Biosynthesis of (1*S*)-7-Methoxy-1,2-dihydrocadalene. Incorporation of ^{2}H- and ^{13}C-Labelled Mevalonates by Cultured Cells of *Heteroscyphus planus*

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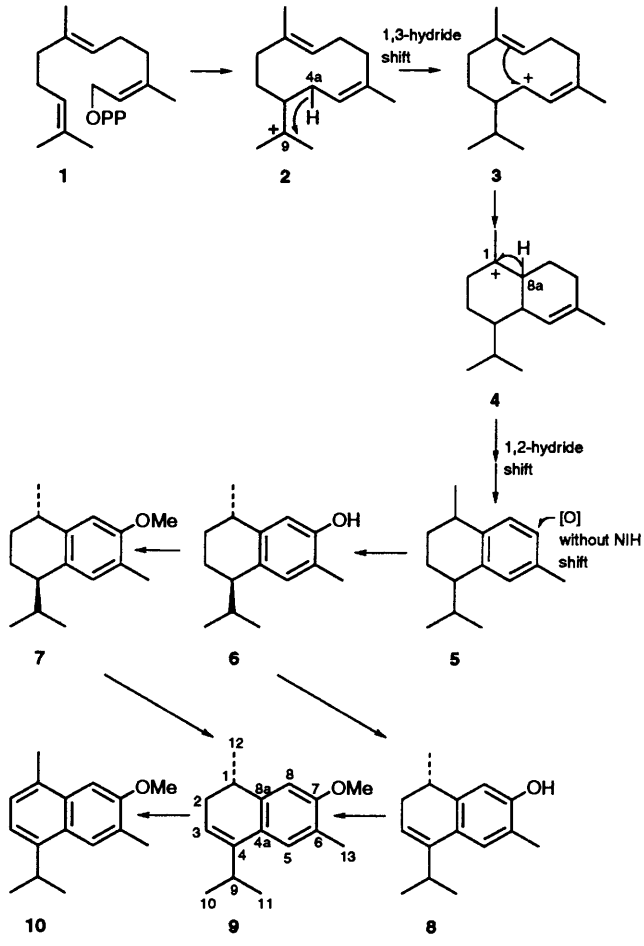
^{2}H and ^{13}C NMR analyses of (1*S*)-7-methoxy-1,2-dihydrocadalene produced by cell cultures of the liverwort *Heteroscyphus planus* in the presence of ^{2}H- and ^{13}C-labelled mevalonates verified 1,2- and 1,3-hydride shifts and the hydroxylation of an aromatic ring without an NIH shift in the biosynthesis of MDC.

Studies on biosynthesis of the lower terpenoids (mono- and sesqui-terpenes) using cell-culture techniques of higher plants have shown that there are differences in terpenoid profiles in cultured cells from those in intact plants.[1] Therefore, except for one report on γ-bisabolene biosynthesis in the calli of *Andrographis paniculata*,[2] there have been no reports of successful biosynthetic experiments involving the feeding of ^{2}H- or ^{13}C-labelled early precursors such as mevalonate on acetate followed by ^{2}H or ^{13}C NMR spectroscopic analysis of the resulting isotopically labelled products. In contrast with cell cultures of higher plants, cultured cells of the liverworts generally accumulate lower terpenes, whose compositions are almost identical with those in plant material, even at significantly high levels.[3,4] Moreover, added precursors such as ^{13}C-labelled acetate[5] and ^{2}H-labelled mevalonate (MVA)[6] have been incorporated into lower terpenes at levels comparable to those normally associated with fungal cultures. We therefore believe that cell cultures of the liverworts must be of increasing value for examining in detail the biosynthesis of lower terpenes. Nevertheless, until the present time, only one group[5] using cell cultures of liverworts has reported a biosynthetic study focusing on biogenetic information gained from $^{2}H/^{13}C$-labelled products incorporating early precursors. Therefore, we[6] studied the biosynthesis of a rare sesquiterpene, (1*S*)-7-methoxy-1,2-dihydrocadalene (MDC) **9** (in Scheme 1), which has been previously isolated from soft corals,[7] in the liverwort *Heteroscyphus planus* by using a cell-culture technique.

The cultured cells and gametophytes of the liverwort *Heteroscyphus planus* accumulated sesquiterpenes of cadinane type (**5**–**10**) with novel chiralities.[4] The biosynthetic sequence of these cadinanes outlined in Scheme 1 has been examined by incorporation of differently deuteriated mevalonate (MVA).[6] Gas–liquid chromatographic–mass spectrometric (GLC-MS) analysis of the isotopically labelled products provided indirect evidence for 1,2- and 1,3-migration of hydrogens, and the hydroxylation of calamenene **5** at C-7 with retention of 8-H to form a novel sesquiterpene, (1*S*,4*R*)-7-hydroxycalamenene (HC, **6**), which is further converted into MDC. In the present report, as a probe of the biosynthetic mechanism of this compound, we administered ^{13}C-labelled mevalonate ([2-^{13}C]- and [4-^{13}C]-MVA), ^{2}H-labelled MVA ([2-$^{2}H_2$]- and [4-$^{2}H_2$]-) and doubly labelled mevalonate ([5-^{13}C, 5-$^{2}H_2$]-) to cultured cell of *H. planus*.[6] We report here that ^{2}H and ^{13}C NMR spectroscopic analyses of compound **9** incorporating isotopically labelled precursors verified the presence of 1,2- and 1,3-hydride shifts and hydroxylation without an NIH shift[8] in its formation.

Results and Discussion

Precursors, Cultures and Isolation.—^{2}H-Labelled and [2-^{13}C]MVAs were prepared by the methods previously reported.[9,10] To avoid a loss of ^{13}C-labelled acetates ([1-^{13}C]- and [2-^{13}C]-) during distillation, ^{13}C-labelled amyl acetates were prepared and used for the preparation of [4-^{13}C]- and [5-^{13}C, 5-$^{2}H_2$]-MVAs.



Scheme 1 Biosynthetic pathway of cadalene derivatives in cultured cells of *H. planus* (this biosynthetic sequence has been proposed by Nabeta *et al.*[6])

Cell cultures of *H. planus* were grown in MSK-4 liquid medium[3] under continuous light at 25 °C. Potassium MVA (0.18 mmol) was fed in separate experiments to the culture (13.5 cm^3). Cells were harvested after 21 days and extracted with MeOH. The enriched MDC **9** in the MeOH extracts was analysed by GLC–MS in order to determine the extent of ^{2}H enrichment before purification. Labelled compound **9** (0.3–0.9 mg) was purified (> 85% purity) by repeated high-performance liquid chromatography (HPLC).

Rate of ^2H *and* ^{13}C *Enrichments.*—The rates of ^2H enrichment of MDC **9** incorporating [2-$^2H_2$]-, [4-$^2H_2$]- and [5-$^2H_2$]-MVA were determined by the relative areas of the selected-ion-monitored peaks in the mass chromatograms. Owing to the complexity of the calculations, the rate of ^2H incorporation of [5-$^2H_2$]MVA was substituted for that of [5-^{13}C, 5-$^2H_2$]MVA, whereas the levels of ^{13}C enrichment in each carbon were determined by comparing the relative peak intensities of the enriched carbons to the MeO-carbon with those of the corresponding carbons in the non-labelled compound **9**. Deviation in the levels of ^2H and ^{13}C enrichment, which are summarized in Table 1, were observed among the differently deuteriated MVAs, *i.e.*, the extent of ^2H and ^{13}C enrichment of compound **9** incorporating the MVAs deuteriated at the C-2, C-4 and C-5 positions was rather lower than that of [6-$^2H_3$]MVA.[6] As discussed below, 2-, 4- and 5-Hs of MVA are lost or migrate in the formation of compound **9**. Therefore, this deviation may be caused by the primary isotopic effect of ^2H substitution.[11]

Labelling Patterns of (1S)-7-*Methoxy*-1,2-*dihydrocadalene* **9**.—The assignment of the quaternary carbons was ambiguous in the previous work.[7] Therefore the complete ^{13}C assignment of MDC was achieved by long-range connectivities observed in the 2D heteronuclear multiple bond coherence (HMBC) spectrum of non-labelled **9** (significant HMBC connectivities, see Fig. 1). The assignment was confirmed by the observed enhancement of the signals of C-5 (δ_C 125.4) and C-8a (δ_C 141.1) in compound **9** from [4-^{13}C]MVA and of that of C-4a (δ_C 126.6) in compound **9** from [5-^{13}C, 5-$^2H_2$]MVA.

Table 2 summarizes the $^{13}C\{^1H\}$ data of compound **9** incorporating [2-^{13}C]-, [4-^{13}C]- and [5-^{13}C, 5-$^2H_2$]-MVA together with that of the natural-abundance parent **9**. Table 3 lists the ^2H signals of the biosynthetically deuteriated compound **9** with the ^1H signals of non-labelled compound **9**.

The hydrogen transfer from C-4a to C-9 in a germacradienyl cation **3** in Scheme 1 and the retention of 8-H during the hydroxylation of an aromatic ring of calamenene **5** to form compound **9** *via* the methylation of HC **6** were confirmed by feeding with [5-^{13}C, 5-$^2H_2$]MVA. The ^{13}C NMR spectrum of doubly labelled compound **9** showed two isotopically shifted triplets,[12] at δ_C 116.5 [C-3, $J(^2H–^{13}C)$ 25.7 Hz, an upfield shift of 0.38 ppm] and at δ_C 108.3 [C-8, $J(^2H–^{13}C)$ 22.0 Hz, 0.32 ppm] with the strongly enriched singlet at δ_C 126.6 (C-4a). Hence the 5-H^a of MVA is incorporated intact at C-3 and C-8 in compound **9**. The ^2H NMR spectrum of the doubly labelled species showed one singlet peak, at $\delta(^2H)$ 2.91 (9-H of **9**), with two doublet peaks at $\delta(^2H)$ 5.63 (3-H) and 6.69 (8-H). Hence the postulated 1,3-hydride shifts from C-4a to C-9 occurred in the cation **3**. Retention of intact 5-H of MVA at C-8 of compound **9** was confirmed indirectly by the fact that no ^2H atom was retained at C-8 of compound **9** incorporating [2-$^2H_2$]MVA (Table 3). Fig. 2 shows the labelling scheme for MDC and MVA.

The putative hydrogen shift from C-8a to C-1 in a bicyclic cation **4** was examined by feeding with [4-$^2H_2$]MVA. The ^2H NMR spectrum of the deuteriated product **9** showed singlet peaks at $\delta(^2H)$ 2.78 (1-H) and $\delta(^2H)$ 7.11 (5-H). This confirmed the postulated 1,2-hydrogen transfer from C-8a to C-1 in the cation **4**.

Table 1 Rates of ^2H and ^{13}C enrichment in isotopically labelled MDCs

Precursor[a]	Atom% excess ^2H Enrichment	Atom% excess ^{13}C Enrichment
[2-$^2H_2$]MVA	6.4	
[4-$^2H_2$]MVA	15.6	
[5-$^2H_2$]MVA	6.9	
[6-$^2H_3$]MVA[b]	19.2	
[2-^{13}C]MVA[c]		10.1
[4-^{13}C]MVA[c]		7.9
[5-^{13}C, 5-$^2H_2$]MVA[d]		5.5

[a] ^2H and ^{13}C enrichment in precursors; see Experimental section. [b] See ref. 6. [c] Average ^{13}C enrichment of the three carbons indicated in Table 2. [d] ^{13}C enrichment at C-4a.

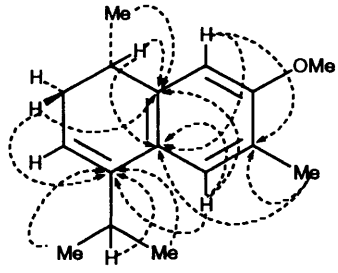


Fig. 1 Significant HMBC long-range connectivities of MDC

Table 2 $^{13}C[^1H]$ NMR data of natural-abundance and biosynthetically ^{13}C-enriched MDCs

Carbon	$\delta(^{13}C)$ Non-labelled	$\delta(^{13}C)$ MVAs[a] [2-^{13}C]-	[4-^{13}C]-	[5-^{13}C, 5-$^2H_2$]-
1	32.6			
2	30.9	30.9 (11.3)		
3	116.8			116.5 (t)
4	141.6		141.6 (7.8)	
4a	126.6			126.6 (5.5)
5	125.4		125.4 (9.2)	
6	123.3			
7	156.3	156.3 (8.4)		
8	108.6			108.3 (t)
8a	141.1		141.1 (6.7)	
9	28.1			
10	22.4	22.4 (10.7)		
11	22.1			
12	19.1			
13	16.1			
OMe	55.4			

[a] Figures in parentheses are ^{13}C enrichments (atom% excess).

Table 3 ^1H and ^2H NMR data of non-labelled and biosynthetically deuteriated MDCs

Hydrogen	δ_H Non-labelled	$\delta(^2H)$ MVAs		
		[2-$^2H_2$]-	[4-$^2H_2$]-	[5-^{13}C, 5-$^2H_2$]-
1	2.78 (m)		2.78	
2α	2.39 (m)	2.37		
2β	2.04 (m)	2.02		
3	5.63 (t)			5.63 (d)
5	7.11 (s)		7.11	
8	6.69 (s)			6.69 (d)
9	2.92 (m)			2.91
10	1.13 (d)	1.14		
11-H_3	1.16 (d)			
12-H_3	1.19 (d)			
13-H_3	2.21 (d)			
OMe	3.85 (d)			

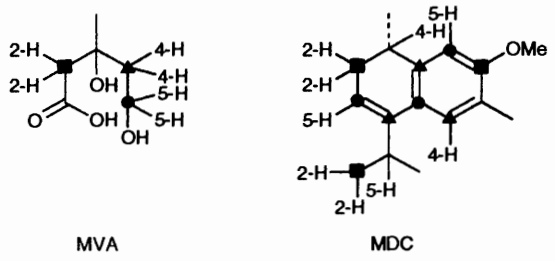
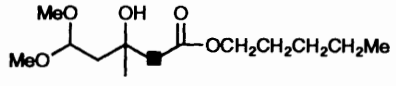
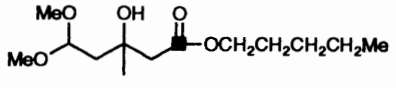


Fig. 2 ^2H and ^{13}C labelling patterns of MDC

In conclusion, the biosynthetic pathway shown in Scheme 1 is proposed for MDC **9** in cell cultures of *H. planus*. An NIH shift [8] often observed during the hydroxylation of aromatic rings of terpenes [1] was not observed in the formation of compound **9** in the cultured cells of *H. planus*, although an alternative sequence of oxygenation at C-7 prior to aromatization may not be excluded.



11 (■ = ^{13}C)



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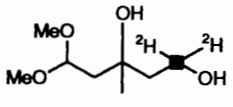

13 (■ = ^{13}C)

Experimental

General Procedure and Materials.—GLC was performed on a Shimadzu GC-14B model (an FS capillary column; Shinwa Chemical Industries Ulbon HR-1; 0.25 mm × 50 m) and detection with a single field ionization detection (FID). GLC–MS was measured on a Hitachi M-80B spectrometer equipped with Ulbon HR-1 column. ^1H NMR (500 MHz) spectra were recorded on a Bruker M-500 spectrometer for solutions in $CDCl_3$ with $CHCl_3$ as internal standard (δ 7.26), and ^2H NMR spectra (76.8 MHz; Bruker M-500 and 41.3 MHz; JEOL EX-270) spectra were recorded for $CHCl_3$ solutions with $CDCl_3$ (δ_D 7.26) as internal standard, respectively. ^{13}C NMR spectra were recorded at 67.8 MHz on a JEOL EX-270 with $^{13}CDCl_3$ as internal standard (δ_C 77.0). Isotopically labelled precursors used for feeding experiments were prepared according to the methods previously described.[9,10] [2-$^2H_2$]- (99.4 atom%), [4-$^2H_2$]- (99.7 atom%) and [5-$^2H_2$]- (99.3%) mevalonolactones have been previously prepared.[6] [2-^{13}C]Mevalonolactone was prepared from ethyl bromo-[2-^{13}C]acetate (99 atom%) by the method reported by Hoffman *et al.*[9] Although [4-^{13}C]- and [5-^{13}C, 5-$^2H_2$]-mevalonolactones are known compounds, we are reporting a slightly experimental procedure that improves their handling.

[4-^{13}C]*Mevalonolactone.*—Sodium [2-^{13}C]acetate (90 atom%; 1.0 g, 11 mmol), 25% (w/w) NaOH (2.53 g) and hexamethylphosphoric triamide (30 cm^3) were mixed. After the solution had been stirred at room temperature for 1 h, amyl iodide (8.73 g, 44 mmol) was added dropwise to it. The mixture was stirred for 2 h, and then was acidified with 5% HCl. The reaction mixture was extracted with diethyl ether. The extracts were washed with water, dried, filtered and evaporated to give amyl [2-^{13}C]acetate (1.13 g, 8.63 mmol, calculated on the basis of the GLC peak areas) in amyl iodide (5.12 g, 25.8 mmol). A solution of diisopropylamine (2.32 g, 22.3 mmol) in anhydrous THF (40 cm^3) was cooled to −78 °C and treated dropwise with BuLi (10 cm^3, 16.6 mmol). The mixture was stirred for 10 min, and then was cooled to −90 °C. To the stirred solution was added amyl acetate (1.13 g) in amyl iodide (5.12 g), and the mixture was then warmed to −78 °C and then treated with 4,4-dimethoxybutan-2-one (11.36 g, 86.07 mmol). After 25 min, the reaction was quenched by the addition of saturated aq. NH_4Cl, and the solution was allowed to warm gradually to room temperature. The whole mixture was evaporated, and the resulting aqueous solution was extracted with diethyl ether. The extracts were washed with brine, dried and evaporated. Chromatography of the residue on silica gel with ethyl acetate–benzene yielded amyl 3-hydroxy-5,5-dimethoxy-3-methyl[2-^{13}C]pentanoate **11** (1.54 g, 64%); δ_H 0.905 (3 H, t, *J* 6.9), 1.30 (3 H, d, J_{CH} 3.9), 1.34–1.64 (6 H, m), 1.91 (2 H, m), 2.36 (1 H, dd, *J* 15, J_{CH} 129), 2.77 (1 H, dd, *J* 15, J_{CH} 129), 3.34 (3 H, s), 3.35 (3 H, s), 3.97 (1 H, d, J_{CH} 1.9), 4.10 (t, *J* 6.9) and 4.64 (1 H, t, *J* 5.6); δ_C 13.9, 22.3, 27.7, 28.1, 28.3, 43.1, 45.6 (90 atom%), 52.9, 53.1, 64.7, 69.5 (d, J_{CC} 38), 102.3 and 172.4 (d, J_{CC} 57).

[4-^{13}C]Mevalonolactone was prepared from compound **11** by a method similar to that reported by Cane and Levin.[10] Compound **11** (1.45 g, 5.52 mmol) was reduced by $LiAlH_4$, acetylated and oxidized with formic acid–hydrogen peroxide–1% sulfuric acid to yield [4-^{13}C]mevalonolactone (0.206 g, ~24% from **11**); δ_H 1.40 (3 H, d, J_{CH} 14, 1.92 [2 H, dm, $J(^{13}CH)$ 128], 2.53 [1 H, dd, J 17, $J(^{13}CCH)$ 1.3], 2.54 [1 H, dd, J 17, $J(^{13}CCH)$ 1.3], 4.34 (1 H, m) and 4.60 (1 H, m); δ_C 29.2, 35.7 (m, 90 atom%), 44.7, 65.3 [d, $J(^{13}CC)$ 35], 68.5 [d, $J(^{13}CC)$ 37 Hz] and 170.2; GLC–MS m/z 72 [CH_2=^{13}CH(CH_3)=O^+H], 58 and 43 (100%).

[5-$^2H_2$,5-^{13}C]*Mevalonolactone*.—Amyl 3-hydroxy-5,5-dimethoxy-3-methyl[1-^{13}C]pentanoate **12** (2.47 g, 9.39 mmol) was prepared from [1-^{13}C]acetic acid (99.5 atom%) by a method similar to that for its regioisomer **11**. Compound **12** had δ_H 0.905 (3 H, t, J 6.9), 1.29 (3 H, s), 1.34–1.64 (6 H, m), 1.91 (2 H, m), 2.48 [1 H, dd, J 15.0, $J(^{13}CCH)$ 6.9], 2.58 [1 H, dd, J 15.0, $J(^{13}CCH)$ 6.9], 3.34 (3 H, s), 3.35 (3 H, s), 4.00 (1 H, br s), 4.10 [2 H, dt, J 6.6, $J(^{13}COCH)$ 2.8] and 4.64 (1 H, t, J 5.3); δ_C 13.9, 22.3, 27.7, 28.0, 28.2, 43.1, 45.1 [d, $J(^{13}CC)$ 57], 52.9, 53.1, 64.8, 69.5, 102.2 and 172.3 (99.5 atom%).

Compound **12** (2.47 g, 9.39 mmol) was reduced with Li^{2}H[$^2H_4$] (0.94 g, 2.24 mmol; 98 atom%) to give 5,5-dimethoxy-3-methyl[1-^{13}C,1-$^2H_2$]pentane-1,3-diol **13** (1.63 g, 95.8%). Compound **13** was acetylated, and the acetate was oxidized with formic acid–hydrogen peroxide–1% sulfuric acid to yield [5-^{13}C, 5-$^2H_2$]mevalonolactone (0.54 g, ~39% from compound **12**). [5-^{13}C, 5-$^2H_2$]Mevalonolactone had δ_H 1.40 (3 H, s), 1.62 (1 H, br s), 1.89 [1 H, d, $J(^{13}CCH)$ 4.9], 2.52 (1 H, d, J 18) and 2.66 (1 H, dd, J 18 and 1.3); δ_C 29.8, 35.7 [d, $J(^{13}CC)$ 35], 44.7, 65.3 (m, 99.5 atom%), 68.5 and 170.2; GLC–MS m/z 74 [$^{13}CD_2$=CHC(CH_3)=O^+H], 58 and 43 (100%).

Feeding Experiment.—The origin of the sample of *H. planus*, and medium and culture conditions for the suspension culture have been described previously.[4] Nine to sixteen cultures, each an average of 200 mg (fresh weight) in MSK-4 medium[3] (13.5 cm^3) were incubated with isotopically labelled MVA (0.18 mmol, 23.8 mg). The liquid suspensions were agitated at 110 rpm at 25 ± 1 °C under continuous light (5000 lux). The cells were harvested 21 days after inoculation to determine the stable isotope enrichment by GLC–MS and to isolate the biosynthetically labelled MDC.

GLC and GLC–MS Analysis.—To determine the composition of each reaction fraction obtained by chromatography, GLC analyses were carried out under the following conditions; column temperature: initial temperature 150 °C, elevated at 2 °C min^{-1} to 220 °C; He flow rate of 1.4 cm^3 min^{-1}. GLC–MS analyses were carried out under the following conditions; column temperature: initial temperature 60 °C (was kept for 5 min), then was elevated at 2 °C min^{-1} to 220 °C; He flow rate of 1.13 cm^3 min^{-1}; ionizing voltage 70 eV.

Isolation of Isotopically Labelled MDC.—Fresh cells were extracted with MeOH (5 v/w, × 2) for 5 h and with pentane for 12 h. The MeOH extracts were combined, and partitioned with pentane. The pentane solutions were combined, concentrated and chromatographed by HPLC on an ODS column (30 cm × 1.5 cm, i.d.) with MeOH at 1 cm^3 min^{-1}. The fractions containing MDC were further separated by chromatography on a silica gel column (24 cm × 1 cm, i.d.) with hexane–chloroform (4:1) to yield MDC (0.3–0.9 mg; 85–91% purity).

Acknowledgements

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