Biosynthesis of Chlorophyll a from ^{13}C-Labelled Mevalonates and Glycine in Liverwort. Nonequivalent Labelling of Phytyl Side Chain

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The phytyl side chain of chlorophyll a derived from exogenous ^{13}C- and ^{2}H-labelled mevalonate in cultured cells of two species of liverworts, *Heteroscyphus planus* and *Lophocolea heterophylla*, is shown to be preferentially labelled in the farnesyl diphosphate-derived portion, while its biosynthesis from exogenous ^{13}C-glycine results in equivalent labelling.

Preferential labelling in the farnesyl diphosphate (FPP)-derived portion in the biosynthesis of diterpenoic acid, heteroscyphic acid A, in cultured cells of *Heteroscyphus planus* suggests that diterpenes are biosynthesized from geranylgeranyl diphosphate (GGPP) *via* the condensation of FPP derived from the exogenously supplied mevalonate (MVA) with endogenous isopentenyl diphosphate (IPP) within chloroplasts in the liverwort.[1] In the biosynthesis of chlorophylls, the phytyl ester formation and the reduction of a initial product to chlorophyll are the last steps that take place at the thylakoids in chloroplasts.[2,3] In this paper we determine whether the nonequivalent labelling observed in the formation of heteroscyphic acid A takes place in the biosynthesis of the phytyl moiety of chlorophyll and the possibility of ubiquitous presence of this type of labelling in the biosynthesis of all compounds which are formed from GGPP in chloroplasts of liverworts.

[2-^{13}C]- (99 atom%), [4,5-$^{13}C_2$]- (both 99 atom%) and [2,2-$^{2}H_2$]-MVA (99.3 atom%) were prepared by the procedure reported previously.[1] Cell cultures of *H. planus* were grown in MSK-medium[4] (8 × 75 ml), to which were fed potassium MVA (1.0 mmol) or [2-^{13}C]glycine (1.0 mmol, 99.2 atom%) under continuous light at 25 °C. [2,2-$^{2}H_2$]MVA was also fed to cell culture of *Lophocolea heterophylla*.[5] Chlorophyll a **1** was isolated from 21 day old cultures by the method reported previously,[6] then hydrolysed by 16.7% aq. Cs_2CO_3 to afford phytol **2**. The ^{2}H{^{1}H} and ^{13}C{^{1}H} NMR spectra of biosynthetically ^{2}H or ^{13}C labelled chlorophyll a and phytol (and its acetate) were recorded at 41.3 MHz ($CDCl_3$ as internal standard: $\delta_{^2H}$ 7.26) and 67.8 MHz ($^{13}CDCl_3$: $\delta_{^{13}C}$ 77.0), respectively. Assignments of all ^{13}C atoms in phytol[7,8] and a carboxy methyl carbon[9] in chlorophyll a were based on the data previously reported. As expected,[9] the ^{2}H and ^{13}C NMR spectra of the labelled chlorophyll a incorporating the labelled MVAs showed that exogenous MVA was not incorporated into the chlorophyllide moiety. The ^{13}C{^{1}H} NMR spectrum of phytol incorporating [2-^{13}C]MVA showed that three ^{13}C signals corresponding to C-8, C-12 and C-16 were intensely enhanced with ^{13}C atoms (av. 6.5 atom% excess, see Table 1 and Fig. 1), while the enrichment factor for C-4 was much less (0.7 atom%). With [4,5-$^{13}C_2$]-MVA as precursor, the ^{13}C{^{1}H} NMR spectrum of phytol showed three pairs of intense ^{13}C–^{13}C coupled resonances between C-5 and C-6 (12.7 atom% excess, $J_{C\text{-}5,C\text{-}6}$ 34.2 Hz), between C-9 and C-10 (8.4, 34.2 Hz) and between C-13 and C-14 (9.4, 34.2 Hz) with one weak pair between C-1 and C-2 (1.6, 47.6 Hz). These findings indeed indicate that the FPP-derived portion of the phytyl side chain was preferentially labelled with exogenously supplied MVA, and that biosynthesis of chlorophyll a can utilize the exogenous MVA more efficiently than that of heteroscyphic acid A (0.9 atom% excess),[1] suggesting that heteroscyphic acid A may be formed at a different site such as envelope in chloroplasts. This preferential labelling was also proven by ^{2}H {^{1}H} NMR of chlorophyll a and phytol incorporating [2,2-$^{2}H_2$]MVA in cell cultures of both *H. planus* and *L. heterophylla*, which showed three broad singlets at δ 1.23 in phytol (1.17 in chlorophyll a), 1.05 (0.99) and 0.87 (0.81), corresponding to phytol labelled with ^{2}H at C-8, C-12 and C-16 but no enhanced peak in phytol at δ 2.00 corresponding ^{2}H at C-4 (assignments of ^{1}H atoms in phytol were acheived by ^{1}H–^{13}C 2D COSY NMR analysis). This result also demonstrates that this type of nonequivalent labelling may take place in a wider range of liverworts.

The ^{13}C{^{1}H} NMR spectrum of chlorophyll a formed in the presence of [2-^{13}C]glycine indicated that a ^{13}C signal at δ 51.5, corresponding to the ester Me at C-13, has increased (4.4 atom% excess), but little or no enrichment has occurred in the chlorophyll macrocycles. This methylation process at C-13 has been previously shown to involve the participation of (*S*)-adenosylmethionine[10] the methyl group of which originates from the C-2 carbon of glycine.[9] ^{13}C enrichment in the phytyl side chain was determined after acetylation of phytol by comparing the relative intensities of the ^{13}C enriched carbons to the acetyl methyl carbon with those of the corresponding carbons in the non-labelled compound. The ^{13}C{^{1}H} NMR spectrum of the acetylated phytol **3** showed sixteen ^{13}C enriched peaks with doublets due to ^{13}C–^{13}C couplings (C-1–C-2, C-3–C-20, C-5–C-6, C-7–C-19, C-9–C-10, C-11–C-18, C-13–C-14 and C-15–C-17) and four intense singlet peaks without

Table 1 ^{13}C enrichments of carbons in phytol and phytyl acetate obtained from chlorophyll a incorporating ^{13}C labelled mevalonates and [2-^{13}C]glycine

Carbon	δ_C in Phytol (in acetate)	^{13}C Enrichment (atom% excess)[a] [2-^{13}C]-MVA	[4,5-$^{13}C_2$]-MVA (J_{CC}/Hz)	[2-^{13}C]glycine (atom% of doublets)
C-1	59.44 (61.40)		1.56 (47.6)	1.20 (0.79)[b]
C-2	123.06 (117.95)			2.09 (1.26)
C-3	140.36 (142.73)			1.79 (0.44)
C-4	39.88 (39.84)	0.7		1.24 —
C-5	25.15 (25.00)		12.67 (34.2)	1.15 (0.39)
C-6	36.66 (36.61)			1.60 (0.84)
C-7	32.69 (32.73)			2.12 (0.76)
C-8	37.29 (37.27)	6.3		0.66 —
C-9	24.48 (24.44)		8.39 (34.2)	1.63 (0.70)
C-10	37.43 (37.40)			0.63 (0.10)
C-11	32.80 (32.76)			1.37 (1.21)
C-12	37.36 (37.32)	6.8		0.29 —
C-13	24.80 (24.78)		9.36 (34.2)	1.96 (0.71)
C-14	39.37 (39.34)			2.42 (2.14)
C-15	27.98 (27.94)			2.18 (0.73)
C-16	22.72 (22.70)	6.3		1.40 —
C-17	22.63 (22.61)			1.78 (0.54)
C-18	19.75 (19.71)			1.99 (0.65)
C-19	19.71 (19.70)			1.93 (0.67)
C-20	16.18 (16.34)			1.78 (0.68)
MeCO	(171.10)			
*Me*CO	(21.03)			
Average				1.52 (0.79[c])

[a] ^{13}C Enrichments in phytol and phytyl acetate were determiend with mevalonate and glycine, respectively, as substrates. [b] Coupling constants in phytyl acetate incorporating ^{13}C-glycine, $J_{^{13}C,^{13}C}$/Hz: C-1–C-2 50.0, C-3–C-20 41.5, C-5–C-6 35.4, C-7–C-19 35.4, C-9–C-10 35.4, C-11–C-18 35.4, C13–C-14 35.3, C-15–C-17 32.9. [c] Average value except for C-4, C-8, C-12 and C-16.

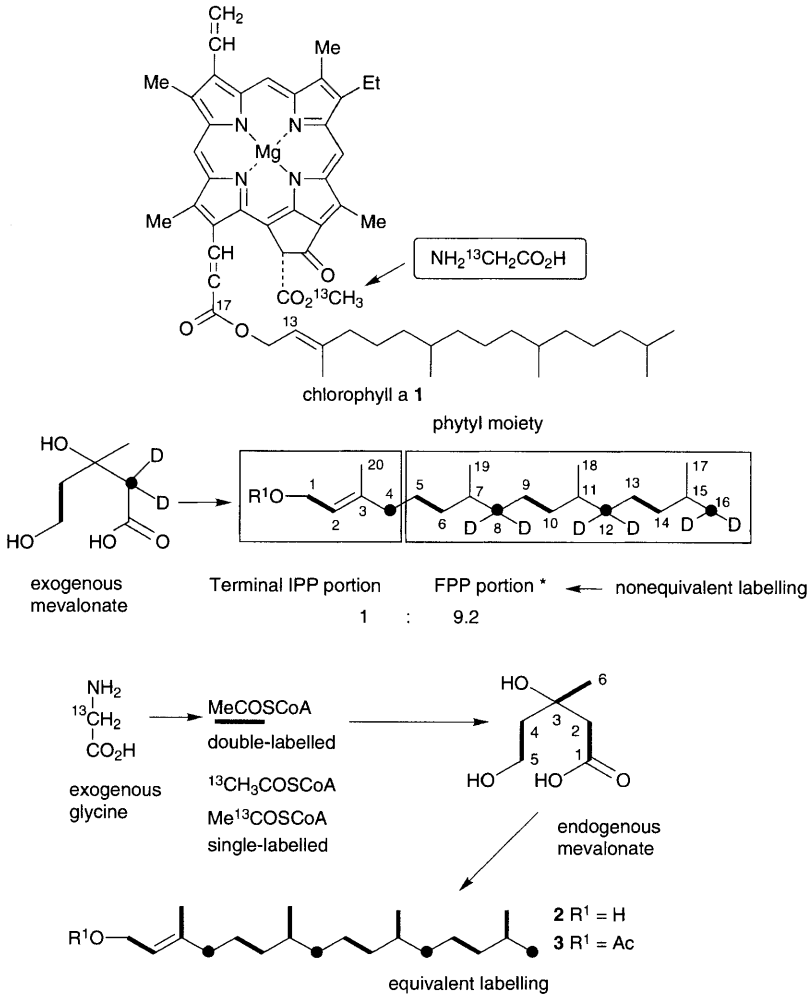

Fig. 1 Labelling patterns of chlorophyll a. Bold lines: ^{13}C–^{13}C couplings were observed. *: Average ^{13}C enrichment of the FPP portion to the terminal IPP in the phytyl side chain of **1** incorporating [2-^{13}C]- and [4,5-$^{13}C_2$]-MVA (Table 1).

doublets at δ 39.9 (C-4), 37.3 (C-8), 37.4 (C-12) and 22.7 (C-16) (see Table 1). The ^{13}C–^{13}C coupled resonances demonstrate that double-labelled acetyl CoA, an obligatory intermediate in chloroplast terpene biosynthesis, was formed from two C-2 carbons of exogenous [2-^{13}C]glycine together with single labelled species (Fig. 1) *via* known metabolic routes for formation of acetyl CoA from the carbon dioxide fixation pathway in chloroplasts,[11] and that it was further converted to MVA. The intense singlet peaks arise from the breaking C-1–C-2 bond in the resulting MVA during the conversion of MVA to IPP. The intensities of ^{13}C enriched peaks with doublet peaks (the sum of those of ^{13}C–^{13}C coupled peaks and centre peaks) and those of the singlet peaks without doublet peaks indicate the equivalent ^{13}C enrichment (an average ^{13}C enrichment: 1.52 atom% excess) in all carbon atoms of phytol. Thus it is clear that the endogenously formed MVA is equivalently incorporated in the phytyl moiety.

These findings suggest that GGPP is biosynthesized by the condensation of FPP from two different sources, extraplastidically formed FPP and FPP arising from photosynthetically fixed carbon dioxide within chloroplasts, with endogenous IPP. Alternatively, nonequivalent labelling might be merely a reflection of the stage in the growth cycle at which FPP and GGPP are biosynthesised within chloroplasts.

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