaena hodgsoniae*

Anna J. Barlow[a], Stephen D. Lorimer[b], Ed R. Morgan[c], Rex T. Weavers[a,*]

[a]*Department of Chemistry, University of Otago, PO Box 56, Dunedin, New Zealand*
[b]*Plant Extracts Research Unit, New Zealand Institute for Crop and Food Research Limited, Department of Chemistry, University of Otago, PO Box 56, Dunedin, New Zealand*
[c]*New Zealand Institute for Crop and Food Research Limited, Private Bag 11600, Palmerston North, New Zealand*

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Abstract

The incorporation of [1-^{13}C] labelled glucose into hodgsonox, a sesquiterpene epoxide with a unique, doubly allylic ether functionality has been studied in axenic cultures of the liverwort *Lepidolaena hodgsoniae*. Quantitative ^{13}C NMR spectroscopic analysis showed that the isoprene units are derived exclusively from the methylerythritol phosphate pathway.

Keywords: *Lepidolaena hodgsoniae*; Lepidolaenaceae; Biosynthesis; Isopentenyl diphosphate; Methylerythritol phosphate pathway; Sesquiterpenoid; Isoprenoids; ^{13}C-labelling; Quantitative ^{13}C NMR spectroscopy; Liverwort tissue culture

1. Introduction

The terpenoids, the largest group of secondary metabolites with over 30,000 known compounds (Dictionary of Natural Products, 2000), are built up by combination of units of isopentenyl diphosphate (IPP), and its double bond isomer dimethylallyl diphosphate (DMAPP). Two biosynthetic pathways are now accepted for the production of isoprenoid units in plants and micro-organisms: the mevalonic acid (MVA) pathway, which has been implicated in the biosynthesis of sesquiterpenoids and steroids; and the methylerythritol phosphate (MEP) pathway, which has been shown to be responsible for the formation of various hemi-, mono- and diterpenoids (for reviews see Eisenreich et al., 1998; Lichtenthaler, 1999; Lichtenthaler et al., 1997a; Rohmer, 1999; Rohmer et al., 1993). The currently accepted hypothesis is that the two pathways operate in different cellular compartments, with the MVA pathway located in the cytoplasm, and the MEP pathway in the plastids (Lichtenthaler et al., 1997b). However, segregation is not necessarily complete, and exchange of metabolites between the two pools has been observed in plants and plant cell cultures (Adam et al., 1999; Adam and Zapp, 1998; Arigoni et al., 1997; Nabeta et al., 1995; Piel et al., 1998).

Recent investigations have shown that the MEP pathway is at least partially responsible for the production of sesquiterpenoids in some organisms. The most compelling example of this is the report that the biosynthesis of the sesquiterpene germacrene D (**1**), isolated from the higher plant *Solidago canadensis*, takes place predominantly via the MEP pathway (Steliopoulos et al., 2002). In addition, several examples of sesquiterpenoids of mixed MVA and MEP biosynthetic origin have been reported (Adam et al., 1999; Adam and Zapp, 1998; Piel et al., 1998). These results suggest further investigation is necessary to determine the extent of operation of the MEP pathway for the production of sesquiterpenoids in plants and micro-organisms.

As a further chapter in this study of sesquiterpene biosynthesis we chose to investigate the origins of the insecticidal sesquiterpenoid hodgsonox (**2**), isolated from the New Zealand liverwort *Lepidolaena hodgsoniae* Grolle. This compound contains a unique doubly allylic ether unit and is the first example of a new class of sesquiterpenoids that may arise from the azulene-type skeleton (**3**) (Ainge et al., 2001). Some natural products have been proposed to have this carbon framework, but

* Corresponding author. Tel.: +64-3479-7925; fax: +64-3479-7906.
E-mail address: rweavers@alkali.otago.ac.nz (R.T. Weavers).

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the structures of most of these have subsequently been disproved and the remainder remain untested (Chiurdoglu and Decot, 1958; Marshall et al., 1967; Marshall and Johnson, 1967; Takahashi, 1968; Weyerstahl et al., 2000a,b,c).

To investigate the biosynthetic origin of isoprenoid building blocks of hodgsonox (**2**), the pathway-independent precursor [1-^{13}C]glucose, which produces distinctly different labelling patterns of the individual isoprene units for the MEP and MVA pathways was employed (Rohmer, 1999). Predicted patterns for these two pathways are shown in Fig. 1.

Axenic cultures of liverworts are useful experimental systems for such biosynthetic studies. Due to their small size, the cultures can be grown fully differentiated in vitro. As has been shown previously, the cultures maintain their capability of synthesising the whole range of isoprenoids, similar to natural conditions. Moreover, labelled precursors can be administered to the plants via the culture medium, similar to cell culture systems. The simple morphology of liverwort tissue cultures supports the uptake of the respective compounds under physiological conditions, in contrast to cell cultures where the dedifferentiated status of the cells might have a drastic impact on many biochemical processes (Becker, 1994).

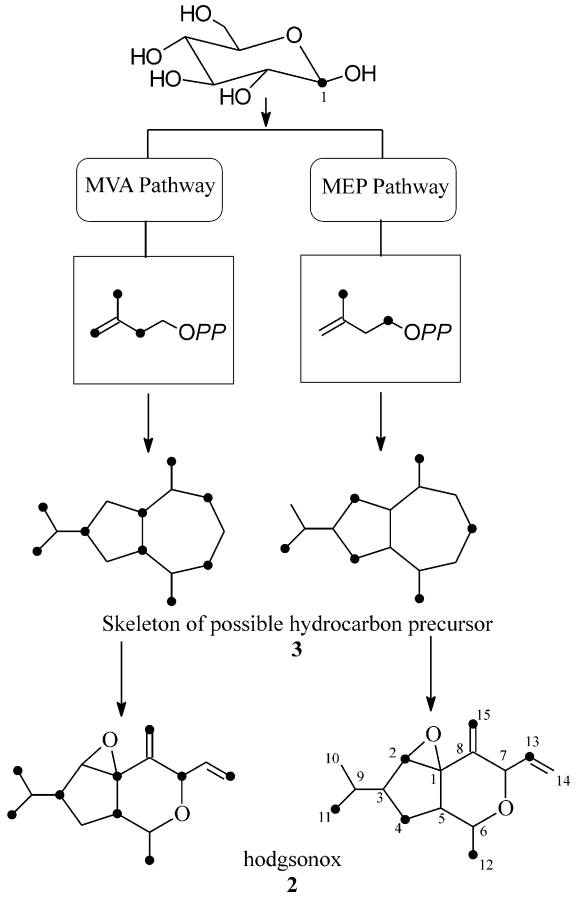
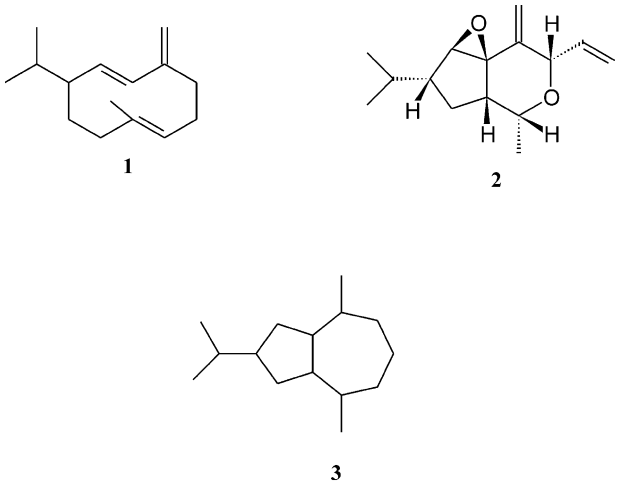


Fig. 1. Possible hodgsonox biosynthesis from [1-^{13}C]glucose showing predicted labelling patterns.

Therefore, ^{13}C labelling experiments on axenic cultures should give a realistic impression of the biosynthetic origin of the sesquiterpene hodgsonox (**2**) in *L. hodgsoniae*.



2. Results and discussion

The biosynthetic feeding experiment with axenic cultures of *L. hodgsoniae* was conducted with [1-^{13}C]glucose following previously established methods (Barlow et al., 2001). The extract from the labelling experiment was separated by silica gel column chromatography as previously described (Ainge et al., 2001); hodgsonox-containing fractions were identified by characteristic purple TLC spots when visualised with vanillin. Fractions were combined on this basis, and the sample of labelled hodgsonox (**2**) was analysed by ^{1}H and ^{13}C NMR spectroscopy. The chemical shifts matched those previously published (Ainge et al., 2001).

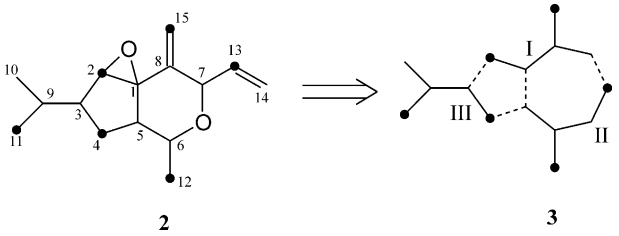
The ^{13}C-labelling pattern for hodgsonox (**2**) was determined by quantitative ^{13}C NMR spectroscopy (Barlow et al., 2001). The absolute percentage of ^{13}C label abundance at C-2 was determined as 5.12% on the basis of ^{13}C satellite analysis for the epoxidic proton (H-2) in the ^{1}H NMR spectrum. The ^{13}C incorporations of the remaining carbons relative to C2 were determined by integration of the ^{13}C NMR spectrum collected in the presence of $Cr(acac)_3$ using the inverse-gated decoupling pulse sequence (Braun et al., 1996). The resulting labelling pattern was confirmed by a second qualitative experiment involving comparison of ^{13}C NMR spectra of samples of the labelled and unlabelled compound prepared under identical conditions.

Hodgsonox (**2**) showed only two labelled carbons per isoprene unit (Table 1, Fig. 2), with an average ^{13}C abundance of 4.85±0.65%. The remaining carbons displayed an average ^{13}C abundance of 1.64±0.52%, compared to the expected natural abundance of 1.11%. This increased background labelling could be explained by complex metabolic turnover of [1-^{3}C]glucose during

Table 1
^{13}C abundances of labelled hodgsonox (**2**)

Carbon atom in IPP-units[a]	Carbon atom	% ^{13}C[b]
1^{I}	2	**5.12**[c]
2^{I}	1	2.18
3^{I}	8	2.08
4^{I}	7	1.51
5^{I}	15	**4.51**
1^{II}	13	**4.90**
2^{II}	14	2.05
3^{II}	6	1.04[d]
4^{II}	5	1.64
5^{II}	12	**4.14**
1^{III}	4	**5.96**
2^{III}	3	1.63
3^{III}	9	0.63[d]
4^{III}	10	1.96
5^{III}	11	**4.44**

[a] I, II, III: denote individual C_5 units (Fig. 2).
[b] ^{13}C abundance obtained from ^{13}C satellite analysis of H-2.
[c] **Bold type**: positions of enrichment.
[d] Close to solvent peaks, ^{13}C level determined with reduced certainty.



2 **3**

Fig. 2. Observed labelling pattern for hodgsonox (**2**).

the growth period, giving rise to give a statistical distribution of labelled carbons in various positions of intermediates of the carbohydrate metabolism (such as metabolism of glucose to carbon dioxide and subsequent assimilation via the Calvin cycle) (Luckner, 1990).

The ratio of ^{13}C levels for specifically labelled:unlabelled carbons was 2.96:1. The labelling pattern demonstrated in Fig. 2 is only consistent with hodgsonox (**2**) being biosynthesised via the MEP pathway, with no detectable contribution from the MVA pathway (Fig. 1). This finding is of considerable importance, signifying hodgsonox (**2**) to be the first example of a sesquiterpenoid biosynthesised via the MEP pathway from a liverwort. It is our plan to further investigate the proposed intermediacy of carbon skeleton (**3**) by conducting experiments with [U–^{13}C]glucose.

3. Experimental

3.1. Spectroscopy

NMR spectra were recorded in $CDCl_3$ at 298 K on a Varian INOVA-500 spectrometer at 11.74 T, operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C respectively, using an Autoswitchable 5 mm Z-axis PFG direct probe. ^{1}H spectra were acquired using sixteen 59° pulses of 5.7 μs and a relaxation delay of 1 s. Spectra were collected over a 6500 Hz window with 32 K data points giving a peak resolution accurate to 0.5 Hz. 1024 scans were obtained. Quantitative ^{13}C NMR measurements were determined using the inverse-gated decoupling pulse sequence employing a 54° pulse of 4.7 μs and relaxation delay of 5 s, in the presence of 0.1 M Cr(acac)$_3$ (Braun et al., 1996). Spectra were collected over a 30 kHz window with 64 K data points, 12,000 scans were obtained. Line broadening was set at 0.5. Chemical shifts are given in ppm on the δ scale and were referenced to $CDCl_3$ solvent peaks at 7.25 ppm (^{1}H) and 77.0 ppm (^{13}C). For integration, the signal:noise ratio was at least 13:1. Qualitative ^{13}C NMR measurements of an unlabelled and a labelled sample of hodgsonox were recorded under exactly the same conditions; labelled carbons were determined by visual comparison of the two spectra.

3.2. Labelled glucose

[1-^{13}C]glucose was purchased from Isotec, Miamisburg, Ohio.

3.3. Plant material

Lepidolaena hodgsoniae (PERU Voucher # 001127-01) was collected from Mt Aspiring National Park, Haast Pass, South Island, New Zealand in November 2000 (Department of Conservation Collection Permit #03, 2000). The gametophyte plants from these collection trips were maintained in Petri dishes of distilled water in the laboratory until used to initiate in vitro cultures.

3.4. In vitro propagation of Lepidolaena hodgsoniae

Apical portions of gametophytes were excised, transferred to distilled water containing Tween 80® and shaken for 3–4 h. The explants were then surface sterilised for 30 s in an 8% (v/v) hypochlorite solution with Tween 80®, then rinsed in sterile distilled water, before transfer to culture media using aseptic techniques. Explants were transferred to a medium similar to that used by Katoh (1998), which consisted of MS (Murashige and Skoog, 1962) salts at half strength supplemented with the vitamins, sugars and organic acids of Kao and Michayluk (1975). Glucose was added at 19.99 g l^{-1}. Media were solidified using 7.5 g l^{-1} agar (Davis). Medium pH was adjusted to 5.7 with either 0.1 mol l^{-1} NaOH or 0.1 mol l^{-1} HCl, prior to autoclaving at 120 °C for 15 min.

Culture vessels containing 25 ml of medium were any of the following: 90×10 mm disposable plastic Petri

dishes, 60×100 mm glass jars or 250 ml Erlenmeyer flasks. Cultures of in vitro plant material were maintained at approximately 19 °C with a 16/8 h light/dark cycle. A light intensity of 32 μmol m^{-2} s^{-1} at shelf level was provided by cool white fluorescent tubes.

3.5. Labelling procedure

For the experiment with [1-^{13}C]glucose, 20% of the unlabelled glucose was replaced by labelled glucose. Plants were transferred on to new medium every 4 weeks, as experience with early cultures showed that, after this period, growth rates slowed and general health of plants decreased. Plants were grown for 3 months.

3.6. Isolation of labelled hodgsonox (2)

Dried plant material (0.507 g) was ground with a mortar and pestle under liquid N_2 and extracted by shaking the plant material in CH_2Cl_2 (50 ml) overnight, followed by Ultra-turrex extraction with fresh CH_2Cl_2 (20 ml). After filtration the solvent was removed by rotary evaporation to yield a green oily extract (78 mg).

The extract (78 mg) was adsorbed on to Si-gel (200 mg) and separated on a Si-gel column (2 g), eluted with a cyclohexane:CH_2Cl_2 gradient. Fractions were combined based on TLC to give five fractions (cyclohexane:CH_2Cl_2 100:0, 86:14, 80:20, 70:30, 50:50). Labelled hodgsonox (**2**) (fraction 4) was eluted with 70:30 cyclohexane:CH_2Cl_2 (7 mg). ^{1}H and ^{13}C spectra matched those previously reported and showed no significant levels of impurities (Ainge et al., 2001). Results from quantitative NMR experiments are listed in Table 1.

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