Biosynthesis of Phytol in the Cyanobacterium *Synechocystis* sp. UTEX 2470: Utilization of the Non-Mevalonate Pathway

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The biosynthesis of phytol in the cyanobacterium *Synechocystis* sp. UTEX 2470 has been examined using 6,6-$^2H_2$-D-glucose, 2-^{13}C−D-glucose, and U-$^{13}C_6$-D-glucose as precursors. Analysis of the isolated phytol using deuterium or ^{13}C NMR showed labeling patterns consistent with incorporation of labeled glucose via the non-mevalonate pathway to terpenes.

The discovery of the non-mevalonate pathway (NMP) to terpenes in bacteria[1,2] and higher plants[3] has led to a reexamination of terpene biosynthesis in a number of organisms. This new route is distinguished by the condensation of pyruvate with glyceraldehyde-3-phosphate (G3P) to form deoxyxylulose-5-phosphate, which is converted into isopentenyl diphosphate (IPP) by an unknown number of steps.[2,4] It has now been demonstrated that a variety of bacteria,[5] higher plants,[6−11] green algae,[10,12] and a red alga[10] can use this alternate route to terpenes. The operation of this mevalonate-independent route in bacteria and in higher plants suggested that cyanobacteria also should synthesize terpenes via this pathway, because they are considered to be the evolutionary precursors of chloroplasts in higher plants.[13] This article describes experiments to confirm that cyanobacteria can utilize the non-mevalonate pathway.

Synechocystis sp. UTEX 2470 (Cyanophyceae)[14] was chosen as representative of the cyanobacteria for its relatively rapid growth rate and its ability to utilize glucose as a carbon source.[15] The use of labeled glucose precursors to support operation of the NMP has been firmly established.[1−3] Phytol, as the phytyl ester in chlorophyll, is the most abundant terpenoid produced by *Synechocystis*, and this metabolite was analyzed for incorporation of labels from glucose. Cultures grown in the presence of either 10 atom % 6,6-$^2H_2$-glucose, 2-^{13}C-glucose, or U-$^{13}C_6$-glucose provided phytol that was sufficiently labeled.

The deuterium atoms from the 6-position of glucose can be incorporated into the 1 and 5 positions of IPP via the NMP and into the 2, 4, and 5 positions via the mevalonate pathway. (Figure 1).[1,3] The deuterium NMR spectrum of isolated phytol (Figure 2) showed significant labeling at H1 (4.15 ppm; 7.6% enrichment; 475 × natural abundance), while H2 (5.41 ppm) and H4 (1.99 ppm) showed no signs of labeling, which is consistent with the NMP. The region upfield of 2 ppm was extensively labeled and represents methyls that are not diagnostic for distinguishing between the two pathways and methylenes that are not resolved. Position 1 of IPP arises from fully oxidized C1 of 3-hydroxy-3-methylglutaryl CoA (HMGCoA), so no deuterium

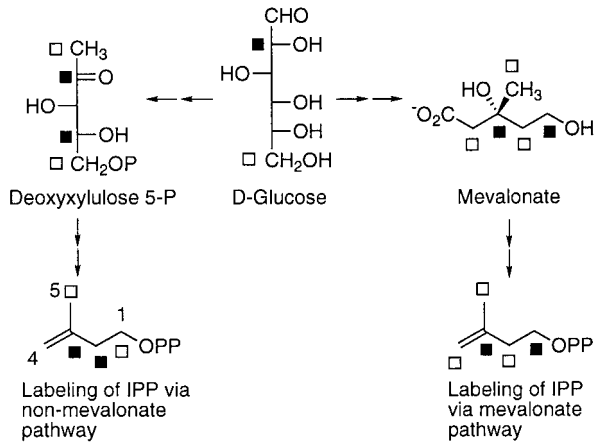

Figure 1. Fates of (■) 2- and (□) 6-labeled glucose via the mevalonate and non-mevalonate (deoxyxylulose 5-phosphate) pathways.

labels would be expected at C1 via the mevalonate pathway. The cycling of deuterium into the NADPH pool and reintroduction to C5 of mevalonic acid via HMGCoA reductase could explain deuterium labeling at C1 of phytol, but the absence of labeling at C2 and C4 does not support the involvement of mevalonate.[16]

The labeling pattern obtained from the 2-^{13}C-glucose feeding is also consistent with the non-mevalonate route.[3] Positions 2 and 3 of IPP will be labeled via the mevalonate-independent pathway, and positions 1 and 3, via the mevalonate pathway (Figure 1). The ^{13}C NMR spectrum revealed that the C2−C3, C6−C7, C10−C11, and C14−C15 pairs of phytol, which correspond to C2 and C3 of IPP, are most extensively labeled (Table 1). Indirect incorporation of label from the 2-^{13}C-glucose precursor after prolonged metabolism may explain the lower level of labeling at the other positions of phytol.

Glucose labeled at C1 was also examined as a potential precursor, but no labeling was observed. *Synechocystis* sp. has been reported to assimilate glucose predominantly via the oxidative pentose phosphate pathway,[17] and if this route of glucose catabolism was operative under the growth conditions used, then C1 of glucose would be lost as $^{13}CO_2$, eliminating direct incorporation into phytol. This result is in contrast to the successful use of 1-^{13}C-glucose to elucidate the operation of the NMP in a number of photosynthetic organisms.[6,11,12]

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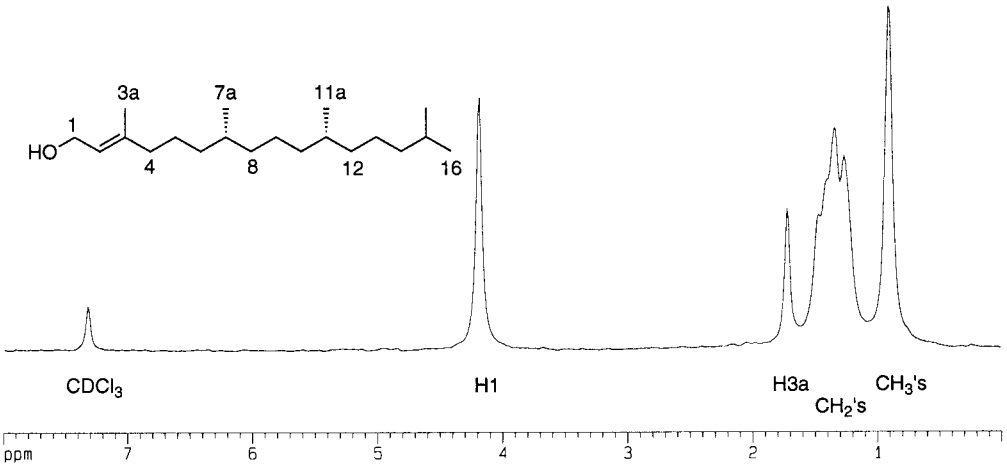

Figure 2. ^{2}H NMR spectrum of labeled phytol obtained from a 6,6-$^{2}H_2$-D-glucose feeding experiment.

Table 1. Intensities of Phytol ^{13}C NMR Peaks from 2-^{13}C-D-Glucose Feeding Experiment

carbon[a]	chemical shift (ppm)[b]	relative ^{13}C abundance[c]
1	59.43	1.2
2	123.10	1.9
3	140.31	2.2
4	39.87	1.0
5	25.14	1.2
6	36.67	1.9
7	32.69	2.2
8	37.36	1.1
9	24.47	1.3
10	37.43	1.9
11	32.79	2.5
12	37.29	1.0
13	24.79	1.3
14	39.37	2.0
15	27.97	2.5
16	22.61	1.0
3a	16.17	1.3
7a[d]	19.71	1.4
11a[d]	19.74	1.6
15a	22.70	1.5

[a] Assignments based on the work of Arigoni et al.[9] [b] Referenced to the $CDCl_3$ centerline at 77.00 ppm. [c] Relative to an average of the abundances of C4, C8, C12, and C16. [d] Interchangeable shifts.

A final experiment in which *Synechocystis* was incubated with U-$^{13}C_6$-D-glucose provided verification of phytol biosynthesis via the NMP. The intact incorporation of a three-carbon fragment (via G3P) derived from glucose is only possible for the NMP.[12] Metabolism of glucose to acetate and incorporation via mevalonate would provide only doubly labeled fragments. Examination of the ^{13}C NMR spectrum revealed small coupling constants (two- and/or three-bond) for carbons C1, C2, and C4, of the same magnitude observed for phytol isolated from the green alga *Scenedesmus obliquus* grown in the presence of $^{13}C_6$-D-glucose.[12] Similar long-range couplings were noted for C5, C9, and C13.[12] The confirmation of the coupling partners was obtained through the use of the modified HMBC experiment.[5] In this experiment, the large $^{1}H-^{13}C$ coupling between H2 and C2 will be observed as satellite peaks in the correlation between H2 and C4 if C2 and C4 are both labeled with ^{13}C in the same molecule (i.e., derived from the same glucose molecule). Figure 3 depicts a portion of the modified HMBC spectrum in which the correlation between H2 (5.41 ppm) and C4 (39.87 ppm) clearly reflects this $^{1}H-^{13}C$ coupling. The correlations H4 (1.99 ppm) to C2 (123.10 ppm) and H1 (4.15 ppm) to C4 (39.87 ppm) similarly reflect large $^{1}H-^{13}C$ couplings, further supporting intact incorporation of a three-carbon fragment. The terminal isoprene unit of phytol also displayed a correlation between H16 and C14, which indicated the derivation of C14 and C16 from the same labeled glucose molecule. The long-range couplings observed are consistent only with the NMP.

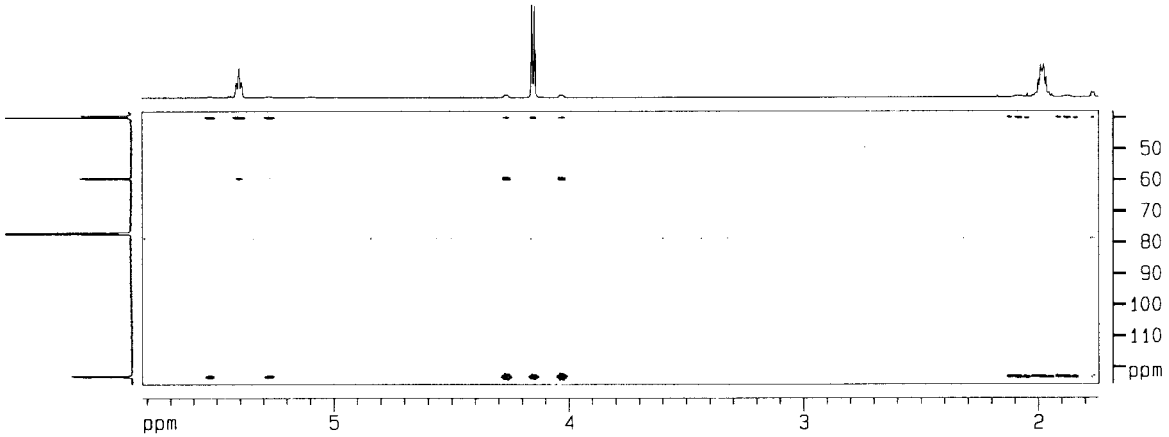

Figure 3. Region of the modified HMBC spectrum depicting the long-range H2−C4, H1−C4, and H4−C2 correlations due to incorporation of an intact three-carbon fragment from U-$^{13}C_6$-D-glucose.

The results from these experiments establish the use of the non-mevalonate pathway to phytol in the cyanobacterium *Synechocystis* sp. The lack of any deuterium labels derived from mevalonate and the complete absence of mevalonate pathway genes in the complete genome of *Synechocystis* sp. PCC 6803[18] suggest that *Synechocystis* produces its terpenoid metabolites exclusively via the non-mevalonate route. The recent identification of a gene in *Synechocystis* sp. PCC 6803 that encodes a putative deoxyxylulose phosphate synthase is also consistent with operation of the NMP.[19]

Experimental Section

General Experimental Procedures. Labeled precursors were obtained from Cambridge Isotope Laboratories, Inc. (6,6-$^2H_2$-D-glucose, 1-^{13}C−D-glucose, and 2-^{13}C−D-glucose) or Martek Biosciences Corporation (U-$^{13}C_6$-D-glucose). NMR spectra were recorded on a Bruker AM400 spectrometer operating at 400.13 MHz for ^{1}H, 100.13 MHz for ^{13}C, and 61.42 MHz for ^{2}H or on a Bruker DRX600 spectrometer (modified HMBC). NMR spectra were referenced to $CHCl_3$ or $CDCl_3$ at 7.26 ppm for ^{1}H and ^{2}H spectra, respectively, and to the centerline of the $CDCl_3$ triplet at 77.00 ppm for ^{13}C spectra. Levels of incorporation from the deuterium NMR spectrum were based on the integration of the natural abundance deuterium signal of $CHCl_3$ (0.016%).

Biological Material. An axenic culture of *Synechocystis* sp. UTEX 2470 was obtained from the University of Texas Culture Collection of Algae and was maintained at 20 °C on a 16 h light/8 h dark cycle on BG-11 medium[20] supplemented with 1 g D-glucose/L.

Biosynthetic Experiments. Seed cultures were inoculated 1% v/v with stock cultures and were grown in BG-11 medium with 1 g/L of D-glucose for 3−4 days under continuous illumination at 30 °C. The seed cultures (1−2% v/v) were used to inoculate the BG-11 production medium containing 1 g/L D-glucose (0.9 g/L unlabeled D-glucose + 0.1 g/L labeled D-glucose). Production cultures of 5 L (deuterium labeling) or 3 L (^{13}C labeling) were aerated and grown at 30 °C under constant illumination with 40 W cool-white fluorescent lights (6−12 μmol of photon m^{-2} s^{-1}). Cultures were harvested by centrifugation at 8700 × *g* after 3.5−4 days, frozen at −80 °C, and lyophilized (dry wt 0.3−0.4 g/L).

Isolation of Phytol. The lyophilized cell material was extracted three times with 2:1 $CHCl_3$−MeOH to provide a crude extract that was loaded onto a flash silica column. Stepwise elution with 20% EtOAc−hexanes, 40% EtOAc−hexanes, and 100% EtOAc provided a chlorophyll-containing fraction in the 40% eluent. This fraction was concentrated and treated with 10 mL of 6% w/v KOH in MeOH overnight at room temperature. The reaction mixture was diluted with 20 mL H_2O and extracted 3 × 20 mL with hexanes. The combined organic phases were washed with 20 mL saturated ammonium chloride solution, then 20 mL H_2O to obtain phytol contaminated with a minor amount of a yellow-orange pigment. The basic aqueous layer was acidified to pH 2−3 with 1N HCl, diluted with 20 mL brine, then extracted 3 × 30 mL with hexanes. The hexanes layer was washed with 30 mL of saturated $NaHCO_3$ solution, then 30 mL H_2O. Although not expected, the hexanes extract from the acidified solution contained phytol that was not fully extracted from the basic solution, even after repeated extractions of the basic layer with hexanes. The pigment was removed from the combined phytol-containing solutions by passing an ether solution of crude phytol through a short activated-charcoal column and eluting with Me_2CO. The purified phytol (2 mg/L avg. yield) was analyzed by GC−MS and NMR to confirm its identity.

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