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Probing the Non-Mevalonate Pathway to Phytol in the Cyanobacterium *Synechocystis* sp. UTEX 2470 Using Deuterium-labeled Glucose

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Abstract: The biosynthesis of phytol in the cyanobacterium *Synechocystis* sp. UTEX 2470 was examined using 1-^{2}H-D-glucose, 2-^{2}H-D-glucose, 2,3,4,6,6-$^2H_5$-D-glucose, and 1,2,3,4,5,6,6-$^2H_7$-D-glucose. Analysis of deuterium NMR spectra indicated that deuterium from the glucose precursors labels C1, C4, and C5 of the IPP units of phytol, while no labeling is observed at C2 of the IPP derived units.

Keywords: biosynthesis; terpenes and terpenoids; isoprenoids; labelling

The non-mevalonate pathway (NMP) has been demonstrated to be an important biosynthetic route to terpenes in numerous organisms,[1,2] including bacteria, plants, algae, and the cyanobacterium *Synechocystis* sp.[2,3] *Synechocystis* sp. UTEX 2470 readily incorporates deuterium from 6,6-$^2H_2$-D-glucose into phytol via the NMP and the extensive labeling suggested that studies using glucose labeled with deuterium at other positions might provide insight into the intermediate steps in the pathway.[3] The NMP is summarized in Figure 1 and begins with the condensation of pyruvate with glyceraldehyde 3-phosphate to form 1-deoxy-D-xylulose 5-phosphate (DXP).[4] DXP is converted directly to 2-*C*-methyl-D-erythritol 4-phosphate,[5] which is further elaborated to isopentenyl diphosphate (IPP) or dimethylallyl diphosphate (DMAPP).[6]

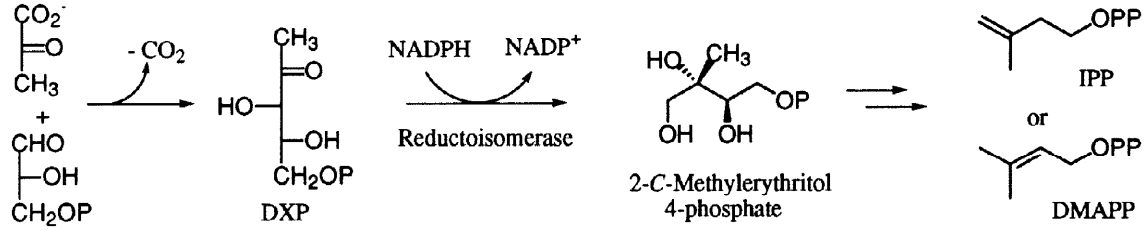

Figure 1. Proposed biosynthesis of IPP/DMAPP via the non-mevalonate pathway.

Our earlier studies demonstrated that deuterium from 6,6-$^2H_2$-D-glucose appears at both positions 1 and 5 of the IPP units in phytol,[3] but the fates of deuterium from other positions of glucose precursors have not been reported. *Synechocystis* sp. PCC6803, a related cyanobacterium, has the full complement of glycolytic enzymes as well as enzymes for the oxidative pentose phosphate pathway.[7] Therefore, interpretations of the labeling patterns from glucose can be made based on catabolism by both pathways.

Four different glucose isotopomers were used to complement the previous experiment with 6,6-$^2H_2$-glucose: 1-^{2}H-D-glucose, 2-^{2}H-D-glucose, 2,3,4,6,6-$^2H_5$-D-glucose, and 1,2,3,4,5,6,6-$^2H_7$-D-glucose.[8] All experiments utilized labeled glucose in a 1:9 ratio with unlabeled glucose. Phytol was isolated after basic

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hydrolysis of the chlorophyll fraction and analyzed by ^2H NMR.[3] The ^2H NMR spectrum of phytol obtained from the pentadeuterated glucose experiment is shown in Figure 2. This spectrum is representative of the spectra obtained with other precursors, except labeling at H4 is not seen with 6,6-$^2H_2$-glucose or 2-^{2}H-D-glucose. Table 1 correlates the levels of enrichment found at the different positions of phytol with the labeled precursors used.

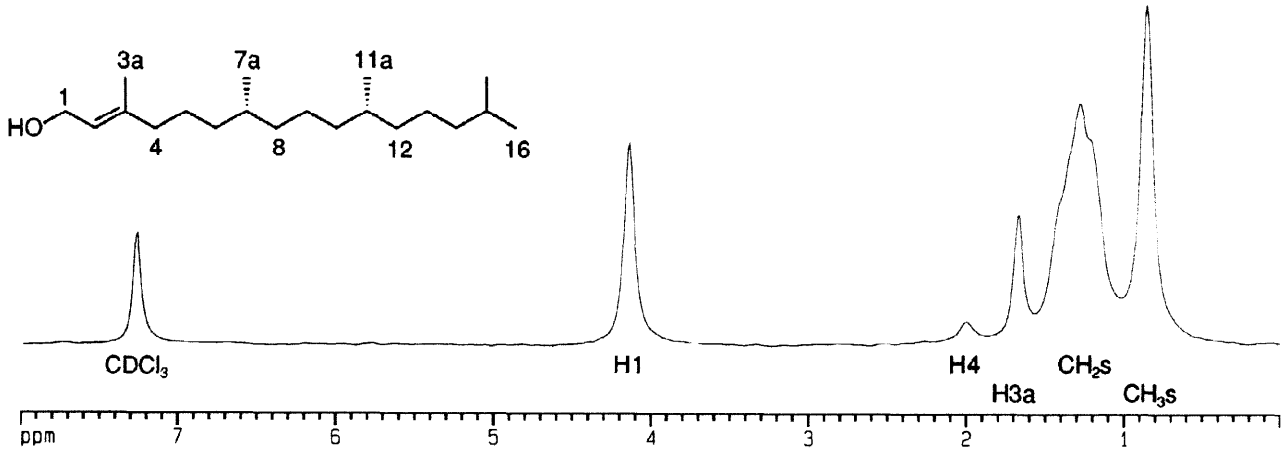

Figure 2. ^2H NMR spectrum of phytol biosynthesized from 2,3,4,6,6-$^2H_5$-glucose.

Deuterium from 6,6-$^2H_2$-glucose is observed at C1 and C3a of phytol, with extensive labeling of unresolved methylenes and the aliphatic methyls (deriving from C1 and C5 of IPP; Figure 3).[3] The 1-^{2}H-glucose experiment provided labeling at C1 and C3a, but also C4 to a lesser extent.[9] The labeling at C4 would not be expected by intact incorporation from the glucose precursor, but can be explained by involvement of a deuterated NADPH pool. The deuterium label at C1 of glucose will be incorporated into NADPH during the conversion of glucose 6-phosphate (G6P) to 6-phosphogluconate by G6P dehydrogenase operating in the oxidative pentose phosphate pathway. This deuterium may ultimately label C4 of IPP by incorporation into C1 of 2-*C*-methylerythritol by the NADPH-dependent deoxyxylulose reductoisomerase.[5] The 2-^{2}H-glucose experiment provided weak labeling at positions derived from C1 and C5 of IPP, consistent with a shift of some deuterium from C2 of G6P to C1 of fructose 6-phosphate (F6P) by G6P isomerase.[10] Using the pentadeuterated glucose, labeling is again observed at positions deriving from C1, C4, and C5 of IPP, although at higher levels than observed with glucose labeled at C1 alone (Table 1). The labels from C2 and C6 of glucose would appear at C1 and C3a of phytol. The deuterium at C4 of phytol arises from C3 and C4 of glucose. Deuterium from C3 of glucose should label the NADPH pool via the 6-phosphogluconate dehydrogenase (decarboxylating) operating in the oxidative pentose phosphate pathway and would be expected to label C4 in the same manner as the deuterium from C1. The deuterium from C4 of glucose could also label C4 by intact incorporation of G3P. The G3P derived from the pentadeuterated glucose should be triply deuterium labeled with a 2:1 ratio of deuterium at C3 to C1. If this fragment is incorporated intact, the labeling in phytol at C1 should be twice that at C4. The observed values were 11% and 1.6% enrichment at the two positions. Correcting for the small contribution of label at C1 of phytol from C2 of glucose and for the contribution at C4 from the NADPH pool discussed above, the ratio is

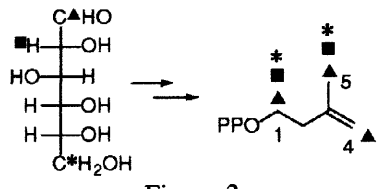

Figure 3.

likely greater than 10:1. This indicates that the deuterium from C4 of glucose is significantly or completely lost during the biosynthesis of phytol. This loss should not be associated with the biochemical interconversions subsequent to the formation of 2-*C*-methylerythritol phosphate because both deuterium atoms at C1 of the erythritol were retained in the biosynthesis of the ubiquinone sidechain in *E. coli*.[11] The loss of label, therefore, presumably occurs prior to the formation of 2-*C*-methylerythritol.[12] The labeling pattern observed for the heptadeuteroglucose is identical to the pentadeuteroglucose pattern, differing only in the magnitude of enrichments (Table 1). This experiment provides evidence that the deuterium from C5 of glucose (and subsequently C2 of G3P) is not retained during the biosynthesis of phytol via the non-mevalonate pathway.

Table 1. Percent deuterium enrichments for different positions of phytol from *Synechocystis* grown in the presence of various deuterated glucose isotopomers.[a]

	H1	H4	H3a	CH_2s	CH_3s
1-$^2H_2$-D-Glucose	1.1	0.6	1.2	5.4	3.9
2-$^2H_2$-D-Glucose	0.5	-	0.6	1.6	1.2
6,6-$^2H_2$-D-Glucose	15	-	8.1	48	29
2,3,4,6,6-$^2H_5$-D-Glucose	11	1.6	6.9	39	25
1,2,3,4,5,6,6-$^2H_7$-D-Glucose	12	2.4	8.3	40	24

[a]Enrichments were calculated relative to 0.016% natural abundance of deuterium in the $CHCl_3$ solvent.

While there is uncertainty about the exact contributions to labeling at C4 of the IPP unit by deuterium from C1, C3, and C4 of glucose, the presence of deuterium at C4 of phytol originating from C1 (and likely C3) of glucose implies the participation of a deuterated NADPH pool. G6P dehydrogenase and 6-phosphogluconate dehydrogenase (decarboxylating) are both Class B dehydrogenases, enzymes in which the pro-*S* hydrogen from NADPH is transferred or hydride is accepted on the *si* face of $NADP^+$.[13] If the deuterium is transferred to the penultimate C4 of IPP via the recently described reductoisomerase, then the stereospecific transfer in this step would be due to delivery of the pro-*S* hydrogen of NADPH. The deuterium transferred by NADPH(D) should be retained in the final terpene product if the previous results with deuterated 2-*C*-methylerythritol in *E. coli* are extended to these studies with *Synechocystis*.[11]

Our experiments suggest that for phytol biosynthesis in *Synechocystis*, deuterium from C4 of glucose is either completely lost or only partially retained at C4 of phytol, probably due to loss of label prior to formation of 2-*C*-methylerythritol 4-phosphate. Also discovered was that there is no deuterium from glucose retained at positions derived from C2 of IPP/DMAPP and that the hydrogen that ultimately resides at C2 of phytol does not arise from NADPH. The most direct explanation for the absence of label at positions derived from C2 of IPP/DMAPP is a dehydration of 2-*C*-methylerythritol 4-phosphate in which the deuterium at C3 is lost along with the hydroxyl at C2 or C3 of the erythritol is oxidized to a ketone with concomitant loss of deuterium. If a parallel to branched chain amino acid biosynthesis is drawn,[10] then the rearrangement of DXP to 2-*C*-methylerythritol phosphate would be followed by elimination of water and tautomerization to yield the keto compound, 1,4-dihydroxy-3-methyl-2-butanone 1-phosphate. This step would fulfill the necessary loss of deuterium from C3 of 2-*C*-methylerythritol 4-phosphate. Experiments are in progress to test this hypothesis.

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6) Isopentenyl diphosphate is the endpoint metabolite of the mevalonate pathway which can be isomerized to dimethylallyl diphosphate (DMAPP). It is not known at this time whether IPP, DMAPP, or both are the true end metabolites of the non-mevalonate pathway.
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8) The growth conditions and procedures for isolation of phytol are as described in ref. 3.
9) The observation of phytol labeling from the 1-^{2}H-D-glucose experiment is in contrast to our earlier experiments with 1-^{13}C-glucose in which we did not observe specific labeling.[3] These earlier experiments with 1-^{13}C-glucose were performed under a 16 hour light/8 hour dark cycle which may have enhanced catabolism of glucose via the oxidative pentose phosphate pathway, leading to less specific labeling via reincorporation of released $^{13}CO_2$ formed upon oxidative cleavage of glucose. A recent report by Lichtenthaler *et al.* has shown that under appropriate growth conditions, phytol from *Synechocystis* can be specifically labeled by 1-^{13}C-glucose via the non-mevalonate pathway.[1]
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12) Part of the loss of label may be explained by interconversions of G3P and 1,3-bisphosphoglycerate (BPG) by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). It has recently been reported that two forms of GAPDH are present in *Synechocystis* sp. PCC 6803. The GAPDH involved in glycolysis utilizes NAD+ as a cofactor, while the GAPDH involved in photosynthesis and gluconeogenesis can use either NADH or NADPH.[14] If deuterium from C1 of G3P were transferred to NAD^{+} upon formation of BPG, then deuterium may or may not be transferred back to G3P at a later stage. This would increase the C1:C4 ratio of deuterium labels seen in the phytol product.
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