Nonequivalent Labeling of the Phytyl Side Chain of Chlorophyll *a* in Callus of the Hornwort *Anthoceros punctatus*

Daisuke Itoh,[†] Renuka Praxide Karunagoda,[†] Takashi Fushie,[†] Kenji Katoh,[‡] and Kensuke Nabeta*[,†]

Department of Bio-organic Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080-8555, Japan, and Shionogi Aburahi Laboratories, Kohga-cho, Shiga-ken 520-3423, Japan

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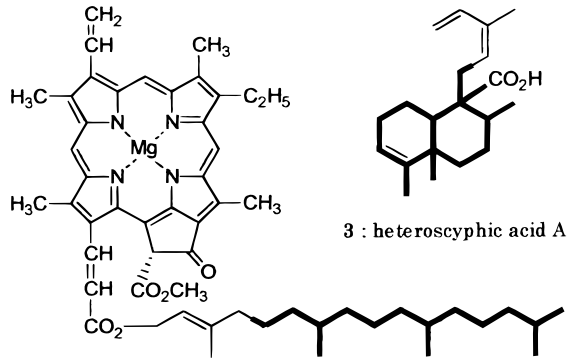
Callus cultures of the hornwort *Anthoceros punctatus* were induced from the apical portions of the gametophytes. Calli can accumulate rosmarinic acid, which is suggested as an intermediate for anthocerotonic acid, a rare phenylpropanoid dimer with a cyclobutane ring, indicating that calli possess the ability to produce secondary metabolites found primarily in intact plants. Biosynthesis of chloroplastidic terpenoids of liverworts showed preferential labeling of the farnesyl diphosphate (FPP)-derived portion in the phytyl side chain of chlorophyll *a* (**1**) when calli of *A. punctatus* are incubated with ^{2}H- and ^{13}C-labeled mevalonate. This finding suggests either that cytoplasmic FPP (or isopentenyl diphosphate, IPP) is taken into chloroplasts and condensed with endogenous IPP derived from a nonmevalonate pathway, or that FPP is synthesized within chloroplasts from extraplastidically formed IPP (or mevalonate) and then condensed with endogenous IPP in a different subplastidic fraction.

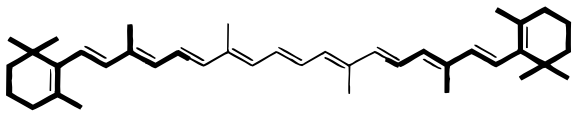
Incorporation of ^{2}H- and ^{13}C-labeled mevalonate (MVA) into chloroplastidic terpenoids such as the phytyl side chain of chlorophyll *a* (**1**),[1,2] *β*-carotene (**2**),[2] and a diterpene [heteroscyphic acid A (**3**)[3,4]] in suspension-cultured cells of liverworts proved that ^{2}H- and ^{13}C-labeled MVA is preferentially incorporated into the farnesyl diphosphate (FPP)-derived portion, but comes also into the terminal isopentenyl diphosphate (IPP) at a low rate (Figure 1). In contrast, remote precursors such as acetate and glycine were incorporated equally into the FPP-derived portion and the terminal IPP portion.[5] The labeling patterns of chloroplastidic terpenoids from exogenous MVA confirmed that intact MVA had been incorporated into both the FPP-derived portion and the terminal IPP portion, demonstrating involvement of the mevalonate pathway in the biosynthesis of terpenoids within chloroplasts of liverworts. However, recent studies have shown that a nonmevalonate pathway[6] (a deoxyxylulose pathway[7]) operates exclusively in the biosynthesis of terpenoids within plastids, including chloroplasts.[8] In fact, [6,6-$^{2}H_2$]glucose and [2-^{13}C]glycerol are incorporated into the phytyl side chain of chlorophyll via the nonmevalonate pathway in the cultured cells of liverworts.[5] Involvement of the nonmevalonate pathway in phytol (**4**) biosynthesis in liverworts has also been reported.[9] This seeming discrepancy may be explained by the fact that extraplastidically synthesized FPP or FPP that has been rapidly synthesized within chloroplasts from extraplastidically synthesized IPP is taken into chloroplasts and condensed with endogeneous IPP. Alternatively, both the mevalonate and the nonmevalonate pathways may operate simultaneously in liverwort chloroplasts.[2]

Hornworts belong to the division Bryophyta that consists of three classes of plants: mosses (class Bryopsida), liverworts (Hepaticopsida), and hornworts (Anthoceropsida). The thallus hornworts are made up of parenchymal cells, and each cell possesses a single large chloroplast with a conspicuous pyrenoid.[10] This characteristic contrasts sharply with the cells of the other bryophytic classes, which have numerous chloroplasts in each cell and no pyrenoids.

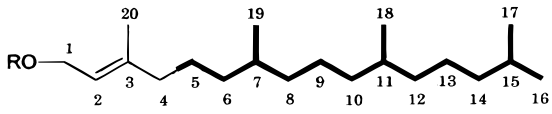


3 : heteroscyphic acid A

1 : chlorophyll a



2 : β-carotene



4 : phytol R=H
5 : phytyl acetate R=CO_2CH_3

Figure 1. Nonequivalent labeling of chloroplastidic terpenoids in cultured cells of liverworts. Bold lines represents FPP-derived portion. (We have previously reported this labeling pattern.[1−4])

Comparatively little work has been done on the secondary metabolites from hornworts. Phenylpropanoids and their derivatives are major secondary metabolites.[11] A rare phenylpropanoid dimer with a cyclobutane ring, anthocerotonic acid, and megacerotonic acid and rosmarinic acid have been isolated from *Anthoceros punctatus*.[12] In this paper, we examine the nonequivalent labeling in the formation of the phytyl side chain in calli of *A. punctatus* that accumulate rosmarinic acid.

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

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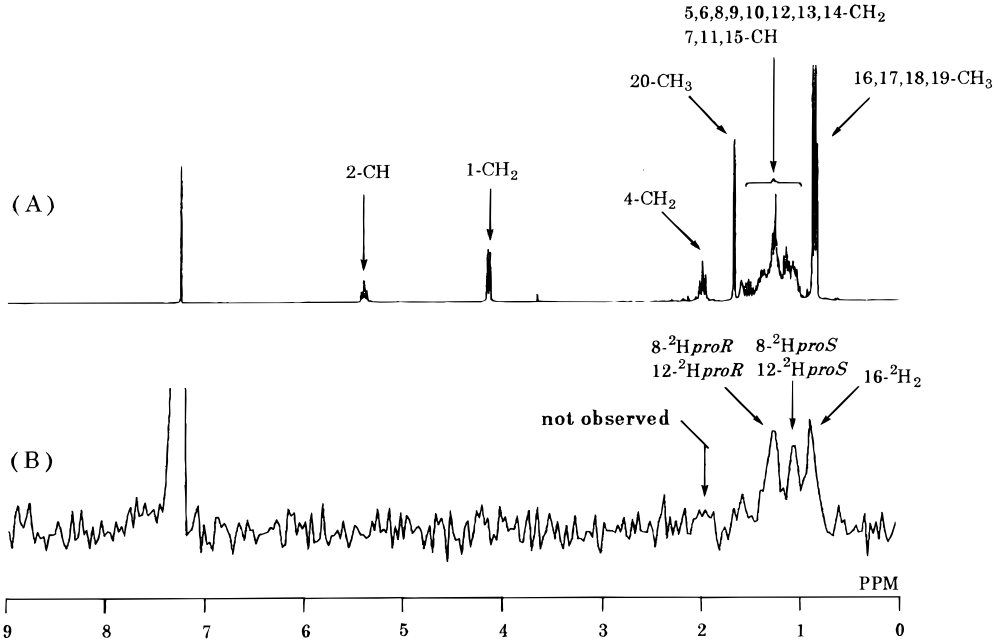

Figure 2. (A) ^{1}H NMR spectrum of nonlabeled phytol (**4**) (270 MHz) and (B) ^{2}H NMR spectrum of **4** (41.3 MHz) formed from [2,2-$^2H_2$]MVA in cultured cells of the hornwort *A. punctatus*.

Results and Discussion

To compare phenolic substances in intact plants and calli, the presence or absence of rosmarinic acid was examined. The MeOH extract from the calli was successively chromatographed on Sephadex LH-20 column and C_{18} HPLC columns. About 3.7 mg of rosmarinic acid was isolated from 244 g (fresh wt) of calli of *A. punctatus*. Isolated rosmarinic acid was identified by direct comparison (^{1}H NMR and $[\alpha]_D$) with an authentic sample of (+)-rosmarinic acid. Thus, calli maintain the capacity for producing a main class of phenolic dimers, including rosmarinic acid, although the presence of anthocerotonic acid and megacerotonic acid found in intact plants[12] has not been determined by the present study. Monoterpenoids and sesquiterpenoids were not detected in calli volatiles by GC−MS analysis.

Labeling of the phytyl side chain of chlorophyll *a* (**1**) was examined by feeding [2,2-$^2H_2$]- and [2-^{13}C]-MVA (10 mM) to a callus culture of *A. punctatus* (70 mL of MSK-3 medium). Calli were harvested 28 days after inoculation. Chlorophyll *a* (**1**) was purified by the method previously reported by Omata and Murata.[13] Biosynthetically labeled phytol (**4**) was obtained by Cs_2CO_3 hydrolysis of isolated **1**. Deuterium and ^{13}C enrichment in the phytyl side chain was determined by ^{2}H and ^{13}C NMR analyses of the resulting **4** before and after acetylation in nonlabeled or [$^2H_6$]acetic anhydride/pyridine.

The preferential labeling of the FPP-derived portion in the phytyl side chain of **1** is shown in the ^{2}H{^{1}H} NMR spectrum of **4**, incorporating [2,2-$^2H_2$]MVA in cell cultures of *A. punctatus*, although ^{2}H enrichment is very low (about 1 atom % excess). Full assignments of the ^{1}H and ^{13}C atoms have been reported previously.[14,15] Figure 2 shows ^{1}H NMR (2A) and ^{2}H[^{1}H] NMR (2B) spectra of **4** incorporating [2,2-$^2H_2$]MVA. The spectra have three broad singlets corresponding to **4** labeled with ^{2}H at C-8, C-12, and C-16, but no enhanced peak corresponding to **4** labeled with ^{2}H at C-4. This nonequivalent labeling has also been observed in the ^{13}C NMR spectrum of **4** incorporating [2-^{13}C]MVA (Table 1). Carbon-13 enrichment was determined by comparing the relative intensities of the ^{13}C-enriched carbons to the acetyl methyl carbon in the labeled compounds with those of the corresponding carbons in the nonlabeled compounds. Table 1 demonstrates that the ^{13}C signals of C-12 (^{13}C enrichment, 0.628 atom % excess), C-8 (1.302 atom % excess), and C-16 (1.126 atom % excess) were enriched with ^{13}C at a relatively high level, but C-4 was labeled at a very low level (0.105 atom % excess), which may be comparable to that of C-15 (^{13}C enrichment, 0.432 atom % excess). Although there are slight differences among the ^{13}C enrichments in C-8, C-12, and C-16, it is difficult to give an explanation for this labeling pattern.

Table 1. ^{13}C Enrichment of Carbons in Phytyl Acetate (**5**) Obtained from Chlorophyll *a* (**1**) Incorporating [2-^{13}C]MVA

carbon	δ_c in phytyl acetate	^{13}C enrichment (atom % in excess)[a]
C-1	61.44	−0.126 ± 0.115
C-2	117.92	0.123 ± 0.058
C-3	142.82	0.086 ± 0.097
C-4	39.84	0.105 ± 0.124
C-5	25.00	0.032 ± 0.085
C-6	36.61	0.076 ± 0.213
C-7	32.65	0.054 ± 0.160
C-8	37.27	**1.302 ± 0.294**
C-9	24.44	0.007 ± 0.259
C-10	37.41	−0.063 ± 0.147
C-11	32.78	−0.103 ± 0.162
C-12	37.34	**0.628 ± 0.297**
C-13	24.78	0.113 ± 0.159
C-14	39.36	0.016 ± 0.080
C-15	27.96	0.432 ± 0.297
C-16	22.61	**1.126 ± 0.116**
C-17	22.72	0.052 ± 0.185
C-18	19.73	0.164 ± 0.165
C-19	19.70	0.018 ± 0.128
C-20	16.35	0.169 ± 0.124
Me*CO*	171.18	
*Me*CO	21.08	
Average	0.211	

[a] Average values of three ^{13}C measurements.

These findings indicate that the FPP-derived portion of the phytyl side chain was preferentially labeled with exogenously supplied MVA (Figure 3). The findings agree with previously reported data for suspension-cultured cells of the liverworts *Heteroscyphus planus* and *Lophocolea*

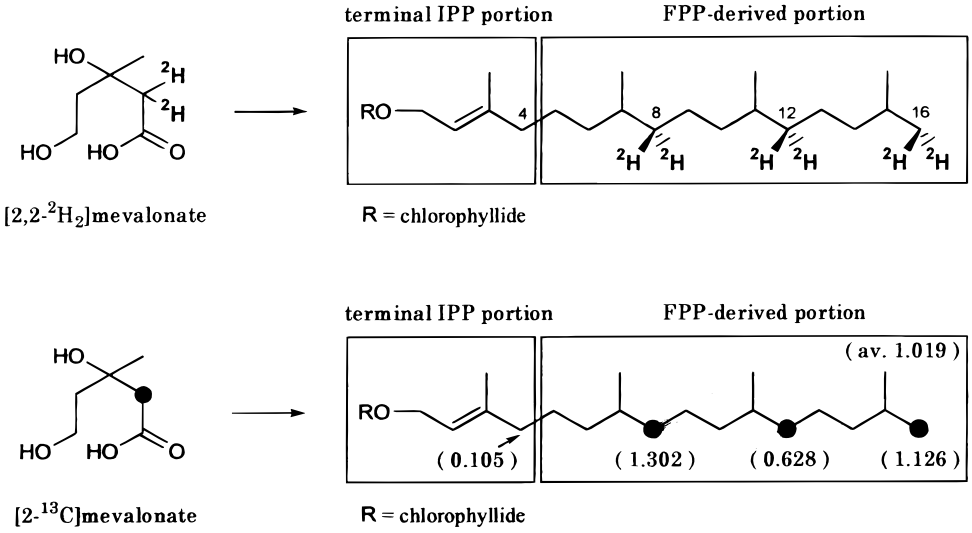

Figure 3. Labeling patterns of the phytyl side chain of chlorophyll *a* (**1**) formed in cultured cells of the hornwort *A. punctatus*.

heterophylla. The IPP-derived portion was less intensely labeled, implying that endogenously biosynthesized IPP had been incorporated into the phytyl side chain of **1**. The degree of efficiency of utilization of exogenous MVA in hornworts is less than that in the liverworts. Preferential labeling can be explained by the fact that cytoplasmic FPP derived from exogenous MVA permeates the chloroplastidic membrane and condenses with the endogenously formed IPP. However, it has been reported that, in the biosynthesis of geranylgeraniol in the chloroplasts of higher plants (spinach), membrane fractions (thylakoid and the envelope membrane) were unable by themselves to synthesize geranylgeraniol.[16] When stromal and membrane fractions were combined, however, the capacity to synthesize geranylgeraniol was restored. Moreover, immunocytochemical staining showed that FPP synthase is localized in the chloroplasts of rice, wheat, and tobacco.[17] Thus, the biosynthesis of FPP from IPP and dimethylallyl diphosphate and the condensation of FPP with IPP to geranylgeranyl diphosphate (GGPP) may occur at different sites within the chloroplasts of hornworts and liverworts. It is possible, therefore, that cytoplasmic IPP may have been taken into chloroplasts and condensed to FPP in a particular subplastidic fraction in chloroplasts, followed by condensation with chloroplastidically synthesized IPP in a different subplastidic fraction.

Experimental Section

General Experimental Procedures. The ^{1}H, ^{2}H{^{1}H}, and ^{13}C{^{1}H} NMR spectra of biosynthetically labeled phytol (**4**) were recorded on a JEOL EX-270 at 270 MHz ($CHCl_3$ in $CDCl_3$ as an internal standard, δ ^{1}H 7.26 or CH_3OD in CD_3OD as the internal standard, δ ^{1}H 3.35), 41.3 MHz ($CDCl_3$ as the internal standard, δ ^{2}H 7.26), and 67.8 MHz ($^{13}CDCl_3$, δ ^{13}C 77.0), respectively. Three ^{13}C NMR measurements were taken. Assignments of all of the ^{13}C atoms in **4** were carried out according to previous data.[14,15] Optical rotations were determined on a JASCO DIP-370 polarimeter. HPLC separations were performed with a JASCO 880 PU equipped with JASCO UVIDEC-100V by monitoring UV at 260 nm. [2-^{13}C]- and [2,2-^{2}H$_2$]-MVA were prepared by the published method.[18] An authentic sample of (+)-rosmarinic acid was purchased from Extrasynthése, France.

Callus Culture and Feeding Experiment. Gametophytes of *A. punctatus* plants were obtained from Sandankyo in Hiroshima Prefecture, Japan. The apical portions (5 mm from the tip) of the gametophytes were sterilized with Tween 80 and NaOCl and grown in MSK (modified Murashige and Skoog)-3 medium[19] supplied with 2% glucose and 0.2% activated charcoal at 25 °C under continuous light of 3000 lux. The calli were subcultured at 40-day intervals over two years prior to the feeding experiment.

Ten or five callus cultures of *A. punctatus* were grown in MSK-3 medium (70 mL each) with 2% glucose, 0.2% activated charcoal, and 10 mM [2-^{13}C]MVA or [2,2-^{2}H$_2$]MVA, respectively. The calli were grown at 25 °C under continuous light of 2500 lux. The calli were harvested 28 days after inoculation, and chlorophyll *a* (**1**) was isolated as described in the following procedure (reported previously[13]).

Extraction and Isolation of Chlorophyll *a* (1). Freshly harvested calli were extracted with 5 vol of MeOH (v/w) for 24 h (× 3). The MeOH solution was concentrated in vacuo and dissolved in Et_2O. The Et_2O solution was concentrated in vacuo, and the residue was redissolved in Me_2CO. The Me_2CO solution was layered on a DEAE-Sepharose CL-6B column and successively eluted with Me_2CO and Me_2CO−MeOH (10:3, v/v). The Me_2CO−MeOH eluate containing **1** was concentrated and chromatographed on a Sepharose CL-6B column 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 (4). The biosynthetically labeled **1** was hydrolyzed according to the following procedure. Aqueous Cs_2CO_3 (0.2 mL of 0.61 mM) was added to the **1**, which was then dissolved in 10 mL of MeOH and stirred at room temperature for 2 h in an N_2 atmosphere. Another 0.4 mL of 0.61 mM aqueous CS_2CO_3 was added, and after further stirring overnight, the reaction solution was extracted with 10 mL of pentane (× 3). The pentane extracts were combined, dried over dry Na_2SO_4, filtered, and concentrated in vacuo. The residue was chromatographed on a Si gel column and eluted with hexane−EtOAc (4:1, v/v) to give pure **4**. Phytol (**4**) incorporating [2-^{13}C]MVA was acetylated in the usual manner. Acetic anhydride and pyridine were removed by evaporation in vacuo at 60 °C to give phytyl acetate (**5**). Phytol (**4**) incorporating [2,2-^{2}H$_2$]MVA was acetylated with [^{2}H$_6$]acetic anhydride.

Extraction and Isolation of Rosmarinic Acid. Fresh calli of *A. punctatus* (244 g fresh wt) were extracted with MeOH (1220 mL × 3) containing a few drops of HOAc. The MeOH extracts were combined and concentrated (5.56 g). The concentrate was chromatographed on a Sephadex LH-20 column (510 mL) eluted with MeOH. Fractions 41−80 (10 g each, 280 mg) contained phenolic substances, including rosmarinic acid, and were further separated by successive chromatography on two C_{18} HPLC columns [one of 300 × 21.5 mm i.d. eluting MeOH−H_2O (13:7, v/v) containing 0.1% HOAc at a flow rate of 1 mL/min, and the second of 250 × 4.6 mm i.d. eluting MeOH−H_2O (3:2, v/v) containing 0.2% HOAc at a flow rate of 1 mL/min] to afford rosmarinic acid (3.7 mg): $[\alpha]^{20}_D$ + 91.0° (*c* 0.10, MeOH); ^{1}H NMR spectrum of isolated rosmarinic acid in CD_3OD containing one drop of HOAc-d_4, was superimposable on that of authentic samples in the same solution.

Supporting Information Available: Figure of possible mechanisms for preferential labeling of the FPP-derived portion in the biosynthesis of chloroplastidic terpenoids. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Nabeta, K.; Kawae, T.; Kikuchi, T.; Saitoh, T.; Okuyama, H. *J. Chem. Soc., Chem. Commun.* **1995**, 2539−2540.
(2) Nabeta, K.; Kawae, T.; Saitoh, T.; Kikuchi, T. *J. Chem. Soc., Perkin Trans. 1* **1997**, 261−267.
(3) Nabeta, K.; Ishikawa, T.; Kawae, T.; Okuyama, H. *J. Chem. Soc., Chem. Commun.* **1995**, 681−682.
(4) Nabeta, K.; Ishikawa, T.; Okuyama, H. *J. Chem. Soc., Perkin Trans. 1* **1995**, 3111−3115.
(5) Nabeta, K.; Saitoh, T.; Adachi, K.; Komuro, K. *Chem. Commun.* **1998**, 671−672.
(6) Rohmer, M.; Knani, M.; Simonin, P.; Sutter, B.; Sahm, H. *Biochem. J.* **1993**, *295*, 517−524.
(7) Arigoni, D.; Sagner, S.; Letzel, C.; Eisenreich, W.; Bacher, A.; Zenk, M. H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10600−10605.
(8) Lichtenhalter, H. K.; Schwender, J.; Disch, A.; Rohmer, M. *FEBS Lett.* **1997**, *400*, 271−274.
(9) Adam, K. P.; Thiel, R.; Zapp, J.; Becker, H. *Arch. Biochem. Biophys.* **1998**, *354*, 181−187.
(10) Weisz, P. B.; Fuller, M. S. *The Science of Botany*; McGraw-Hill: New York, 1962; p 187.
(11) Asakawa, Y. *Progress in the Chemistry of Organic Natural Products*; Herz, W., Kirby, G. W., Moore, R. E., Steglich, W., Tamm, Ch., Eds.; Springer-Verlag: New York, 1995; Vol. 65, pp 460−464.
(12) Takeda, R.; Hasegawa, J.; Shimazaki, M. *Tetrahedron Lett.* **1990**, *31*, 4159−4163.
(13) Omata, T.; Murata, N. *Photochem. Photobiol.* **1980**, *31*, 183−185.
(14) Boxer, S. G.; Closs, G. L.; Katz, J. J. *J. Am. Chem. Soc.* **1974**, *96*, 7058−7066.
(15) Arigoni, D.; Eisenreich, W.; Latzel, C.; Sagner, S.; Radykewicz, T.; Zenk, M. H.; Bacher, A. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1309−1314.
(16) Block, M. A.; Joyard, J.; Bouge, R. *Biochim. Biophys. Acta* **1980**, *631*, 210−219.
(17) Sanmiya, M.; Ueno, O.; Matsuoka, M.; Yamamoto, N. *Plant Cell Physiol.* **1999**, *40*, 348−354.
(18) Hoffmann, C. H.; Wagner, A. F.; Wilson, A. N.; Walton, E.; Shunk, C. H.; Wolf, D. E.; Holly, F. W.; Folkers, K. *J. Am. Chem. Soc.* **1957**, *79*, 2316−2318.
(19) Katoh, K. *Methods in Bryology*; Glime, J. M., Ed.; Hattori Botanical Laboratory: Nichinan, Japan, 1988; pp 99−105.

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