Synthesis of 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate and kinetic studies of *Mycobacterium tuberculosis* IspF, a potential drug target

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SUMMARY

Many pathogenic bacteria utilize the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway for the biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate, two major building blocks of isoprenoid compounds. The fifth enzyme in the MEP pathway, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (ME-CPP) synthase (IspF), catalyzes the conversion of 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate (CDP-ME2P) to ME-CPP with a corresponding release of cytidine 5-monophosphate (CMP). Since there is no ortholog of IspF in human cells IspF is of interest as a potential drug target. However, study of IspF has been hindered by a lack of enantiopure CDP-ME2P. Herein, we report the first synthesis of enantiomerically pure CDP-ME2P from commercially available D-arabinose. Cloned, expressed, and purified *M. tuberculosis* IspF was able to utilize the synthetic CDP-ME2P as a substrate, a result confirmed by mass spectrometry. A convenient, sensitive, *in vitro* IspF assay was developed by coupling the CMP released during production of ME-CPP to mononucleotide kinase, which can be used for high throughput screening.

INTRODUCTION

Mycobacterium tuberculosis is the etiological agent of tuberculosis (TB) and, as of 2007, roughly 1/3 of the world's population was infected with tubercle bacilli (World Health Organization, WHO Report 2007). This, and the fact that both multi-drug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) are rapidly spreading(Zhao et al., 2009) has renewed the urgency for the development of new treatments for this disease. Here we report continued efforts to characterize and identify inhibitors of isoprenoid biosynthesis in pathogenic bacteria.

Isoprenoid compounds, such as polyprenols, polyprenyl phosphates, carotenoids, sterols, monoterpenes, sesquiterpenes, lipoquinones, etc., are found in all living organisms(Edwards and Ericsson, 1999), and a number of isoprenoid compounds are found in mycobacteria that are essential for bacterial survival(Brennan and Crick, 2007). To date, two different

biosynthetic pathways are known to lead to the synthesis of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), universal precursors of isoprenoids(Rohmer, 1999; Brennan and Crick, 2007). The mevalonate pathway(Buhaescu and Izzedine, 2007), first identified in mammals, and the non-mevalonate or methylerythritol phosphate (MEP) pathway found in plants (MEP pathway in plants is constricted to the chloroplasts), apicomplexan protozoa, and many eubacteria, including human pathogens such as *M. tuberculosis, Escherichia coli*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Salmonella enterica* Serovar Typhi, *Mycobacterium leprae*, *Staphylococcus aureus* and *Plasmodium falciparum* (Rohmer, 1999; Skorupinska-Tudek et al., 2008; Rohmer, 2007; Eoh et al., 2008).

In the MEP biosynthetic pathway (Fig. 1), 1-deoxy-D-xylulose 5-phosphate **3** (Dxp) is made by condensing pyruvate **1** and glyceraldehyde 3-phosphate **2** catalysed by Dxp synthase (Dxs). Subsequently **3** undergoes intramolecular rearrangement and reduction by Dxp reductoisomerase (IspC) enzyme to synthesize **4. 4** is coupled with cytidine triphosphate (CTP) using MEP cytidyltransferase (IspD) to synthesize **5** as the major product. **5** is subsequently phosphorylated by CDP-ME kinase (IspE) at the 2-hydroxyl position to form 4-diphosphocytidyl-2-*C*-methyl-D-erythritol-2-phosphate **6** (CDP-ME2P) which is cyclized by ME-CPP synthase (IspF)(Buetow et al., 2007;Campbell and Brown, 2002;Fellermeier et al., 2001;Rohdich et al., 2001;Steinbacher et al., 2002;Ramsden et al., 2009;Richard et al., 2002;Lehmann et al., 2002) to form 2-*C*-methyl-D-erythritol 2, 4-cyclodiphosphate **7** (ME-CPP). The cyclic diphosphate undergoes reductive elimination to form 1-hydroxy-2-methyl-2-*E*-butenyl 4-diphosphate **8** (HMBPP), a reaction catalysed by IspG and IspH (LytB) is utilized subsequently to generate IPP **9** and DMAPP **10**.

Since the MEP pathway is not found in human cells it is generally considered to be a source of good targets for the development of antimicrobials(Illarionova et al., 2006), antimalarials (Giessmann et al., 2008), and herbicidal agents(Ershov, 2007), a hypothesis being explored by many researchers. However, a major difficulty faced by researchers in this area is the lack of availability of pure substrates. Access to MEP pathway intermediates and their analogues is essential to ongoing biochemical investigations and development of new antibiotics targeting the respective enzymes. Recently we reported the synthesis of CDP-ME(Narayanasamy et al., 2008), ME-CPP(Narayanasamy and Crick, 2008) and kinetic studies of mycobacterial Dxs (Bailey et al., 2002), IspC(Dhiman et al., 2003), IspD(Eoh et al., 2007), and IspE (Narayanasamy et al., 2008). In order to extend our research to include IspF, we were in need of compound **6**; however the previously reported enzymatic synthesis of **6** is tedious, expensive and leads to low yields(Illarionova et al., 2006; Herz et al., 2000; Luttgen et al., 2000). Herein, we report the first chemical synthesis of enantiomerically pure **6** and its use as a substrate to initiate studies of IspF.

RESULTS AND DISCUSSION

Interestingly, although reported for the synthesis of **4** and **5**, there is no procedure for synthesis of enantiomerically pure **16**, **21** or **6** from commercially available D-arabinose. To initiate the reactions leading to **6**, we extended the studies on the synthesis of methylerythritol described in the literature(Urbansky et al., 2004). Thus, the tertiary hydroxyl group of **13** is phosphorylated by PCl$_3$ followed by benzylation using benzyl alcohol to yield dibenzyl phosphite and subsequent oxidation to dibenzyl phosphate, **19** using a hydrogen peroxide solution. The TBS group in **19** is deprotected by triethylamine hydrofluoride in good yield and the primary hydroxyl group of **20** is phosphorylated using dimethyl phosphochloridate in a n-BuLi solution. The methyl in the dimethyl phosphate **21** is deprotected using TMSI as previously reported(Zygmunt et al., 1978) before coupling to give **22** in stable form. We assumed that the benzyl protected methylerythritol **22** could be efficiently coupled with

cytidine monophosphate (CMP) to give the final product **23**; unfortunately, this reaction only afforded an 8 % yield of **23**, presumably due to high stearic hindrance. Hydrogenolysis of **23** using 20 % Pd(OH)$_2$/C yielded pure **6**, but the overall yield for this route to **6** is unacceptably low. Therefore an alternate route was developed. Initially, an attempt was made to use dibenzyl phosphochloridate in pyridine to selectively protect the primary hydroxyl of **14**, but that reaction was unsuccessful, so **14** was activated with n-BuLi at low temperature and then reacted with freshly prepared dibenzyl phosphochloridate to yield **15**. The free tertiary hydroxyl group was phosphorylated using PCl$_3$ at low temperature, followed with ethanol to give diethyl phosphite, which was subsequently oxidized to diethyl phosphate, **16**, in good yield (Fig. 2). Subsequently the benzyl deprotection was carried out by hydrogenolysis in one step using 20 % Pd(OH)$_2$/C in the presence of hydrogen to yield **17**.

Cytidine monophosphate was titrated with triethylamine leading to the formation of the corresponding triethylammonium cytidine 5′-monophosphate. The phosphoester moiety was activated by triflouroacetyl anhydride followed by conversion to phosphoramide by treatment with methylimidazole and then coupling with the tributylammonium salt of **17;** this reaction was completed in 4 h and quenched with 1 M aq. NH$_4$HCO$_3$. The crude material was purified by passing through a benzyl-DEAE cellulose column using a gradient of 10–500 mM aq. NH$_4$HCO$_3$ and fractions containing the product, **18,** were collected and lyophilized giving 40 % yield. Examination of **18** was done by ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR and MS (see supplemental information, SI). The phosphate moiety of **18** was deprotected using TMSI as reported in literature and purification by column chromatography on Whatman fibrous cellulose using 2-propanol/methanol/water (4:2:4) solution(Illarionova et al., 2006) gave an 84 % yield of **6.** Since the deprotected **6** is not stable it was subjected to analysis by MS and used immediately for the kinetic study of IspF. The nature and structure of product **6** was confirmed by MS, ^{1}H-NMR, ^{31}P-NMR (see **SI**) and utilization by pure recombinant enzyme.

An amino acid sequence alignment of Rv3581c with *E. coli* IspF(Herz et al., 2000) showed 40% overall similarity and suggested that Rv3581c is the *M. tuberculosis* ortholog of IspF (Fig. 3A). *Rv3581c* is 480 bp in length, encoding a polypeptide of 159 amino acids with a molecular weight of 17.7 kDa, which is predicted to be cytosolic(Sgraja et al., 2008). *M. tuberculosis* IspF expression in the heterologous host, *E. coli,* was confirmed by Western blot analysis using an anti–His antibody (Fig. 3B) and purified by immobilized metal affinity chromatography. Interestingly, all purification steps require the inclusion of 1 mM Zn^{2+} in order to maintain activity (see **SI**).

Initially, a radioisotope based assay was utilized to confirm the activity of the recombinant protein. When synthesized CDP-ME and [γ-^{32}P]ATP were incubated in presence of IspE, TLC analysis of the reaction mixture clearly showed the formation of a product with the reported chromatographic properties of CDP-ME2P (Fig. 4, lane 2). Addition of *M. tuberculosis* IspF to the mixture resulted in the formation of a new product that had chromatographic properties on TLC and mass spectral data similar to those previously reported for ME-CPP generated by an assay in which IspE and IspF were coupled (Testa et al., 2006) (Fig. 4, lane 3). In addition, crude reaction mixtures containing enantiomerically pure CDP-ME2P and *M. tuberculosis* IspF were analyzed by mass spectrometry in the absence of CDP-ME and IspE. In these assays, a decrease in the CDP-ME2P molecular ion and the appearance of a molecular ion corresponding to ME-CPP (Narayanasamy and Crick, 2008;Urbansky et al., 2004) was observed (data not shown). Although this protocol confirmed the formation of product, it is cumbersome for the analysis of multiple samples.

In general, IspF catalyzes an unusual cyclization reaction producing ME-CPP and releasing CMP (Herz et al., 2000); in previous reports analysis of IspF assay products were based on TLC or HPLC (Herz et al., 2000; Rohdich et al., 2001), which is not suitable for high throughput

screening. To develop a more convenient assay, which is applicable to high throughput screening and to determine kinetic properties of *M. tuberculosis* IspF, CMP generation was coupled to nucleotide monophosphate kinase (NMK) and the ADP Quest HS Kinase Assay Kit (GE Healthcare, UK) to monitor ADP formation (Figure S1 in SI). The activity of pure recombinant IspF was linear with increasing protein concentration up to 281.5 pmol and reaction time up to 30 minutes. The coupled assay showed classic saturation kinetics with increasing concentration of CDP-ME2P (Fig. 3C). From this data the $K_m^{CDP-ME2P}$ and V_{max} values of 81.1 μM and 81.6 nmolS^{-1} respectively were calculated. The constants, k_{cat} and k_{cat}/K_m for *M. tuberculosis* IspF were calculated to be 7.3×10^{-3} S^{-1} and 5.4×10^{-4} μM^{-1}min^{-1} respectively.

SIGNIFICANCE

We successfully synthesized enantiopure **6** by two routes. Although this compound was unstable under usual storage conditions, the derivative **18** can be stored at −20° C and easily deprotected to form **6**. In the synthetic scheme of **6** reported here, radiolabeling also can be easily introduced during the methylation, reduction and/or coupling steps if required. To determine the kinetic properties of *M. tuberculosis* IspF using **6**, the enzyme was identified bioinformatically, overexpressed, purified in the presence of 1 mM Zn^{2+} and utilized to develop a spectrophotometry based *in vitro* assay. This assay was used to generate the first kinetic characterization of an IspF enzyme and, importantly, it can be used for high throughput screening to identify IspF inhibitors. We also observed that the k_{cat}/K_m value 5.4×10^{-4} μM^{-1}min^{-1} of *M. tuberculosis* IspF was much lower than that reported for *M. tuberculosis* IspD and IspE(Eoh et al., 2007) suggesting that the catalytic efficiency of IspF is lower than the preceding two steps on the biosynthetic pathway.

EXPERIMENTAL PROCEDURES

M. tuberculosis H$_{37}$Rv strain genomic DNA was provided by Dr. John T. Belisle of Colorado State University (NIH/NIAID Contract N01-AI-75320, "Tuberculosis Research Material and Vaccine Testing"). All PCR reagents and cloning materials were purchased from Qiagen (Valencia, CA). His-select nickel affinity resin, ATP and reagents were obtained from Sigma-Aldrich (St. Louis, MO). Nucleotide monophosphate kinase (NMK) and phosphorylase inhibitor were purchased from Roche. The ADP Quest HS Kinase Assay Kit was purchased from GE healthcare Bio–Sciences Corp. UK. All other reagents and solvents were of at least analytical grade. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham Biosciences (Pittsburgh, PA).

Procedure for synthesis of 16

To alcohol **15** (0.275 mmol) in dcm (6 mL), pyridine (0.91 mmol) was added at −13 °C, followed by addition of phosphorus trichloride (0.28 mmol). After 2 h of stirring at 13 °C, completion of reaction was checked by TLC and then dry ethanol (0.605 mmol) was added at 13 °C and stirred at 16 °C for 2 h. After the completion of the reaction it was cooled to 0 °C and hydrogen peroxide (0.99 mmol) solution was added. The reaction mixture was stirred at room temperature for 4 h and then diluted with dichloromethane. Subsequently the organic phase was washed with 10 % sodium metabisulfite solution, dilute HCl and brine solution. The organic phase was dried with MgSO$_4$, solvents were evaporated at reduced pressure and the crude mixture was purified by flash column chromatography using 80:20 (EtOAc: hexane) as eluent and gave a 68 % yield of **16**.

Procedure for synthesis of 18

To CMP (0.05 mmol) in acetonitrile (0.5 mL), *N*, *N*, dimethyl aniline (0.21 mmol) and triethylamine (0.05 mmol) were added consequently at 0 °C. Trifluoroacetic anhydride (0.26 mmol) in acetonitrile was added slowly to the above mixture and stirred for 15 minutes. Excess TFA and anhydride was removed under reduced pressure. Then 1-methyl imidazole (0.15 mmol) and triethylamine (0.26 mmol) in acetonitrile was slowly added and the mixture was stirred for 30 more minutes. The activated CMP obtained was added to 0.04 mmol of **16** and activated 4 A° molecular sieves in acetonitrile at 0 °C and stirred for 4h. The mixture was then extracted with chloroform and the aqueous layer was lyophilized. The dried compound was dissolved in 100 mM aq. ammonium bicarbonate and purified through Bio-Gel® P-2 gel fine column using 100 mM aq. ammonium bicarbonate followed by further purification on a benzyl DEAE cellulose anion exchange column, eluted by a gradient of 0–0.5 M aq. ammonium bicarbonate leading to a 40 % yield of **18** as the major product.

In vitro radiochemical IspF assay

IspF assays were performed in reaction mixtures containing 50 mM Tris-Cl (pH 7.0), 100 μM [γ-^{32}P]ATP (10 dpm/nmol), 2 mM DTT, 100 μM CDP-ME, 5 mM MgCl$_2$, 97.2 pmol *M. tuberculosis* IspE, and 112.6 pmol *M. tuberculosis* IspF in a 50 μl final reaction volume. Reactions were initiated by addition of purified *M. tuberculosis* IspF, incubated at 37 °C for 30 min, and terminated by the addition of 10 mM of EDTA (pH 8.0). TLC analysis was performed by transferring 10 μl of the reaction mixture to a TLC plate (Polygam Sil N-HR, Macherey and Nagel) and developing with n-propanol/ethyl acetate/H$_2$O (6:1:3, v/v/v). Distribution of the radioactivity on the TLC plates was analyzed using a Molecular Dynamics Typhoon 8600 Phosphoimager.

MS based *in vitro* IspF assay

The IspF activity was also assessed by monitoring ME-CPP formation by MS. Reaction mixture contained 50 mM MOPS (pH 8.0), 5 mM of MgCl$_2$, 100 μM CDP-ME2P, 1 mM phosphatase inhibitor, in a final volume of 50 μl. In all cases, the reactions were started by the addition of 112.6 pmol purified IspF, incubated at 37 °C for 30 minutes and terminated by the addition of 10 mM EDTA. The reaction mixture was completely dried, dissolved in methanol/H$_2$O (1:1) and analysed on an Agilent 6210 mass spectrometer.

Spectrophotometry based *in vitro* IspF assay

The IspF activity was assessed by monitoring CMP release, which was coupled to NMK to generate ADP (Fig. 2 in SI). The ADP Quest HS Kinase Assay Kit was used to detect ADP formation. Reactions were performed in 96-well black microplates with clear bottoms (Costar, Bethesda, MD); each reaction mixture contained 50 mM MOPS (pH 8.0), 5 mM of MgCl$_2$, 100 μM CDP-ME2P, 1 mM phosphatase inhibitor, 0.05 U of NMK, and 200 μM ATP in a final volume of 50 μl. In all cases, the reactions were started by the addition of 112.6 pmol of purified IspF, incubated at 37 °C for 30 minutes and terminated by the addition of 10 mM EDTA. Subsequently, 25 μl of reagent A and 50 μl of reagent B of the ADP Quest HS Kinase Assay Kit were added and incubated at room temperature for 15 min. Fluorescence was measured by Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc. Winooski, VT) with an excitation wavelength of 530 nm and emission wavelength of 590 nm.

Kinetic characterization of Rv3581c

The effect of CDP-ME2P concentrations on reaction rates was determined by adding various concentrations of the compound to the reactions. Results presented are the average of duplicate experiments. The K_m and V_{max} values of substrates for the enzyme were calculated by non-linear regression analysis using SigmaPlot V.8.02A.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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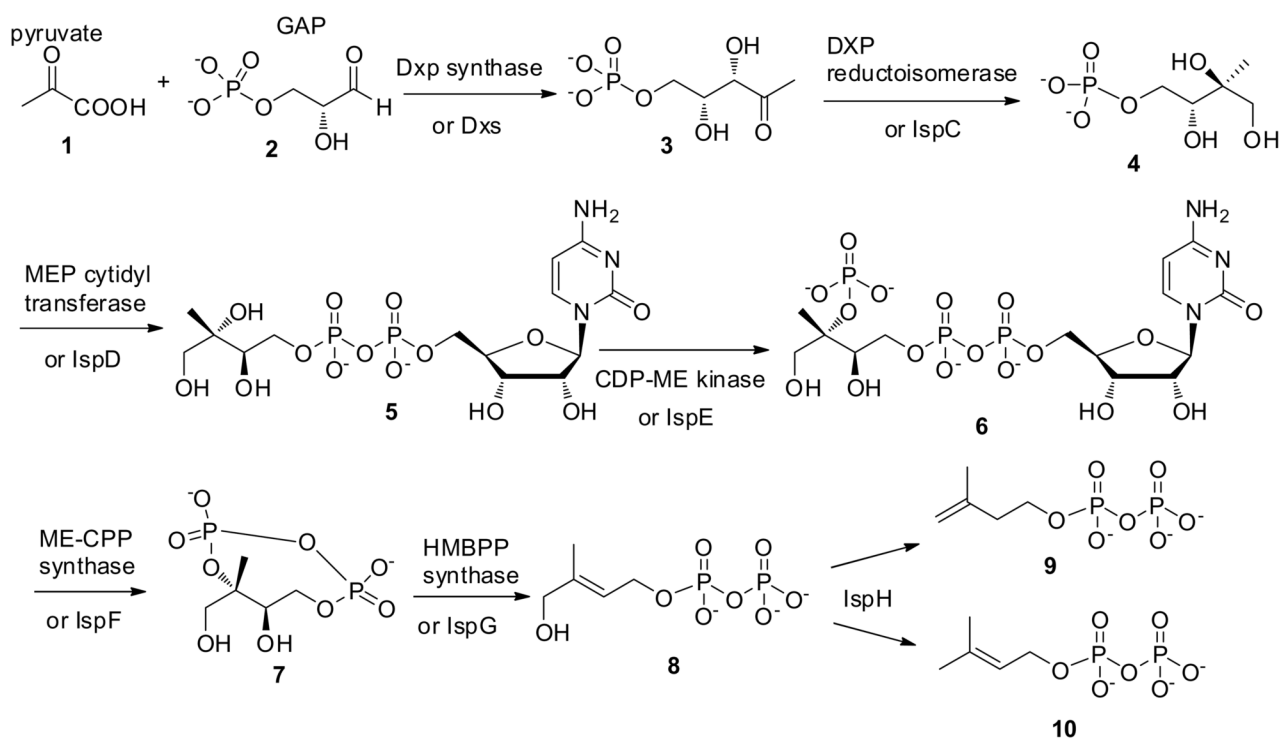Zygmunt J, Kafarski P, Mastalerz P. Preparation of oxoalkanephosphonic acids. Synthesis-Stuttgart 1978:609–612.
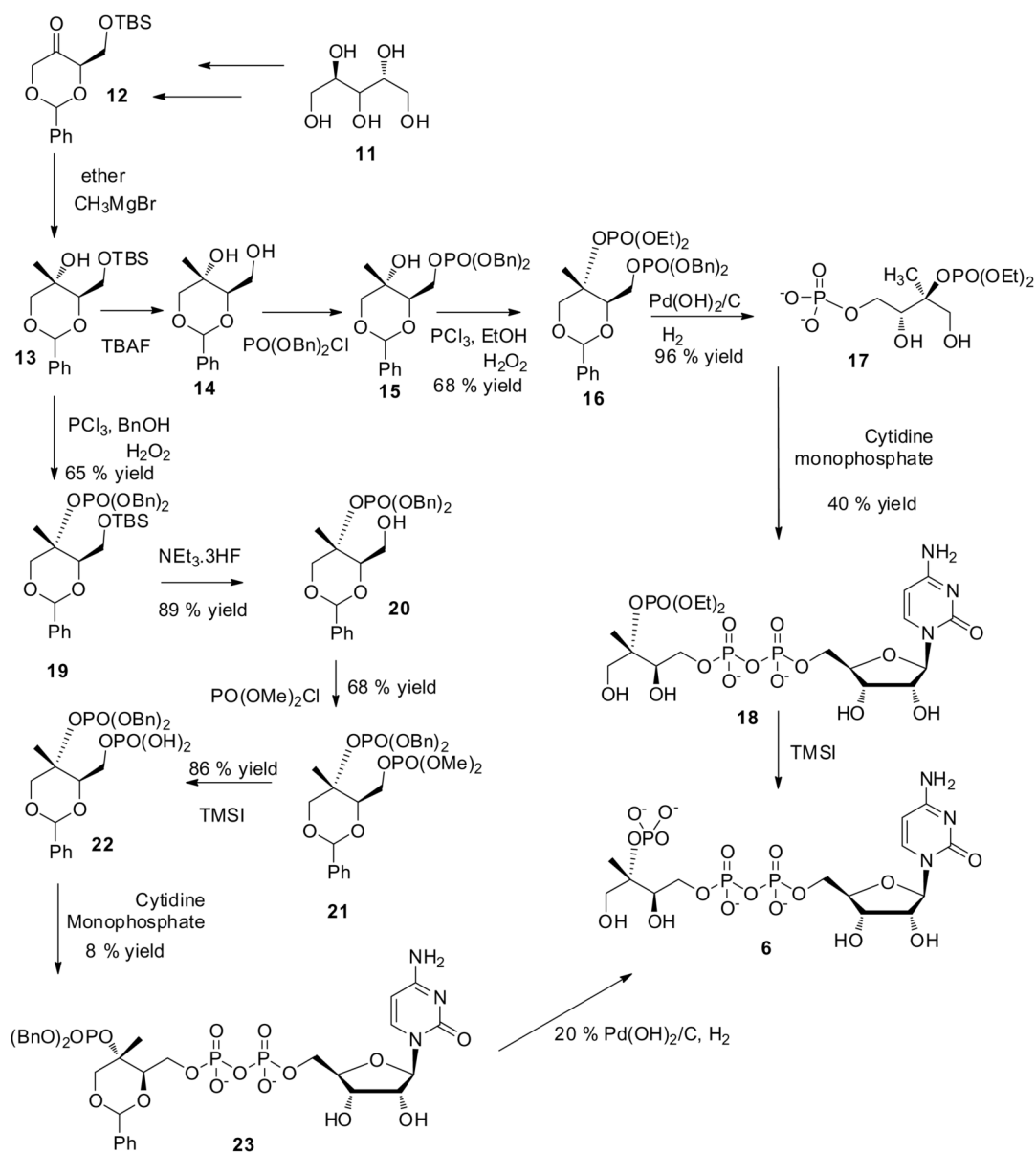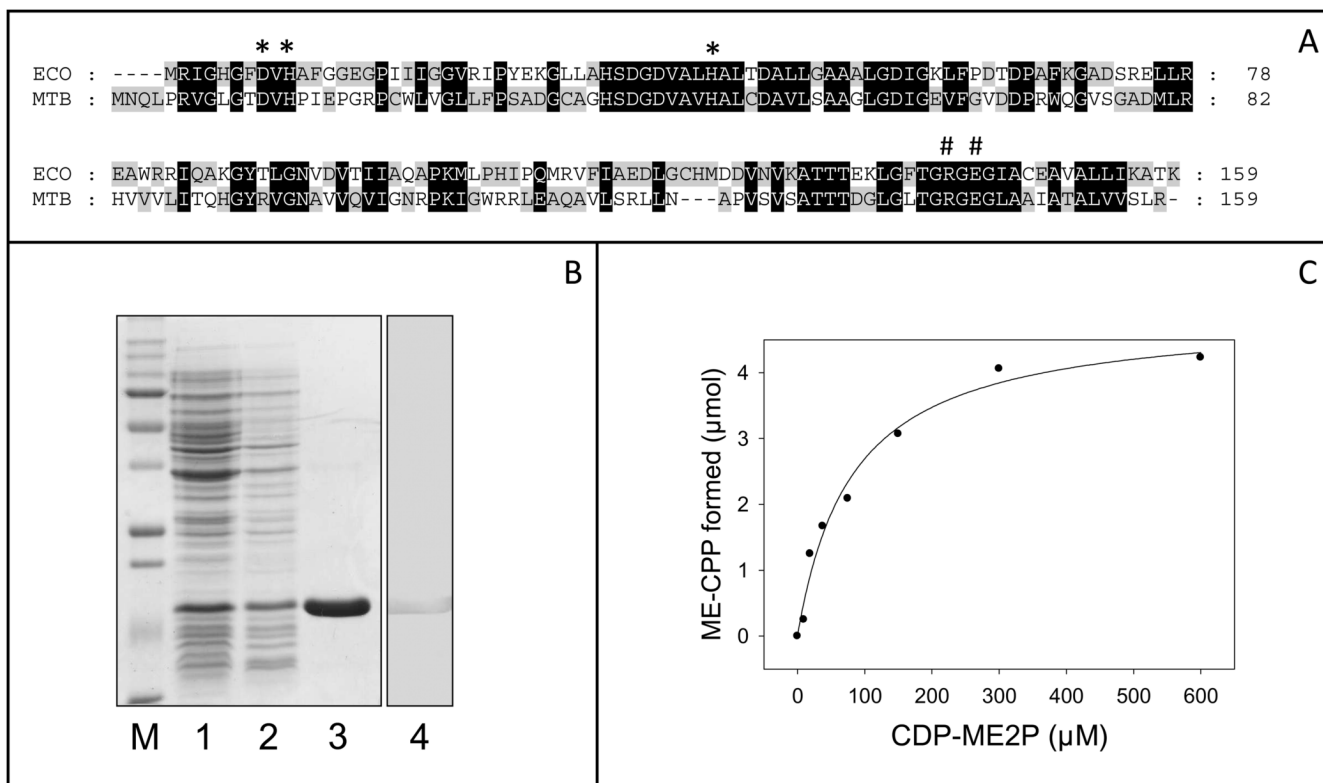
Figure 1.
MEP biosynthetic pathway.

Figure 2.
Enantiomeric synthesis of **6.**

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Figure 3.
Partial alignment, purification and characterization of IspF. Panel A: Partial alignment of putative *M. tuberculosis* (MTB) IspF and *E. coli* (ECO) IspF. Identities are indicated in black boxes and similarities in gray. Conserved amino acids reported to be involved in substrate specificity (#) and the Zn^{2+} binding (*) are indicated. Panel B: Expression and purification of His tagged *M. tuberculosis* IspF. SDS-PAGE and Western blot analysis of protein fractions from *E. coli* transfomed with pET28a(+)::Rv3581c. Lane 1, cell lysate prior to IPTG induction. Lane 2, cell lysate after IPTG treatment. Lane 3, purified His-tagged IspF visualized by Coomassie Brilliant Blue 250R. Lane 4, Western blot analysis of purified IspF using an anti-His antibody. Panel C: The effect of CDP-ME2P concentration on *M. tuberculosis* IspF activity. Reaction mixtures are described in the Experimental Procedures. ADP generated from this reaction was detected using ADP Quest HS Kinase kit and a Synergy™ HT Multi-Detection Microplate Reader with an excitation wavelength of 520 nm and emission wavelength of 590 nm.
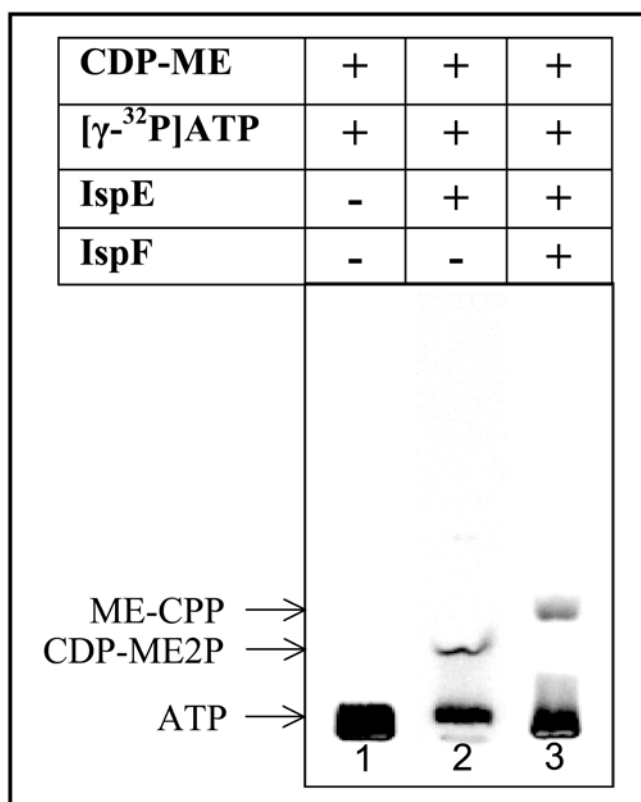
CDP-ME	+	+	+
$[\gamma\text{-}^{32}P]ATP$	+	+	+
IspE	-	+	+
IspF	-	-	+

Figure 4.
Determination of activity of recombinant *M. tuberculosis* IspF. Three different reaction mixtures containing the indicated compounds were analyzed. The arrows indicate the migration of authentic compounds.