Biosynthesis of Heteroscyphic Acid A in Cell Cultures of *Heteroscyphus planus*: Nonequivalent Labelling of C-5 Units in Diterpene Biosynthesis

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Preferential labelling in the farnesyl diphosphate (FPP)-derived portion and a 1,2-migration of the methyl (C19) originating at C-6 of mevalonate in the biosynthesis of heteroscyphic acid A {(12*Z*)-5,10-*trans*-cleroda-3,12,14-trien-20-oic acid} incorporating [2-^{13}C]- and [4,5-$^{13}C_2$]-mevalonates are evident from the ^{13}C-enrichment and the complete ^{13}C-^{13}C coupling pattern.

Earlier *in vivo* tracer studies of lower terpene (mono- and sesqui-terpene) biosynthesis using isotopically labelled precursors such as mevalonate (MVA) or acetate have provided valuable information on the lower terpenoid biosynthesis and its regulation, such as translocation, compartmentation and the metabolic turnover, in higher plants.[1,2] The biosynthesis of lower terpenes from exogenous ^{14}C-labelled MVA in tissues from higher plants usually yielded preferentially labelled terpenes in the isopentenyl diphosphate (IPP)-derived portion of molecules, suggesting that preferential labelling results from the condensation of IPP derived from exogenous ^{14}C-labelled MVA with dimethylallyl diphosphate (DMAPP) that is mainly present in a metabolic pool.

Heteroscyphic acid A **1**[3] and 7-methoxy-1,2-dihydrocadalene (**2**, MDC)[4] are the main diterpene and sesquiterpene, respectively, from the suspension cultures of *H. planus*. Compound **2** biosynthesized from exogenous ^{2}H- and ^{13}C-labelled MVA was shown to be equivalently labelled at the higher label (4–19 atom % excess), and thus the labelling pattern was determined by GC-MS[5] and ^{2}H and ^{13}C NMR analyses.[6] As an extension of this study, we now describe studies using ^{13}C-labelled MVAs, which verified the most unexpected feature of the ^{13}C-labelling pattern, that is, preferential labelling in the FPP-derived portion of heteroscyphic acid A.

[4,5$^{13}C_2$]- and [2-^{13}C]-MVA were prepared from 3-methylbutyl [1,2-$^{13}C_2$] acetate (both 99 atom %)[6] and ethyl [2-^{13}C]-bromoacetate (99 atom %),[7] respectively, by the method reported previously. Cell cultures of *H. planus* were grown in MSK-4 medium[8] (2 × 75 ml), to which were fed 1.0 mmol of potassium MVA, under continuous light at 25 °C. Heteroscyphic acid A enriched with [2-^{13}C]-MVA was obtained by the

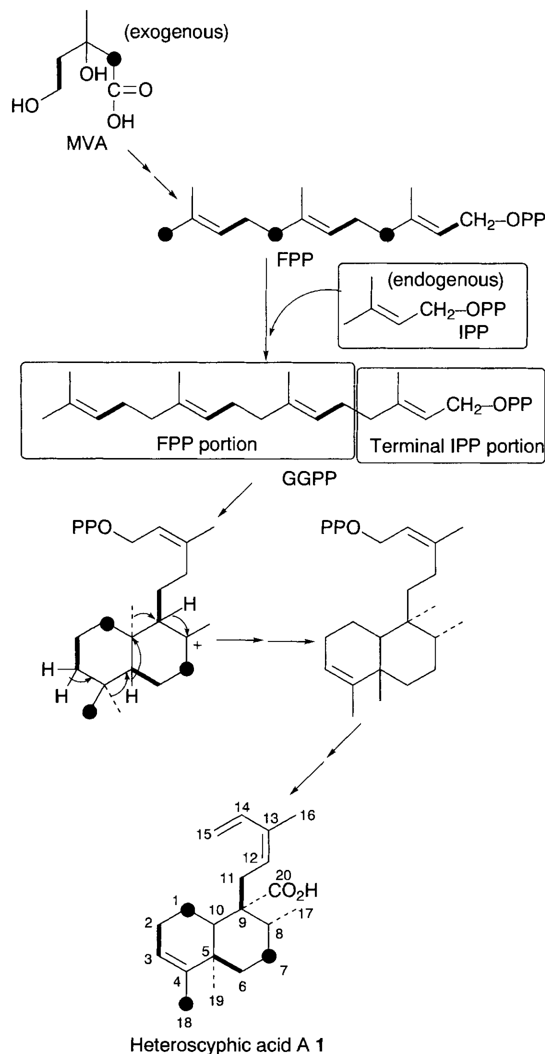
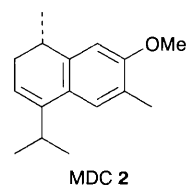


Fig. 1 Biosynthetic pathway of heteroscyphic acid A

Table 1 Isotopically enriched resonances in the ^{13}C{^{1}H}-NMR spectra of heteroscyphic acid A isolated from the suspension cultures of *Heteroscyphus planus* supplemented with potassium [2-^{13}C] and [4,5-$^{13}C_2$]-mevalonates

		[2-^{13}C]-MVA	[4,5-$^{13}C_2$]-MVA[a]	
Carbon	δ_C	Atom % excess	$J_{^{13}C,^{13}C}$/Hz	Relative intensity[b]
1	20.6	0.9		
2	27.2	—	$J_{^{13}C-2,^{13}C-3}$ = 41.5	0.28
3	121.0	—		
4	143.6	—		
5	38.5	—	$J_{^{13}C-5,^{13}C-6}$ = 34.2	0.29
6	37.1	—		
7	27.6	0.7		
8	37.6	—		
9	50.7	—	$J_{^{13}C-9,^{13}C-11}$ = 32.9	0.28
10	48.8	—		
11	32.2	—		
12	127.0	—		
13	136.3	—		
14	141.7	—	Not observed	
15	111.1	—		
16	12.1	—		
17	17.1	—		
18	18.1	1.1		
19	17.5	—		
20	176.4	—		
Average		0.9		0.28

[a] ^{13}C {^{1}H}-NMR was measured for the methyl ester. [b] Relative peak intensity of ^{13}C–^{13}C coupled resonances to natural abundant resonance.



repeated HPLC as described previously,[3] while that with [4,5-$^{13}C_2$]-MVA was further purified by HPLC after methylation. The resulting $^{13}C\{^1H\}$ NMR (67.8 MHz) analysis of heteroscyphic acid A (and its methyl ester) clearly showed the labelling pattern as illustrated in Fig. 1.

Although the incorporation of [2-^{13}C]-MVA into heteroscyphic acid A (average: 0.9 atom % excess, Table 1) was considerably lower than that into MDC (10.1 atom % excess), the ^{13}C signals corresponding to C-1, C-7 and C-18 were apparently enhanced with the ^{13}C atom, while the intensity of C-12 was much less than that expected from equivalent labelling of diterpenoid biosynthesis. In the $^{13}C\ \{^1H\}$ NMR spectrum of heteroscyphic acid A (as methyl ester; purity: > 98.5%) incorporating [4,5-$^{13}C_2$]-MVA the ^{13}C–^{13}C coupling patterns were observed between C-2 and C-3 (the relative peak intensity of ^{13}C–^{13}C coupled resonances to the natural abundant resonance: 0.28, $J_{C\text{-}2, C\text{-}3}$ = 41.5 Hz), 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, indeed confirming 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 at C-2 of MVA at the C-18 position provides indirect evidence for a 1,2-methyl (originating a 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 at C-2 of mevalonate shifts to the C-5 position in the biosynthesis of the *cis*-clerodane skeleton.[9]

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 that may be localized in certain subcellular organelles of the liverwort, or merely by the slower synthesis of geranylgeranyl diphosphate in comparison with the overall utilization of MVA.

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