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Direct Formation of 2-C-Methyl-D-Erythritol 4-Phosphate from 1-Deoxy-D-Xylulose 5-Phosphate by 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase, a New Enzyme in the Non-Mevalonate Pathway to Isopentenyl Diphosphate

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Abstract.

1-Deoxy-D-xylulose 5-phosphate is biotransformed to 2-C-methyl-D-erythritol 4-phosphate in a single step in the presence of NADPH by a new recombinant enzyme named 1-deoxy-D-xylulose 5-phosphate reductoisomerase purified from *Escherichia coli*. © 1998 Elsevier Science Ltd. All rights reserved.

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Since the initial discovery of the mevalonate pathway in the 1950s, it was a widely accepted view that isopentenyl diphosphate (IPP), the fundamental unit in terpenoid biosynthesis, was only formed in all living organisms by condensation of acetyl coenzyme A through the ubiquitous mevalonate pathway. However, it has been disclosed recently that some organisms including many bacteria, green algae and chloroplasts of higher plants can utilize an alternative mevalonate-independent pathway (non-mevalonate pathway)¹⁻⁴ for the formation of IPP. Several recent experimental findings lend additional support to the operation of this novel non-mevalonate pathway in terpenoid biosynthesis.⁵⁻⁸

According to Rohmer and co-workers, the initial step of this pathway is the formation of 1-deoxy-D-xylulose 4-phosphate (DXP) **1** by condensation of pyruvate and glyceraldehyde 3-phosphate. In the second step the intramolecular rearrangement of **1** to a hypothetical rearranged intermediate 2-C-methyl-D-erythrose 4-phosphate **2** is proposed to occur and is

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followed by an unspecified reduction process of this intermediate to afford 2-C-methyl-D-erythritol 4-phosphate **3**. Precise details about the reductive mechanism of formation of **3**, however, were not disclosed nor made available from these earlier studies.

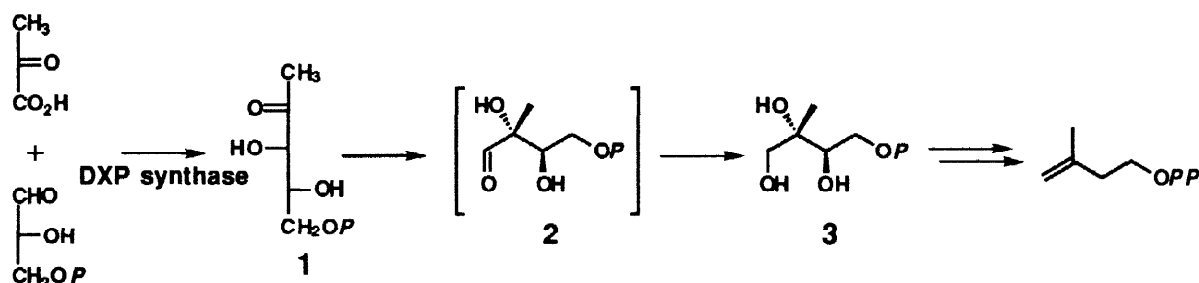


Figure 1. Hypothetical alternative non-mevalonate pathway for IPP biosynthesis

To further elucidate the details of this interesting mechanism of **3** formation, we initiated studies on the cloning of the gene responsible for synthesis of **3**. To this end, we employed the useful strategy of preparing and selecting mutants of *Escherichia coli* possessing a metabolic block(s) between **1** and **3**. Thus these mutants would require **3** for their growth and survival. Three mutants selected from *ca.* 20,000 colonies screened showed the expected properties; addition of the free alcohol of **3**⁹, but not of 1-deoxyxylulose, to the minimal medium M9¹⁰ facilitated the growth of these mutants.¹¹ With these mutants in hand, we cloned a gene which complemented IPP biosynthesis coding region of these blocked mutants.¹¹ To demonstrate that the cloned gene was actually involved in IPP biosynthesis, we constructed a plasmid for the overexpression of this gene product and succeeded in preparation of the recombinant enzyme in quantities sufficient for its characterization.¹² The purified recombinant enzyme afforded a homogeneous protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a subunit size of 42 kDa. The enzyme had a molecular mass of 165 kDa on native polyacrylamide gel electrophoresis. Thus the enzyme appears to form a homotetramer.

Incubation of this enzyme with enzymatically synthesized **1**¹³ (0.15 mM) in the presence of 0.3 mM NADPH at 37 °C for 30 min in 200 ml of 100 mM Tris-HCl (pH 7.5) containing 1 mM MgCl₂ resulted in utilization of NADPH. Replacement of NADPH with NADH decreased the reaction rate to *ca.* 1/100 indicating that the enzyme utilizes only NADPH as a coenzyme for this reaction. The enzyme required Co²⁺, Mg²⁺ or Mn²⁺ as well; no effect was observed with other divalent cations such as Ca²⁺, Cu²⁺, Fe²⁺, Ni²⁺ and Zn²⁺.

The reaction product was purified with the assistance and guidance of ³¹P NMR spectroscopy. The reaction mixture was passed through an activated carbon column (2.2 X 10 cm) and after dilution to 1000 ml with H₂O, subjected to Dowex 1-X8 (Cl⁻ type, 3.5 X 20 cm) chromatography and eluted with 1 % NaCl (400 ml). All fractions showing a ³¹P-signal was combined, concentrated and chromatographed on a Sephadex G-10 column (1.8 X 100 cm) and eluted with water only. Fractions showing a ³¹P NMR signal were combined and freeze-dried to afford the pure reaction product.

The structure of the purified product was deduced by spectroscopic methods. The molecular formula was determined to be C₅H₁₂O₇P by HR-FABMS [*m/z* 215.0276 (M-H)⁻, Δ-4.5 mmu]. The ¹H and ¹³C NMR spectral features of the compound¹⁴ showed close similarities to those of a

chemically synthesized sample of 2-*C*-methylerythritol.¹⁵ The isolated compound, however, showed one ³¹P NMR signal at 4.1 ppm which was coupled to C-4 ($J_{C_4-P}=4.6$ Hz) and C-3 ($J_{C_3-P}=6.5$ Hz). Treatment of the reaction product with bacterial alkaline phosphatase (Takara) gave a free alcohol with ¹H and ¹³C NMR spectral features in full accord with those of chemically synthesized 2-*C*-methylerythritol. The optical rotation¹⁶ of the dephosphorylated product ($[\alpha]_D^{21} +6.0$ (c 0.050, H₂O)) was identical with that of 2-*C*-methyl-D-erythritol ($[\alpha]_D^{25} +7.0$ (c 0.13, H₂O)).⁷ These spectral data unequivocally establish the structure of the reaction product produced by the recombinant enzyme as 2-*C*-methyl-D-erythritol 4-phosphate. Thus, the hypothetical rearranged intermediate **2** suggested by Rohmer was not yielded as the reaction product by the enzyme. This result clearly demonstrates that 2-*C*-methyl-D-erythritol 4-phosphate is synthesized in the presence of NADPH by rearrangement and reduction of **1** in a single step. We propose to designate this enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

Precedent for this biosynthetic rearrangement reaction is found in the biosynthesis of valine, isoleucine and leucine. The enzyme involved in this reaction, ketol acid reductoisomerase (EC1.1.1.86) catalyzes rearrangement of 2-acetolactate to yield 2,3-dihydroxyisovalerate with simultaneous reduction in the presence of NADPH (Figure 2).¹⁷⁻²⁰ It is to be noted that a hypothetical compound (Figure 2) with a keto function corresponding to **2** had been excluded as a possible reaction product intermediate because of its inhibitory action on this reaction.¹⁸

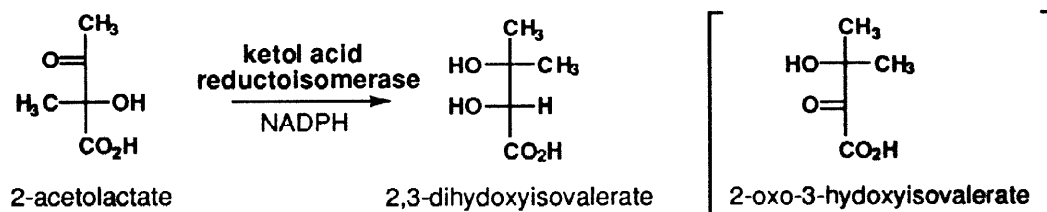


Figure 2. ketol acid reductoisomerase reaction

1 is a biosynthetic intermediate not only for IPP but also for thiamine and pyridoxol in *E. coli*.²¹⁻²³ Therefore, our result on the *in vitro* formation of **3** from **1** provides the first evidence that the reaction catalyzed by DXP reductoisomerase is the committed step in the IPP formation.

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12. Protein expression and purification of the recombinant DXP reductoisomerase. The coding region of the *E. coli* DXP reductoisomerase gene¹¹ was cloned into the expression vector pQE30 (Qiagen) to give pQEDXR. *E. coli* M15²⁴ containing pREP4 [*neo*, *lacI*] (Qiagen) was used as a host for expression of the DXP reductoisomerase gene. *E. coli* M15 (pREP4, pQEDXR) was cultured at 37 °C in 100 ml of Luria-Bertani medium¹⁰ containing kanamycin and ampicillin for 5 hrs with 0.2 mM isopropylthiogalactoside upon reaching an optical density at 660 nm of 0.8. Cells were harvested by centrifugation and resuspended in 100 mM Tris-HCl (pH 8.0). After brief sonication, the lysate was centrifuged at 10,000 X g for 20 min and the supernatant was collected. A 50% slurry of Ni-NTA resin (Qiagen) was added into the supernatant and stirred on ice for 60 min. The resin was washed with 50 mM imidazole in 100 mM Tris-HCl (pH 8.0) and then the protein which binds to the Ni-NTA resin was eluted with 200 mM imidazole in 100 mM Tris-HCl (pH 8.0). The eluate was used as the purified DXP reductoisomerase in the subsequent experiments.
13. Enzymatic synthesis of 1-deoxy-D-xylulose 5-phosphate. The coding region of the *E. coli* DXP synthase gene²⁵ was cloned into the expression vector to give pQEDXS. *E. coli* M15 containing pREP4 was used as a host for expression of the DXP synthase gene. Protein expression and purification of the recombinant DXP synthase were done according to the identical procedures to those of DXP reductoisomerase. Production and purification of 1 were monitored at 195 nm by HPLC with a Senshu pak NH2-1251-N (4.6 X 250 mm) HPLC column (Senshu), eluted with 100 mM KH₂PO₄ (pH 3.5). The DXP synthase reaction was started by adding the enzyme solution at 37 °C for 12 hrs in 20 ml of 100 mM Tris-HCl (pH 7.5) containing 10 mM sodium pyruvate, 30 mM DL-glyceraldehyde 3-phosphate, 1.5 mM thiamine diphosphate, 10 mM MgCl₂ and 1 mM DL-dithiothreitol. The reaction mixture was diluted to 300 ml by adding H₂O and subjected to a column of activated carbon (2.2 X 8 cm). The passing fraction was applied to Dowex 1-X8 (Cl⁻ type, 3.5 X 25 cm) chromatography eluting with 1 % NaCl (500 ml). The eluate was concentrated and then chromatographed on Sephadex G-10 (1.8 X 100 cm) eluting with H₂O. The fractions containing 1 were freeze-dried to give a white powder of 1 as the disodium salt (α . 50 mg). Then, the enzymatically synthesized 1 was analyzed by NMR spectroscopy. The chemical shifts and coupling constants of ¹H and ³¹P NMR spectra (A-500 instrument, JEOL) of the enzymatic product were identical to those reported previously.²⁵
14. ¹H NMR (D₂O, 500 MHz): δ 4.03 (ddd, *J* = 11.5, 6.5, 2.5 Hz, 1H), 3.84 (ddd, *J* = 11.5, 8.0, 6.5 Hz, 1H), 3.78 (dd, *J* = 8.0, 2.5 Hz, 1H), 3.60 (d, *J* = 12.0 Hz, 1H), 3.50 (d, *J* = 12.0 Hz, 1H), 1.15 (s, 3H); ¹³C NMR (D₂O, 125 MHz): δ 75.1 (C-2), 74.8 (d, *J* = 6.5, C-3), 67.4 (C-1), 65.9 (d, *J* = 4.6, C-4), 19.4 (2-Me).
15. ¹H NMR (D₂O, 500 MHz): δ 3.83 (dd, *J* = 11.5 and 2.5 Hz, 1H), 3.66 (dd, *J* = 8.5 and 2.5 Hz, 1H), 3.59 (dd, *J* = 11.5 and 8.5 Hz, 1H), 3.58 (d, *J* = 11.5 Hz, 1H), 3.47 (d, *J* = 11.5 Hz, 1H), 1.12 (s, 3H); ¹³C NMR (D₂O, 125 MHz): δ 76.1 (C-3), 75.2 (C-2), 67.4 (C-1), 63.1 (C-4), 19.5 (2-Me).
16. The optical rotation of the reaction product (2-C-methyl-D-erythritol 4-phosphate): $[\alpha]_D^{21} +6.4$ (*c* 0.10, H₂O).
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