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Chemoenzymatic synthesis of 4-diphosphocytidyl-2-*C*-methyl-D-erythritol: a substrate for IspE

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ABSTRACT

Enantiomerically pure 2-*C*-methyl-D-erythritol 4-phosphate **1** (MEP) is synthesized from 1,2-*O*-isopropylidene-α-D-xylofuranose via facile benzylation in good yield. Subsequently, **1** is used for enzymatic synthesis of 4-diphosphocytidyl-2-*C*-methyl-D-erythritol **2** (CDP-ME) using 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase (IspD). The chemoenzymatically synthesized **2** can be used as substrate for assay of IspE and for high throughput screening to identify IspE inhibitors.

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About 450,000 people a year are infected with multi-drug resistant tuberculosis (MDR-TB), which is resistant to the main first-line drugs isoniazid and rifampin. In addition extensively drug resistant tuberculosis (XDR-TB), which is resistant to isoniazid and rifampin and resistant to any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin) has been reported in 37 countries in all regions of the world since 2006. Moreover human immunodeficiency virus—tuberculosis (HIV-TB) co-infection is also a big challenge besides the XDR-TB.[1] Yet no new anti-TB drug has been introduced since the 1960s. In this context, designing and developing a new anti-TB drug is very important.

To date two different biosynthetic pathways have been reported leading to isopentenyl diphosphate, the universal precursor of isoprenoids. The mevalonate pathway[2] is found in animals, whereas the non-mevalonate or methylerythritol phosphate (MEP) pathway is found in many bacteria, some protozoa, and plants (Scheme 1).[3] In the MEP pathway, 1-deoxy-D-xylulose 5-phosphate **5** (DXP) is made by condensing pyruvate **3** and glyceraldehyde 3-phosphate **4** catalyzed by DXP synthase (Dxs). Subsequently, **1** is synthesized by intramolecular rearrangement and reduction of **5** catalyzed by IspC. Then **1** is coupled with cytidine triphosphate (CTP) using IspD to produce **2**. **2** is subsequently phosphorylated by 4-diphosphocytidyl-2-*C*-methyl-D-erythritol-2-phosphate synthase (IspE) to form 4-diphosphocytidyl-2-*C*-methyl-D-erythritol-2-phosphate **6** (CDP-ME2P) and cyclized by IspF to form 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate **7** (ME-CPP). The cyclic diphosphate is transformed into 1-hydroxy-2-methyl-2-*E*-butenyl 4-diphosphate **8** (HMBPP) by IspG. IspH (LytB) catalyzes the synthesis of isopentenyl diphosphate **9** (IPP) and its isomer dimethylallyl diphosphate **10** (DMAPP).

Since the MEP pathway is not found in mammalian cells, it is considered an attractive target for the development of antimicrobials, antimalarials, and herbicidal agents,[3] a hypothesis that is being explored by an increasing number of researchers. A major difficulty hindering this research is the shortage of pure substrates. In this regard, access to MEP pathway intermediates and their analogues is essential to ongoing biochemical investigations and development of high throughput screens to attempt to identify leads for synthesis of new therapeutics. Recently, we have described assays for mycobacterial Dxs, IspC, and IspD.[4] In order to study mycobacterial IspE, we were in need of compound **2**.

However, the reported chemical synthesis of **2** is only 50% enantiomerically pure.[5] Whereas when synthesized enzymatically starting with the formation of **5** by condensation of **3** and **4** catalyzed by Dxs, the ultimate yield of **1** is very low.[6] In addition this enzymatic method is time consuming and expensive. Herein, we report a chemoenzymatic method to synthesize **2** in good yield.

To initiate the synthesis of **2** (Scheme 2), we synthesized enantiopure **1**. Many procedures are available for the synthesis of **1**. However, only one procedure is reported for synthesis of enantiomerically pure **1** from commercially available 1,2-*O*-isopropylidene-α-D-xylofuranose.[7]

Dibenzyl phosphochloridate[8] was synthesized by a modified procedure of chlorinating dibenzyl phosphite **11**, in toluene and benzene using *N*-chlorosuccinamide (NCS). Dibenzyl phosphochloridate **12** in pyridine was used to selectively protect the primary alcohol of 1,2-*O*-isopropylidene-α-D-xylofuranose **13** yielding 5-dibenzylphosphate-1,2-*O*-isopropylidene-α-D-xylofuranose **14**. Then the free secondary alcohol **14** was oxidized to ketone **15** quantitatively with pyridinium dichromate (PDC).[7]

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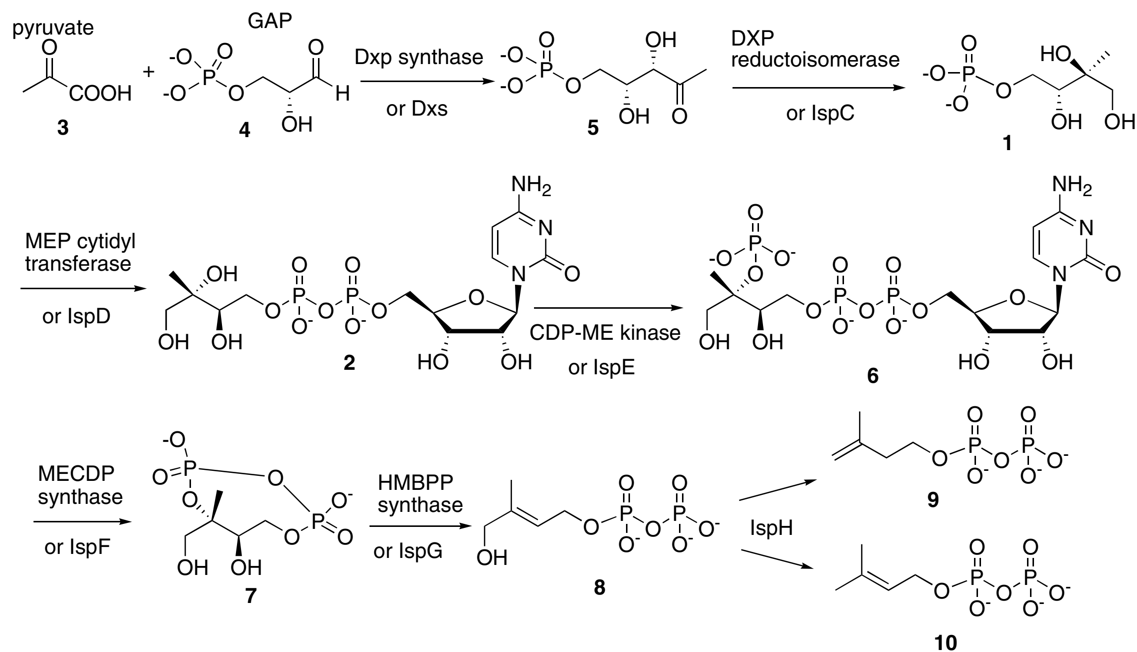
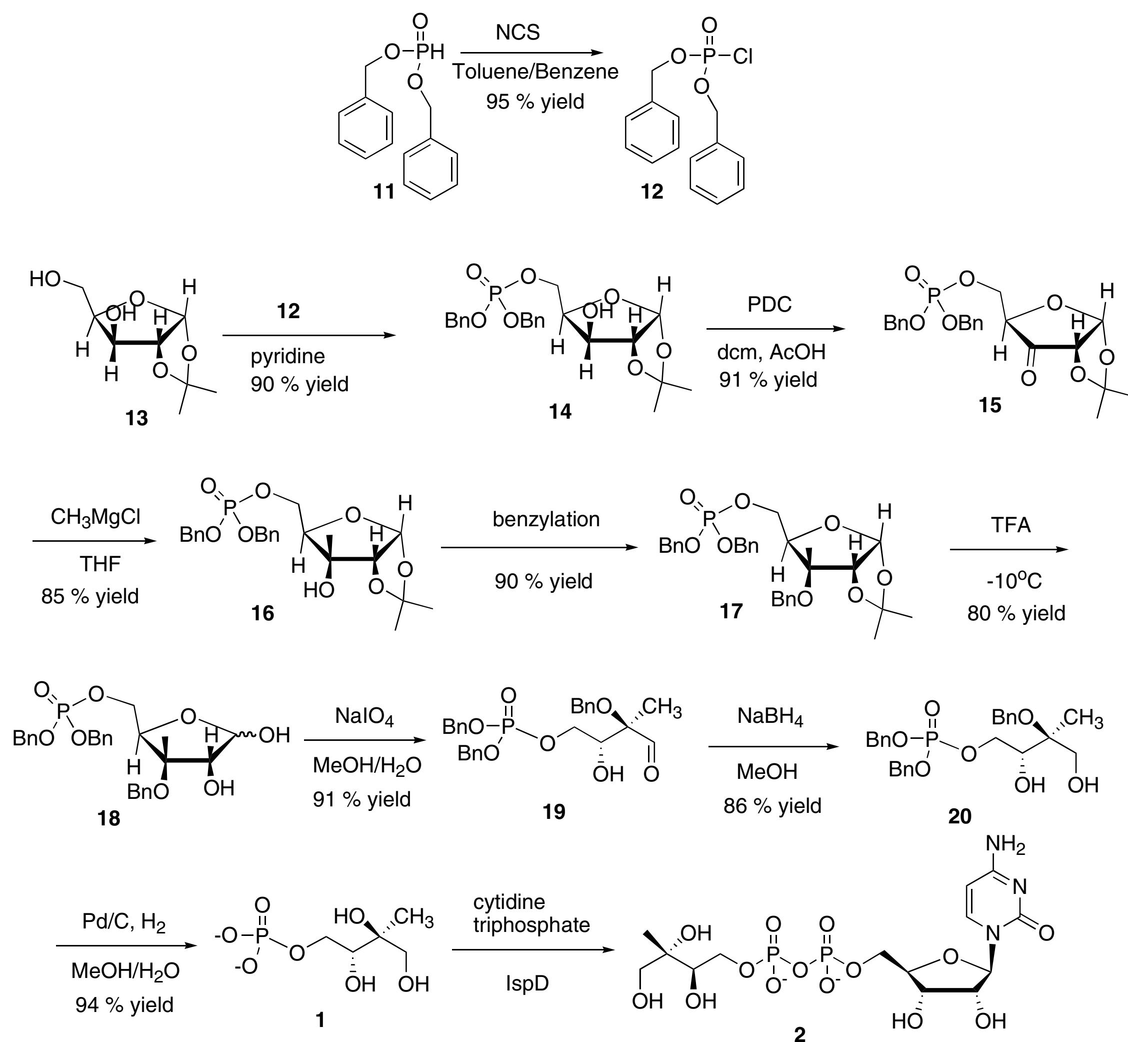


Scheme 1. Isoprenoid biosynthesis via the MEP pathway.



Scheme 2. Chemoenzymatic synthesis of CDPME.

Previously, 3-*C*-alkyl ribofuranoses were obtained by a stereoselective addition reaction with the alkyl group on the β-face of the carbohydrate ring.[9] Accordingly, in the ketone **15**, addition of the methyl group occurs from the less hindered β-face, leading to the tertiary alcohol **16** with the desired stereochemistry. The alcohol **16** was protected by using benzyl bromide, after activating the hydroxyl group to yield 90% of benzylated **17**.[10]

Acetonide deprotection was carried out using 90% aq trifluoroacetic acid giving rise to two anomers **18**, which underwent a sodium metaperiodate-mediated glycol oxidative cleavage to give the aldehyde **19**. The aldehyde was reduced with sodium borohydride forming the MEP precursor,[11] alcohol **20**, followed by hydrogenolysis in water/methanol medium, without acid workup leading to enantiomerically pure **1** (Scheme 2). The chemically synthesized **1** was characterized by NMR, MS, and optical rotation, and the data were found to be identical with those previously reported in the literature.[7,12,13] Subsequently, chemically synthesized **1** was used as a substrate for the enzymatic synthesis of **2**.

Recombinant *Rv3582c*, *M. tuberculosis* IspD, was prepared as previously described.[4a] Briefly, *Rv3582c* was amplified using PCR primers, and Expand High Fidelity PCR system (Roche Molecular Biochemicals, Indianapolis, Indiana, USA) (Rv3582c–F: CAT ATG AGG GAA GCG GGC GAA GTA G and Rv3582c–R: CTC GAG TCA CCC GCG GAG TAT AGC TTG), containing *Nde*I and *Xho*I restriction enzyme sites (underlined), respectively. The PCR products were digested and ligated into the pET28a(+) vector (EMD Biosciences, Inc., San Diego, CA) and the ligation mixtures were used to transform *E. coli* DH5α cells (Life Technologies, Rockville, MD) creating DH5α[pET28a(+)::*Rv3582c*] for amplification. The recombinant plasmids harboring *Rv3582c* were isolated using a Plasmid Miniprep Kit (Qiagen, Valencia, CA) and the sequences of the plasmids were confirmed by Macromolecular Resources (Colorado State University). Transformation of BL21 (DE3) (Novagen, Madison, WI) with pET28a(+)::*Rv3582c* afforded the recombinant strain BL21(DE3)[pET28a(+)::*Rv3582c*]. Protein expression was induced in the presence of 0.5 mM isopropyl–β–D–thiogalactopyranoside (IPTG) at 20 °C for 10 h. The recombinant protein carrying a hexa–histidine tag was purified by immobilized metal affinity chromatography on HIS-select™ Nickel affinity gel from Sigma–Aldrich (St. Louis, MO) using a linear gradient of 50–200 mM imidazole in washing buffer [50 mM 4–morpholine propane sulfonic acid (MOPS) (pH 7.9), 1 mM $MgCl_2$, 10% glycerol, and 1 mM β-mercaptoethanol].

Compound **2** was synthesized enzymatically[14] with a maximum yield of 25% after incubation at 37 °C for 1 h. Formation of **2** is followed by monitoring the formation of PP*i* released during catalysis by IspD (EnzChek® Phosphate Assay Kit, Invitrogen) although this is not required in production of **2**. When examined by MS and ^{1}H NMR the product was found to generate spectra identical to reported data.[5,13,15]

Thus, we successfully synthesized enantiomerically pure **1**, which could be utilized by mycobacterial IspD to synthesize **2** in satisfactory yields. Radiolabel could be introduced during the methylation or reduction steps if required. Pure **2** can be used to study the kinetic properties of IspE and for high throughput screening to identify IspE inhibitors. Experiments for *M. tuberculosis* IspE inhibitors are in progress.

Acknowledgment

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11. Data for **20**: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.27–7.19 (m, 15H), 5.00–4.96 (m, 4H), 4.43–4.41 (m, 2H), 4.26 (t, 1H, *J* = 9.9 Hz), 3.98–3.94 (m, 2H), 3.62–3.60 (m, 2H), 1.10 (*s*, 3H).; ^{13}C NMR ($CDCl_3$, 75 MHz): 138.5, 135.7, 135.6, 128.6, 128.4, 128.1, 128.0, 127.6, 127.3, 78.2, 73.0, 69.6, 64.8, 64.2, 15.4.; IR (neat, cm^{-1}): 3588, 2966, 2362, 2336, 1652, 1614. HRMS (ESI) $C_{26}H_{32}O_7P$ ($M+H^+$) calcd 487.1880 and found 487.1870; $[\alpha]_D$ 10.0 (*c* 0.5, $CHCl_3$).
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14. Reaction mixtures contained 50 mM Tris–HCl (pH 7.4), 200 μM 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG), 1 mM DTT, 100 μM 2-*C*-methyl-D-erythritol 4-phosphate (MEP), 100 μM CTP, 0.03 U inorganic pyrophosphatase, 1U purine nucleoside phosphorylase and 11.6 pmols of purified *M. tuberculosis* IspD per 50 μl of reaction volume. Reaction mixtures were incubated at room temperature for 30 min after which their endpoint absorbance at 360 nm was determined. Chemically produced **1** was compared with authentic **1**. Synthesized **2** was found to generate spectra identical to reported data.
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