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Brief Communication

Synthesis of 4-Diphosphocytidyl-2-*C*-Methyl-D-Erythritol 2-Phosphate and Kinetic Studies of *Mycobacterium tuberculosis* IspF

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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). Because 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, to our knowledge, 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 one third of the world's population was infected with tubercle bacilli (World Health Organization, 2007). This and the fact that both multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) are rapidly spreading (Zhao et al., 2009) have 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, and lipoquinones, 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), which are universal precursors of isoprenoids (Rohmer, 1999; Brennan and Crick, 2007). The mevalonate pathway (Buhaescu and Izzedine, 2007), which was first identified in mammals, and the non-mevalonate, or methylerythritol phosphate (MEP), pathway are found in plants (the 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 (Figure 1), 1-deoxy-D-xylulose 5-phosphate **3** (Dxp) is made by condensing pyruvate **1** and glyceraldehyde 3-phosphate **2** catalyzed 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 catalyzed by IspG, and IspH (LytB) is utilized subsequently to generate IPP **9** and DMAPP **10**.

Because 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 analogs is essential to ongoing biochemical investigations and development of new antibiotics

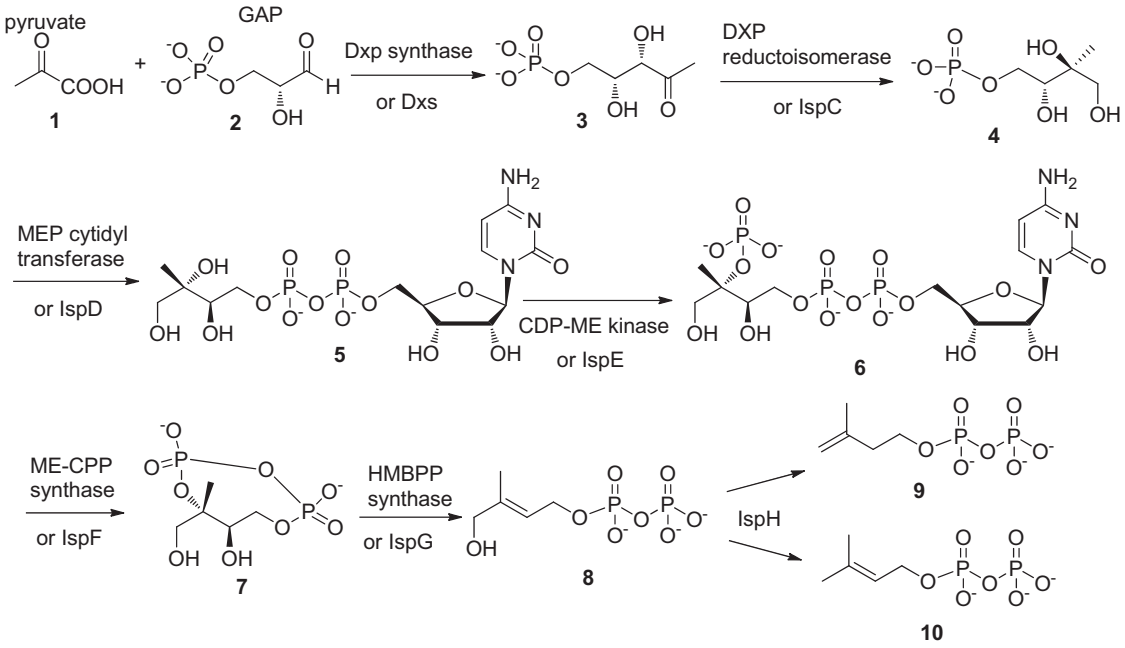

Figure 1. MEP Biosynthetic Pathway

targeting the respective enzymes. Recently, we reported the synthesis of CDP-ME (Narayanasamy et al., 2008) and ME-CPP (Narayanasamy and Crick, 2008) and kinetic studies of mycobacterial Dxs (Bailey et al., 2002), IspC (Dhiman et al., 2005), IspD (Eoh et al., 2007), and IspE (Narayanasamy et al., 2008). To extend our research to include IspF, we needed compound **6**; however, the previously reported enzymatic synthesis of **6** is tedious and expensive and leads to low yields (Illarionova et al., 2006; Herz et al., 2000; Luttgen et al., 2000). Here, we report the first, to our knowledge, 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 reported elsewhere (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 as a result of 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 (Figure 2). Subsequently, the benzyl deprotection was performed 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 hr and quenched with 1 M aqueous NH_4HCO_3. The crude material was purified by passing through a benzyl-DEAE cellulose column using a gradient of 10–500 mM aqueous NH_4HCO_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 available online). 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, and water (in a ratio of 4:2:4) solution (Illarionova et al., 2006) gave an 84% yield of **6.** Because the deprotected **6** is not stable, it was subjected to analysis by MS and used immediately for the kinetic study of IspF. The nature

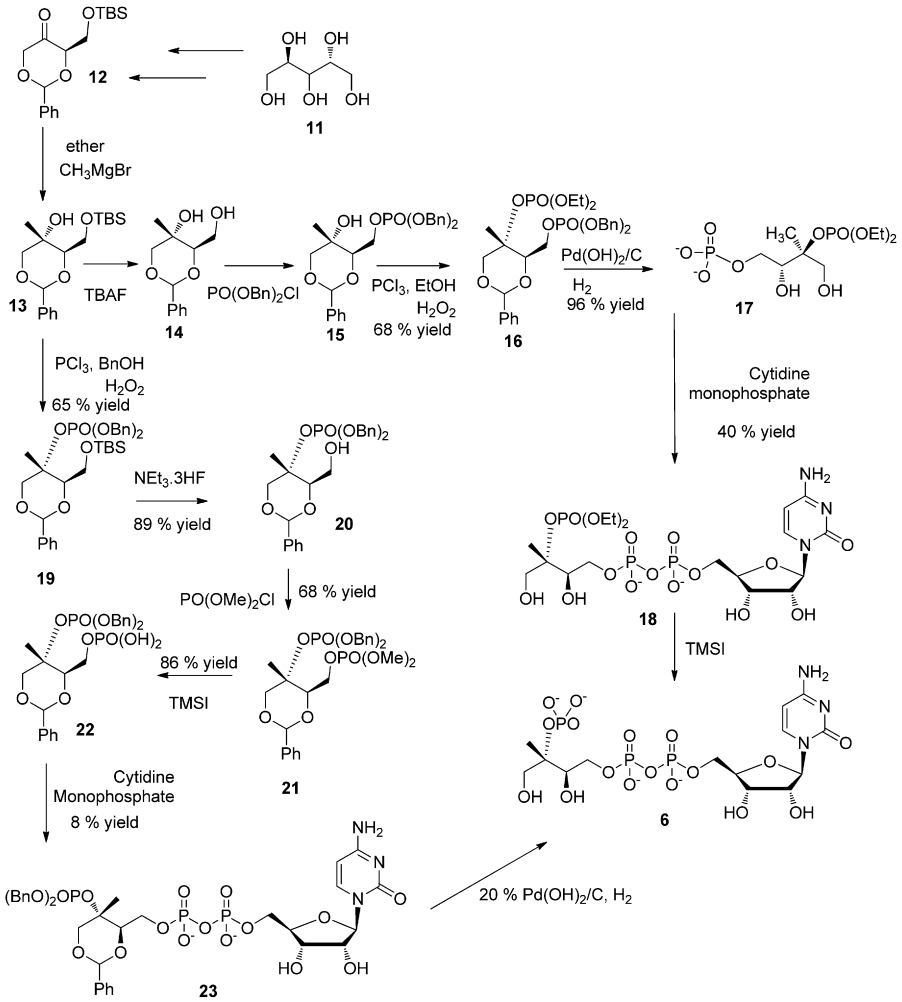

Figure 2. Enantiomeric Synthesis of 6

and structure of product **6** was confirmed by MS, ^{1}H-NMR, ^{31}P-NMR (see Supplemental Information), 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 (Figure 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 (Figure 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 Supplemental Information).

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 (Figure 4 lane 2). The 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) (Figure 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 was 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

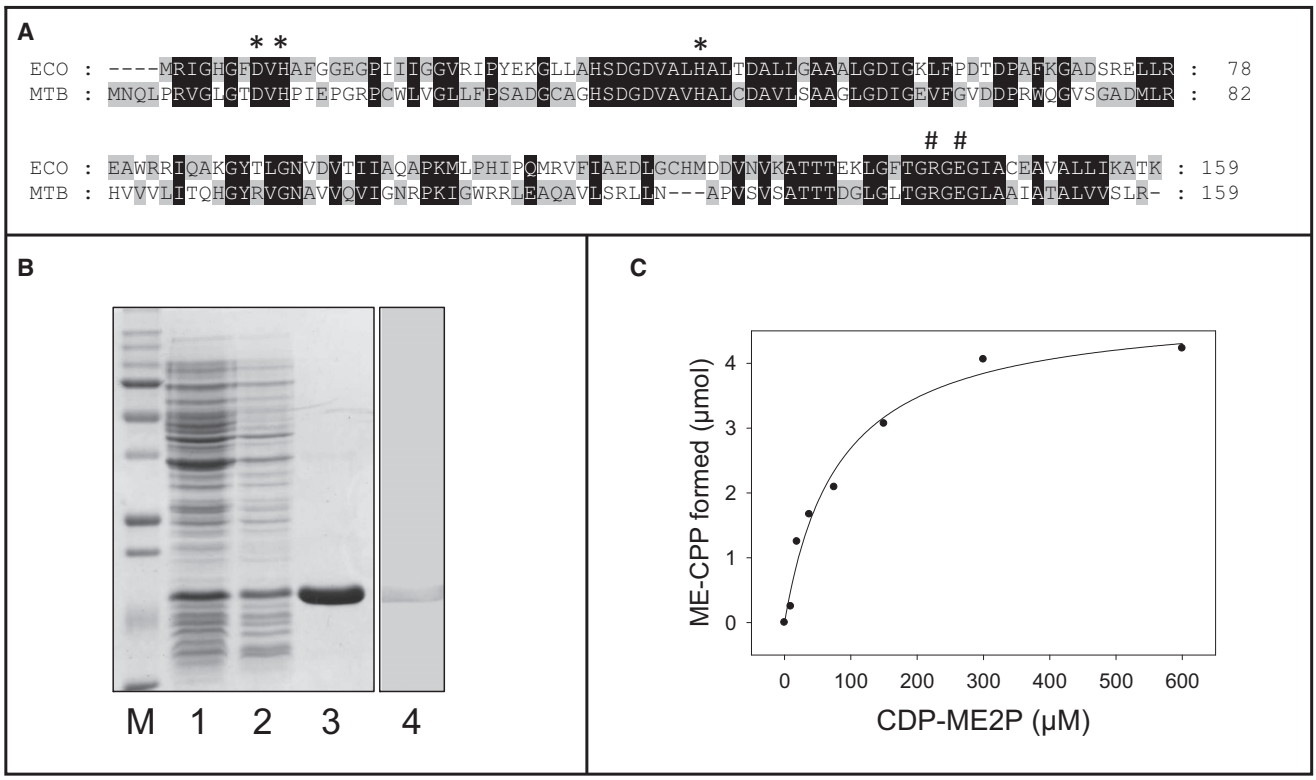

Figure 3. Partial Alignment, Purification, and Characterization of IspF

(A) Partial alignment of putative *M. tuberculosis* (MTB) IspF and *E. coli* (ECO) IspF. Identities are indicated in black boxes, and similarities are indicated in gray. Conserved amino acids reported to be involved in substrate specificity (#) and the Zn^{2+} binding (*) are indicated.

(B) Expression and purification of His-tagged *M. tuberculosis* IspF. SDS-PAGE and western blot analysis of protein fractions from *E. coli* transformed 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.

(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.

(GE Healthcare, UK) to monitor ADP formation (Figure S1). The activity of pure recombinant IspF was linear with increasing protein concentration up to 281.5 pmol and reaction time up to 30 min. The coupled assay showed classic saturation kinetics with increasing concentration of CDP-ME2P (Figure 3C). From these data, the $K_m^{CDP\text{-}ME2P}$ 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} $\mu M^{-1}min^{-1}$, 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 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} $\mu M^{-1}min^{-1}$ 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).

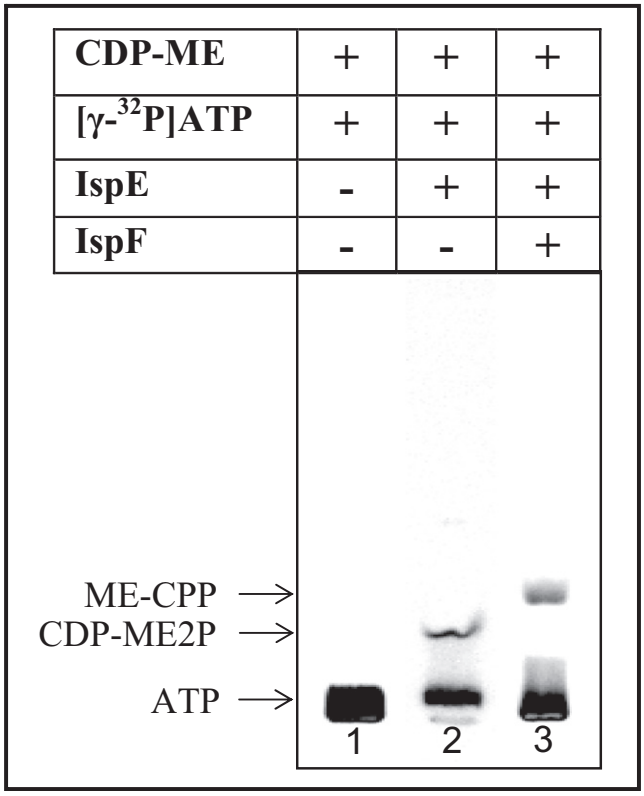

Figure 4. Determination of Activity of Recombinant *M. tuberculosis* IspF

Three different reaction mixtures containing the indicated compounds were analyzed. The arrows indicate the migration of authentic compounds.

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 hr 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 hr. 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 hr 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 min. 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 **17** 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 aqueous ammonium bicarbonate and purified through Bio-Gel® P-2 gel fine column using 100 mM aqueous ammonium bicarbonate followed by further purification on a benzyl DEAE cellulose anion exchange column, eluted by a gradient of 0–0.5 M aqueous 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_2O (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 $MgCl_2$, 100 μM CDP-ME2P, and 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 min, and terminated by the addition of 10 mM EDTA. The reaction mixture was completely dried, dissolved in methanol/H_2O (1:1), and analyzed 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 (Figure S2). 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 $MgCl_2$, 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 min 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 nonlinear regression analysis using SigmaPlot V.8.02A.

SUPPLEMENTAL INFORMATION

Supplemental Information contains one figure and Supplemental Experimental Procedures and may be found with this article online at doi:10.1016/j.chembiol.2010.01.013.

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