Biosynthesis of Chloroplastidic and Extrachloroplastidic Terpenoids in Liverwort Cultured Cells: ^{13}C Serine as a Probe of Terpene Biosynthesis via Mevalonate and Non-mevalonate Pathways

Daisuke Itoh,[†] Kyouhei Kawano,[‡] and Kensuke Nabeta*[,‡]

The United Graduate School of Agricultural Science, Iwate University, Morioka 020-8550, Japan, and Department of Agricultural and Life Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan

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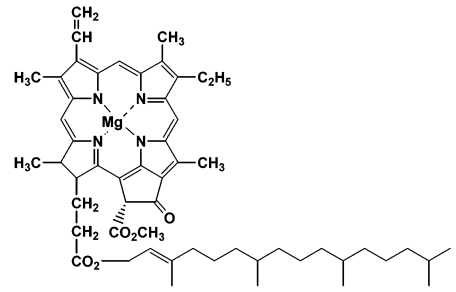
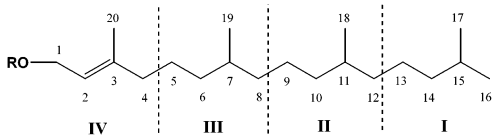
Two terpenoid biosynthetic pathways, the mevalonate and non-mevalonate (glyceraldehyde phosphate-pyruvate) routes, were examined by feeding ^{13}C-labeled serines ([1-^{13}C]- and [3-^{13}C]-) to the cultured cells of the liverwort, *Heteroscyphus planus*. The labeling patterns observed in the isoprenoid unit of the biosynthetically ^{13}C-labeled stigmasterol corresponded to those expected from the mevalonate pathway, while those of the phytyl side chain corresponded to those from the non-mevalonate pathway. Thus, serine is a potential probe to determine the origin of terpenoid biosynthesis, in either the mevalonate or non-mevalonate pathway.

Isoprenoids are derived by the consecutive condensations of isopentenyl diphosphate (IPP, **10** in Figure 1) and its isomer dimethylallyl diphosphate (DMAPP).[1,2] The acetate/mevalonate (MVA) pathway has been accepted for many years as the sole pathway to IPP in the isoprenoid biosynthesis in plants, until the glyceraldehyde phosphate-pyruvate route (non-MVA pathway) was discovered.[3,4] The more recently identified non-MVA pathway occurs in plants,[5−7] protozoa,[8] most bacteria,[3,8,9] and algae.[6,8,10] In vascular plants and liverworts, the non-MVA pathway occurs in plastids, where monoterpenes (C_{10}), diterpenes (C_{20}), carotenoids (C_{40}), plastoquinones, and phytol (**2**) conjugates such as chlorophylls and tocopherols are biosynthesized, while the MVA pathway occurs in the cytoplasm/endoplasmic reticulum, where sesquiterpenes (C_{15}) and triterpenes (C_{30}) are produced.[6,11,12] The non-MVA pathway can be distinguished from the MVA pathway on the basis of different labeling patterns of the isoprenoid units resulting after the incorporation of ^{13}C-labeled precursors (glucose,[5,8,10−12] acetate,[9,10,13−15] erythrose,[16] and pyruvate[3,8]). The segregation of the two compartments, however, is not necessarily absolute, and the exchange of precursors for terpenoid synthesis between the two compartments has been reported.[6,7,17] When ^{2}H- or ^{13}C-labeled acetates were administered to the cultured cells of liverworts, extensive scrambling of labels was observed in the formation of chloroplastid terpenoids,[15] while the acetate labels were incorporated into the expected position of the sesquiterpenes according to the classical MVA pathway and no scrambling of the isotopic enrichment occurred.[13,14] Thus, a direct and uninterrupted carbon flow from the intermediates of cytoplasmic IPP synthesis to the chloroplastid IPP synthesis appears doubtful, and the intermediates for chloroplastid IPP synthesis may not be channeled into the MVA pathway in the cytoplasm.

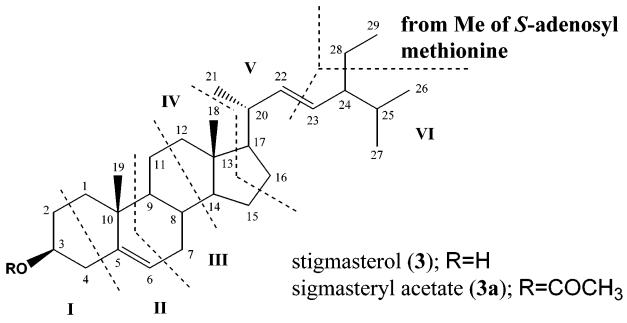
In this report, in an attempt to distinguish between the MVA and non-MVA pathways, we administered ^{13}C serines (**4**) ([1-^{13}C]- and [3-^{13}C]-) to the cultured cells of liverwort, *Heteroscyphus planus* (Mitt.) Schiffn (Geocalycaceae). We report herein that serine is a potential probe to determine the origin of terpenoid biosynthesis, either the MVA or non-MVA pathway, and can be used to support the hypothesis that IPP biosynthesis from serine in the plastids is not channeled to the IPP synthesis in the cytoplasm/endoplasmic reticulum.



chlorophyll *a* (**1**)



phytol (**2**); R=H
phytyl acetate (**2a**); R=$COCH_3$



stigmasterol (**3**); R=H
sigmasteryl acetate (**3a**); R=$COCH_3$

Results and Discussion

The cell cultures of *H. planus* were grown in MSK-4 medium[18] (4 × 75 mL), to which were fed 0.38 mmol of

* To whom correspondence should be addressed. Tel: +81-155-49-5540. Fax: +81-155-49-5544. E-mail: knabeta@obihiro.ac.jp.
[†] Iwate University.
[‡] Obihiro University of Agriculture and Veterinary Medicine.

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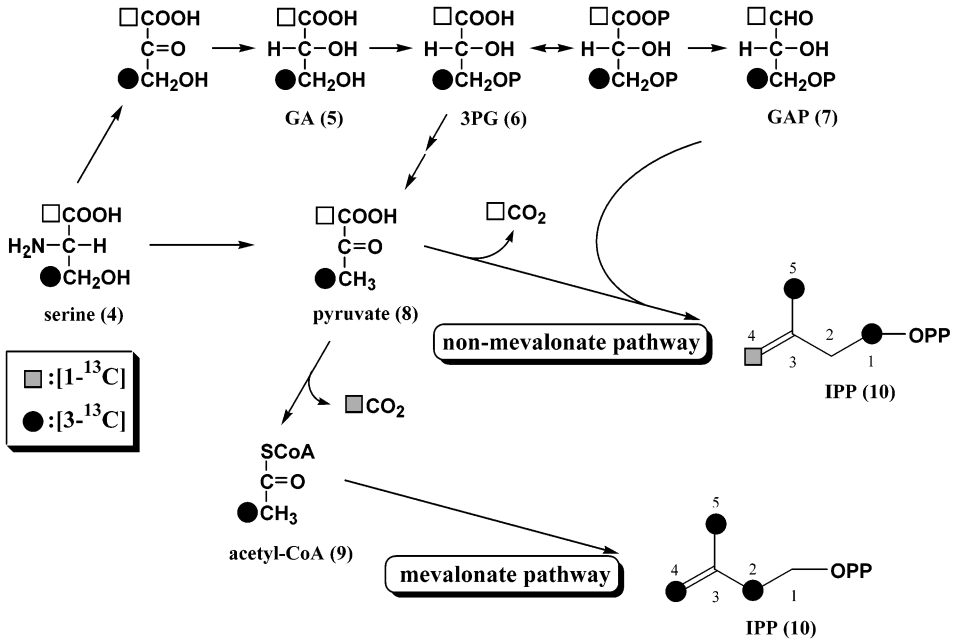

Figure 1. Biosynthesis of IPP from ^{13}C-labeled serine via the MVA pathway and the non-MVA pathway.

Table 1. ^{13}C Enrichment in Carbons of Phytyl Acetate (**2a**) Derived from Suspended Cultured Cells of *H. planus* Administered [1-^{13}C]Serine and [3-^{13}C]Serine

carbon atom	carbon atom in IPP units[a]	δ^{13}C in **2a**	^{13}C atom % excess[b]	
			[1-^{13}C]serine	[3-^{13}C]serine
C-1	1_{IV}	61.44	0.58 ± 0.05	**1.16 ± 0.26**
C-2	2_{IV}	117.92	0.89 ± 0.09	0.43 ± 0.04
C-3	3_{IV}	142.81	0.77 ± 0.17	0.23 ± 0.20
C-4	4_{IV}	39.84	**2.31 ± 0.10**	0.48 ± 0.16
C-5	1_{III}	25.00	1.08 ± 0.09	**0.87 ± 0.57**
C-6	2_{III}	36.61	1.04 ± 0.11	0.18 ± 0.31
C-7	3_{III}	32.65	1.11 ± 0.04	0.81 ± 0.25
C-8	4_{III}	37.34	**1.26 ± 0.16**	−0.01 ± 0.07
C-9	1_{II}	24.44	0.84 ± 0.10	**0.62 ± 0.51**
C-10	2_{II}	37.40	0.67 ± 0.12	0.10 ± 0.04
C-11	3_{II}	32.78	0.59 ± 0.06	0.52 ± 0.34
C-12	4_{II}	37.27	**2.20 ± 0.07**	0.73 ± 0.18
C-13	1_{I}	24.78	1.27 ± 0.04	**1.56 ± 0.08**
C-14	2_{I}	39.36	0.51 ± 0.13	0.33 ± 0.20
C-15	3_{I}	27.96	1.49 ± 0.05	0.89 ± 0.14
C-16	4_{I}	22.61	**1.91 ± 0.18**	0.16 ± 0.23
C-17	5_{I}	22.72	0.70 ± 0.07	**1.58 ± 0.29**
C-18	5_{II}	19.73	1.26 ± 0.19	**1.46 ± 0.47**
C-19	5_{III}	19.70	1.28 ± 0.05	**1.79 ± 0.36**
C-20	5_{IV}	16.35	0.86 ± 0.09	**0.95 ± 0.60**
average			1.13±0.10	0.74 ± 0.26
carbonyl C		171.16		
methyl C		21.08		

[a] The positions of isopentenyl units are given by arabic numbers, and their numbering is given by roman numerals. [b] Average value of three ^{13}C measurements.

Table 2. ^{13}C Enrichment in Carbons of Stigmasteryl Acetate (**3a**) Derived from Suspended Cultured Cells of *H. planus* Administered [1-^{13}C]Serine and [3-^{13}C]Serine

carbon atom	carbon atom in IPP units[a]	δ^{13}C in **3a**	^{13}C atom % excess[b]	
			[1-^{13}C]serine	[3-^{13}C]serine
C-1	4_{II}	36.98	0.49 ± 0.15	**1.36 ± 0.17**
C-2	1_{I}	27.75	0.49 ± 0.13	0.43 ± 0.16
C-3	2_{I}	73.98	0.50 ± 0.15	**1.27 ± 0.15**
C-4	3_{I}	38.10	0.46 ± 0.06	0.26 ± 0.09
C-5	2_{II}	139.64	0.69 ± 0.13	**1.79 ± 0.21**
C-6	1_{II}	122.62	0.21 ± 0.04	0.05 ± 0.04
C-7	4_{III}	31.86[c]	not calcd	not calcd
C-8	3_{III}	31.86[c]	not calcd	not calcd
C-9	2_{III}	50.03	0.17 ± 0.08	**0.87 ± 0.13**
C-10	3_{II}	36.59	0.17 ± 0.06	0.08 ± 0.10
C-11	1_{III}	20.99	0.26 ± 0.12	0.23 ± 0.08
C-12	1_{IV}	39.61	0.01 ± 0.02	0.02 ± 0.16
C-13	2_{IV}	42.19	0.09 ± 0.06	**0.66 ± 0.15**
C-14	3_{IV}	56.77	−0.03 ± 0.09	−0.07 ± 0.1
C-15	4_{IV}	24.33	0.03 ± 0.08	**0.62 ± 0.08**
C-16	1_{V}	28.90	0.00 ± 0.06	−0.09 ± 0.06
C-17	2_{V}	55.90	−0.06 ± 0.08	**0.68 ± 0.17**
C-18	5_{IV}	12.02	0.02 ± 0.04	**0.74 ± 0.08**
C-19	5_{II}	19.30	0.42 ± 0.10	**1.48 ± 0.26**
C-20	3_{V}	40.49	−0.02 ± 0.05	−0.19 ± 0.07
C-21	5_{V}	21.21	−0.02 ± 0.05	**0.62 ± 0.17**
C-22	4_{V}	138.31	0.02 ± 0.07	**0.55 ± 0.15**
C-23	1_{VI}	129.25	−0.03 ± 0.11	−0.11 ± 0.09
C-24	2_{VI}	51.21	−0.07 ± 0.07	**0.41 ± 0.11**
C-25	3_{VI}	31.86[c]	not calcd	not calcd
C-26	4_{VI}	21.08	0.15 ± 0.09	**0.47 ± 0.15**
C-27	5_{VI}	18.96	0.05 ± 0.04	**0.71 ± 0.15**
C-28	Met	25.39	0.09 ± 0.07	**1.65 ± 0.16**
C-29	Met	12.24	0.08 ± 0.02	**1.95 ± 0.11**
average			0.16±0.08	0.63 ± 0.13
carbonyl C		170.55		
methyl C		21.44		

[a] The positions of isopentenyl units are given by arabic numbers, and their numbering is given by roman numerals. [b] Average value of three ^{13}C measurements. [c] Overlapped peak.

DL-[1-^{13}C]- (99 at. % ^{13}C) or DL-[3-^{13}C]- (99 at. % ^{13}C) serine (**4**). Chlorophyll *a* (**1**) and stigmasterol (**3**) were isolated from 21-day-old cultures. Chlorophyll *a* was hydrolyzed by aqueous Cs_2CO_3 to afford phytol (**2**). ^{13}C[^{1}H] NMR spectra of phytol, stigmasterol and their acetates (**2a** and **3a**) were recorded at 67.5 MHz. Assignments of all ^{13}C atoms in phytol, stigmasterol, and their acetates were reported previously.[19−21] The extents of ^{13}C enrichment in the acetates of phytol and stigmasterol incorporating ^{13}C-labeled serines were determined by comparing the relative peak intensities ($RPA_{labeled}$) of the enriched carbons to acetyl methyl carbon with those (RPA_{nat}) of the corresponding carbons in the nonlabeled compounds using eq 1.[22]

$$\text{Enrichment (at. \% excess)} = 1.08 \times (RPA_{labeled}/RPA_{nat} - 1) \quad (1)$$

The ^{13}C enrichments of phytol and stigmasterol are sum-

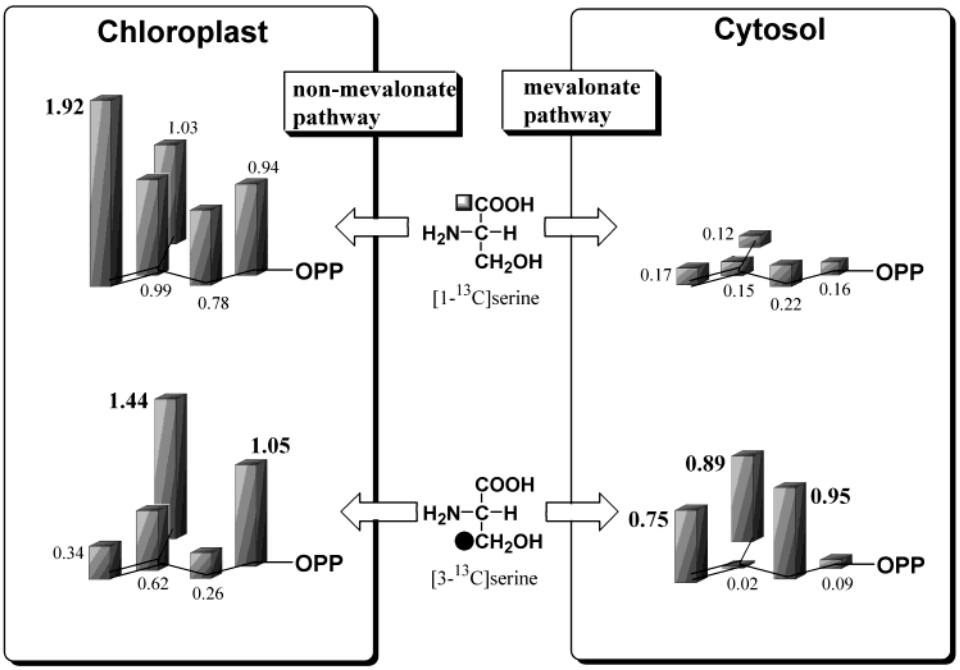

Figure 2. Average ^{13}C enrichment in carbon atoms of the C_5 isoprenic skeleton of the phytyl side chain and stigmasterol incorporating [1-^{13}C]-serine and [3-^{13}C]serine.

marized in Tables 1 and 2, respectively, showing that chloroplastidic phytol was slightly increased when labeled with the ^{13}C serines (average ^{13}C enrichments: 1.13 at. % excess with [1-^{13}C]serine and 0.74% with [3-^{13}C]serine) than cytoplasmic stigmasterol (0.16 at. % excess and 0.63%, respectively). The hydroxymethyl carbon (C-3) of serine was incorporated into both phytol and stigmasterol at an almost identical level.

As can be seen in Table 1, the carboxy carbon (C-1) of serine was universally incorporated into all carbon atoms of phytol (**2**). The carbons (average ^{13}C enrichment: 1.92 at. % excess) at C-4, C-8, C-12, and C-16 of the phytyl moiety, however, were labeled twice as intensely as other carbon atoms (0.93 at. % excess) in phytol. Thus, the C-4 position of the isopentenyl moiety of phytol was predominantly enriched with the carboxy carbon of serine (Figure. 2). In contrast, carbon atoms at C-1, C-5, C-9, and C-13, which correspond to the C-1 position (1.05 at. % excess) of the isopentenyl moiety, and those at C-17, C-18, C-19, and C-20, to the C-5 position (1.44 at. % excess) of the isopentenyl moiety, were labeled from the hydroxymethyl carbon of serine. The carbon atoms at the C-1 and the C-5 positions of the isopentenyl moiety of phytol were much more intensely labeled from the hydroxymethyl carbon (C-3) of serine, although the other carbon positions of the isopentenyl moiety (C-1, C-2, and C-3, average ^{13}C enrichment: 0.41 at. % excess) were also enriched with the hydroxymethyl carbon.

The incorporation of ^{13}C serine into stigmasterol is summarized in Table 2. The ^{13}C enrichments in the stigmasterol (**3**) incorporating [1-^{13}C]serine demonstrate that the carboxy carbon atom was not actually utilized in the sterol biosynthesis. The ^{13}C label of the hydroxymethyl carbon of serine was incorporated into the carbons at C-3, C-5, C-9, C-13, C-17, and C-24 of stigmasterol, corresponding to the C-2 position (an average ^{13}C enrichment: 0.95 at. % excess, Figure 2) of the isopentenyl moiety, those at C-1, C-15, C-22, and C-26 (^{13}C enrichment of C-7 is not calculated because of overlapping peaks), corresponding to the C-4 position (0.75 at. % excess), and those at C-18, C-19, C-21, and C-27, corresponding to the C-5 position (0.89 at. % excess). The C-28 (1.65 at. % excess) and C-29 (1.95 at. % excess) carbons of stigmasterol were intensely enriched with ^{13}C of [3-^{13}C]serine, which is incorporated into 5-methyltetrahydrofolic acid (5-methyl THF, **14**) and is the carbon source for the *S*-adenosyl methionine (**18**) methyl group (Figure 3).[23,24] The data support that the C-28 and C-29 carbon atoms of stigmasterol arise from the *S*-adenosyl methionine methyl group during the alkylation of the steroid side chain.[21] Carbon-13 enrichment at the C-1 and C-3 positions of the isoprenyl unit of stigmasterol with the hydroxymethyl carbon of serine is not actually observed. Thus, labeling patterns obtained after incorporation of ^{13}C-labeled serine into the stigmasterol in *H. planus* cells via the MVA pathway were clear, and no scrambling had occurred.

Some of the exogenously supplied serine is postulated to penetrate the mitochondrial membrane and is then converted to glyceric acid (GA, **5**) after deamination and then reduction within peroxisomes. Glyceric acid is further reduced to pyruvate (**8**) via 3-phosphoglycerate (3PG, **6**) and phosphoenolpyruvate by the Embden−Mayerhof pathway. In addition, 3PG is biotransformed to glyceraldehyde-3-phosphate (GAP, **7**) via the reductive pentose-phosphate cycle during the photorespiration process (Figure 1).[25−27] The IPP framework via the non-MVA pathway is then formed by condensation of the C_2 units derived from the pyruvate decarboxylation on the C-2 carbon of GAP followed by a transposition. Thus, the carboxy carbon of serine is incorporated into the C-4 position of the IPP framework via the non-MVA pathway, while the hydroxymethyl carbon is incorporated into the C-1 and C-5 positions.[4,6] Acetyl CoA (**9**) is formed from pyruvate by decarboxylation, and then three molecules of acetyl CoA are assembled to MVA, which is converted to IPP via the generally understood IPP biosynthesis.[1] Thus, the hydroxymethyl carbon is incorporated into the C-2, C-4, and C-5 positions of the isoprenic unit via the MVA pathway, while the carboxy carbon of serine is not incorporated into any position of the isoprenoid unit. The labeling patterns of the phytyl moiety

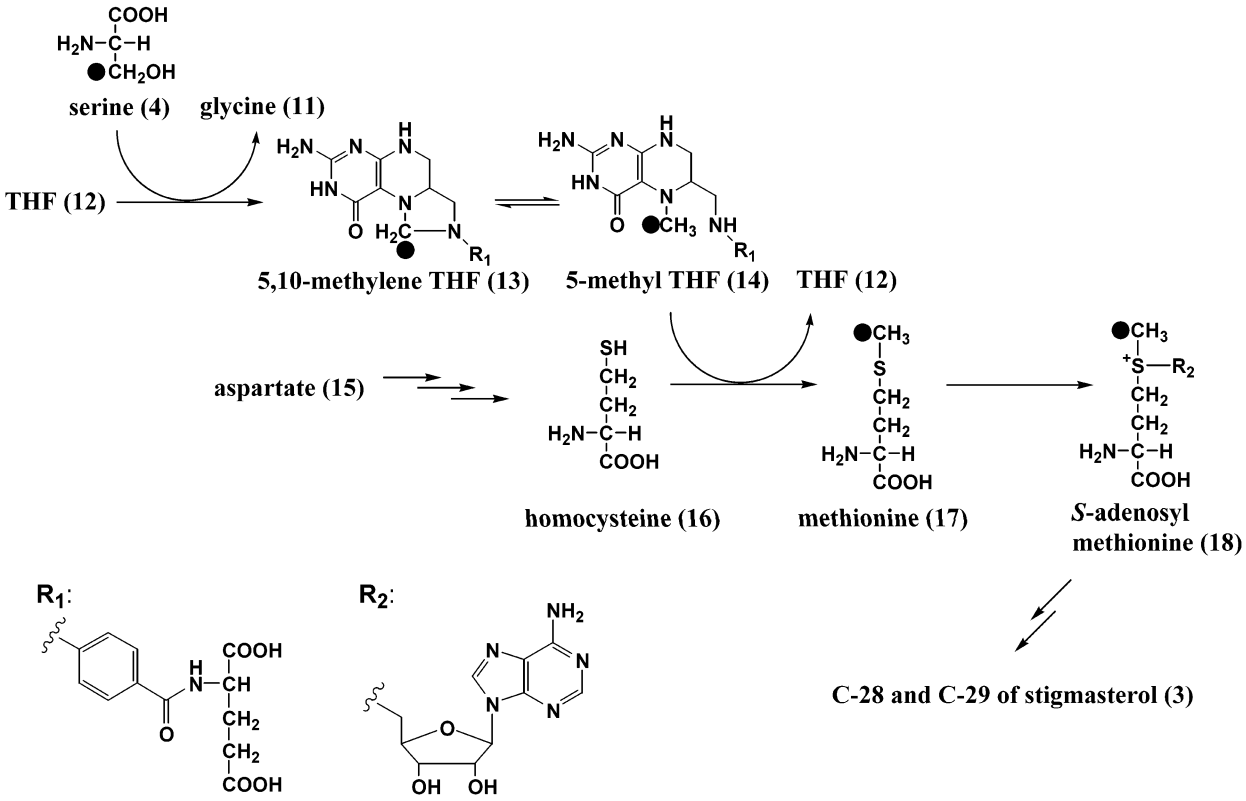

Figure 3. Incorporation of the C-3 atom of serine into the *S*-adenosyl methionine group.

of chlorophyll *a* and stigmasterol incorporating [1-^{13}C]- and [3-^{13}C]serine demonstrate that sterols are biosynthesized via the MVA pathway and the phytyl moiety via the non-MVA pathway.

The coexistence in two cell compartments of two biosynthetic routes for isoprenoid biosynthesis in liverwort cells with the MVA route present in the cytoplasm and the non-MVA route confined to the plastid was demonstrated for the first time by labeling experiment using ^{2}H and ^{13}C acetates and ^{2}H glucose into sesquiterpene (β-barbatene)[14] and the phytyl side chain of chlorophylls[15] in cultured cells of *H. planus* and ^{13}C glucose into sesquiterpenes (cubebanol and ricciocarpin A), stigmasterol, and phytol in those of *Ricciocarpos natans* and *Conocephalum conicum*.[11] The same dichotomy in the formation of hopanes and the plastidic terpenoids was recently observed in the labeling experiment using suspension cultured cells of *Fossombronia alaskana*.[12] However, some scrambling of ^{13}C label of [1-^{13}C]glucose was observed in the formation of the cytosolic isoprenoids, which may be caused by intricated metabolic turnover, while no scrambling was observed by labeling the cytosolic terpenoids with ^{13}C serine.

Although preferential incorporation of the carboxy and the hydroxymethyl carbons of serine into the phytyl side chain via the non-MVA pathway was observed, these carbons were universally incorporated into all carbon atoms of the phytyl moiety. Universal labeling can be rationalized by the explanation that carbon dioxide evolving from the carboxy carbon of serine via pyruvic acid was reutilized to biosynthesize isoprenoids in the chloroplasts through the tricarboxylic acid cycle (Krebs cycle), the reductive pentose cycle, and the glycolic acid pathway, in mitochondoria, chloroplasts, and peroxisomes, respectively.[25−27] The equivalently labeled glyceraldehyde-3-phosphate and pyruvate may be rebuilt with the ^{13}C atom of [1-^{13}C]serine as observed in the formation of phytol from the exogenous supplied acetate.[15] The label of hydroxymethyl of serine can also be randomized by several passages through the glycolic acid pathways and incorporated into the phytol molecule.

The above-described labeling experiments using ^{13}C-labeled serine have therefore led to the unambiguous detection of the occurrence of two distinct biosynthetic routes of the IPP in *H. planus* cultured cells: the MVA pathway in the cytoplasm, and the non-MVA pathway in the chloroplasts, demonstrating that IPP formed in the chloroplasts was not utilized in the terpenoid biosynthesis in the cytosol, while that in the cytosol was utilized in the terpenoid biosynthesis in the plastids.[5]

Experimental Section

General Experimental Procedures. The ^{1}H and ^{13}C[^{1}H] NMR spectra were recorded on a JEOL EX-270 NMR spectrometer at 270 MHz ($CHCl_3$ in $CDCl_3$ as an internal standard, δ ^{1}H 7.26) and 67.5 MHz ($^{13}CDCl_3$, δ ^{13}C 77.0), respectively. Assignments of all of the ^{13}C atoms in phytol (**2**), phytyl acetate (**2a**), and stigmasterol (**3**) were carried out according to previous data.[19,20] Assignments of the ^{13}C atoms in stigmasteryl acetate (**3a**) were carried out according to previous data[21] and CH-COSY analysis. Three ^{13}C NMR measurements were taken. GC-EIMS was measured on a Hitachi M-80B spectrometer equipped with a DB-WAX column (J&W Scientific, Folsom, CA; 30 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, used for **2** and **2a**) or a Silicone OV-1 column (Shimadzu GLC, Japan; 1 m × 3.4 mm i.d., isothermal at 250 °C, used for **3** and **3a**). The ionizing voltage employed was 70 eV. DL-[1-^{13}C]-Serine and DL-[3-^{13}C]serine (99 atom % ^{13}C) were purchased from Isotec, Miamisburg, OH.

Cell Culture and Feeding Experiments. The origin of *H. planus*, the medium, and the conditions for the suspension cultures have been described previously.[28] Four suspension cultures of *H. planus* were grown in MSK-4 medium[18] (75 mL each) with 4% glucose, 0.5 mmol of nonlabeled serine, and 0.5 mmol of DL-[1-^{13}C]serine or DL-[3-^{13}C]serine, respectively. The

suspension cultured cells were grown at 25 °C under continuous light of 2500 lux. There was no significant growth within 3 days after inoculation, and then cells grew rapidly until the 21st day (growth index: 2.5 on the packed cell volume basis) under these conditions. The cells were harvested 21 days after inoculation.

Extraction and Isolation of Chlorophyll *a* (1). Chlorophyll *a* (**1**) was isolated as described in the following procedure.[29] Freshly cultured cells were extracted with 5 volumes of MeOH for 24 h (× 3). The MeOH extracts was concentrated in vacuo and extracted with Et_2O. The Et_2O solution was concentrated in vacuo, and the residue was dissolved in Me_2CO. The Me_2CO solution was layered on a DEAE-Sepharose CL-6B column (Amersham Biosciences, Piscataway, NJ) and successively eluted with Me_2CO and Me_2CO−MeOH (10:3, v/v). The Me_2CO−MeOH elute containing **1** was concentrated and chromatographed on a Sepharose CL-6B column (Amersham Biosciences) and eluted first with hexane−propan-2-ol (20:1, v/v) and then with hexane−propan-2-ol (10:1, v/v). Chlorophyll *a* (**1**) was eluted in the hexane−propan-2-ol (20:1) fraction, and it was concentrated to dryness to yield pure **1**.

Hydrolysis of Chlorophyll *a* (1) and Acetylation of Phytol (2). The biosynthetically labeled **1** was hydrolyzed according to the following procedure. Aqueous Cs_2CO_3 [0.6 mL of 20% (w/v)] was added portionwise to **1** in 10 mL of MeOH and stirred at room temperature for 6 h in an N_2 atmosphere. Another 0.4 mL of aqueous Cs_2CO_3 was added, and after further stirring overnight, the reaction solution was extracted with 10 mL of pentane (× 4). The pentane extracts were combined, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was chromatographed on a silica gel column and eluted with hexane−EtOAc (4:1, v/v) to give pure **2**. Phytol (**2**) incorporating DL-[1-^{13}C]- and DL-[3-^{13}C]serine was acetylated overnight in the usual manner (**2**−acetic anhydride−pyridine, 1:3:9, by weight). Acetic anhydride and pyridine were removed by evaporation in vacuo at 60 °C to give **2a**. Compound **2a** was isolated on a silica gel column and eluted with hexane−EtOAc (4:1, v/v).

Phytol (2): colorless oil; ^{1}H NMR ($CDCl_3$, 270 MHz) δ 5.40 (1H, t, J = 6.9 Hz, H-2), 4.15 (2H, d, J = 6.9 Hz, H-1), 1.96 (2H, t, J = 7.4 Hz, H-4), 1.67 (3H, s, H-20), 0.83−0.88 (12H, m, H-16,17,18, and 19); EIMS m/z 278 $[M - H_2O]^+$ (1) 196 (2), 151 (2), 123 (27), 109 (7), 95 (17), 81 (29), 71 (100).

Phytyl acetate (2a): colorless oil; ^{1}H NMR ($CDCl_3$, 270 MHz) δ 5.34 (1H, t, J = 7.3 Hz, H-2), 4.58 (2H, d, J = 7.3 Hz, H-1), 2.06 (3H, s, COCH_3), 2.00 (2H, t, J = 7.6 Hz, H-4), 1.67 (3H, s, H-20), 0.83−0.88 (12H, m, H-16,17,18, and 19); EIMS m/z 278 $[M - AcOH]^+$ (6), 179 (2), 151 (2), 123 (46), 109 (12), 95 (41), 81 (44), 71 (38), 43 (100).

Separation and Acetylation of Stigmasterol (3). The DEAE Me_2CO eluate was concentrated and chromatographed on a silica gel column and eluted with hexane−EtOAc (2:1, v/v). The fraction containing sterol was rechromatographed on a silica gel column and eluted with benzene−CH_3CN (4:1, v/v). The **3**-containing fraction was further separated by HPLC [TSK-GEL Silica-60 column, Tosoh, Japan; hexane−EtOAc (3:1)] and acetylated as described above. The **3a** was separated by a silica gel column [hexane−EtOAc (3:1, v/v)] and further purified by preparative argentation TLC [silica gel 60 F_{254}-15% $AgNO_3$, Merck, Germany; hexane−Et_2O (19:1, v/v)]. The purified **3a** confirmed the presence of about 10% of campesteryl acetate (calculated by GC peak area monitored at m/z 382 and 394).

Stigmasterol (3): colorless crystals; ^{1}H NMR ($CDCl_3$, 270 MHz) δ 5.34 (1H, m, H-6), 4.96−5.19 (2H, m, H-22 and 23), 3.52 (1H, m, H-3), 1.02 (3H, d, J = 6.3 Hz, H-21), 1.01 (3H, s, H-19), 0.84 (3H, d, J = 6.6 Hz, H-26), 0.80 (3H, t, J = 7.3 Hz, H-29), 0.79 (3H, d, J = 6.6 Hz, H-27), 0.69 (3H, s, H-18); EIMS m/z 412 $[M]^+$ (19), 379 (5), 351 (9), 300 (12), 255 (30), 213 (12), 83 (64), 55 (100).

Stigmasteryl acetate (3a): colorless crystals; ^{1}H NMR ($CDCl_3$, 270 MHz) δ 5.37 (1H, m, H-6), 4.96−5.20 (2H, m, H-22 and 23), 4.60 (1H, m, H-3), 2.03 (3H, s, COCH_3), 1.02 (3H, s, H-19), 1.02 (3H, d, J = 6.6 Hz, H-21), 0.84 (3H, d, J = 6.6 Hz, H-26), 0.80 (3H, t, J = 7.3 Hz, H-29), 0.79 (3H, d, J = 6.6 Hz, H-27), 0.69 (3H, s, H-18); EIMS m/z 394 $[M - AcOH]^+$ (68), 379 (9), 351 (12), 255 (54), 213 (14), 83 (64), 55 (100).

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