Sesqui- and di-terpene biosynthesis from ^{13}C labelled acetate and mevalonate in cultured cells of *Heteroscyphus planus*

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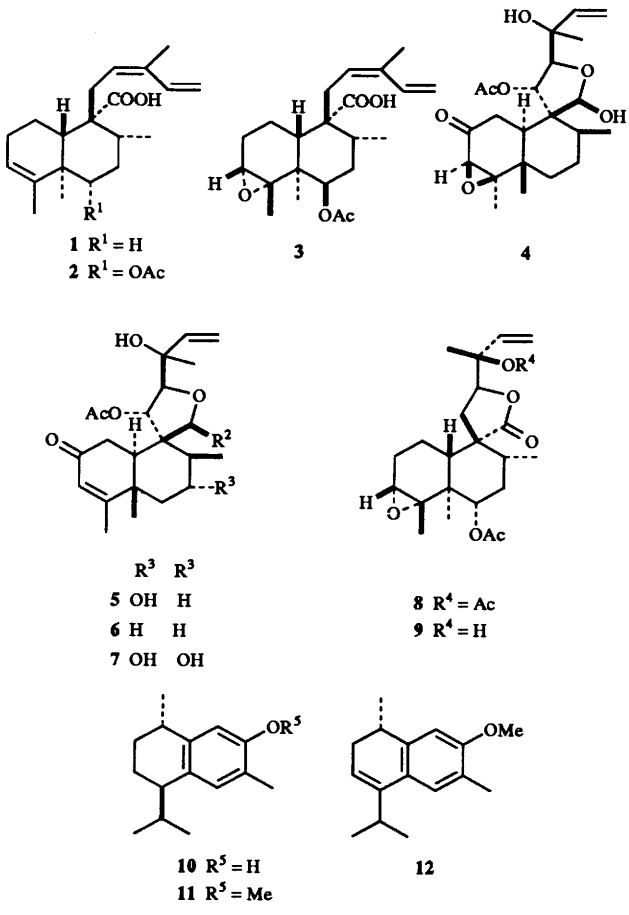
Cultured cells of the liverwort, *Heteroscyphus planus*, accumulate a wide variety of sesquiterpenes and diterpenes of the clerodane type. ^{13}C Atoms of [2-^{13}C]acetate and [2-^{13}C]- and [4,5-$^{13}C_2$]-mevalonates (MVAs) are incorporated into sesquiterpenes more extensively than into diterpenes by cultured cells of *H. planus*. (1*S*)-7-Methoxy-1,2-dihydrocadalene, a predominant sesquiterpene, and heteroscyphic acid A, a main diterpene, incorporating ^{13}C labelled precursors were analysed by ^{13}C NMR spectroscopy. Preferential labelling of the farnesyl diphosphate (FPP)-derived portion of heteroscyphic acid A was confirmed by ^{13}C enriched peaks and ^{13}C–^{13}C couplings, while sesquiterpenes were labelled equivalently. From these results, it appears that separate sites for sesquiterpene and diterpene biosynthesis exist in cultured cells of *H. planus* as could be observed in higher plants. Since the same type of nonequivalent labelling was observed in the phytyl side chain of chlorophyll by other experiments, it is suggested that all compounds, which are formed from GGPP within chloroplasts, are biosynthesized partly *via* the condensation of FPP derived from exogenous MVA with endogenous isopentenyl diphosphate in chloroplasts. A 1,2- methyl migration of the methyl group originating at C-3 of MVA is also confirmed in the biosynthesis of the *trans*-clerodane skeleton.

A number of labelled compounds including CO_2, glucose, sucrose, acetate and mevalonate† (MVA) were tested as mono- and sesqui-terpene precursors in a search for substrates which might reach isolated sites of terpenoid biosynthesis, using whole higher plants or cuttings of plants.[1] These *in vivo* experiments revealed that the exogenously supplied precursors were poorly incorporated; glucose and CO_2 were relatively efficient substrates in monoterpene biosynthesis, and acetate and MVA were less effective. However, in sesquiterpene biosynthesis, acetate and MVA were much more effective precursors than CO_2 and glucose.[2] These findings suggested that the separate sites for monoterpene and sesquiterpene biosynthesis were compartmentalized, and isolated from the rest of higher terpene (di-, tri- or tetra-terpenes) biosynthesis in the plants.[2] Additionally the biosynthesis of lower terpenes from exogenous ^{14}C-labelled MVA in tissues from higher plants usually yielded terpenes which were preferentially labelled in the isopentenyl diphosphate (IPP)-derived portion of the molecules, suggesting that preferential labelling results from the condensation of IPP (derived from exogenous ^{14}C-labelled MVA) with dimethylallyl diphosphate (DMAPP) which is mainly present in a metabolic pool.[3] However, diterpenes, tetraterpenes (carotenoids and xanthophylls) and the phytyl moiety of chlorophyll, all of which are formed from geranylgeranyl diphosphate (GGPP), are recognized to be biosynthesized in the chloroplast and related plastids.[4] However, nonequivalent incorporation of MVA into di- and tetra-terpenes has not yet been reported.

The cultured cells and gametophytes of the liverwort, *Heteroscyphus planus* produced diterpenoic acids of clerodane-type including heteroscyphic acid A **1**, B **2** and C **3**,[5] the lactols heteroscyphone A **4**, B **5**, C **6** and D **7**,[6] and the lactones heteroscypholide A **8** and heteroscypholide B **9**[7] together with sesquiterpenes of cadinane-type such as (1*S*,4*R*)-7-hydroxy-calamenene **10**, (1*S*,4*R*)-7-methoxycalamenene **11** and (1*S*)-7-methoxy-1,2-dihydrocadalene **12**.[8]

Previous studies demonstrated that tracers (^2H and ^{13}C) in the isotopically labelled mevalonate were incorporated into sesquiterpenes **10** and **12** at the higher levels in the cultured cells of the liverwort *H. planus*.[9,10] We very recently observed that the differentiated, cultured gametophytes of *Aneura pinguis* also



† Mevalonate is the trivial name for the salt of 3,5-dihydroxy-3-methylpentanoic acid.

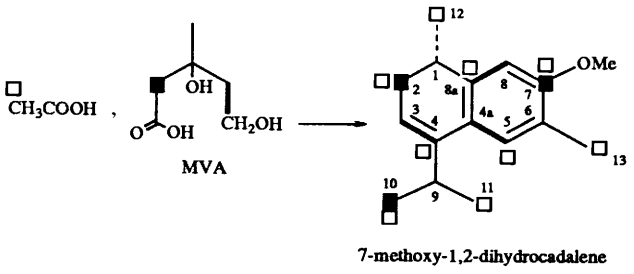


Fig. 1 Labelling pattern of 7-methoxy-1,2-dihydrocadalene incorporating [2-^{13}C]acetate, and [2-^{13}C]- and [4,5-$^{13}C_2$]-MVA

accumulated the highly ^{13}C-enriched pinguisone, after administration of [2-^{13}C]acetate.[11] Labels from ^{2}H- and ^{13}C-enriched acetate or MVA had been incorporated into the specific positions of sesquiterpenes, indicating that long incubation (28 days) did not lead to significant randomization of tracers in acetate and MVA. Thus the positions of ^{2}H and ^{13}C atoms were determined by gas chromatography–mass spectrometry (GLC–MS),[9] and ^{2}H and ^{13}C NMR analyses.[10]

In a recent communication of diterpene biosynthesis in cultured cells of *H. planus*,[12] we demonstrated that exogenous MVA was unexpectedly incorporated into the FPP-derived portion of heteroscyphic acid A, while the terminal IPP-derived moiety of the compound was not labelled. The present study was directed toward determining the extent and the ^{13}C-labelled positions of incorporation of the labelled acetate and mevalonates into the sesquiterpene 7-methoxy-1,2-dihydrocadalene and the diterpene heteroscyphic acid A, and attempting to explain that the compartmentation of diterpene biosynthesis may be a related phenomenon to the nonequivalent labelling of heteroscyphic acid A.

Results and discussion

The acetate and MVA labelling patterns of 7-methoxy-1,2-dihydrocadalene

A full assignment of the natural abundance $^{13}C\{^{1}H\}$ NMR spectrum of 7-methoxy-1,2-dihydrocadalene has been made previously.[10] Potassium [2-^{13}C]acetate (0.1, 0.5 and 1.0 mmol) and [4,5-$^{13}C_2$]MVA (1.0 mmol) were fed separately to 75 cm^3 of the cell cultures of *H. planus*. After the 21-day culture periods, 7-methoxy-1,2-dihydrocadalene was extracted with diethyl ether and subjected to GLC–MS analysis. The ^{13}C enriched 7-methoxy-1,2-dihydrocadalenes were isolated (purities: over 94%) by reversed-phase high-pressure liquid chromatography (HPLC) for GLC–MS and ^{13}C NMR analyses. The dosages of acetate significantly retarded growth (growth index for 21 days: without acetate, 4.87; with 0.1, 0.5 and 1.0 mmol acetate, 3.0, 3.2 and 3.3, respectively), and the green cells changed to brown cells at a higher concentration (1.0 mmol).

Since separation of the isotopically labelled compounds by HPLC may lead to substantial errors for quantification of ^{13}C enrichments,[13] the enrichments of the resulting ^{13}C-labelled 7-methoxy-1,2-dihydrocadalenes were determined by GLC–MS analysis (Ulbon HR-1 column, methyl silicone type) using the areas (PAs) of the selected ion monitored (SIM) peaks before and after purification of the ^{13}C enrichment of 7-methoxy-1,2-dihydrocadalene and compared with those determined by ^{13}C NMR analysis. The ^{13}C enrichments were estimated thus ^{13}C enrichment (%) = $\{100 \times (PA_1/PA_0) - \text{natural abundance of } [M^+ + 1]\}/9 + 2\{100 \times (PA_2/PA_0) - \text{natural abundance of } [M^+ + 2]\}/9 + 100\ [(3 \times PA_3) + (4 \times PA_4) + (5 \times PA_5) + (6 \times PA_6) + (7 \times PA_7) + (8 \times PA_8) + (9 \times PA_9)]/(9 \times PA_0)$ where PA_0 and PA_ns are the peak areas of non-labelled (*m/z* 230) and [$^{13}C_n$]-labelled compounds (*m/z* 231 to 239), respectively. In the case of 7-methoxy-1,2-dihydrocadalene in the diethyl ether extract from the cultured cells fed 0.5 mmol acetate, PA_0, PA_1, PA_2, PA_3, PA_4, PA_5, PA_6, PA_7, PA_8 and PA_9 were estimated to be 389 631, 85 806, 24 444, 11 735, 4141, 6207, 3825, 4192, 2416 and 1638, respectively, with computer supported evaluation. Thus the ^{13}C enrichment of 7-methoxy-1,2-dihydrocadalene before purification by HPLC was calculated to be 6.31%, while that of 7-methoxy-1,2-dihydrocadalene after purification was 3.10%. Although 7-methoxy-1,2-dihydrocadalene incorporating 1.0 mmol [2-^{13}C]acetate was labelled at a higher level (6.89%), no significant ^{13}C enrichment was observed in 7-methoxy-1,2-dihydrocadalene incorporating 0.1 mmol acetate.

The ^{13}C enrichment of the purified 7-methoxy-1,2-dihydrocadalene incorporating 0.5 mmol [2-^{13}C]acetate together with the labelling patterns (Fig. 1) was also determined by ^{13}C NMR analysis on the basis of the relative peak intensities of ^{13}C enriched peaks to an average intensity of non-labelled carbons (C-1, C-3, C-4a, C-6, C-8 and C-9) and are listed in Table 1 which demonstrates the equivalently enhanced carbons at C-2, C-4, C-5, C-7, C-8a, C-10, C-11, C-12 and C-13. An average ^{13}C enrichment (3.41%) conformed to that determined by GLC–MS. It is of interest to note that the methoxy carbon of 7-methoxy-1,2-dihydrocadalene is significantly enhanced (0.41%), suggesting that the methyl carbon of acetate is partially incorporated into 7-methoxy-1,2-dihydrocadalene after prior degradation of acetate.

The ^{13}C enrichment and the ^{13}C–^{13}C coupling patterns of the purified 7-methoxy-1,2-dihydrocadalene incorporating [4,5-$^{13}C_2$]MVA (1.0 mmol) are also given in Table 1. The ^{13}C–^{13}C coupling patterns were observed between C-3 and C-4 ($J_{C\text{-}3,C\text{-}4}$ 70.8 Hz), between C-4a and C-5 ($J_{C\text{-}4a,C\text{-}5}$ 59.8 Hz) and between C-8 and C-8a ($J_{C\text{-}8,C\text{-}8a}$ 61.1 Hz) as could be expected from the established biosynthetic pathway for cadinanes.[9,10,14,15] An average ^{13}C enrichment (4.0%) which was determined on the basis of a relative peak intensity of ^{13}C–^{13}C coupled resonances to the natural abundance resonance is slightly higher than those of 7-methoxy-1,2-dihydrocadalene incorporating [2-^{13}C]acetate.

In comparison with the low incorporation observed in whole higher plants, the high incorporation of exogenous acetate and MVA into sesquiterpenes in cultured cells and gametophytes of liverworts suggests that sites for sesquiterpene biosynthesis in liverworts are subjected to a lesser degree of compartmentalization than those in higher plants, although it may be merely attributable to the efficient uptake of the cultured cells and the gametophytes.

The acetate and MVA labelling patterns of heteroscyphic acid A

The ^{13}C enrichments of heteroscyphic acid A incorporating [2-^{13}C]acetate (0.5 and 1.0 mmol) were determined by SIM GLC–MS at *m/z* 302 to 314 using a CBP-1 wide-bore capillary column in the same manner as in the case of 7-methoxy-1,2-dihydrocadalene before purification by HPLC, and estimated to be 0.69 and 1.36 atom% excess. These values were 8.8 to 5.5 times lower than those in 7-methoxy-1,2-dihydrocadalene. Heteroscyphic acid A enriched with [2-^{13}C]acetate and [2-^{13}C]- and [4,5-$^{13}C_2$]-MVA were purified by repeated HPLC as described previously.[7] The average ^{13}C enrichment of heteroscyphic acid A incorporating [2-^{13}C]acetate (see Table 2) and [2-^{13}C]-MVA was estimated to be 0.3 and 0.9 atom% excess,[12] respectively, by ^{13}C NMR measurement after methylation. The rather poor incorporation of acetate and MVA into heteroscyphic acid A may be explained as follows. The activities responsible for GGPP synthesis and its conversion into macrocyclic and polycyclic diterpenes,[16,17] the phytyl side chain of chlorophyll[18] and tetraterpene[19] have

Table 1 ^{13}C enrichments of carbons in 7-methoxy-1,2-dihydrocadalene derived from [2-^{13}C]acetate and [2-^{13}C]- and [4,5-$^{13}C_2$]-MVA in the cultured cell of *H. planus*

Carbon	^{13}C Enrichments [2-^{13}C]Acetate 0.5 mmol atom% excess[a]	[2-^{13}C]-MVA 1.0 mmol atom% excess[a,b]	[4,5-$^{2}C_{13}$]-MVA 1.0 mmol relative intensity[c] ($J_{^{13}C-^{13}C}$ coupling)
OMe	1.42	1.02	
C-1	0.63	0.84	
C-2	4.00	12.2	
C-3	0.94	0.87	5.7 (70.8 Hz)
C-4	3.19	ND	
C-4a	1.27	ND	2.8 (59.8 Hz)
C-5	2.89	1.13	
C-6	ND	ND	
C-7	2.74	8.72	
C-8	1.10	1.00	3.4 (61.1 Hz)
C-8a	2.54	ND	
C-9	1.07	1.13	
C-10	3.79	11.9	
C-11	4.19	1.07	
C-12	3.59	1.01	
C-13	3.73	0.95	
Average	3.41	10.9	4.0
GLC–MS	3.10	9.80	

[a] ^{13}C Enrichments in each carbon were determined by comparing the relative peak intensities of ^{13}C enriched carbons to the non-labelled carbons with those of the corresponding carbons in the non-labelled 7-methoxy-1,2-dihydrocadalene. [b] See ref. 10 (in ref. 10, the values were calculated on the basis of ^{13}C enrichment of O*Me* = 1.0). [c] Relative peak intensity of ^{13}C–^{13}C coupled resonances to naturally abundant resonance. ND not determined.

been proven to be localized in the chloroplast and its related plastids, such as ethioplast, chromoplasts and proplastides, in higher plants. It has been suggested that acetyl CoA, an obligatory intermediate in chloroplast terpene biosynthesis, was formed from photosynthetically fixed carbon dioxide.[20,21] Thus, the exogenously supplied MVA and acetate were rather poorly incorporated into tetraterpenes compared with the precursors of chloroplastidic acetyl CoA in higher plants.[22] In suspension cultures of liverwort, the cells contain well-developed chloroplasts and the levels of chlorophyll were generally significantly high.[23,24] Thus, acetyl CoA for diterpene biosynthesis might be sufficiently furnished by photosynthetic fixation which eventually leads to poor incorporation of MVA and acetate into diterpenes in cultured cells of *H. planus*. Poor incorporation of MVA may be also explained by the known relative impermeability of the chloroplast membrane to MVA,[21] while extraplastidic sesquiterpenoids were formed in significant amounts from exogenously supplied acetate and MVA.

Heteroscyphic acid A incorporating [4,5-$^{13}C_2$]-MVA was further purified by HPLC after methylation, which afforded the purified heteroscyphic acid A methyl ester (purity: 98.5% on GLC chromatograph). The resulting $^{13}C\{^1H\}$ NMR (67.8 MHz) analysis of heteroscyphic acid A (and its methyl ester) incorporating ^{13}C-labelled MVA clearly showed the incorporation and labelling pattern as indicated in Fig. 2, whereas heteroscyphic acid A incorporating [2-^{13}C]acetate did not give any significantly ^{13}C enriched carbons (Table 2). This indicates that the methyl carbon in acetate was incorporated randomly into heteroscyphic acid A after degradation.

Although the incorporation of [2-^{13}C]-MVA into heteroscyphic acid A was considerably lower than that into 7-methoxy-1,2-dihydrocadalene (10.1 atom% excess[10]), the ^{13}C signals corresponding to C-1, C-7 and C-18 were apparently enhanced with the ^{13}C atom, whereas the intensity of C-12 was much less than expected from an equivalent labelling of diterpenoid biosynthesis. Moreover, in the ^{13}C NMR spectrum of heteroscyphic acid A methyl ester incorporating [4,5-$^{13}C_2$]-MVA, the ^{13}C–^{13}C coupling patterns were observed between

Table 2 ^{13}C enrichments of carbons in heteroscyphic acid A methyl ester derived from [2-^{13}C]acetate (0.5 mmol) in the cultured cell of *H. planus*

Carbon	Relative intensity[a]	Carbon	Relative intensity
COO*Me*	1.0	C-11	1.3
C-1	1.6	C-12	1.8
C-2	1.0	C-13	1.3
C-3	0.87	C-14	1.4
C-4	1.5	C-15	1.2
C-5	1.2	C-16	2.2
C-6	1.4	C-17	1.9
C-7	1.4	C-18	1.3
C-8	1.3	C-19	1.6
C-9	0.95	C-20	1.4
C-10	1.3	Average	1.4 (*ca.* 0.3 atom% excess)

[a] Relative intensity = relative peak intensity of labelled C-n to COO*Me*/relative peak intensity of non-labelled C-n to COO*Me*.

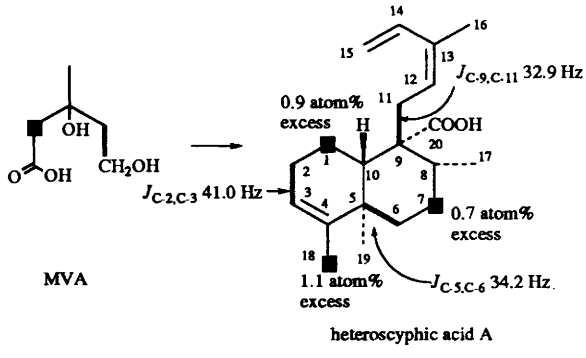


Fig. 2 Labelling pattern of heteroscyphic acid A incorporating [2-^{13}C]- and [4,5-$^{13}C_2$]-MVA

C-2 and C-3 (the relative peak intensity of ^{13}C–^{13}C coupled resonances to the natural abundant resonance: 0.28, $J_{C-2,C-3}$

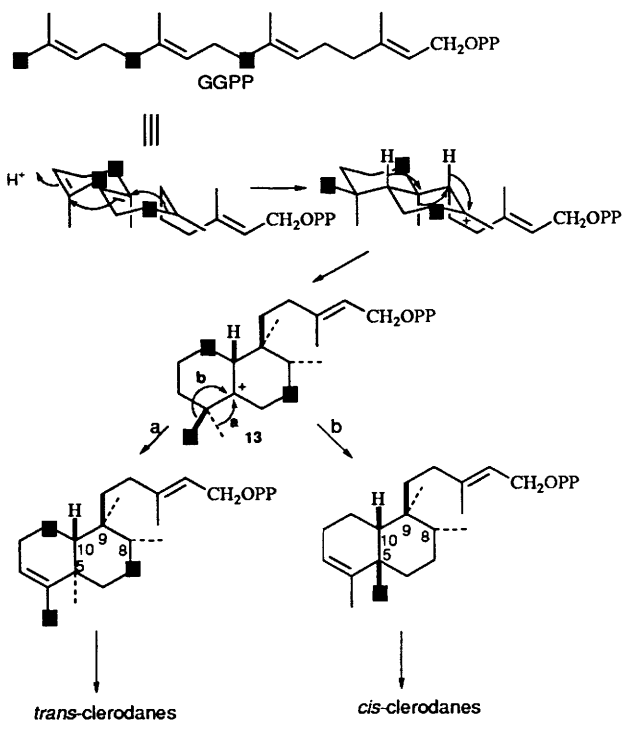


Fig. 3 Presumed conformation of GGPP leading to *trans*- and *cis*-clerodanes

41.5 Hz, see Fig.2), between C-5 and C-6 (0.29, $J_{C\text{-}5,C\text{-}6}$ 34.2 Hz) and between C-9 and C-11 (0.28, $J_{C\text{-}9,C\text{-}11}$ 32.9 Hz) but not between C-14 and C-15. These findings indeed confirmed the preferential labelling in the FPP-derived portion of heteroscyphic acid A. This labelling pattern is sharply in contrast to the preferential labelling in the IPP-derived portion in mono- and sesqui-terpene biosyntheses.

The retention of the methyl group originating from the methylene at C-2 of MVA at the C-18 position provides the indirect evidence for a 1,2-methyl (originating from the methyl group of MVA) migration from the C-4 to C-5 position in the biosynthesis of the *trans*-clerodane skeleton, whereas the methyl originating from C-2 of the mevalonate shifts to the C-5 position in the biosynthesis of the *cis*-clerodane skeleton (Fig. 3).[24] Since *cis*-and *trans*-clerodanes possess the common absolute stereochemistry at C-8, C-9 and C-10,[25] these compounds are postulated to be formed from a common cation **13** which is synthesized from GGPP *via* stereospecific pathways as indicated in Fig. 3.

The observed nonequivalent labelling in heteroscyphic acid A may be explained by the condensation of FPP derived from exogenous labelled MVA with endogenous IPP in a metabolic pool, or merely by the slower synthesis of geranylgeranyl diphosphate (GGPP) in comparison with the overall utilization of MVA. Since heteroscyphic acid A was produced synchronously with 7-methoxy-1,2-dihydrocadalene (levels of heteroscyphic acid A (10.1 mg g^{-1} fresh weight) and 7-methoxy-1,2-dihydrocadalene (0.92 mg) were at a maximum in 3 and 6 day-old cells, respectively, dropped within 6 to 15 days and increased again after 15 days) the latter explanation might be excluded. In a preliminary study of the chlorophyll a biosynthesis in cultured cells of several liverworts, we found that the phytyl moiety of chlorophyll was also labelled nonequivalently.[26] This suggests that this type of nonequivalent labelling takes place in the biosynthesis of all compounds which are formed from GGPP within chloroplasts in the liverworts. Additional investigations of the permeability of membranes of intact chloroplasts to FPP and the properties and functions of GGPP synthethases and other prenyl transferases in chloroplasts may help to answer this nonequivalent labelling question.

Experimental

General procedure and materials

GLC–MS was measured on a Hitachi M-80B spectrometer equipped with a Shinwa Chemical Ulbon HR-1 column, or a Shimadzu CBP-1 column. All $^{13}C\{^1H\}$ NMR spectra of 7-methoxy-1,2-dihydrocadalene and heteroscyphic acid A and its methyl ester incorporating ^{13}C labelled precursors were recorded on a JEOL EX-270 NMR spectrometer with $^{13}CDCl_3$ (0.40 cm^3) as internal standard (77.0 ppm) using the JEOL experimental mode (BCM) with the following parameters: spectral frequency 67.80 MHz; spectral width 20 KHz; observation frequency offset 135.0 KHz; observation frequency fine offset 5200 Hz; data points 32 768; acquisition time 0.819 sec; pulse delay 2.181 s; pulse width 7.3 μs (90°); exponential broadening factor 1.22 Hz; numbers of scans 12 639–40 000 (the signals to noise ratios of the most intense signals of the biosynthetically ^{13}C labelled compounds and non-labelled compound *ca.* 30 to 40) and numbers of measurements ⩾2. *J* Values are given in Hz.

[4,5-$^{13}C_2$]Mevalonolactones [4,5-$^{13}C_2$]

MVA was prepared from [1,2-$^{13}C_2$]acetate (99 atom%) by the method previously reported by us.[10] [4,5-$^{13}C_2$]-MVA: δ_H 1.41 (d, 3 H, $J_{^{13}CCCH}$ 4.0), 1.9 (dm, 2 H, $J_{^{13}CH}$ 128), 2.53 (dd, 1 H, *J* 17.5, $J_{^{13}CCH}$ 1.7), 2.54 (dd, 1 H, *J* 17.5, $J_{^{13}CCH}$ 1.7), 4.34 (dm, 1 H, $J_{^{13}CH}$ 151) and 4.60 (dm, 1 H, $J_{^{13}CH}$ 151); δ_C 29.9, 36 (d, $J_{^{13}CC}$ 35, 99 atom%), 44.7, 65.3 (d, $J_{^{13}CC}$ 35, 99 atom%), 68.5 (d, $J_{^{13}CC}$ 18.3) and 170.2; GLC–MS: *m/z* 73 ($^{13}CH_2$-$^{13}CH_2$=OH^+), 58 and 43 (100%).

Feeding experiment, and determination of heteroscyphic acid A and 7-methoxy-1,2-dihydrocadalene contents

The origin of the *H. planus*, medium and culture conditions for the suspension culture have been described previously.[8] Two cultures, each 2.7 g on average fresh weight in MSK-4 medium (75 cm^3) were incubated with [4,5-$^{13}C_2$]-MVA (132 mg, 1 mmol). The feeding experiment of [2-^{13}C]-MVA was carried out previously. Other cultures were separately incubated with 0.1 (6.1 mg), 0.5 (30.5 mg) and 1.0 mmol (61 mg) of [2-^{13}C]acetate. The liquid suspensions were agitated at 110 rpm at 25 ± 1 °C under continuous light of 5000 lux. The cells were harvested 21 days after inoculation to determine the ^{13}C enrichment by GLC–MS and to isolate the biosynthetically labelled 7-methoxy-1,2-dihydrocadalene. To determine the changes in heteroscyphic acid A and 7-methoxy-1,2-dihydrocadalene contents during growth, cultured cells (average; 250 mg fresh weight) were inoculated in 10 cm^3 MSK-4 medium, and harvested every 3 days until day 21. The harvested cells were extracted with methanol (5 × volume). The methanol extracts were added to a known amount of eicosane and analysed by GLC–MS using the CBP-1 column. The average contents (means of 3 independent determinations) were estimated on the basis of the ratios of their total ion monitored peak areas to a known amount of eicosane.

GLC–MS Analysis

GLC–MS analyses were carried out for determining heteroscyphic acid A and 7-methoxy-1,2-dihydrocadalene contents and the ^{13}C enrichments using Ulbon HR-1 (50 m × 0.25 mm i.d., initial temperature of 60 °C was kept for 5 min and then elevated at 2 °C min^{-1} to 220 °C; flow rate of He, 1.13 cm^3 min^{-1}) and CBP-1 columns (methyl silicone type, 12 m × 1.0

mm i.d., initial temperature of 60 °C was kept for 5 min and then elevated at 2 °C min^{-1} to 220 °C; flow rate of He, 21.4 cm^3 min^{-1}). The ionizing voltage employed was 70 eV. The following ions were monitored together with total ions: 7-methoxy-1,2-dihydrocadalene incorporating [2-^{13}C]acetate, *m/z* 230–239; heteroscyphic acid A incorporating [2-^{13}C]-MVA, *m/z* 304–314.

Isolation of isotopically labelled 7-methoxy-1,2-dihydrocadalene and heteroscyphic acid A

The fresh cells were extracted with 5 volumes of MeOH (v/w, × 2) for 5 h and pentane for 12 h. The MeOH extracts were combined, concentrated to dryness and the residue was dissolved in diethyl ether (5 × the volume of the cultured cells, v/w). The ether solutions were divided into the acidic and the neutral–basic fractions in the usual manner. The acidic fractions were combined, concentrated and chromatographed by HPLC on a ODS column (30 cm × 1.5 cm i.d.) with MeOH at 1 cm^3 min^{-1}. The fractions containing heteroscyphic acid A were combined and concentrated to yield 0.5–0.7 mg of heteroscyphic acid A (91–94% purity). In the case of heteroscyphic acid A incorporating [4,5-$^{13}C_2$]-MVA, heteroscyphic acid was methylated with diazomethane and further separated by chromatography on a silica gel column (24 cm × 1 cm i.d.) with hexane–chloroform (4:1) at 1 cm^3 min^{-1} to yield heteroscyphic acid A methyl ester (98.5% purity, examined by GLC–MS). The differently labelled 7-methoxy-1,2-dihydrocadalenes (0.3–0.9 mg, 85–91% purity) were purified from the neutral–basic fractions by the ODS column with MeCN 1 cm^3 min^{-1}.

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