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Formation of 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol from 2-*C*-methyl-D-erythritol 4-phosphate by 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase, a new enzyme in the nonmevalonate pathway

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Abstract

2-*C*-Methyl-D-erythritol 4-phosphate is transformed to 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol in the presence of cytidine 5′-triphosphate by a novel *Escherichia coli* enzyme, 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase, involved in the nonmevalonate pathway.

Keywords: isopentenyl diphosphate; 2-*C*-methyl-D-erythritol 4-phosphate; 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase.

Since the initial discovery of the mevalonate pathway, it had widely been accepted that isopentenyl diphosphate (**1**), the fundamental unit in terpenoid biosynthesis, was only formed through the ubiquitous mevalonate pathway. It was recently revealed, however, that **1** is synthesized through the nonmevalonate pathway in many bacteria, green algae and chloroplast of higher plants (Fig. 1).[1] The initial step of this new pathway is the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) (**2**) by condensation of pyruvate and glyceraldehyde 3-phosphate catalyzed by DXP synthase. In the second step the intramolecular rearrangement and reduction of **2** occur simultaneously to yield 2-*C*-methyl-D-erythritol 4-phosphate (MEP) (**3**).[2,3] We succeeded in the first cloning and overexpression of the *dxr* gene (formerly *yaeM*) encoding DXP reductoisomerase from *Escherichia coli*, and showed that the recombinant enzyme catalyzes the formation of **3** from **2** in the presence of NADPH and a divalent cation such as Mn^{2+}, Mg^{2+} or Co^{2+}.[3] We also demonstrated that the enzyme activity is strongly and specifically inhibited by fosmidomycin (FR-31564), an antibiotic possessing the formyl and phosphonate functions in the molecule (Fig. 1).[4] However, the following reactions leading to **1** from **3** remain unknown.

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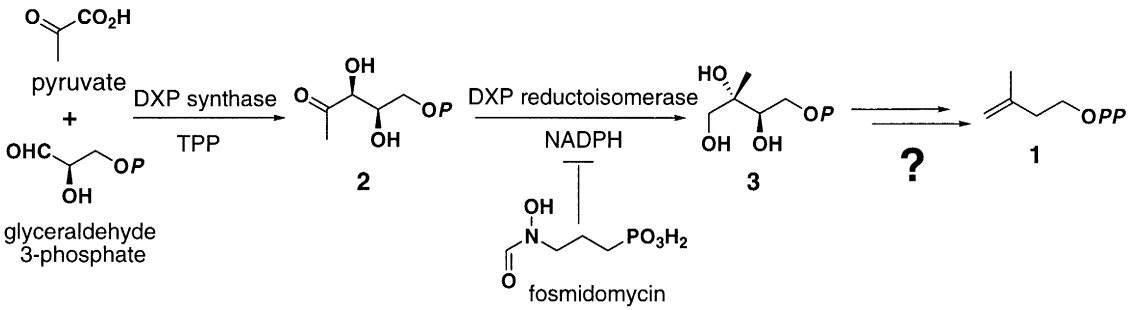

Fig. 1. The nonmevalonate pathway for IPP biosynthesis, and the structure of a DXP reductoisomerase inhibitor, fosmidomycin

To elucidate the remaining reactions leading to **1** from **3**, we initiated studies on the cloning of the genes responsible for conversion of **3** into **1**. To this end, we employed a unique strategy for preparation of mutants of *E. coli* possessing a metabolic block(s) between **3** and **1**. Since such mutations would be lethal, we constructed an *E. coli* transformant possessing an additional biosynthetic pathway for **1**, the mevalonate pathway.[‡] Using this transformant as the parent strain, we prepared mutants with an obligatory requirement of mevalonate for growth and survival. Thirty-three mutants from ca. 60 000 colonies screened showed the expected phenotypes; addition of mevalonate to the minimal medium M9 facilitated the growth of these mutants.[5] Thus, these phenotypic features of the mutants unequivocally demonstrated that these mutants have a defect(s) in the pathway leading to **1** from **3**. With these mutants in hand, we could clone several genes that complemented the defects of these blocked mutants in synthesizing **1** from **3**. To identify the functions of these genes, we constructed plasmids for overexpression of these gene products and succeeded in preparation of purified enzymes in quantities sufficient for their characterization.[5] We found that one of these gene products converted **3** into an unknown product in the presence of cytidine 5′-triphosphate (CTP).[§]

Incubation of this enzyme (4 mg[¶]) with **3** (10 mM) in the presence of 10 mM CTP at 37°C for 4 h in 25 ml of 100 mM Tris–HCl (pH 8.0) containing 5 mM·$MnCl_2$ resulted in appearance of a new peak in HPLC with an Aminex HPX-87H column (7.8×300 mm, Bio-Rad).[‖] The reaction product corresponding to this peak was purified with the assistance and guidance of this HPLC. Thus, the reaction mixture was diluted to 250 ml with H_2O, subjected to Dowex 1-X8 (Cl^- type, 2×6 cm) chromatography and eluted with

[‡] This transformant with mevalonate kinase, phosphomevalonate kinase and pyrophosphomevalonate decarboxylase activities can utilize the partial mevalonate pathway for IPP biosynthesis only in the presence of mevalonate. The genes encoding these enzymes were cloned from *Streptomyces* sp. strain CL190.[6] Thus mevalonate added into the growth medium is converted by these three enzymes into IPP to be utilized as a precursor of terpenoids by the transformant.

[§] This gene product can utilize ATP or UTP as well, but not GTP or TTP. ATP or UTP was less effective than CTP.

[¶] Protein expression and purification of the MEP cytidylyltransferase: The coding region of the *E. coli* MEP cytidylyltransferase gene[5] was cloned into the expression vector pQE30 (Qiagen) to give pQEMECT. *E. coli* M15 containing pREP4 [*neo*, *lacI*] (Qiagen) was used as a host for expression of the MEP cytidylyltransferase gene. *E. coli* M15 (pREP4, pQEMECT) was cultured at 37°C in 100 ml of Luria–Bertani medium containing 25 μg/ml kanamycin and 200 μg/ml ampicillin for 5 h with 0.1 mM isopropyl ß-D-thiogalactoside 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 MEP cytidylyltransferase in the subsequent experiments.

[‖] Formation of the reaction product was monitored at 270 nm with an Aminex HPX-87H column (Bio-Rad), eluted with 5 mM H_2SO_4 at the flow rate of 0.6 ml/min at room temperature.

1% NaCl aq. (100 ml). All fractions showing the peak with a retention time of 6.3 min was combined, concentrated and chromatographed on a Sephadex G-10 column (1.8×100 cm) and elution was made with water only. A fraction showing only the peak with the same retention time was freeze-dried to afford a pure reaction product (1.1 mg).

The structure of the purified product was deduced by spectroscopic methods. The molecular formula was determined to be $C_{14}H_{23}O_{14}N_3P_2Na_2$ by HR-FABMS [*m/z* 542.0594 $(M-Na)^-$, Δ +4.1 mmu]. The ^1H and ^{13}C NMR spectral features of the purified product showed close similarities to those of **3** and cytidine 5′-diphosphate (CDP) (Table 1). The purified compound, however, showed two ^{31}P NMR signals at –10.6 and –11.2 ppm which coupled each other (20.2 Hz). In addition, these ^{31}P NMR signals were coupled to C-4 (J_{C4-P}=5.8 Hz) and C-3 (J_{C3-P}=6.7 Hz) in the MEP moiety, and to C-5′ ($J_{C5'-P}$=5.8 Hz) and C-4′ ($J_{C4'-P}$=8.6 Hz) in the CDP moiety. All these coupling constants indicated that a cytidylyl function generated from CTP bound to 4-phosphate in the MEP molecule. The UV spectral feature of the compound (λ_{max}=271 nm (ε 9100) at pH 7, 280 nm (ε 13000) at pH 2) was identical to that of cytidine. These spectral data unequivocally established the structure of the reaction product as 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME) (**4**). Thus, **3** proved to be converted into **4** in the presence

Table 1

^1H, ^{13}C and ^{31}P NMR spectra data of the reaction product, MEP and CDP

	reaction product **4**	MEP **3**	CDP
^1H-NMR	MEP moiety		
	H-1a 3.37 (d, 11.5)	H-1a 3.50 (d, 12.0)	
	H-1b 3.49 (d, 11.5)	H-1b 3.60 (d, 12.0)	
	2-Me 1.03 (s)	2-Me 1.15 (s)	
	H-3 3.73 (dd, 8.5, 2.5)	H-3 3.78 (dd, 8.0, 2.5)	
	H-4a 3.86 (m)	H-4a 3.84 (ddd, 11.5, 6.5, 2.5)	
	H-4b 4.11 (m)	H-4b 4.03 (ddd, 11.5, 6.5, 2.5)	
	CDP moiety		
	H-5 6.03 (d, 7.5)		H-5 6.02 (d, 7.6)
	H-6 7.88 (d, 7.5)		H-6 7.89 (d, 7.6)
	H-1' 5.89 (d, 4.3)		H-1' 5.87 (d, 4.0)
	H-2' 4.20 (dd, 5.0, 4.3)		H-2' 4.21 (dd, 5.0, 4.0)
	H-3' 4.25 (dd, 5.0, 5.0)		H-3' 4.30 (dd, 5.5, 5.0)
	H-4' 4.16 (m)		H-4' 4.15 (m)
	H-5'a 4.09 (m)		H-5'a 4.13 (m)
	H-5'b 4.16 (m)		H-5'b 4.13 (m)
^{13}C-NMR	MEP moiety		
	C-1 67.3	C-1 67.4	
	C-2 74.8	C-2 75.1	
	2-Me 19.1	2-Me 19.4	
	C-3 74.3 (d, 6.7)	C-3 74.8 (d, 6.5)	
	C-4 67.8 (d, 5.8)	C-4 65.9 (d, 4.6)	
	CDP moiety		
	C-2 158.7		C-2 158.5
	C-4 167.1		C-4 167.0
	C-5 97.5		C-5 97.3
	C-6 142.4		C-6 142.4
	C-1' 90.1		C-1' 90.2
	C-2' 75.2		C-2' 75.1
	C-3' 70.2		C-3' 69.7
	C-4' 83.6 (d, 8.6)		C-4' 83.5 (d, 9.3)
	C-5' 65.5 (d, 5.8)		C-5' 64.8 (d, 5.5)
^{31}P-NMR	-10.6 (d, 20.2)		
	-11.2 (d, 20.2)		

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

of CTP by the enzyme (Fig. 2). This result clearly demonstrates that a cytidylyl transfer to **3** is the reaction after the formation of **3** in the nonmevalonate pathway for IPP biosynthesis. We propose to designate this enzyme 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase (MEP cytidylyltransferase).

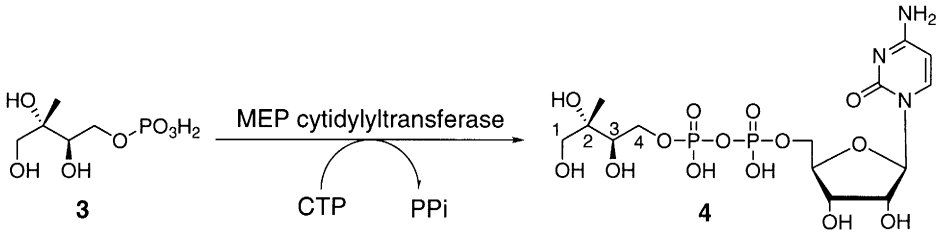

Fig. 2. MEP cytidylyltransferase reaction

As mentioned above, we have already cloned several genes that encode the enzymes responsible for synthesizing **1** from **3**. One of these gene products converted **4** into an unknown product. The structure elucidation of this product is now under way.

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