We have recently shown that open reading frame \textit{Rv}1086 of the \textit{Mycobacterium tuberculosis} H37Rv genome sequence encodes a unique isoprenyl diphosphate synthase. The product of this enzyme, \textit{\(\omega,\omega,\omega\)-farnesyl diphosphate synthase}, is an intermediate for the synthesis of decaprenyl phosphate, which has a central role in the biosynthesis of most features of the mycobacterial cell wall, including peptidoglycan, arabinan, linker unit galactan, and lipoarabinomannan. We have now purified \(\omega,\omega,\omega\)-farnesyl diphosphate synthase to near homogeneity using a novel mycobacterial expression system. \(\omega,\omega,\omega\)-Farnesyl diphosphate synthase catalyzed the addition of isopentenyl diphosphate to \(\omega,\omega\)-geranyl diphosphate or \(\omega,\omega,\omega\)-neryl diphosphate yielding \(\omega,\omega,\omega,\omega\)-farnesyl diphosphate and \(\omega,\omega,\omega,\omega,\omega\)-farnesyl diphosphate, respectively. The enzyme has an absolute requirement for a divalent cation, an optimal pH range of 7–8, and \(K_m\) values of 124 \(\mu\text{M}\) for isopentenyl diphosphate, 38 \(\mu\text{M}\) for geranyl diphosphate, and 16 \(\mu\text{M}\) for neryl diphosphate. Inhibitors of the \(\omega,\omega,\omega\)-farnesyl diphosphate synthase were designed and chemically synthesized as stable analogs of \(\omega,\omega\)-geranyl diphosphate in which the labile diphosphate moiety was replaced with stable moieties. Studies with these compounds revealed that the active site of \(\omega,\omega,\omega\)-farnesyl diphosphate synthase differs substantially from \(\omega,\omega,\omega\)-farnesyl diphosphate synthase from pig brain (\textit{Sus scrofa}).

Isoprenyl diphosphate synthases catalyze the condensation of an allylic diphosphate with isopentenyl diphosphate (IPP, \(\text{C}_5\)) via an electrophilic alkylation reaction to produce longer allylic diphosphates (1, 2). Chain elongation continues until a physiologically appropriate chain length is reached, at which time the molecule may undergo further modifications (dephosphorylation, cyclization, or head-to-head condensation reactions). Polyprenyl phosphate (Pol-P) is formed by dephosphorylation of an allylic prenyl diphosphate chain. The predominant form of prokaryotic Pol-P is \(\omega,\omega,\omega\)-polyZ-undecaprenyl phosphate (C\textsubscript{25}); however, there are documented exceptions in \textit{Paracoccus denitriticans} (3) and in \textit{Mycobacterium} sp. (4–8). \textit{M. smegmatis} contains heptaprenyl diphosphate (8) (\(\text{C}_{35}\), four saturated, three double bonds) and decaprenyl diphosphate (5) (\(\text{C}_{50}\), one \(E\), and eight \(Z\) double bonds), whereas \textit{M. tuberculosis} contains only decaprenyl phosphate (6). Although the stereochemistry of decaprenyl phosphate from \textit{M. tuberculosis} has not been determined, our enzymatic studies suggest that it has similar stereochemistry to decaprenyl phosphate from \textit{M. smegmatis} (9).

Pol-P is central to prokaryotic cell wall synthesis as a sugar carrier, and it has been reported that the levels of Pol-P may be rate-limiting for \textit{in vivo} cell wall synthesis (10–13). Our laboratory has shown that Pol-P is instrumental in the synthesis of each component of the covalently linked peptidoglycan-arabino-galactan-mycolic acid cell wall core of mycobacteria, and other noncovalently associated macromolecules such as lipo- mannan and lipoarabinomannan (5, 14, 15). The importance of Pol-P is also demonstrated \textit{in vivo} by the fact that \textit{M. tuberculosis} (16) (and other \textit{Mycobacterium} sp.)\textsuperscript{3} are sensitive to the antibiotic bacitracin, which specifically binds isoprenyl diphosphate (17) intermediates in Pol-P synthesis, inhibiting both chain elongation and dephosphorylation reactions.

Evolutionarily, there appear to be two independent families of isoprenyl diphosphate synthases, based on the type of stereochernistry (\(E\) or \(Z\)) introduced at the products’ new double bond. \(E\)-Isoprenyl diphosphate synthases are capable of catalyzing the chain elongation of a range of substrates, the smallest one being dimethylallyl diphosphate (DMAPP, \(\text{C}_5\)) with IPP to form \(\omega,\omega\)-geranyl diphosphate (\(\omega,\omega\)-GPP, \(\text{C}_{10}\)). Other short-chain isoprenyl diphosphates (\(\omega,\omega,\omega\)-farnesyl diphosphate (\(\omega,\omega,\omega,\omega\)-FPP, \(\text{C}_{15}\)) and \(\omega,\omega,\omega,\omega,\omega\)-geranylgeranyl diphosphate (\(\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega\)-GGPP, \(\text{C}_{20}\))) are generated by a similar mechanism with additional molecules of IPP. Medium-chain \(E\)-isoprenyl diphosphate synthases use the short-chain products as allylic substrates to produce compounds that are \(\text{C}_{35}\) to \(\text{C}_{50}\) in length. Medium-chain \(E\)-isoprenyl diphosphate synthases are homologous to the short-chain \(E\)-isoprenyl diphosphate synthases, because both types contain two signature aspartate motifs (DDXXY\textsubscript{1}, \textsubscript{2}D).

Thus far, only seven protein sequences have been biochemically correlated with \(Z\)-isoprenyl diphosphate synthase activity, including undecaprenyl diphosphate synthases from \textit{Micrococcus luteus}, \textit{Escherichia coli}, \textit{Hemophilus influenzae}, \textit{Streptococcus pneu-

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\textsuperscript{1} The abbreviations used are: IPP, isopentenyl diphosphate; Cit-PP, citronellyl diphosphate; DMAPP, dimethylallyl diphosphate; \(\omega,\omega\)-GPP, \(\omega,\omega\)-geranyl diphosphate; \(\omega,\omega,\omega\)-GGPP, \(\omega,\omega,\omega,\omega\)-geranylgeranyl diphosphate; \(\omega,\omega,\omega\)-FPP, \(\omega,\omega,\omega\)-farnesyl diphosphate; \(\omega,\omega,\omega,\omega,\omega\)-FPP, \(\omega,\omega,\omega,\omega,\omega,\omega\)-farnesyl diphosphate; \(\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega\)-GGPP, \(\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega\)-GGPP; Pol-P, polypropenyl phosphate; Z-FPP synthase, \(\omega,\omega,\omega\)-farnesyl diphosphate synthase; E-FPP synthase, \(\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega,\omega\)-farnesyl diphosphate synthase; \(\omega,\omega\)-Z-NPP, \(\omega,\omega\)-neryl diphosphate; MS, mass spectrometry; PCR, polymerase chain reaction; bp, base pair(s); MES, 4-morpholineethane-sulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

\textsuperscript{2} The stereochernical configuration of the isoprene units is always listed starting at the omega (\(\omega\)) end of the molecule.

\textsuperscript{3} M. C. Schulbach, and D. C. Crick, unpublished.
**EXPERIMENTAL PROCEDURES**

**Synthesis**

All oxygen- and water-sensitive reactions (Scheme 1) were performed under dry argon atmosphere. 1H NMR spectra of all compounds were obtained with a Varian Gemini 200 operating at 200 MHz in approximately 2% solution of CDCl₃ or D₂O, using Me₄Si or Me₃Si(CH₂)₃SO₃Na as the internal standard. Column chromatographies were performed using 230- to 400-mesh silica gel (Merck) or reverse phase silica gel (Macherey-Nagel Polygosil 60-4063 C₁₈). Mass spectra were recorded on a VG 70-25S mass spectrometer or an HP-5988 A spectrometer. Reagents and solvents were purchased from Aldrich or Fluka.

**Purification and Enzymatic Characterization of Z-FPP Synthase**

E. coli, which provides a solid understanding of how the active site determines the chain length of the product (22, 23). However, little is known about Z-isoprenyl diphosphate synthases. We have purified and enzymatically characterized the short-chain Z-isoprenyl diphosphate synthase from *M. tuberculosis*, which catalyzes the first committed step in the synthesis of decaprenyl diphosphate and farnesyl diphosphate synthases. We have also determined the crystal structure of the short-chain Z-isoprenyl diphosphate synthase (avian FPP synthase) has been determined (9), all known Z-isoprenyl diphosphate synthases utilized ω,E,E-FPP or ω,E,E-GGPP as the allylic substrate, added multiple units of IPP, and released long-chain (C₄₅ and greater) isoprenyl diphosphate molecules with mixed stereochemistry (2).

**Synthesis of ω,E-geranyl diphosphate analogs, Compounds 1–4.** Reagents and conditions: (i) N-hydroxyphthalimide, diethyl azodicarboxylate, triphenylphosphine, anhydrous tetrahydrofuran, 18 h, room temperature; (ii) NH₄OH, ethanol, 18 h, room temperature; (iii) 1-hydroxybenzotriazole, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, anhydrous tetrahydrofuran, 18 h, room temperature; (iv) a, bromotrimethylsilane, 2,4,6-collidine, dichloromethane, 18 h, room temperature; b, KOH 1 N, 3 h, room temperature; (v) butyl lithium, 1.6 M 1-hydroxybenzotriazole, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, anhydrous tetrahydrofuran, 18 h, room temperature; (vi) 1-hydroxybenzotriazole, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, anhydrous tetrahydrofuran, 18 h, room temperature; (vii) butyl lithium, 1.6 M hexane solution, anhydrous tetrahydrofuran, 1 h, –78 °C.

**Scheme 1. Synthesis of ω,E-geranyl diphosphate analogs, Compounds 1–4.**
of 1:1 in the case of 1:1 or 2:1. The resulting crude residue was purified by column chromatography on reverse phase silica gel, eluting with methanol-water (1:4) and collecting 2-ml fractions. The appropriate fractions were combined, evaporated, lyophilized, and used as an enzyme source for the final purification.

#### Purification of Z-FPP Synthase

Open reading frame Rv1086 (26) was initially cloned into a commercially available protein expression vector pET22 (New England Biolabs). However, due to the apparent toxic effects of the Rv1086 fusion protein to the E. coli cells harboring the plasmid, the cloning sequence that encoded the complete insert/fusion was moved into a mycobacterial expression vector pET22b (New England Biolabs). 1H NMR (D_{2}O): 6.087 (t, J = 7.2 Hz, CH_{3}C=H), 1.62 (s, 3H, CH_{3}), 1.69 (s, 3H, CH_{3}), 1.71 (s, CH_{3}), 2.01–2.29 (m, 6H, 2 × CH_{2}), 2.41–2.62 (m, 1H, CH), 4.40 (d, 2H, J = 7.2 Hz, CH_{2}), 5.18 (br, 3H, 5 × CH), 5.40 (t, J = 7.2 Hz, CH); MS (FAB\') m/e 396 (M+H\').

#### Diethyl (E)-1-ethyl-1-(4,8-dimethyl-3,7-nonenol)arylpropyl Phosphonate (16)

Butyl lithium (2.85 ml of 1.6 M hexane solution, 4.56 mmol) was added dropwise to a stirred solution of diethyl 1-[ethoxymethyl]phosphoryl]-1-ethylpropylphosphonic acid 15 (25) (1.200 g, 3.81 mmol) in anhydrous tetrahydrofuran (15 ml) and cooled at -78 °C under an argon atmosphere. After stirring for 1 h at -78 °C, the ethoxymethyl bromide 14 (0.990 g, 4.56 mmol) was added dropwise and the mixture was stirred for an additional 1 h at -78 °C. The reaction was quenched with acetic acid (0.546 g, 9.12 mmol), diluted with CH_{2}Cl_{2} (60 ml), and washed with brine. The organic phase was dried and evaporated under reduced pressure. The residue was purified by silica gel column chromatography, eluting with ethyl acetate-hexane (3:1). The appropriate fractions were combined, evaporated, and used as an enzyme source for the final purification.

#### Enzymatic Assays and Product Characterization

In vitro Z-FPP synthase assays, the enzymatic treatment of reaction products, and the purification of products were done as described previously (9, 27). Assays were conducted under conditions that were linear for time and protein concentration. In the metal ion dependence studies, the major of the endogenous divalent cations were removed by incubating the enzyme preparation with Bio-Rex 70 200–400 mesh (sodium form, Bio-Rad) on ice for 20 min. The enzymatic activity was reduced to a basal level, but it was not completely abolished, indicating that residual divalent cations were present. To determine which divalent cations supported activity, CaCl_{2}, MgCl_{2}, MnCl_{2}, or ZnCl_{2} were added to the assay mixtures containing all of the other metals tested, but no activity was supported by these divalent cations. The study of the pH dependence of Z-FPP synthase activity, a broad-range buffer containing 250 mM Tris-HCl, 125 mM MES, and 125 mM acetic acid was used. The pH was adjusted with tetraethylammonium hydroxide.

Preparation of Sus scrofa E-FPP Synthase—Porcine brain cytosol was prepared by homogenizing pig gray matter in 10 mM HEPES (pH 7.4) and 0.25 M sucrose using 18 passes of a Dounce homogenizer. The homogenates were centrifuged at 9000 × g for 15 min at 4 °C. The supernatant was decanted and centrifuged at 140,000 × g for 1 h. The supernatant was decanted, divided into 1-ml aliquots, stored at -70 °C, and used as an enzyme source for the eukaryotic-synthesis assays. The
protein concentration was estimated with a BCA protein assay kit (Pierce).

Other Materials—The sources of all materials have been described previously (9) with some exceptions. Citronellyl diphosphate, ω,E,E-farnesyl diphosphate, and ω,E-geranyl diphosphate were synthesized as described by Davisson et al. (28). Authentic prenols and prenyl phosphates of various chain lengths were purchased from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). ω,Z-Neryl diphosphate was a gift from Drs. J. S. Rush and C. J. Waechter (University of Kentucky).

RESULTS

Purification of Z-FPP Synthase—Z-FPP synthase was expressed in M. smegmatis as a fusion protein with a C-terminal chitin binding domain. Affinity chromatography on a chitin column permitted a simple one-step protein purification (New England BioLabs). After the cells were harvested and disrupted, the 20,000 × g supernatant was applied to the chitin column and washed. Subsequent elution with a dithiothreitol-containing buffer cleaved the Z-FPP synthase from the chitin binding domain via an internal intein site. The column eluate was collected and subjected to SDS-polyacrylamide gel electrophoresis analysis (Fig. 1). The eluate was also assayed for [14C]IPP incorporation into butanol-extractable material with five different allylic primers, DMAPP (C₅), ω,E,E-GPP (C₁₀), ω,Z-neryl diphosphate (ω,Z-NPP; C₁₀), ω,E,E-FPP (C₁₅), and ω,E,E,E-GGPP (C₂₀). ω,E-GPP and ω,Z-NPP were the only functional substrates (data not shown). Assays with the other allylic primers did not produce any detectable radioactive product. [14C]IPP incorporation into butanol-extractable material in assays primed with ω,E-GPP or ω,Z-NPP was linear for at least 40 min (data not shown). The product of the ω,E-GPP assay was analyzed for chain length and stereochemistry by TLC (Fig. 2), confirming that the protein fraction shown in Fig. 1 (lane 4) synthesized ω,E,Z-FPP. The product of the ω,Z-NPP assay was also analyzed by TLC, demonstrating the enzyme’s ability to synthesize a C₁₅ molecule, presumably ω,Z,Z-FPP (data not shown).

Z-FPP Synthase Reaction Requirements—Z-FPP synthase was absolutely dependent on the presence of divalent cation for activity, and addition of 10 mM EDTA abolished the enzymatic activity (Table I). Z-FPP synthase activity was supported by the addition of MgCl₂ or MnCl₂. The optimal concentration of MgCl₂ and MnCl₂ fell between 0.01 and 1.0 mM. Higher concentrations of MgCl₂ (between 1.0 and 5.0 mM) reduced (with respect to optimal concentration) the activity, whereas high concentrations of MnCl₂ (5.0 mM) strongly inhibited it. The enzyme was not stimulated by ZnCl₂ and CaCl₂ at the concentrations tested (0.01–5 mM). Z-FPP synthase was also tested for optimal activity over a range of pH (5.5–9.5 in 0.5 increments). The enzyme had a broad peak of activity over pH 7–8 (data not shown). When the rate of Z-FPP synthesis was measured in the presence of saturating ω,E-GPP and varying concentrations of [14C]IPP (Fig. 3A), a Kₘ value of 124 μM was calculated for the
isoprenyl donor by nonlinear regression (Table II). When the concentration of IPP was fixed and the concentration of $\omega$E-GPP or $\omega$Z-NPP was varied, Michaelis constants of 38 and 16 $\mu$M were calculated (Fig. 3 and Table II).

**Inhibition of Z-FPP Synthase**—Compounds 1–4 (Scheme 1) and citronellyl diphosphate (Cit-PP, Fig. 4A) were tested for the ability to inhibit the synthesis of $\omega$E-Z-FPP by the Z-FPP synthase from *M. tuberculosis* and the synthesis of $\omega$E-E-FPP by the E-FPP synthase from pig brain (*S. scrofa*). Z-FPP synthase was inhibited by compound 4 and Cit-PP (Fig. 4B). The respective IC$_{50}$ values were estimated to be 300 and 350 $\mu$M in the presence of 100 $\mu$M GPP. E-FPP synthase was inhibited only by Cit-PP with an IC$_{50}$ of 125 $\mu$M under similar assay conditions (Fig. 4C). Compounds 1, 2, and 3 had no effect on either of the FPP synthases at the concentrations tested (data not shown).

**DISCUSSION**

Little is known about Z-isoprenyl diphosphate synthases. Some early partial purification and characterization work has been published for bacterial (29, 30) and eukaryotic (31–35) Z-isoprenyl diphosphate synthases, but it was not until Shimizu et al. (36) purified undecaprenyl diphosphate synthase from *M. luteus* in 1998 that a gene and protein sequence were correlated with the biochemical data. The complete sequencing of the *M. tuberculosis* H37Rv genome (and other genomes) has since revealed a wealth of information. Apfel et al. (37) was able to find 28 homologs of *M. luteus* undecaprenyl diphosphate synthase using comparative genome queries. *M. tuberculosis* is unique among organisms that have had their genomes sequenced in that there are two genes with homology to Z-isoprenyl diphosphate synthases. *M. tuberculosis* synthase using comparative genome queries. *M. tuberculosis* synthase using comparative genome queries.

**Purification and Enzymatic Characterization of Z-FPP Synthase**

The enzyme preparation was preincubated with Bio-Rex 70 200–400 mesh (sodium form, Bio-Rad) on ice for 20 min. Z-FPP synthase activity was tested for dependence on divalent cations in mixtures containing 50 mM MOPS (pH 7.9), 10 mM sodium orthovanadate, 2.5 mM dithiothreitol, 0.3% Triton X-100, 100 $\mu$M geranyl diphosphate, 30 $\mu$M $[^{14}C]$isopentenyl diphosphate, and 60 $\mu$g of protein in a final volume of 50 $\mu$L. MgCl$_2$, MnCl$_2$, or EDTA was added to the assay mixtures at the indicated concentrations. Reactions were incubated for 10 min, stopped by the addition of water saturated with NaCl, and extracted with n-butanol saturated with water.

### TABLE I

| Concentration | Divalent cation activity | 
|---------------|--------------------------|
|               | MgCl$_2$ | MnCl$_2$ |
| 0             | 219      | 219      |
| 0.01          | 352      | 699      |
| 0.1           | 664      | 1409     |
| 1.0           | 704      | 872      |
| 5.0           | 326      | 20       |
| EDTA (10mM)   | 0        | 0        |

**Effect of divalent cations on Z-FPP synthase activity**

The enzyme preparation for $[^{14}C]$isopentenyl diphosphate synthesis by Z-FPP synthase. Assays contained 50 mM MOPS (pH 7.9), 10 mM sodium orthovanadate, 0.05 mM MgCl$_2$, 2.5 mM dithiothreitol, 0.3% Triton X-100 and 60 $\mu$g of protein in a final volume of 50 $\mu$L. The results shown in A are from assays containing 100 $\mu$M $\omega$E-geranyl diphosphate and increasing amounts of $[^{14}C]$isopentenyl diphosphate (structure inset). The assay mixtures for B contained 30 $\mu$M isopentenyl diphosphate and increasing amounts of $\omega$E-geranyl diphosphate (structure inset). The assay mixtures for C contained 30 $\mu$M isopentenyl diphosphate and increasing amounts of $\omega$Z-nerlyl diphosphate (structure inset). Following incubation at 73 °C for 10 min, the reactions were stopped with 1 ml of water saturated with NaCl, and extracted with 1 ml of butanol saturated with water. An aliquot was taken for liquid scintillation spectrometry.

### TABLE II

**Kinetic constants of Z-FPP synthase**

Experimental conditions are as described for Fig. 3. The data were subjected to nonlinear regression analysis using SigmaPlot for Windows version 4.01 (SPSS Inc.).

| Substrate | IPP | GPP | NPP |
|-----------|-----|-----|-----|
| $K_m$ ($\mu$M) | 124 | 38  | 16  |
| $V_{max}$ (pmol/mg/min) | 4328 | 2827 | 1098 |
| Coefficient of determination | 0.99 | 0.99 | 0.99 |
FIG. 4. Inhibition of Z-FPP synthase and E-FPP synthase with \( \omega \)-E-GPP Analogos. Assay mixtures contained a buffer (Tris-HCl, MES, and acetic acid, pH 7.0), 10 mM sodium orthovanadate, 0.05 mM MgCl\(_2\), 2.5 mM dithiothreitol, 0.5% Triton X-100, and 100 \( \mu \)M \( \omega \)-E-GPP in a final volume of 50 \( \mu \)l. Results shown in B are from assays containing 60 \( \mu \)g of pure Rv1086 protein. Results shown in C are from assays containing 60 \( \mu \)g of cytosolic protein prepared from pig brain (S. scrofa). Increasing concentrations of each inhibitor, citronellyl diphosphate (triangles), and compound 4 (circles) were added, and the reactions were allowed to incubate for 20 min at 37°C. The products were extracted with butanol saturated with water, and an aliquot was taken for liquid scintillation spectrometry.

**Purification and Enzymatic Characterization of Z-FPP Synthase**

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synthesizing \( \omega \)-E,F-FPP from DMAPP, the enzyme completes two condensation reactions with IPP, releasing only trace amounts of the intermediate \( \omega \)-E-GPP (3). In contrast, DMAPP was not a functional substrate for the Z-FPP synthase. \( \omega \)-E-GPP and \( \omega \)-Z-NPP were the only effective allylic substrates tested, supporting the synthesis of \( \omega \)-E,Z-FPP and \( \omega \)-Z,Z-FPP, respectively. Although little is known about the intracellular concentrations of IPP or the allylic substrates, \( \omega \)-E-GPP or \( \omega \)-Z,NPP, in *M. tuberculosis*, the observed Michaelis constants for these substrates are 124, 38, and 16 \( \mu \)M, respectively. Long-chain Z-isoprenyl diphosphate synthases have been shown to be capable of utilizing allylic primers with different stereoechemistries as substrates (3, 44). If the Pol-P from *M. tuberculosis* is structurally similar to the Pol-P from *M. smegmatis* (\( \omega \)-E,polyZ-decaprenyl phosphate), \( \omega \)-E-GPP is the natural substrate of the Z-FPP synthase. It is possible that \( \omega \)-Z,NPP is a precursor to an, as yet, undescribed isoprenoid molecule in *M. tuberculosis*. Nevertheless, the Z-FPP synthase is a multifunctional enzyme, and a separate enzyme must exist in *M. tuberculosis* that synthesizes either \( \omega \)-E,GPP or \( \omega \)-Z,NPP from DMAPP and IPP.

A comparison of the mycobacterial Z-FPP synthase with the E-FPP synthase from *S. scrofa* brain was of interest, because the two enzymes catalyze the addition of IPP to \( \omega \)-E,GPP but release products with opposite stereocchemistry at the newly formed double bond. Substrate analogs such as Cit-PP have been previously shown to inhibit pig liver E-FPP synthase (45, 46). Cit-PP and other substrate analogs are thought to bind the enzyme active site through nonspecific lipophilic forces and a diphosphate binding force (45). The enzyme activity is inhibited, because Cit-PP lacks the allylic double bond in the 2 position (Fig. 4A) and is not able to undergo the typical electrophilic alkylation reaction. As shown in Fig. 4 (B and C), Cit-PP was effective at inhibiting both the E- and the Z-FPP synthases.

Compounds 1–4 (Scheme 1) were designed as analogs of \( \omega \)-E-GPP in which the labile phosphoacetamide moiety was replaced by moieties that can act as stable isosteres that possess different conformational and stereoelectronic characteristics such as an unsubstituted phosphonoacetamidoxymethylene (compound 1), an \( \alpha \)-ethyl-substituted phosphonoacetamidoxymethylene (compound 2), a phosphonoacetamido group (compound 3), or an \( \alpha \),\( \alpha \)-diethyl-substituted \[methylene(hydroxy)phosphoryl]-methylphosphonic moiety (compound 4). These isosteric moieties were chosen bearing in mind previous studies on isosteres of the diphosphate group of prenyl diphosphates as squalene synthase (25), protein:farnesyl transferase (47), and protein:geranylgeranyl transferase inhibitors (48, 49). Compounds 1–3 had no effect on either the Z-FPP synthase or the E-FPP synthase (data not shown). Compound 4 specifically inhibited the Z-FPP synthase (Fig. 4B) but had no effect on the activity of the E-FPP synthase (Fig. 4C). Despite its relatively high \( IC_{50} \), the \( \alpha \),\( \alpha \)-diethyl-substituted \[methylene(hydroxy)phosphoryl\]methylphosphonic moiety of compound 4 seems to possess the correct conformational and stereoelectronic features to selectively interact with the active site of the mycobacterial Z-FPP synthase, suggesting that the study of the active site of this previously uncharacterized enzyme could lead to the development of novel chemotherapeutic agents for the treatment of multiple drug-resistant tuberculosis.
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