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# Studies on the nonmevalonate pathway: formation of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate from 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol

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**Abstract**

2-Phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol was transformed to 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate by a novel *Escherichia coli* enzyme involved in the nonmevalonate pathway. 

*Keywords:* isopentenyl diphosphate; 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase.

Isopentenyl diphosphate **1**, a building block of isoprenoids, is biosynthesized through the nonmevalonate pathway in many eubacteria, green algae and the chloroplasts of higher plants (Fig. 1).[1] The initial step of this pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) **2** by condensation of pyruvate and D-glyceraldehyde 3-phosphate catalyzed by DXP synthase.[2–4] In the second step **2** is converted to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) **3** by DXP reductoisomerase as demonstrated in our study.[5,6] Compound **3** is then cytidylylated by MEP cytidylyltransferase to give 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME) **4**,[7,8] which is phosphorylated by CDP-ME kinase to afford 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME2P) **5**.[9,10] We have recently succeeded in the cloning of MEP cytidylyltransferase and CDP-ME kinase genes from *Escherichia coli* and demonstrated unequivocally that these enzymes are essential for the formation of **1** in the nonmevalonate pathway.[8,10] The following reactions leading to **1** from **5**, however, remain unknown.

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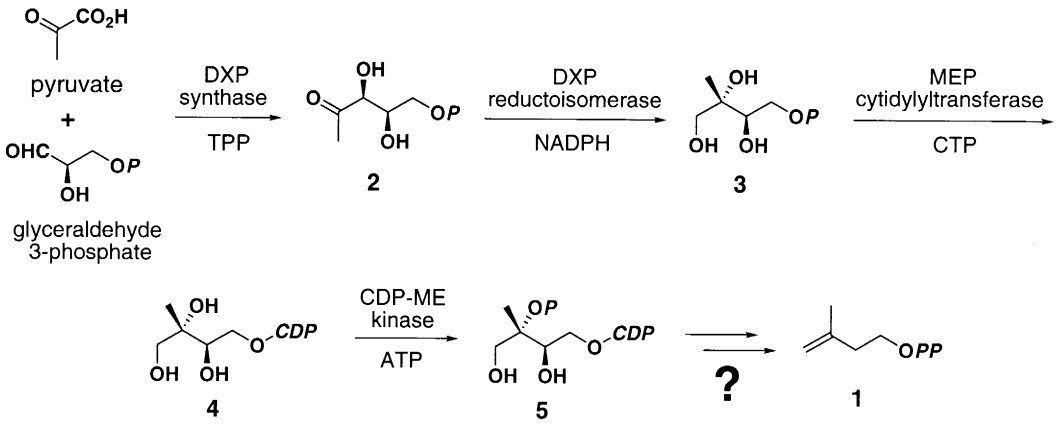


Fig. 1. The nonmevalonate pathway for IPP biosynthesis

In previous papers[8,10] we reported the cloning of several *E. coli* genes that complemented the defects of blocked mutants in synthesizing **1** from **3**.[‡] This paper shows conversion of **5** into 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP) **6** by an enzyme encoded by one of these genes, *ygbB* (DDBJ/EMBL/GenBank accession number AB038256), which is located at 61.8 min on the chromosome map of *E. coli*.

Incubation of the *ygbB* gene product (2 mg)[§] with **5** (50 mg) at 37°C for 2 h in 50 ml of 10 mM Tris–HCl (pH 8.0) containing 1 mM $MgCl_2$ resulted in the decrease of **5** and appearance of a new peak identified as cytidine 5′-monophosphate (CMP) in HPLC with an Asahipak GS-320 HQ column (7.8×300 mm, Showa Denko, Tokyo, Japan).[¶] This decrease of **5** was proportional to the increase of CMP formation and occurred only in the presence of the *ygbB* gene product.

The reaction product was purified with the assistance and guidance of $^1$H NMR spectroscopy. The reaction mixture was diluted to 100 ml with $H_2O$, subjected to Dowex 1-X8 ($Cl^-$ type, 2×6 cm) chromatography and eluted with 1% NaCl aq. (100 ml). All fractions showing a methyl proton singlet at 1.43 ppm were combined, concentrated and chromatographed on a Sephadex G-10 column (1.8×100 cm) and eluted with water only. Fractions showing this proton singlet were combined and freeze-dried to afford a pure reaction product (1.4 mg).

‡ These mutants were derived from an *E. coli* transformant with the mevalonate kinase, phosphomevalonate kinase and pyrophosphomevalonate decarboxylase activities. Thus, this transformant can utilize the partial mevalonate pathway for IPP biosynthesis only by supplementation of mevalonate. Therefore, these mutants, which can grow only in the presence of mevalonate, are concluded to be deficient in the nonmevalonate pathway (see Ref. 8).

§ Protein expression and purification of the *ygbB* gene product: The *ygbB* gene was cloned into the expression vector pQE30 (Qiagen) to give pQECMEP. *Escherichia coli* M15 containing pREP4 [*neo*, *lacI*] (Qiagen) was used as a host for expression of the *ygbB* gene. *Escherichia coli* M15 (pREP4, pQECMEP) was cultured at 37°C in 100 ml of a Luria–Bertani medium containing 50 μg/ml kanamycin and 200 μg/ml ampicillin for 5 h with 0.1 mM isopropyl-β-D-thiogalactopyranoside 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×g for 20 min and the supernatant was collected. A 50% slurry of Ni-nitrilotriacetic acid (NTA) agarose 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 bound to the Ni-NTA agarose resin was eluted with 200 mM imidazole in 100 mM Tris–HCl (pH 8.0). The eluate was used as the purified *ygbB* gene product in the subsequent experiments. The purified enzyme afforded a homogeneous protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a subunit size of 22 kDa.

¶ Decrease of **5** was monitored at 280 nm with an Asahipak GS-320 HQ column, eluted with 10 mM $KH_2PO_4$ (pH 2.5) at the flow rate of 1.0 ml/min at 30°C. In this HPLC condition, CMP and **5** ware eluted at 9.6 and 11.6 min, respectively.

The structure of the purified product was deduced by spectroscopic methods. The molecular formula was determined to be $C_5H_{10}O_9P_2Na_2$ by HR-FABMS [*m/z* 344.9481 $(M+Na)^+$, Δ-1.2 mmu]. The $^1H$, $^{13}C$ and $^{31}P$ NMR spectra of the purified product unequivocally established its structure as 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate **6** [$^1H$ NMR (500 MHz): $\delta$ 4.21 (m, H-4a), 4.13 (m, H-4b), 4.13 (m, H-3), 3.77 (d, *J*=12.5 Hz, H-1a), 3.62 (d, *J*=12.5 Hz, H-1b), 1.43 (s, 2-Me); $^{13}C$ NMR (125 MHz): $\delta$ 84.3 (d, *J*=8.5 Hz, C-2), 68.9 (d, *J*=<3.0 Hz, C-3), 67.4 (*J*=4.5 Hz, C-1), 66.2 (d, *J*=6.5, C-4), 16.8 (*J*=5.5 Hz, 2-Me); $^{31}P$ NMR (202 MHz): $\delta$ −10.9 (d, *J*=23.2 Hz), −14.9 (d, *J*=23.2 Hz)].‖ Thus, the 2-*C*-methylerythritol skeleton in **6** was proved to be present as in **5** by $^1H$ and $^{13}C$ NMR spectral analysis. The large $^{13}C$–$^{31}P$ splittings observed with C-2 and C-4 signals indicated that these positions were phosphorylated. The characteristic high field $^{31}P$ signals at −10.9 and −14.9 ppm showing a $^{31}P$–$^{31}P$ coupling with each other proved the presence of a diphosphate diester function in **6**. It is interesting to note that **6** had been isolated from the fermentation broth of a Gram-negative anaerobe, *Desulfovibrio desulfuricans*.[11] The NMR spectral data of **6** are in complete agreement with the reported values.

These results clearly demonstrate that the elimination of the CMP moiety from the CDP-ME2P molecule is concomitant with the formation of the cyclic diphosphate **6** (Fig. 2). We propose to designate this enzyme 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECDP synthase).

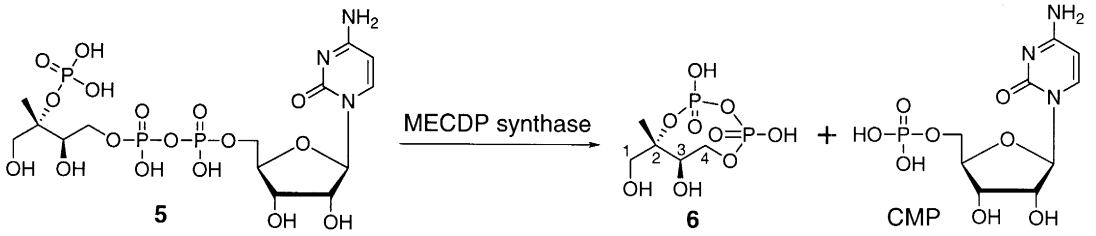


Fig. 2. MECDP synthase reaction

Although an accumulation of **6** was also found in some bacteria including *Corynebacterium ammoniagenes* under oxidative stress conditions caused by treatment of benzyl viologen,[12] no reports have been available to date on the role of **6** in the nonmevalonate pathway. We have clearly showed in this study that **6** is an intermediate in the nonmevalonate pathway for IPP biosynthesis.

The *ygbB* gene product showed significant homologies to hypothetical proteins with unknown functions present in *Aquifex aeolicus*, *Thermotoga maritima*, *Synechocystis*, *Bacillus subtilis*, *Mycobacterium tuberculosis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Treponema pallidum*, *Chlamydia trachomatis* and *Chlamydia pneumoniae* which are known to utilize the nonmevalonate pathway for IPP biosynthesis. Similar distribution patterns in eubacteria are observed with DXP synthase, DXP reductoisomerase, MEP cytidylyltransferase and CDP-ME kinase, which are involved in the nonmevalonate pathway. These data corroborate that the *ygbB* gene is essential for this pathway.

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‖ All NMR data were collected with an A500 NMR spectrometer (JEOL, Tokyo, Japan) in $D_2O$. The $^{31}P$ chemical shifts are relative to phosphoric acid as an external standard at 0 ppm.

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## References

1. Rohmer, M. In *Comprehensive Natural Products Chemistry*; Barton, D.; Nakanishi, K., Eds. Isoprenoids including carotenoids and steroids. Elsevier: Amsterdam, 1999; Vol. 2, pp. 45–67.
2. Sprenger, G. A.; Schörken, U.; Wiegert, T.; Grolle, S.; deGraaf, A. A.; Taylor, S. V.; Begley, T. P.; Bringer-Meyer, S.; Sahm, H. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 12857–12862.
3. Lois, L. M.; Campos, N.; Putra, S. R.; Danielsen, K.; Rohmer, M.; Boronat, A. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2105–2110.
4. Lange, B. M.; Wieding, M. R.; McCaskill, D.; Croteau, R. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2100–2114.
5. Kuzuyama, T.; Takahashi, S.; Watanabe, H.; Seto, H. *Tetrahedron Lett*. **1998**, *39*, 4509–4512.
6. Takahashi, S.; Kuzuyama, T.; Watanabe, H.; Seto, H. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 9879–9884.
7. Rohdich, F.; Wungsintaweekul, J.; Fellermeier M.; Sagner, S.; Herz, S.; Kis, K.; Eisenreich, W.; Bacher, A.; Zenk, M. H. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11758–11763.
8. Kuzuyama, T.; Takagi, M.; Kaneda, K.; Dairi, T.; Seto, H. *Tetrahedron Lett*. **2000**, *41*, 703–706.
9. Lüttgen, H.; Rohdich, F.; Herz, S.; Wungsintaweekul, J.; Hecht, S.; Schuhr, C. A.; Fellermeier, M.; Sagner, S.; Zenk, M. H.; Bacher, A.; Eisenreich, W. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1062–1067.
10. Kuzuyama, T.; Takagi, M.; Kaneda, K.; Dairi, T.; Seto, H. *Tetrahedron Lett*. **2000**, *41*, 2925–2928.
11. Turner, D. L.; Santos, H.; Fareleira P.; Pacheco, I.; Legall, J.; Xavier, A. V. *Biochem. J.* **1992**, *285*, 387–390.
12. Ostrovsky, D.; Diomina, G.; Lysak, E.; Matveeva, E.; Ogrel, O.; Trutko, S. *Arch. Microbiol*. **1998**, *171*, 69–72.