Published in final edited form as:

J Org Chem. 2018 September 07; 83(17): 9580–9591. doi:10.1021/acs.joc.8b00686.

Synthesis and Evaluation of Fluoroalkyl Phosphonyl Analogs of 2-C-Methylerythritol Phosphate as Substrates and Inhibitors of IspD from Human Pathogens

David Bartee, Michael J. Wheadon, and Caren L. Freel Meyers*

Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD, 21205, United States

Abstract

Targeting essential bacterial processes beyond cell wall, protein, nucleotide, and folate syntheses holds promise to reveal new antimicrobial agents and expand the potential drugs available for combination therapies. The synthesis of isoprenoid precursors, isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), is vital for all organisms; however, humans use the mevalonate pathway for production of IDP/DMADP while many pathogens, including *Plasmodium falciparum* and *Mycobacterium tuberculosis*, use the orthogonal methylerythritol phosphate (MEP) pathway. Toward developing novel antimicrobial agents, we have designed and synthesized a series of phosphonyl analogs of MEP and evaluated their abilities to interact with IspD, both as inhibitors of the natural reaction and as antimetabolite alternative substrates that could be processed enzymatically to form stable phosphonyl analogs as potential inhibitors of downstream MEP pathway intermediates. In this compound series, the *S*-monofluoro MEP analog displays the most potent inhibitory activity against *Escherichia coli* IspD and is the best substrate for both the *E. coli* and *P. falciparum* IspD orthologs with a K_m approaching that of the natural substrate for the *E. coli* enzyme. This work represents a first step toward the development of phosphonyl MEP antimetabolites to modulate early isoprenoid biosynthesis in human pathogens.

Graphical Abstract

*Please address correspondence to C. Freel Meyers (Tel 410-502-4807; FAX: 410-955-3023; cmeyers@jhmi.edu).

Associated Content

The Supporting Information is available free of charge on the ACS Publication website <http://pubs.acs.org/>.

Additional HPLC chromatograms and mass spectra of IspD-catalyzed reactions of **1** – **5a,b** and CTP. Michaelis-Menten plots of **1** – **5a,b** as substrates of *E. coli* IspD. Preliminary evaluation of inhibitory activity of **1** – **5a,b**. IC₅₀ curves of **5b** against *E. coli* and *P. falciparum* IspD. NMR characterization of **1** – **5a,b** and **7** – **21a,b**.

Introduction

The methylerythritol phosphate (MEP) pathway produces the essential 5-carbon isoprenoid precursors isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) (Scheme 1).¹ It is present in many human pathogens, including *Mycobacterium tuberculosis*², *Plasmodium falciparum*³, *Escherichia coli*, and most gram-negative bacteria⁴. Isoprenoid precursors are processed downstream to produce essential components of the bacterial cell wall,⁵ electron transport chain,⁶ and secondary metabolites including virulence factors.⁷ Thus, interrupting the production of isoprenoids represents an attractive strategy for the development of novel antimicrobial agents.

Despite the great need for new antibiotics, development of inhibitors targeting the MEP pathway has been relatively slow and focused mainly on inhibiting IspC-catalyzed production of MEP, the first committed pathway intermediate.^{8,9,10,11} IspD catalyzes the second committed step in the MEP pathway, a condensation of cytidine triphosphate (CTP) and MEP to produce cytidine diphosphate – methylerythritol (CDPME). Bacteria which utilize the MEP pathway require IspD for growth.¹² Recently, IspD inhibitors from the Malaria Box¹³ have been shown to inhibit the growth of *P. falciparum*, supporting IspD as a viable target for the development of antimicrobial agents.^{14,15,16} However, despite their potent activity against *P. falciparum*, these compounds are inactive against *E. coli* or *M. tuberculosis* IspD.

Developing selective inhibitors of MEP pathway enzymes is challenging, in part, because of the polar active sites of these enzymes which have evolved to accommodate hydrophilic phospho-sugar intermediates.¹⁷ As such, conventional small molecule drug libraries containing mostly hydrophobic compounds have been largely unsuccessful for identifying selective inhibitors of these enzymes.⁹ With this challenge in mind, we have sought to develop close structural analogs of MEP that could serve as rationally designed inhibitors of IspD and antimetabolites for the MEP pathway. By virtue of their close resemblance to MEP, these phosphonate analogs could compete with MEP in the IspD active site to block CDPME production and undergo enzyme-catalyzed transformation to the corresponding phosphoryl-CDPME. In an *in vivo* context, successive processing through the MEP pathway would ultimately lead to phosphoryl analogs of the essential isoprenoids IDP and DMADP that would be unable to undergo the essential electrophilic chemistry of the natural diphosphates. Not only would this result in potentially toxic phosphonate antimetabolites, it would also provide an additional metabolic stress by the overall consumption of two molecules of NTP and two reducing equivalents of NADPH per MEP analog further impeding cell growth.

Toward this goal, the present study aimed to develop MEP analogs as rational inhibitors and alternative substrates of IspD from bacterial and apicomplexan species. Phosphonyl analogs of MEP were synthesized and evaluated against IspD from several pathogens. Analogs were kinetically characterized as substrates for the *E. coli* ortholog of IspD and as inhibitors of IspD from *E. coli*, *P. falciparum*, and *M. tuberculosis*. All analogs act as alternative substrates for IspD with the *S*-monofluoro MEP analog **5b** emerging as the best alternative substrate for *E. coli* IspD and the most potent inhibitor of all IspD orthologs. Perhaps more

intriguing, the focused SAR presented herein reveals that even subtle changes to the MEP scaffold result in a profound decrease in catalytic efficiency. While we observed only modest inhibition, the *S*-monofluorophosphono-MEP scaffold could provide a foundation for the development of broad spectrum selective inhibitors of IspD.

Results

Design and Synthesis of Phosphonyl MEP analogs

Since MEP is the first committed metabolite in the bacterial isoprenoid pathway, developing analogs of MEP is an ideal approach to implement an antimetabolite strategy for pharmacological intervention of isoprenoid biosynthesis. Analysis of available crystal structures of IspD bound to its product, CDPME, reveals that all hydroxyl groups of the methylerythritol portion of CDPME make hydrogen bonding contacts to the enzyme suggesting that their substitution would be detrimental to IspD affinity.¹⁸ The oxygen bridging the phosphate and sugar, however, has no apparent contacts to the enzyme; therefore, we sought to synthesize a small set of phosphonyl analogs of MEP substituting the bridging oxygen for carbon. (Figure 1)

Compounds **1** – **3**, and **5a,b** (Figure 1) were synthesized from benzylidene-protected methylerythritol **6**¹⁹ (Schemes 2 and 4). Alcohol **6** was converted to the aldehyde **7** through a 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO)-catalyzed oxidation²⁰ which provided access to the key intermediates **8a** and **8b** through a Horner-Wadsworth-Emmons olefination with either tetramethyl bisphosphonate or tetramethyl fluoro-bisphosphonate, respectively (Scheme 2). At this point the syntheses diverge to provide compounds **1** – **3**. Vinyl phosphonates **2** and **3** were accessed by acid-catalyzed removal of the benzylidene protecting group to give **10a** and **10b** followed by de-esterification of the phosphonate by trimethylsilyl bromide (TMS-Br). Deprotection of dimethyl ester **8a** with TMS-Br yielded phosphonate **9**, which was then simultaneously reduced and hydrogenolyzed to yield analog **1**.

A fundamentally different approach was pursued for the synthesis of difluorophosphonyl analog **4**, starting with dimethyl allyl bromide (Scheme 3). The difluoromethyl phosphonate was installed by displacement of the bromide by cuprous difluoromethyl (diethyl)phosphonate.²¹ With the carbon skeleton intact, SeO₂-mediated allylic oxidation and subsequent NaBH₄ reduction gives allylic alcohol **13**. Sharpless epoxidation of **13** provided asymmetric epoxide **14** in 90 % ee.²² Acid-catalyzed ring opening of **14** inverted the tertiary stereocenter to provide the diethyl phosphonyl methylerythritol **15**.²³ The regioselectivity of this transformation was assessed by ¹⁸O labeling revealing 97% selectivity for the more substituted C₂ carbon (Figure S1). Under the same reaction conditions, the methyl erythritol precursor (*R,R*)-4-benzyloxy-2,3-epoxy-2-methylbutanol was converted to its corresponding triol with 89% regioselectivity (78% ee)²³ suggesting that the electron-withdrawing difluoromethyl phosphonate disfavors C₃ attack by water during epoxide ring opening. Deprotection of **15** with TMS-Br afforded difluorophosphonyl analog **4**.

Accessing stereochemically pure monofluoro analogs **5a** and **5b** was less straightforward (Scheme 4). Initial attempts to reduce olefin **8b** to a mixture of diastereomeric alkyl fluorides **16a** and **16b** under standard conditions (H_2 (1 atm), Pd/C, MeOH, 23 °C) resulted in undesired loss of the benzylidene group in addition to olefin reduction to give **17a,b**. Close monitoring of the reaction revealed a mixture of benzylidene-deprotected vinyl fluoride **10b**, desired benzylidene-protected alkyl fluoride **16a,b**, and benzylidene-deprotected alkyl fluoride **17a,b** after one hour. The concurrence of desired products **16a,b** and vinyl fluoride **10b** demonstrates there was little preference for olefin reduction over benzylidene deprotection under these conditions. Moreover, while epimers **16a** and **16b** could be resolved by silica chromatography, epimers **17a** and **17b** were inseparable. Hence, a selective reduction of **8b** to **16a** and **16b** was necessary to access epimers **5a** and **5b** in a diastereomerically pure fashion. Fortunately, cooling the standard hydrogenation conditions from 23 °C to –5 °C resulted in preferential reduction of the alkene with no observable loss of the benzylidene group over the course of the reaction cleanly affording the alkyl fluorides **16a** and **16b** in 72% overall yield (28% and 44% yield for **16a** and **16b**, respectively). The epimers were separated by silica chromatography and then sequentially deprotected by TMS-Br and hydrogenolyzed to provide monofluoro phosphonyl analogs **5a** and **5b**.

Establishing the stereochemistry of Mono-fluoro MEP analogs **5a** and **5b**

While diastereomers **16a** and **16b** were easily separated by silica chromatography, their absolute stereochemical configurations were unknown. Thus, fused bicycles **21a** and **21b**, which provide fixed chair-chair confirmations that can be probed using ^1H - ^1H NOESY NMR, were prepared to establish the relative stereochemistry of alkyl fluorides **16a** and **16b**. To access these fused bicycles, alkyl fluorides **16a** and **16b** were monodeprotected with thiophenol and diisopropylethylamine to provide monomethyl phosphonate esters **19a** and **19b**. Subsequent HATU-promoted cyclization yielded the bicyclic diastereomeric phosphonate esters **20a** and **20b**. The new P-stereocenter was formed in an approximately 4:3 ratio of diastereomers with the equatorial OMe predominating; this ratio was the same for both epimers at C_5 . Finally, the cyclized monomethyl phosphonates were deprotected rendering the P-atom achiral, and thus providing single diastereomers of each C_5 epimer, **21a** and **21b**.

The strong equatorial preference of the phenyl group of the benzylidene moiety gives an axial acetal proton (H_a , Figure 2) providing a useful reference by which to assign the remaining proton peaks to each face of the chair conformation. With the known axial protons assigned, the fluorine stereochemistry can be determined by defining the facial orientation of its C_5 proton (H_5). A NOESY cross-peak was observed between H_5 and H_3 in **21a** which was absent in **21b** (Figure 2, S2 – S3), which, coupled with knowledge of the absolute stereochemistry of benzylidene-protected starting material **6**¹⁹, provides strong evidence that **5a** and **5b** have *R* and *S*-configurations at C_5 , respectively.

MEP analog turnover by *E. coli* IspD

Due to their close structural similarity with the natural substrate, MEP, these analogs are expected to bind to the IspD active site and, upon binding, they can: 1) occupy the active site

and engage in IspD-catalyzed chemistry to produce analogs of CDPME, or 2) remain bound in the active site, acting as inhibitors. Given the potential for unnatural products of CDPME to have interesting activity of their own, we sought to evaluate **1** – **5a,b** as alternative substrates for *E. coli* IspD.

Initial qualitative analysis of **1** – **5a,b** showed time- and enzyme-dependent turnover of these analogs to give the corresponding unnatural CDPME products (Figure 3, Figures S4 – S10). By HPLC, each new product was observed to have a λ_{max} of 272 nm corresponding to the cytidine chromophore. LC-MS analysis confirmed a time-dependent accumulation of products with the predicted m/z for each analog tested (Figures S11 – S17). IspD shows a clear preference for saturated analogs **1**, **4** – **5a,b** over vinyl phosphonates **2** and **3**, which is corroborated by the Michaelis-Menten parameters obtained from detailed kinetic analysis (Table 1, Figure S17). Catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) for the saturated analogs **1**, **4** – **5a,b** is 2.5 to 17-fold greater than the unsaturated analogs **2** and **3**. However, the best substrate from this series, monofluoro analog **5b**, displays a catalytic efficiency of $7.29 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$, which is 250-fold lower than the natural substrate, MEP ($k_{\text{cat}}/K_{\text{m}} = 1.81 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$).

The poor catalytic efficiency of these analogs appears to be driven primarily by low k_{cat} , implying that the chemical step is more sensitive to alterations at C₅, α to the phosphonyl, than binding to form the enzyme-analog complex. In particular, **5b** has a substantially lower K_{m} than its epimer **5a** while maintaining a similar k_{cat} suggesting that the stereochemical placement of the fluorine atom is more important for binding than turnover.

The stereochemical preference for *S* (**5b**) over *R* (**5a**) can potentially be explained by an examination of the CDPME-bound crystal structure of *E. coli* IspD (PDB 1INI) (Figure 4).¹⁸ When the phosphonate analogs are modeled into the active site, the *S*-epimer places the fluorine atom 3.2 angstroms from Lys213, suggesting the possibility for an enthalpically favorable H-bonding interaction. The *R*-epimer, on the other hand, places the fluorine atom within 3 angstroms of the C₃-hydroxyl, which in the natural substrate engages in a hydrogen-bonding interaction with the backbone carbonyl of Thr189. It is possible that the fluorine atom acts as a hydrogen-bond acceptor for the C₃-hydroxyl in the unbound state, a hydrogen-bonding interaction that would have to be overcome to enable binding to the enzyme, thus imposing an enthalpic cost.

Monofluoro analog **5b** is a modest inhibitor of IspD

As alternative substrates, the MEP analogs demonstrated their ability to bind the IspD active site and undergo catalysis. Expanding on this finding, we sought to determine whether the analogs were able to inhibit the natural reaction by IspD with MEP. Because IspD is present in many pathogens of great interest for public health, analogs **1** – **5a,b** were initially evaluated at 1 mM as inhibitors of the IspD orthologs from *E. coli*, *P. falciparum*, and *M. tuberculosis* (Table 2, Figure S19). Mono-fluoro analog **5b** emerged as the most potent inhibitor for *E. coli* and *P. falciparum* IspD while no significant inhibition was observed against *M. tuberculosis* IspD by any analog. IC₅₀ values for **5b** were determined to be $0.7 \pm 0.1 \text{ mM}$ and $1.3 \pm 0.7 \text{ mM}$ for the *E. coli* and *P. falciparum* enzymes, respectively (Figure S20).

Discussion

With the prevalence of antibiotic resistant infections is steadily increasing, there is a growing need for new antimicrobial agents. Six phosphonate analogs of MEP were designed and synthesized in an effort to develop novel compounds capable of modulating flux through the MEP pathway to isoprenoids, essential for growth in many human pathogens. These analogs were designed to act as potential inhibitors and/or alternative substrates of IspD. In addition, it was reasoned that the unnatural alternative products, also mimicking downstream MEP pathway intermediates, would provide multiple opportunities to inhibit isoprenoid biosynthesis, potentially resulting in stable phosphonyl analogs of IDP and DMADP. Here, in addition to their synthesis and characterization, we have demonstrated that **1** – **5a,b** are substrates for *E. coli* IspD with the most efficient substrate also being the best inhibitor in the series. More generally, we discovered that the ability of IspD to produce CDPME analogs is very sensitive to even minor changes in the MEP scaffold.

MEP analogs **1** – **5a,b** were carefully designed to closely resemble the natural substrate of IspD, MEP. This molecular mimicry was intended to produce analogs that could productively bind to the highly polar active site of IspD.¹⁷ An analysis of the CDPME-bound crystal structure of *E. coli* IspD reveals that all three hydroxyls of the methylerythritol scaffold are engaged in hydrogen bonding interactions and the 2C-methyl has van der Waals interactions with Thr165. The oxygen bridging the phosphate and ME scaffold, however, appears to be without clear protein interactions making it a potentially suitable site for chemical manipulation that could retain enzyme affinity. However, much to our intrigue, this conservative approach failed to produce alternative substrates that interacted with IspD with efficiencies comparable to MEP. Nevertheless, two key findings emerge from this study of alternative substrates: 1) *E. coli* IspD preferentially turns over the *S*-monofluorophosphonate **5b** compared to its *R*-fluoro-epimer, and 2) low k_{cat} appears to be primarily responsible for the diminished catalytic efficiency.

Deeper insight into why such seemingly conservative changes to the MEP scaffold result in dramatic reductions in substrate turnover was gained through detailed kinetic analysis on alternative substrates **1**–**5a,b**. The analogs are inferior to MEP primarily in their ability to undergo the chemical step of catalysis. This becomes clear when comparing the relative k_{cat} and K_{m} values of the phosphonyl analogs and MEP. For example, while **5b** has a K_{m} only 2.4-fold higher than MEP, a 100-fold lower k_{cat} is measured for this conversion relative to MEP. Krasutsky et al.²⁵ reported 1-amino- and 4-thio-MEP analogs as precursors to inhibitors of IspG and IspH, respectively. Their study showed that IspD was capable of processing MEP analogs when incubated with enzyme at high concentration and for long incubation times, so long as the stereochemistry of the MEP scaffold was unaltered. A quantitative analysis of alternative substrate turnover was not reported, limiting the conclusions that can be drawn relating the turnover efficiencies of those MEP analogs to that of the natural substrate.

The focused SAR presented here suggests that the diminished turnover efficiencies are due to non-productive binding to the active site rather than electronic alterations from replacement of the phosphate with a phosphonate. While mono-fluorophosphonates have

pK_a values similar to phosphates at about 6.5, phosphonates and difluorophosphonates are markedly different at 7.5 and 5.5, respectively.²⁶ Assuming the same protonation state at the active site, the analog with the highest pK_a should be the most reactive and the lowest pK_a should be the least reactive; however, pK_a does not appear to be correlated with k_{cat} for these analogs. Hence, these data support a model where the analogs are able to bind to the enzyme active site, but may assume conformations that do not provide adequate orbital overlap to enable catalysis.^{27,28} While this finding is undesirable for the development of alternative substrates for IspD, it indicates that the *S*-monofluorophosphonyl methylerythritol scaffold may be useful as a nonhydrolyzable methylerythritol binding moiety in more elaborated inhibitors of IspD.

Experimental

General

Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. Dimethyl allyl difluoro diethyl phosphonate²¹ (**12**) and D-1,3-benzylidene-2-C-methyl-erythritol¹⁹ (**6**) were synthesized as previously described. Dichloromethane (DCM) and diisopropylethylamine were distilled after drying on CaH₂. Yields of all reactions refer to the purified products. Silica chromatography was carried out in the indicated solvent system using pre-packed silica gel cartridges for use on the Biotage Isolera® Purification System. TLC silica gel 60 F₂₅₄ plates sold by Millipore were used for thin layer chromatography (TLC) and developed with KMnO₄ except where otherwise noted. ¹H, ¹³C, and ³¹P NMR spectra were acquired on a Bruker Avance III 500 spectrometer operating at 500 MHz for ¹H, 126 MHz for ¹³C, and 202 MHz for ³¹P. Chemical shift values are reported as δ (ppm) relative to CHCl₃ at δ 7.27 ppm, MeOH at δ 3.31 ppm, and DMSO at δ 2.50 ppm for ¹H NMR and CHCl₃ at δ 77.0 ppm, MeOH at δ 49.15 ppm, and DMSO at δ 39.51 ppm for ¹³C NMR. ³¹P chemical shifts are reported relative to triphenylphosphine oxide at δ 0 ppm as an external standard. High resolution mass spectrometry analysis was carried out The Johns Hopkins University School of Medicine, Baltimore, MD using a Thermo Q-exactive Orbitrap mass spectrometer with electrospray ionization. All compounds examined in bioassays (**1** – **5a,b**) were determined to be 95% pure as determined by HPLC-MS analysis (Figures S21 – S26).

Overexpression and purification of IspD from *E. coli*.²⁹

LB growth media (2 × 2 L) was treated with ampicillin (100 μ g/mL) and inoculated with 20 mL of a saturated overnight culture of *E. coli* BL21 (DE3) harboring an *E. coli* IspD-containing pET-37b vector. Cultures were grown with shaking at 37 °C to an OD₆₀₀ of 0.6 and then induced with isopropyl β -D-thiogalactoside (IPTG, 200 μ M) and incubated at 37 °C with shaking for 5 h. Cells were harvested by centrifugation (10,000 rpm, 10 min, 4 °C) and then resuspended in 50 mL of protein purification buffer (25 mM Tris, 160 mM glycerol, 20 mM MgCl₂, 10% v/v glycerol, pH 7.4) with 1 mM PMSF, 1× Protease Inhibitor Cocktail, and DNase added. Cells were lysed by sonication and the cell debris was pelleted by centrifugation (20,000 rpm, 30 min, 4 °C). The cell lysate (30 mL) was batch-bound to Ni-NTA resin (1.5 g) and rocked at 4 °C for 3 h. The resin was collected by centrifugation (300 rpm, 5 min, 4 °C) and resuspended in protein purification buffer with added imidazole

(3 mL, 5 mM imidazole). The His₆-tagged protein was eluted from the resin using a stepwise gradient of imidazole (20 – 500 mM). Fractions were analyzed by SDS-PAGE (12%) and stained with colloidal Coomassie. Selected fractions were combined and dialyzed with 1 L of dialysis buffer (50 mM Tris, 100 mM NaCl, glycerol, 10 % v/v, pH 7.4) with added EDTA (1 mM) at 4 °C for 12 h and then subjected to second dialysis with 1 L of dialysis buffer with added TCEP (1 mM) at 4 °C for 4 h. Protein concentration was determined by Bradford assay.

Overexpression and purification of IspD from *M. tuberculosis*

LB growth media (3 × 2 L) was treated with ampicillin (100 µg/mL) and inoculated with 20 mL of a saturated overnight culture of Artic Express (DE3) RP *E. coli* cells (Stratagene) harboring a *M. tuberculosis* IspD-containing BG1861 vector. Cultures were grown with shaking at 37 °C to an OD₆₀₀ of 0.6 and then cooled to 4 °C. After 90 minutes, the cells were induced with isopropyl β-D-thiogalactoside (IPTG, 200 µM) and incubated at 8 °C with shaking for 40 h. The cells were collected by centrifugation (10,000 rpm, 10 min, 4 °C) and then resuspended in 50 mL of a buffer containing Tris (25 mM, pH 8), NaCl (160 mM), glycerol (10 % v/v), MgCl₂ (20 mM), PMSF (1 mM), Protease Inhibitor Cocktail (1X), and DNase. Cells were lysed by French Press and the cell debris was pelleted by centrifugation (20,000 rpm, 30 min, 4 °C). The cell lysate was combined and purified as described above for the *E. coli* enzyme.

HPLC assays to determine substrate turnover for analogs²⁹

An HPLC assay was used to observe alternative substrate turnover with *E. coli* IspD. IspD reaction mixtures containing buffer (100 mM Tris, 7.5 mM MgCl₂, 1 mM DTT, pH 7.4) with 150 µM CTP, 500 µM MEP or analog, and 0.125 U/mL inorganic pyrophosphatase were preincubated at 37 °C for 10 min, and reactions were then initiated with 4 µM IspD. At specified timepoints, aliquots (40 µL) were quenched in cold 0.1% SDS (80 µL). The quenched samples were then passed through spin columns with a 3 kDa MW-cutoff (purchased from VWR). The filtrate was then analyzed by C₁₈-RP-HPLC. (Solvents - A: aqueous monobasic potassium phosphate (100 mM, pH 6) and tetrabutylammonium hydrogensulfate (5 mM); B: aqueous monobasic potassium phosphate (100 mM, pH 6), tetrabutylammonium hydrogensulfate (5 mM), and acetonitrile (30% v/v); Gradient – 0 to 50% B over 9 minutes, 50 to 100% B over 1 minute, 100% B for 3 minutes.)

LCMS assays to confirm identity of IspD products from analogs

The conditions were the same as described above for the HPLC assays except each timepoint (75 µL) was quenched into an equal volume of MeOH (75 µL, 0 °C) before filtration through the 3 kDa MW-cutoff spin column. Each sample was analyzed by LC-MS. The LC method was as follows: 0 – 4 min, 2% B; 4 – 5 minutes, 2 – 100% B; 5 – 8 minutes, 100% B.

Kinetic assays for characterization of analogs as alternative substrates for *E. coli* IspD¹⁴

IspD activity was evaluated by a continuous spectrophotometric coupled assay that measured the formation of CDPME or its corresponding phosphonyl analog via the purine

nucleoside phosphorylase (PNP)-catalyzed consumption of MESG by reaction with phosphate resulting in the production of ribose-1-phosphate and 2-amino-6-mercapto-7-methylpurine ($\lambda_{\text{max}} = 360 \text{ nm}$).^{14,24} IspD reaction mixtures containing buffer (100 mM Tris, 7.5 mM MgCl₂, 1 mM DTT, 1 mg/mL BSA, pH 7.4), 500 μM CTP, 0.01 – 4.0 mM MEP or analog, 0.2 U/mL inorganic pyrophosphatase, 1 U/mL PNP, and 150 μM MESG were preincubated at 37 °C for 10 min prior to addition of IspD (4 – 400 nM) to initiate the reaction.

Inhibition assays for *E. coli*, *P. falciparum*, and *M. tuberculosis* IspD

To screen for the ability of substrate analogs to act as inhibitors of IspD, the MESG assay was utilized as described above.^{14,24} IspD reaction mixtures containing buffer (100 mM Tris, 7.5 mM MgCl₂, 1 mM DTT, 1 mg/mL BSA, pH 7.4), 500 μM CTP, MEP (1 X K_m: *E. coli* – 100 μM , *P. falciparum* - 10 μM , *M. tuberculosis* - 800 μM) and analog (1.0 mM), 0.2 U/mL inorganic pyrophosphatase, 1 U/mL PNP, and 150 μM MESG were preincubated at 37 °C for 10 min. Enzymatic reactions were initiated by addition of IspD (*E. coli* – 15 nM, *P. falciparum* - 75 nM, *M. tuberculosis* - 15 nM). Initial rates were measured upon addition of IspD. An additional control was conducted for these experiments that excluded MEP to ensure that the observed rates were not primarily due to analog turnover (Figures S19, S27).

Modeling of **5b** into the active site of *E. coli* IspD

The crystal structure of *E. coli* IspD bound to CDPME (PDB: 1INI)¹⁸ was used to generate figure 4. The bound CDPME was altered in Pymol using the “builder” functionality to delete the cytidine monophosphate portion of CDPME leaving only MEP. The MEP portion was further altered to covert MEP to monofluorophosphonate **5b** by substituting the bridging O to (*S*)-CHF. Atom distances were measured using the distance wizard functionality within Pymol.

D-1,3-benzylidene-2-C-methyl-4-aldo-erythritol (7)—D-1,3-benzylidene-2-C-methyl-erythritol (**6**) (100 mg, 0.45 mmol) was dissolved in anhydrous DCM (1 mL) and cooled to 0 °C. Trichloroisocyanuric acid (TCICA) (110 mg, 0.47 mmol) was added followed by addition of TEMPO (1 mg, 6 μmol). The mixture was then removed from the ice bath and allowed to stir at ambient temperature for 0.5 h. The mixture was then passed through a pipette packed with Celite to remove solids. The Celite was washed with DCM (2 $\times$ 1 mL). The filtrate was then transferred to a separatory funnel and washed with saturated Na₂CO₃ (10 mL), 1M HCl (10 mL), and then brine (10 mL). The solution was dried over Na₂SO₄, filtered, and condensed under reduced pressure. Drying *in vacuo* yielded the desired aldehyde as an amorphous white solid. Yield: 69 mg, 70 %. ¹H NMR (500 MHz, CDCl₃) δ 9.79 (s, 1H), 7.55 (m, 2H), 7.40 – 7.44 (m, 3H), 5.61 (s, 1H), 4.14 (s, 1H), 3.93 (d, J = 10.9 Hz, 1H), 3.76 (d, J = 10.9 Hz, 1H), 2.71 (br. s, 1H), 1.48 (s, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 203.7, 136.8, 129.4, 128.4, 126.2, 102.1, 85.8, 76.3, 67.2, 20.9. HRMS (ESI) calcd for C₁₂H₁₅O₄ [M + H]⁺ 223.0965, found 223.0962

D-1,3-benzylidene-methylerythritol vinyl phosphonate dimethyl ester (8a)—

D-1,3-benzylidene-2-C-methyl-erythritol (**6**) (500 mg, 2.23 mmol) was dissolved in anhydrous DCM (5 mL) and cooled to 0 °C. TCICA (544 mg, 2.34 mmol) was added

followed by TEMPO (4 mg, 0.02 mmol). The mixture was then removed from the ice bath and allowed to stir at ambient temperature for 10 min. The mixture was then passed through a pipette packed with Celite to remove solids. The Celite was washed with DCM (2×6 mL). The filtrate was then transferred to a separatory funnel and washed with saturated Na_2CO_3 (10 mL), 1M HCl (10 mL), and then brine (10 mL). The solution was dried over MgSO_4 , filtered, and condensed under reduced pressure. Drying *in vacuo* gave the desired aldehyde which was dissolved in anhydrous acetonitrile (4 mL). In a separate flask, tetramethyl methylene bisphosphonate (0.393 g, 1.69 mmol) was dissolved in acetonitrile (8 mL). LiCl (0.072 g, 1.69 mmol) and DBU (0.23 mL, 1.54 mmol) were added. The aldehyde solution was then added dropwise to the bisphosphonate solution at ambient temperature. Upon stirring overnight, white precipitate formed. The precipitate was removed by vacuum filtration and the acetonitrile was removed from the filtrate under reduced pressure. The resulting residue was purified by silica flash chromatography (EtOAc to 9:1 EtOAc:MeOH) to yield **8a** as a white wax. Yield: 0.305 g, 42% yield. ^1H NMR (500 MHz, CDCl_3) δ 7.52 (d, $J=6.1$ Hz, 2 H) 7.39 (d, $J=6.8$ Hz, 3 H) 7.24 (m, $J=23.4$, 17.3, 1.7 Hz, 1 H) 6.04 (dd, $J=21.7$, 18.4 Hz, 1 H) 5.59 (s, 1 H) 4.78 (s, 1 H) 4.41 (br. s., 1 H) 3.95 (d, $J=10.7$ Hz, 1 H) 3.79 (d, $J=10.7$ Hz, 1 H) 3.72 (d, $J=11.2$ Hz, 6 H) 1.35 (s, 3 H). ^{13}C NMR (126 MHz, CDCl_3) δ 149.5 (d, $J=7.3$ Hz), 137.6 (s), 129.0 (s), 128.2 (s), 126.2 (s), 115.2 (d, $J=188.0$ Hz), 101.4 (s), 83.0 (d, $J=20.0$ Hz), 77.1 (s), 65.9 (s), 52.4 (d, $J=11.8$ Hz), 52.4 (d, $J=12.7$ Hz), 20.1 (s). ^{31}P NMR (202 MHz, CDCl_3) δ ppm -3.34 (s, 1 P).

HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{22}\text{O}_6\text{P}$ $[\text{M} + \text{H}]^+$ 329.1149, found 329.1148.

D-methylerythritol vinyl phosphonate dimethyl ester (10a)—Vinyl phosphonate **8a** (125 mg, 0.38 mmol) was dissolved in MeOH (2 mL), and *p*-toluene sulfonic acid monohydrate (7 mg, 0.04 mmol) was added. The solution was stirred overnight at ambient temperature. Silica gel (1.0 g) was added to the solution. The volatiles were removed under reduced pressure and the crude reaction adsorbed to silica was then purified by silica flash chromatography (9:1 DCM:MeOH) to yield **10a** as a colorless oil. Yield: 43 mg, 47%. ^1H NMR (500 MHz, $\text{MeOD}-d_4$) δ 7.10 (ddd, $J=23.0$, 17.1, 3.8 Hz, 1 H) 6.04 (ddd, $J=23.0$, 17.3, 2.0 Hz, 1 H) 4.31 (dd, $J=5.3$, 3.6 Hz, 1 H) 3.73 (d, $J=11.2$ Hz, 6 H) 3.61 (d, $J=11.2$ Hz, 1 H) 3.43 (d, $J=11.2$ Hz, 1 H) 1.03 (s, 3 H). ^{13}C NMR (126 MHz, $\text{MeOD}-d_4$) δ 156.5 (d, $J=6.4$ Hz), 115.6 (d, $J=188.9$ Hz), 75.5 (s), 75.3 (d, $J=20.9$ Hz), 68.7 (s), 53.4 (d, $J=6.4$ Hz, 2 C) 19.2 (s). ^{31}P NMR (202 MHz, $\text{MeOD}-d_4$) δ -1.73 (s, 1 P). HRMS (ESI) calcd for $\text{C}_8\text{H}_{18}\text{O}_6\text{P}$ $[\text{M} + \text{H}]^+$ 241.0836, found 241.0835

D-1,3-benzylidene-methylerythritol vinyl phosphonate (9)—Vinyl phosphonate **8a** (0.125 g, 0.38 mmol) was dissolved in anhydrous DCM (4 mL) and pyridine (0.31 mL, 3.8 mmol) was added followed by TMS-Br (0.25 mL, 1.9 mmol). The reaction was stirred overnight at ambient temperature. Volatiles were removed under reduced pressure. The residue was treated with MeOH (3 mL) containing pyridine (0.25 mL) for 1 h. The volatiles were removed again under reduced pressure. The residue was purified by C_{18} -RP-silica chromatography to yield a colorless oil (solvents - A: 50 mM triethylammonium acetate, B: acetonitrile) Gradient - 0–2.5 column volumes (CV), 2% B, 2.5–4.5 CV 2–100% B; 4.5–7.5 CV, 100% B. Yield: mono-triethylammonium salt, 107 mg, 70%. ^1H NMR (500 MHz,

MeOD- d_4) δ 7.46 – 7.51 (m, 2 H) 7.34 – 7.38 (m, 3 H) 6.59 (ddd, $J=20.8, 17.6, 4.1$ Hz, 1 H) 6.16 (td, $J=17.8, 1.1$ Hz, 1 H) 5.58 (s, 1 H) 4.25 (br. s., 1 H) 3.82 (d, $J=10.5$ Hz, 1 H) 3.71 (d, $J=10.7$ Hz, 1 H) 3.11 (q, $J=7.4$ Hz, 6 H) 2.02 (s, 1 H) 1.30 (s, 3 H) 1.27 (t, $J=7.3$ Hz, 12 H). ^{13}C NMR (126 MHz, MeOD- d_4) δ 139.8 (s), 139.4 (d, $J=5.5$ Hz), 130.0 (s, 2 C) 129.24 (s, 4 C) 127.6 (s, 5 C) 127.9 (d, $J=176.2$ Hz), 102.8 (s), 85.0 (d, $J=19.1$ Hz), 78.2 (s), 67.3 (s), 47.4 (s, 5 C) 9.2 (s). ^{31}P NMR (202 MHz, MeOD- d_4) δ -13.50 (s, 1 P). HRMS (ESI) calcd for $\text{C}_{13}\text{H}_{18}\text{O}_6\text{P}$ $[\text{M} + \text{H}]^+$ 301.0836, found 301.0836.

D-methylerythritol phosphonate (1)—Phosphonate **9** (0.10 g, 0.25 mmol) was dissolved in MeOH (3 mL) and formic acid (3 drops) was added followed by $\text{Pd}(\text{OH})_2/\text{C}$ (0.040 g). The solution was subjected to an H_2 atmosphere (80 psi, Parr shaker) and shaken overnight. Solids were removed by vacuum filtration and the filtrate was condensed under reduced pressure. The resultant residue was purified by C_{18} -RP-silica chromatography to yield a colorless oil (solvents - A: 50 mM triethylammonium acetate, B: acetonitrile) Gradient - 0–2.5 CV, 2% B, 2.5–4.5 CV 2–100% B; 4.5–7.5 CV, 100% B. Yield: 54 mg, 100%. ^1H NMR (500 MHz, D_2O) δ 3.51 (d, $J=11.9$ Hz, 1 H) 3.42 (d, $J=11.5$ Hz, 1 H) 3.11 (q, $J=7.3$ Hz, 3 H) 1.77 (s, 2 H) 1.40 – 1.55 (m, 2 H) 1.18 (t, $J=7.3$ Hz, 8 H) 1.03 (s, 3 H). ^{13}C NMR (126 MHz, D_2O) δ 75.0 (d, $J=16.3$ Hz), 74.8 (s), 66.4 (s), 46.6 (s), 24.9 (d, $J=133.5$ Hz), 24.2 (s), 17.7 (s), 8.2 (s). ^{31}P NMR (202 MHz, D_2O) δ 0.94 (s, 1 P). HRMS (ESI) calcd for $\text{C}_6\text{H}_{14}\text{O}_6\text{P}^-$ $[\text{M}]^-$ 213.0533, found 213.0536.

D-methylerythritol vinyl phosphonate (2)—Vinyl phosphonate **10** (36 mg, 0.15 mmol) was dissolved in anhydrous DCM (1.5 mL) and pyridine (0.12 mL, 1.5 mmol) was added followed by TMS-Br (0.1 mL, 0.75 mmol). The reaction was stirred overnight at ambient temperature. Volatiles were removed under reduced pressure. The residue was treated with MeOH (3 mL) containing pyridine (0.25 mL) for 1 h. The volatiles were removed again under reduced pressure. The residue was purified by C_{18} -RP-silica chromatography (solvents - A: 50 mM triethylammonium acetate, B: acetonitrile) Gradient - 0–2.5 CV, 2% B, 2.5–4.5 CV 2–100% B; 4.5–7.5 CV, 100% B. The resulting residue was treated with Dowex- H^+ resin to obtain the free acid as a colorless oil. Yield: 26 mg, 82%. ^1H NMR (500 MHz, D_2O) δ 6.67 (ddd, $J=23.0, 17.8, 5.1$ Hz, 1 H) 6.00 (ddd, $J=21.4, 17.3, 1.7$ Hz, 1 H) 4.17 (ddd, $J=4.6, 3.1, 1.8$ Hz, 1 H) 3.49 (d, $J=11.8$ Hz, 1 H) 3.36 (d, $J=11.8$ Hz, 1 H) 0.96 (s, 3 H). ^{13}C NMR (126 MHz, D_2O) δ 148.2 (d, $J=5.4$ Hz), 120.0 (d, $J=181.7$ Hz), 74.6 (s), 74.2 (d, $J=20.9$ Hz), 66.2 (s), 18.1 (s). ^{31}P NMR (202 MHz, D_2O) δ -8.26 (s, 1 P). HRMS (ESI) calcd for $\text{C}_6\text{H}_{12}\text{O}_6\text{P}$ $[\text{M} - \text{H}]^-$ 211.0376, found 211.0379.

D-1,3-benzylidene-methylerythritol fluorovinyl phosphonate dimethyl ester (8b)—Tetramethyl fluoromethylene bisphosphonate (1.31 g, 5.24 mmol) was dissolved in acetonitrile (30 mL). LiCl (0.267 g, 6.29 mmol) and DBU (0.78 mL, 5.24 mmol) were added. The aldehyde **7** (1.16 g, 5.24 mmol) was dissolved in acetonitrile (20 mL) and added dropwise to the bisphosphonate solution at ambient temperature. Upon stirring overnight, white precipitate formed. The precipitate was removed by vacuum filtration and the acetonitrile was removed from the filtrate under reduced pressure. The resulting residue was purified by silica flash chromatography (7:3 EtOAc:Hexanes) to provide the desired vinyl phosphonate as a colorless oil. Yield 1.043 g, 57%. ^1H NMR (500 MHz, CDCl_3) δ 7.49 (d, J

= 7.2 Hz, 2H), 7.38 (d, J = 6.1 Hz, 3H), 6.04 (td, J = 8.2, 39.0 Hz, 1H), 5.57 (s, 1H), 4.75 (d, J = 8.2 Hz, 1H), 3.99 (d, J = 10.9 Hz, 1H), 3.85 (d, J = 11.0 Hz, 3H), 3.84 (d, J = 11.1 Hz, 3H), 3.79 (d, J = 10.9 Hz, 1H), 1.48 (s, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 151.1 (dd, J = 284.3, 236.1 Hz), 137.2 (s), 128.9 – 129.2 (m), 127.9 – 128.4 (m), 126.1 (s), 121.1 (dd, J = 28.2, 1.8 Hz), 101.6 (s), 78.1 (dd, J = 11.8, 1.8 Hz), 76.9 (s), 66.2 (s), 53.6 (t, J = 5.9 Hz), 19.8 (s). ^{31}P NMR (202 MHz, CDCl_3) δ -18.78 (d, J_{PF} = 102 Hz, 1P). HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{21}\text{FO}_6\text{P}$ $[\text{M} + \text{H}]^+$ 347.1054, found 347.1053.

D-methylerythritol fluorovinyl phosphonate dimethyl ester (10b)—Vinyl phosphonate **8b** (121 mg, 0.35 mmol) was dissolved in MeOH (1.8 mL) and *p*-toluene sulfonic acid monohydrate (7 mg, 0.04 mmol) was added. The solution was stirred overnight at ambient temperature. Silica gel (1.0 g) was added to the solution. The volatiles were removed under reduced pressure and the crude reaction adsorbed to silica was then purified by silica flash chromatography (9:1 DCM:MeOH) to yield a white amorphous solid. Yield: 49 mg, 54%. ^1H NMR (500 MHz, $\text{MeOD}-d_4$) δ 6.10 (dt, J = 39.5, 8.5 Hz, 1 H) 4.60 (dd, J = 9.2, 1.3 Hz, 1 H) 3.83 (dd, J = 11.3, 2.8 Hz, 6 H) 3.52 (d, J = 10.8 Hz, 1 H) 3.45 (d, J = 10.8 Hz, 1 H) 1.17 (s, 3 H). ^{13}C NMR (126 MHz, $\text{MeOD}-d_4$) δ 150.8 (dd, J = 276.1, 238.0 Hz), 127.7 (dd, J = 26.3, 2.7 Hz), 75.4 (s), 69.2 (dd, J = 11.8, 4.5 Hz), 68.0 (s), 54.5 (t, J = 5.4 Hz), 19.9 (s). ^{31}P NMR (202 MHz, $\text{MeOD}-d_4$) δ -16.54 (d, J = 107.0 Hz, 1 P). HRMS (ESI) calcd for $\text{C}_8\text{H}_{17}\text{FO}_6\text{P}$ $[\text{M} + \text{H}]^+$ 259.0741, found 259.0742.

D-methylerythritol fluorovinyl phosphonate (3)—Vinyl phosphonate **10b** (65 mg, 0.25 mmol) was dissolved in anhydrous ACN (2.5 mL). Pyridine (0.20 mL, 2.5 mmol) was added and the solution was cooled to 0 °C. TMS-Br (0.33 mL, 2.52 mmol) was added dropwise and the solution was allowed to warm to ambient temperature and stirred overnight. The reaction was quenched after 16 h by addition of aq NH_4OH (10%, 10 mL) and stirred for 1 h. The aqueous layer was washed with ether (2 $\times$ 10 mL). The aqueous layer was collected to which was added ACN (20 mL) and solvents were removed under reduced pressure. The residue was resuspended in water and passed through Dowex cation exchange NH_4^+ -form (2 g) to provide the diammonium salt of **3** as a white wax after lyophilization. Yield: 35 mg, 53%. ^1H NMR (500 MHz, D_2O) δ 5.60 (ddd, J = 39.8, 9.4, 7.2 Hz, 1 H) 4.50 (dd, J = 9.4, 1.4 Hz, 1 H) 3.46 (d, J = 11.8 Hz, 1 H) 3.38 (d, J = 11.5 Hz, 1 H) 1.07 (s, 3 H). ^{13}C NMR (126 MHz, D_2O) δ 157.5 (dd, J = 277.9, 219.8 Hz), 116.6 (dd, J = 25.4, 4.5 Hz, 2 C) 74.6 (s), 67.7 (dd, J = 10.0, 6.4 Hz), 65.9 (s), 18.3 (s). ^{31}P NMR (202 MHz, D_2O) δ -25.32 (d, J = 95.1 Hz, 1 P). HRMS (ESI) calcd for $\text{C}_6\text{H}_{11}\text{FO}_6\text{P}$ $[\text{M} - \text{H}]^-$ 229.0283, found 229.0283.

(E)-(2-methyl-4-difluoromethylphosphonate diethyl ester) allyl alcohol (13)—A flask was charged with SeO_2 (0.147 g, 1.32 mmol), anhydrous DCM (5 mL) was added, and then it was cooled to 0 °C. Tert-butyl hydrogen peroxide (0.95 mL, 5.6 M in decane, 5.3 mmol) was added and the solution was stirred at 0 °C for 10 min. In a separate flask, alkene **12** (0.678 g, 2.65 mmol) was dissolved in anhydrous DCM (3 mL) and then added dropwise to the SeO_2 solution at 0 °C. After an additional 10 min at 0 °C, the ice bath was removed, and the reaction was stirred overnight at ambient temperature. The reaction was quenched with brine (10 mL) and diluted with DCM (10 mL). $\text{Na}_2\text{S}_2\text{O}_3$ (10% w/v, 10 mL) was added,

and the reaction was transferred to a separatory funnel. The DCM layer was collected and dried over MgSO_4 , then filtered, and condensed under reduced pressure. The resulting residue was dissolved in methanol (6.5 mL) and cooled to 0 °C at which point sodium borohydride (0.121 g, 3.18 mmol) was added in one portion. The ice bath was removed, and the reaction was stirred at ambient temperature for 1 h. The reaction was quenched with sat'd NH_4Cl (10 mL), transferred to a separatory funnel and extracted with DCM (3×10 mL). The DCM layer was dried over MgSO_4 , filtered, and condensed under reduced pressure. Purification by silica flash chromatography (4:6 Hexanes:EtOAc) yielded a colorless oil. Yield: 0.353 g, 49%. ^1H NMR (500 MHz, CDCl_3) δ 5.55 (tq, $J=7.3, 1.4$ Hz, 1 H) 4.28 (quind, $J=7.3, 7.3, 7.3, 7.3, 0.8$ Hz, 4 H) 4.07 (d, $J=5.7$ Hz, 2 H) 2.86 (tt, $J=19.6, 6.8$ Hz, 2 H) 1.71 (s, 3 H) 1.39 (td, $J=7.0, 0.5$ Hz, 6 H). ^{13}C NMR (126 MHz, CDCl_3) δ 141.0 (s), 120.1 (td, $J=258.9, 214.3$ Hz), 113.2 (d, $J=4.5$ Hz), 68.0 (s), 64.4 (d, $J=7.3$ Hz), 32.8 (td, $J=21.8, 15.4$ Hz), 16.4 (d, $J=5.4$ Hz), 13.9 (s). ^{31}P NMR (202 MHz, CDCl_3) δ -17.90 (t, $J=107.8$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{10}\text{H}_{19}\text{F}_2\text{O}_4\text{PNa}$ $[\text{M} + \text{Na}]^+$ 295.0881, found 295.0882

(2R, 3R)-(1-hydroxy-2-methyl-4-difluoromethylphosphonate diethyl ester) 2,3-epoxybutane (14)—A flame-dried flask was charged with powdered 4 angstrom molecular sieves (0.460 g) and anhydrous DCM was added (10 mL). The flask was cooled to -20 °C and (-)-D-diethyltartrate (0.05 mL, 0.293 mmol) was added, followed by titanium (IV) isopropoxide (0.03 mL, 0.11 mmol), and then *tert*-butyl hydrogen peroxide (0.60 mL, 5.5 M in decane, 3.3 mmol). Allylic alcohol **13** (0.300 g, 1.1 mmol) dissolved in DCM (1 mL) was added dropwise. The flask was then placed a -20 °C freezer for 24 h. The flask was then transferred back to a -20 °C bath and quenched with aqueous Rochelle's salt (10% w/v, 20 mL). The mixture was stirred at -20 °C for 5 min, 0 °C for 10 min, and then ambient temperature for 15 min. The mixture was transferred to a separatory funnel and extracted with DCM (3×15 mL). The DCM layer was washed with MgSO_4 , filtered, and condensed under reduced pressure. The resulting residue was purified by silica flash chromatography (6:4 EtOAc:Hexanes) to yield a colorless oil. Yield: 0.329 g, 52% yield, 90% ee. NOTE: TLC stain (*p*-anisaldehyde) was particularly useful for monitoring this reaction. The alkene starting material stains blue/purple and the desired product stains yellow. ^1H NMR (500 MHz, CDCl_3) δ 4.31 (sxt, $J=7.5$ Hz, 4 H) 3.67 (dd, $J=11.9, 9.1$ Hz, 1 H) 3.59 (dd, $J=11.8, 3.5$ Hz, 1 H) 3.33 (dd, $J=7.1, 5.2$ Hz, 1 H) 2.40 – 2.55 (m, 1 H) 2.26 (dd, $J=9.3, 3.8$ Hz, 1 H - OH) 2.22 – 2.36 (m, 1 H) 1.40 (td, $J=7.1, 2.6$ Hz, 6 H) 1.35 (s, 3 H). ^{13}C NMR (126 MHz, CDCl_3) δ 119.5 (td, $J=259.8, 216.2$ Hz), 65.8 (s), 64.8 (dd, $J=30.9, 7.3$ Hz), 60.5 (s), 53.8 (q, $J=6.4$ Hz), 33.9 (td, $J=20.9, 15.4$ Hz), 16.3 (dd, $J=5.4, 3.6$ Hz), 14.4 (s). ^{31}P NMR (202 MHz, CDCl_3) δ -19.42 (dd, $J=107.8, 102.6$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{10}\text{H}_{20}\text{F}_2\text{O}_5\text{P}$ $[\text{M} + \text{H}]^+$ 289.1011, found 289.1006.

Percent enantiomer excess was determined by the synthesis of the *S*-Mosher's acid derivative of the allylic epoxide **14**.²² Briefly, DMAP (0.020 g, 0.17 mmol) was dissolved in anhydrous DCM (0.5 mL) and diisopropylethylamine (0.01 mL, 0.57 mmol) was added. The epoxide (0.050 g, 0.17 mmol) was added neat immediately followed by addition of *R*-(-)-Mosher's Acid Chloride (0.035 mL, 0.19 mmol). After stirring for 10 min, the reaction was quenched with ethanolamine (0.05 mL) and volatiles were removed under reduced pressure.

Purification by silica flash chromatography (6:4 Ethyl Acetate: Hexanes) yielded the product as a clear oil. Yield: 0.073 g, 85%. ^1H NMR (500 MHz, CDCl_3) δ 7.49 – 7.56 (m, 2 H) 7.39 – 7.43 (m, 3 H) 4.48 (d, $J=11.8$ Hz, 1 H) 4.28 (quind, $J=7.2, 7.2, 7.2, 7.2, 1.7$ Hz, 2 H) 4.28 (quin, $J=7.2$ Hz, 2 H) 4.16 (d, $J=11.8$ Hz, 1 H) 3.55 (s, 2 H) 3.23 (t, $J=6.0$ Hz, 0 H) 2.21 – 2.47 (m, 2 H) 1.38 (t, $J=7.1$ Hz, 3 H) 1.38 (t, $J=7.1$ Hz, 3 H) 1.30 (s, 3 H).

D-methylerythritol difluoromethyl phosphonate diethyl ester (15)—Epoxide **14** (0.115 g, 0.400 mmol) was dissolved in THF (4.5 mL). Aqueous H_2SO_4 (0.5 mL, 5 M, 2.5 mmol) was added at ambient temperature and the solution was stirred for 20 min then quenched with diisopropylethylamine (0.87 mL, 5.0 mmol) and diluted with diethyl ether (5 mL). The mixture was transferred to a separatory funnel and extracted with ethyl acetate (3×10 mL). The organic layer was washed with brine (15 mL) and dried with MgSO_4 , filtered, and condensed under reduced pressure. The resulting residue was purified by silica flash chromatography (EtOAc) yielding a colorless oil. Yield 0.050 g, 41%. ^1H NMR (500 MHz, MeOD-d_4) δ 4.30 (quin, $J=7.3$ Hz, 4 H) 4.06 (d, $J=9.6$ Hz, 1 H) 3.58 (d, $J=11.2$ Hz, 1 H) 3.44 (d, $J=11.2$ Hz, 1 H) 2.55 (quind, $J=14.3 \times 4, 6.1$ Hz, 1 H) 2.04 – 2.24 (m, 1 H) 1.39 (t, $J=7.1$ Hz, 6 H) 1.07 (s, 3 H). ^{13}C NMR (126 MHz, MeOD-d_4) δ 122.4 (td, $J=258.9, 218.0$ Hz), 75.0 (s), 68.5 (s), 66.3 (t, $J=6.8$ Hz), 58.5 (s), 36.8 (td, $J=19.3, 14.1$ Hz), 18.3 (s), 16.9 (d, $J=4.5$ Hz). ^{31}P NMR (202 MHz, MeOD-d_4) δ -17.25 (t, $J=110.7$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{10}\text{H}_{22}\text{F}_2\text{O}_6\text{P}$ $[\text{M} + \text{H}]^+$ 307.1117, found 307.1113.

Regioselectivity was determined using a method identical to the one listed above expect the 5M H_2SO_4 solution was prepared with ^{18}O enriched water. ^{13}C NMR (126 MHz, CHLOROFORM-d) δ ppm 120.7 (td, $J=259.9, 215.1$ Hz), 73.76 (s, ^{16}O -bonded), 73.73 (s, ^{18}O -bonded), 68.8 (br. s.), 67.2 (s), 65.3 (dd, $J=27.0, 6.9$ Hz), 37.4 (td, $J=19.6, 15.2$ Hz), 18.3 (s), 16.3 (d, $J=5.2$ Hz),

D-methylerythritol difluoromethyl phosphonate (4)—Diethyl phosphonate **15** (0.050 g, 0.163 mmol) was dissolved in acetonitrile (1.7 mL). Pyridine (0.13 mL, 1.63 mmol) was added and the solution was cooled to 0 °C. TMS-Br (0.22 mL, 1.63 mmol) was added dropwise. The solution was allowed to warm to ambient temperature and stirred overnight. Volatiles were removed under reduced pressure and the resulting residue was cooled to 0 °C and aqueous NH_4OH (10% w/v, 10 mL) was added. After stirring at ambient temperature for 1 h, the aqueous solution was transferred to a separatory funnel and washed with DCM (3×10 mL). Water was removed under reduced pressure and the resulting residue was redissolved in aqueous NH_4HCO_3 (25 mM, 5 mL) and passed through Dowex cation exchange ammonium-form (5 g) and then a C_{18} solid phase extraction column to remove any residual pyridine. The collected fractions were then lyophilized to provide the difluoroMEP analog **4** as an amorphous white solid. Yield 45 mg, 97%. ^1H NMR (500 MHz, D_2O) δ ppm 3.99 (d, $J=9.6$ Hz, 1 H) 3.53 (d, $J=11.8$ Hz, 1 H) 3.41 (d, $J=11.8$ Hz, 1 H) 2.35 (dddd, $J=30.5, 15.4, 11.9, 3.8$ Hz, 1 H) 2.07 (dddd, $J=37.7, 15.7, 11.0, 3.6$ Hz, 1 H) 1.01 (s, 3 H). ^{13}C NMR (126 MHz, D_2O) δ ppm 122.2 (td, $J=257.9, 199.8$ Hz, C) 74.3 (s), 68.0 (dd, $J=8.2, 5.4$ Hz), 66.27 (s), 34.7 (td, $J=19.8, 14.1$ Hz), 17.1 (s). ^{31}P NMR (202 MHz, D_2O) δ -19.7 (t, $J=97.0$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_6\text{H}_{12}\text{F}_2\text{O}_6\text{P}$ $[\text{M} - \text{H}]^-$ 249.0345, found 249.0347.

(5R)-D-1,3-benzylidene-methylerythritol monofluoromethyl phosphonate dimethyl ester (16a) and (5S)-D-1,3-benzylidene-methylerythritol monofluoromethyl phosphonate dimethyl ester (16b)—Alkene **8b** (1.04 g, 3.00 mmol) was dissolved in methanol (120 mL) and cooled to -10 – (-5) °C in an ice-brine bath. Pd/C (10%, 0.40 g) was added and the flask was filled with hydrogen (1 atm, balloon). The temperature was maintained between -10 °C and -5 °C for 3 h. The catalyst was removed by vacuum filtration and the filtrate was condensed under reduced pressure to provide a mixture of epimers. The stereoisomers were purified by silica flash chromatography (7:3 EtOAc:Hexanes) with the *S*-epimer eluting first. *R*-epimer **16a**, a colorless oil. Yield 0.292 g, 28%. ^1H NMR (500 MHz, MeOD- d_4) δ 7.46 (d, $J=5.2$ Hz, 2 H) 7.32 – 7.36 (m, 3 H) 5.52 (s, 1 H) 5.15 (dt, $J=46.0$, 6.5 Hz, 1 H) 3.92 (d, $J=9.0$ Hz, 1 H) 3.86 (d, $J=11.5$ Hz, 3 H) 3.83 (d, $J=10.8$ Hz, 3 H) 3.68 (d, $J=10.4$ Hz, 1 H) 2.32 – 2.49 (m, 0 H) 2.02 – 2.17 (m, 0 H) 1.35 (s, 3 H). ^{13}C NMR (126 MHz, CDCl_3) δ 137.6 (s), 128.9 (s), 128.2 (s), 126.1 (s), 101.7 (s), 86.5 (dd, $J=180.7$, 168.9 Hz), 80.3 (t, $J=6.4$ Hz), 77.4 (s), 66.1 (s), 53.8 (dd, $J=91.7$, 6.4 Hz), 30.4 (d, $J=21.8$ Hz), 18.9 (s). ^{31}P NMR (202 MHz, MeOD- d_4) δ -4.20 (d, $J=78.0$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{23}\text{FO}_6\text{P}$ [$\text{M} + \text{H}$] $^+$ 349.1211, found 349.1211. *S*-epimer **16b**, a colorless oil. Yield 0.458 g, 44%. ^1H NMR (500 MHz, MeOD- d_4) δ 7.47 (d, $J = 5.3$ Hz, 1H), 7.30 – 7.40 (m, 3H), 5.55 (s, 1H), 5.1 (dd, $J = 12.0$, 47.0 Hz, 1H), 3.88 (d, $J = 11.2$ Hz, 3H), 3.84 (d, $J = 11.0$ Hz, 3H), 3.68 (d, $J = 10.2$ Hz, 1H), 2.37 (quin, $J = 12.0$ Hz, 1H), 1.87 (td, $J = 13.0$, 43.0 Hz, 1H), 1.34 (s, 3H). ^{13}C NMR (126 MHz, CDCl_3) δ 137.6 (s), 128.8 – 129.1 (m), 128.1 – 128.3 (m), 126.1 (s), 101.6 – 101.8 (m), 84.5 (dd, $J=178.9$, 172.6 Hz), 78.4 (dd, $J=13.6$, 2.7 Hz), 77.4 (s), 65.6 (s), 53.6 (dd, $J=77.2$, 7.3 Hz), 29.3 (d, $J=20.0$ Hz), 19.2 (s). ^{31}P NMR (202 MHz, MeOD- d_4) δ -3.56 (d, $J = 76.0$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{23}\text{FO}_6\text{P}$ [$\text{M} + \text{H}$] $^+$ 349.1211, found 349.1211.

Attempt to selectively reduce 8b to 16a,b at 23 °C resulting in byproduct formation 17ab—Alkene **8b** (23 mg, 66 μmol) was dissolved in methanol (2.6 mL) at ambient temperature. Pd/C (10%, 9 mg) was added and the flask was filled with hydrogen (1 atm, balloon). The reaction progress was monitored by ^{31}P NMR. After 1h, all starting material had been consumed resulting in a mixture of **16a**, **16b**, **10b**, and **17ab** (^{31}P NMR δ -2.70 (d, $J=75.9$ Hz) -3.35 (d, $J=78.4$ Hz).

(5R)-D-1,3-benzylidene-methylerythritol monofluoromethyl phosphonate (18a)—Diester **16a** (56 mg, 0.15 mmol) was dissolved in anhydrous DCM (2 mL) and cooled to 0 °C. Pyridine (0.12 mL, 1.5 mmol) was added followed by dropwise addition of TMS-Br (0.10 mL, 0.75 mmol). The reaction was stirred at ambient temperature overnight. Volatiles were removed under reduced pressure and the resulting residue was treated with a methanolic solution of pyridine (1%, 3.6 mL) and stirred for 1 h at ambient temperature. After removing volatiles under reduced pressure, the residue was purified by C_{18} -RP flash chromatography yielding an amorphous white solid (Solvents - A: 50 mM triethylammonium acetate, B: acetonitrile; Gradient - 0–2 CV, 10% B, 2–12 CV 10–100% B; 12–14 CV, 100% B). The resulting solution was lyophilized and used immediately in the next reaction. ^1H NMR (500 MHz, D_2O) δ 7.42 – 7.50 (m, 2 H) 7.33 – 7.40 (m, 3 H) 5.64 (s, 1 H) 4.78 (ddt, $J=43.1$, 8.1, 4.1, 4.1 Hz, 1 H) 4.02 (dd, $J=8.3$, 3.0 Hz, 1 H) 3.85 (d, $J=10.7$ Hz, 1 H) 3.71 (d, $J=10.7$ Hz, 1 H) 3.10 (q, $J=7.2$ Hz, 19 H) 2.16 – 2.34 (m, 1 H) 1.97 (ddd,

$J=26.3, 17.3, 9.1$ Hz, 1 H) 1.33 (s, 3 H) 1.18 (t, $J=7.3$ Hz, 29 H). ^{31}P NMR (202 MHz, MeOD- d_4) δ -8.21 (d, $J=75.9$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{13}\text{H}_{19}\text{FO}_6\text{P}$ $[\text{M} + \text{H}]^+$ 321.0898, found 321.0897.

(5S)-D-1,3-benzylidene-methylerythritol monofluoromethyl phosphonate (18b)

—The *S*-epimer was prepared using the same procedure was the *R*-epimer **18a**, an amorphous white solid. ^1H NMR (500 MHz, D_2O) δ 7.44 – 7.50 (m, 2 H) 7.34 – 7.41 (m, 3 H) 5.66 (s, 1 H) 4.73 (dd, $J=46.8, 11.8$ Hz, 33 H) 3.96 (d, $J=10.8$ Hz, 1 H) 3.87 (d, $J=10.8$ Hz, 1 H) 3.72 (d, $J=10.8$ Hz, 1 H) 3.11 (q, $J=7.2$ Hz, 18 H) 2.10 – 2.23 (m, 1 H) 1.85 (dt, $J=46.7, 14.8$ Hz, 11 H) 1.32 (s, 3 H) 1.18 (t, $J=7.2$ Hz, 24 H) ^{31}P NMR (202 MHz, MeOD- d_4) δ -7.89 (d, $J=73.4$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{13}\text{H}_{19}\text{FO}_6\text{P}$ $[\text{M} + \text{H}]^+$ 321.0898, found 321.0896.

(5R)-D-methylerythritol monofluoromethyl phosphonate (5a)—Benzylidene protected analog **18a** (18 mg, 0.057 mmol) was dissolved in methanol (2 mL) and Pd/C (10%, 9 mg) was added followed by formic acid (1 drop). The flask was then filled with hydrogen (1 atm, balloon) and stirred vigorously overnight. The catalyst was removed by vacuum filtration and removal of solvent under reduced pressure yielded the desired analog **5a** as a colorless oil. Yield: 13 mg, 37%. ^1H NMR (500 MHz, D_2O) δ 4.72 (ddt, $J=46.8, 7.7, 3.5, 3.5$ Hz, 11 H) 3.75 (dd, $J=9.7, 1.9$ Hz, 1 H) 3.52 (d, $J=11.8$ Hz, 1 H) 3.42 (d, $J=11.3$ Hz, 1 H) 3.10 (q, $J=7.3$ Hz, 3 H) 2.08 – 2.26 (m, 1 H) 1.78 – 1.94 (m, 1 H) 1.18 (t, $J=7.3$ Hz, 5 H) 1.04 (s, 3 H). ^{13}C NMR (126 MHz, D_2O) δ 91.3 (dd, $J=172.6, 159.9$ Hz, 2 C) 74.7 (s), 73.1 (dd, $J=11.4, 2.3$ Hz), 66.4 (s), 46.6 (s), 32.0 (d, $J=18.2$ Hz), 17.4 (s), 8.2 (s). ^{31}P NMR (202 MHz, D_2O) δ -11.89 (d, $J=69.7$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_6\text{H}_{13}\text{FO}_6\text{P}$ $[\text{M} - \text{H}]^-$ 231.0439, found 231.0440.

(5S)-D-methylerythritol monofluoromethyl phosphonate (5b)—The *S*-epimer was prepared using the same procedure was the *R*-epimer **5a**. Analog **5b**, a colorless oil Yield: 13 mg, 68%. ^1H NMR (500 MHz, D_2O) δ 4.71 (dd, $J=46.7, 11.9$ Hz, 8 H) 3.69 (d, $J=10.8$ Hz, 1 H) 3.52 (d, $J=11.5$ Hz, 1 H) 3.43 (d, $J=11.6$ Hz, 1 H) 3.09 (q, $J=7.3$ Hz, 6 H) 2.04 (ddd, $J=21.5, 13.5, 12.7$ Hz, 1 H) 1.73 (dt, $J=44.3, 12.4$ Hz, 1 H) 1.17 (t, $J=7.3$ Hz, 9 H) 1.04 (s, 3 H). ^{13}C NMR (126 MHz, D_2O) δ 88.3 (dd, $J=171.7, 158.9$ Hz, 2 C) 74.5 (s), 69.5 (d, $J=10.9$ Hz), 66.4 (s), 46.6 (s), 31.7 (d, $J=20.0$ Hz), 17.6 (s), 8.2 (s). ^{31}P NMR (202 MHz, D_2O) δ -11.44 (d, $J=63.4$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_6\text{H}_{13}\text{FO}_6\text{P}$ $[\text{M} - \text{H}]^-$ 231.0439, found 231.0440.

(5R)-D-1,3-benzylidene-methylerythritol monofluoromethyl phosphonate monomethyl ester (19a)—Alkyl fluoride **16a** (0.229 g, 0.66 mmol) was dissolved in anhydrous THF (3.3 mL) and diisopropylethylamine (0.69 mL, 3.94 mmol) was added followed by thiophenol (0.22 mL, 1.97 mmol). After stirring overnight at ambient temperature, the starting material was consumed. The reaction solution was added directly to a silica column for purification (Solvent A: 1% TEA in DCM; Solvent B: methanol) to provide **19a** as an amorphous white solid. The gradient was as follows 0 – 10% B over 10 CV, 10 – 50% B over 1 CV, 50% B over 5 CV. A TLC stain (2,4-DNP) was necessary to visualize this compound. Yield: 0.183 g, 100% yield. ^1H NMR (500 MHz, MeOD- d_4) δ

7.46 (d, $J=5.8$ Hz, 2 H) 7.33 (d, $J=6.3$ Hz, 3 H) 5.53 (s, 1 H) 4.84 (dt, $J=32.0, 6.0$ Hz, 1 H) 3.99 (d, $J=6.1$ Hz, 1 H) 3.80 (d, $J=10.5$ Hz, 1 H) 3.66 (d, $J=10.2$ Hz, 3 H) 3.20 (q, $J=7.1$ Hz, 6 H) 2.35 – 2.50 (m, 0 H) 1.95 – 2.09 (m, 0 H) 1.34 (s, 3 H) 1.31 (t, $J=7.2$ Hz, 9 H). ^{13}C NMR (126 MHz, MeOD- d_4) δ 139.9 (s), 129.8 (s), 129.2 (s), 127.6 (s), 103.1 (s), 90.3 (dd, $J=175.3, 161.7$ Hz), 83.0 (dd, $J=9.1, 5.4$ Hz), 78.8 (s), 67.1 (s), 53.1 (d, $J=7.3$ Hz), 48.0 (s), 32.5 (d, $J=22.7$ Hz), 19.4 (s), 9.4 (s). ^{31}P NMR (202 MHz, MeOD- d_4) δ -9.87 (d, $J=68.0$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{21}\text{FO}_6\text{P}$ $[\text{M} + \text{H}]^+$ 335.1054, found 335.1053.

(5S)-D-1,3-benzylidene-methylerythritol monofluoromethyl phosphonate monomethyl ester (19b)—The *S*-epimer was prepared using the same procedure as the *R*-epimer **19a**. Analog **19b**, an amorphous white solid. Yield 0.242 g, 84%. ^1H NMR (500 MHz, MeOD- d_4) δ 7.46 (d, $J=5.2$ Hz, 3 H) 7.29 – 7.38 (m, 5 H) 5.54 (s, 1 H) 4.85 (dd, $J=49.0, 12.1$ Hz, 1 H) 3.88 (d, $J=10.8$ Hz, 1 H) 3.82 (d, $J=10.5$ Hz, 1 H) 3.66 (t, $J=11.0$ Hz, 3 H) 3.19 (q, $J=7.2$ Hz, 6 H) 2.25 – 2.40 (m, 1 H) 1.96 (dt, $J=46.0, 12.0$ Hz, 1 H) 1.35 (s, 3 H) 1.30 (t, $J=7.2$ Hz, 9 H). ^{13}C NMR (126 MHz, MeOD- d_4) δ 139.9 (s), 129.9 (s), 129.2 (s), 127.6 (s), 103.2 (s), 87.7 (dd, $J=175.3, 163.5$ Hz), 80.7 (d, $J=12.7$ Hz), 78.6 (s), 66.6 (s), 53.0 (d, $J=6.4$ Hz), 48.0 (s), 31.5 (d, $J=17.3$ Hz), 19.5 (s), 9.3 (s). ^{31}P NMR (202 MHz, MeOD- d_4) δ -9.65 (d, $J=65.0$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{21}\text{FO}_6\text{P}$ $[\text{M} + \text{H}]^+$ 335.1054, found 335.1052.

(5R)-D-1,3-benzylidene-methylerythritol monofluoromethyl phosphonolactone methyl ester (20a)—Monomethylphosphonate **19a** (0.156 g, 0.34 mmol) was dissolved in anhydrous acetonitrile (6.5 mL) and diisopropylethylamine (0.06 mL, 0.34 mmol) was added followed by HATU (0.142 g, 0.37 mmol). The solution stirred at room temperature overnight. After stirring overnight, acetonitrile was removed under reduced pressure and the resulting residue was purified by silica flash chromatography (1:1 Hexanes:Ethyl Acetate) to yield both diastereomers as amorphous white solids. Yield: 0.069 g, 64% (both stereoisomers). *R_p*-(Methoxy equatorial) ^1H NMR (500 MHz, CDCl_3) δ 7.44 – 7.49 (m, 2 H) 7.37 – 7.42 (m, 3 H) 5.56 (s, 1 H) 4.91 (dddd, $J=47.0, 12.1, 7.3, 3.3$ Hz, 1 H) 4.06 (d, $J=10.4$ Hz, 1 H) 3.90 (d, $J=11.2$ Hz, 3 H) 3.79 (dt, $J=12.0, 2.0$ Hz, 1 H) 3.75 (d, $J=10.4$ Hz, 1 H) 2.50 – 2.74 (m, 2 H) 1.89 (s, 3 H). ^{13}C NMR (126 MHz, CDCl_3) δ 136.4 (s), 129.5 (s), 128.4 (s), 126.1 (s), 103.0 (s), 84.9 (dd, $J=190.7, 150.8$ Hz), 77.1 (dd, $J=11.8, 4.5$ Hz), 76.8 (d, $J=8.2$ Hz), 75.7 (d, $J=10.9$ Hz), 54.3 (dd, $J=7.3, 1.8$ Hz), 31.2 (dd, $J=20.0, 1.8$ Hz), 16.9 (s). ^{31}P NMR (202 MHz, CDCl_3) δ -10.07 (d, $J=74.5$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{19}\text{FO}_5\text{P}$ $[\text{M} + \text{H}]^+$ 317.0949, found 317.0949. *S_p*-(Methoxy axial) ^1H NMR (500 MHz, CDCl_3) δ 7.44 – 7.49 (m, 2 H) 7.37 – 7.41 (m, 3 H) 5.57 (s, 1 H) 5.07 (dddd, $J=45.5, 11.5, 7.3, 2.8$ Hz, 1 H) 4.04 (d, $J=10.5$ Hz, 1 H) 3.95 (d, $J=10.8$ Hz, 3 H) 3.84 (dt, $J=12.9, 2.8$ Hz, 1 H) 3.81 (d, $J=10.5$ Hz, 1 H) 2.65 (dddd, $J=33.1, 12.3, 7.2, 6.9, 3.1$ Hz, 1 H) 2.41 (dt, $J=18.0, 11.9, 11.9, 6.0, 6.0$ Hz, 1 H) 1.74 (s, 3 H). ^{13}C NMR (126 MHz, CDCl_3) δ 136.4 (s), 129.5 (s), 128.5 (s), 126.2 (s), 103.1 (s), 83.6 (dd, $J=189.8, 153.5$ Hz), 77.7 (dd, $J=10.9, 1.8$ Hz), 76.2 (d, $J=4.5$ Hz), 75.7 (d, $J=10.9$ Hz), 53.7 (d, $J=5.4$ Hz), 31.0 (d, $J=20.0$ Hz), 17.6 (s). ^{31}P NMR (202 MHz, CDCl_3) δ -13.68 (d, $J=68.5$ Hz, 1 P). HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{19}\text{FO}_5\text{P}$ $[\text{M} + \text{H}]^+$ 317.0949, found 317.0949.

(5S)-D-1,3-benzylidene-methylerythritol monofluoromethyl phosphonolactone methyl ester (20b)—The *S*-epimer was prepared using the same procedure as the *R*-epimer **20a**. Both diastereomers of **20b** were amorphous white solids. Yield 0.023 g, 26% (both stereoisomers). *R_p*-(Methoxy equatorial) ¹H NMR (500 MHz, CDCl₃) δ 7.45 – 7.51 (m, 2 H) 7.37 – 7.42 (m, 3 H) 5.62 (s, 1 H) 5.17 (dq, *J*=47.5, 3.5 Hz, 1 H) 4.21 (dd, *J*=12.0, 4.8 Hz, 1 H) 4.05 (d, *J*=10.2 Hz, 1 H) 3.91 (d, *J*=10.8 Hz, 3 H) 2.46 – 2.67 (m, 2 H) 1.86 (s, 3 H). ¹³C NMR (126 MHz, CDCl₃) δ 136.6 (s), 129.5 (s), 128.5 (s), 126.2 (s), 103.3 (s), 85.1 (dd, *J*=188.0, 149.0 Hz), 76.6 (d, *J*=4.5 Hz), 75.8 (d, *J*=10.0 Hz), 75.3 (d, *J*=10.0 Hz), 54.7 (dd, *J*=6.4, 2.7 Hz), 30.8 (d, *J*=20.0 Hz), 17.6 (s). ³¹P NMR (202 MHz, CDCl₃) δ –15.72 (d, *J*= 66.0 Hz, 1 P). HRMS (ESI) calcd for C₁₄H₁₉FO₅P [M + H]⁺ 317.0949, found 317.0949. *S_p*-(Methoxy axial) ¹H NMR (500 MHz, CDCl₃) δ 7.44 – 7.51 (m, 2 H) 7.36 – 7.43 (m, 3 H) 5.63 (s, 1 H) 5.03 (dq, *J*=46.0, 3.4 Hz, 1 H) 4.26 (dd, *J*=12.8, 3.2 Hz, 1 H) 4.02 (d, *J*=10.4 Hz, 1 H) 3.91 (d, *J*=10.5 Hz, 1 H) 3.87 (d, *J*=10.8 Hz, 3 H) 2.31 – 2.65 (m, 2 H) 1.70 (s, 3 H). ³¹P NMR (202 MHz, CDCl₃) δ –16.15 (d, *J*=87.0 Hz, 1 P). HRMS (ESI) calcd for C₁₄H₁₉FO₅P [M + H]⁺ 317.0949, found 317.0949.

(5R)-D-1,3-benzylidene-methylerythritol monofluoromethyl phosphonolactone (21a)—Phosphonolactone **20a** (0.039 g, 0.123 mmol) was dissolved in anhydrous THF (0.9 mL) to which was added diisopropylethylamine (0.13 mL, 0.74 mmol) and thiophenol (0.04 mL, 0.37 mmol). The solution was stirred at ambient temperature overnight. The reaction solution was added directly to a silica column for purification (Solvent A: 1% TEA in DCM; Solvent B: methanol) to yield **21a** as an amorphous white solid. Gradient: 0 – 10 CV, 0 – 10% B; 10–11 CV, 10 – 50% B; 11–16 CV, 50% B. A TLC stain (CAM) was necessary to visualize this compound. Yield: 50 mg, 100%. ¹H NMR (500 MHz, MeOD-*d*₄) δ 7.42 – 7.49 (m, 2 H) 7.31 – 7.39 (m, 3 H) 5.61 (s, 1 H) 4.72 – 4.87 (m, 1 H) 3.89 (d, *J*=10.1 Hz, 1 H) 3.85 (dt, *J*=12.4, 2.5 Hz, 1 H) 3.72 (d, *J*=10.2 Hz, 1 H) 3.19 (q, *J*=7.4 Hz, 6 H) 2.24 – 2.52 (m, 2 H) 1.73 (s, 3 H) 1.31 (t, *J*=7.3 Hz, 9 H). ¹³C NMR (126 MHz, MeOD-*d*₄) δ 139.3 (s), 130.2 (s), 129.3 (s), 127.6 (s), 104.1 (s), 88.1 (dd, *J*=181.7, 149.0 Hz), 80.1 (d, *J*=11.8 Hz), 77.9 (d, *J*=10.9 Hz), 74.1 (d, *J*=5.4 Hz), 48.0 (s), 33.1 (d, *J*=20.9 Hz), 17.9 (s), 9.3 (s), ³¹P NMR (202 MHz, MeOD-*d*₄) δ –17.42 (d, *J*=67.2 Hz, 1 P). HRMS (ESI) calcd for C₁₃H₁₅FO₅P [M – H][–] 301.0647, found 301.0648.

(5S)-D-1,3-benzylidene-methylerythritol monofluoromethyl phosphonolactone (21b)—The *S*-epimer was prepared using the same procedure as the *R*-epimer **21a**. Analog **21b**, an amorphous white solid Yield: 0.019 g, 63%. ¹H NMR (500 MHz, MeOD-*d*₄) δ 7.41 – 7.50 (m, 2 H) 7.32 – 7.39 (m, 3 H) 5.63 (s, 1 H) 4.83 (d, *J*=49.0 Hz, 1 H) 4.15 (d, *J*=12.6 Hz, 1 H) 3.83 – 3.90 (m, 1 H) 3.72 – 3.81 (m, 1 H) 3.18 (q, *J*=7.2 Hz, 6 H) 2.22 – 2.57 (m, 2 H) 1.71 (s, 3 H) 1.30 (t, *J*=7.2 Hz, 9 H). ¹³C NMR (126 MHz, MeOD-*d*₄) δ 139.4 (s), 130.2 (s), 129.3 (s), 127.6 (s), 104.4 (s), 87.9 (dd, *J*=181.7, 147.1 Hz), 77.9 (t, *J*=10.4 Hz), 74.2 (d, *J*=6.4 Hz), 47.9 (s), 32.7 (d, *J*=19.1 Hz), 30.8 (d, *J*=37.2 Hz), 17.8 (s, 0 C) 9.3 (s). ³¹P NMR (202 MHz, MeOD-*d*₄) δ –19.87 (d, *J*=65.9 Hz, 1 P). HRMS (ESI) calcd for C₁₃H₁₅FO₅P [M – H][–] 301.0647, found 301.0649.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge Leah Imlay and Audrey Odom John for a generous gift of purified IspD from *P. falciparum* and plasmids containing IspD from *P. falciparum* and *M. tuberculosis*. We also thank Carley Heck and Namandje Bumpus for help with high resolution mass spectrometry analyses. This work was supported by funding from the National Institutes of Health (GM084998 for C.L.F.M. and D.B., T32 GM08018901 for M. W. and D.B.). We also wish to acknowledge support from the Johns Hopkins University School of Medicine, Institute for Basic Biomedical Sciences for C.L.F.M. and D.B.

References

1. Rohmer M, Knani M, Simonin P, Sutter B, Sahm H. Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. *Biochem J.* 1993; 295(Pt 2):517–524. DOI: 10.1042/BJ2950517 [PubMed: 8240251]
2. Eoh H, Brown AC, Buetow L, et al. Characterization of the Mycobacterium tuberculosis 4-Diphosphocytidyl-2-C-Methyl-d-Erythritol Synthase: Potential for Drug Development. *J Bacteriol.* 2007; 189(24):8922–8927. DOI: 10.1128/JB.00925-07 [PubMed: 17921290]
3. Cassera MB, Gozzo FC, D’Alexandri FL, et al. The Methylerythritol Phosphate Pathway Is Functionally Active in All Intraerythrocytic Stages of Plasmodium falciparum. *J Biol Chem.* 2004; 279(50):51749–51759. DOI: 10.1074/jbc.M408360200 [PubMed: 15452112]
4. Heuston S, Begley M, Gahan CGM, Hill C. Isoprenoid biosynthesis in bacterial pathogens. *Microbiology.* 2012; 158(Pt 6):1389–1401. DOI: 10.1099/mic.0.051599-0 [PubMed: 22466083]
5. van Heijenoort J. Lipid intermediates in the biosynthesis of bacterial peptidoglycan. *Microbiol Mol Biol Rev.* 2007; 71(4):620–635. DOI: 10.1128/MMBR.00016-07 [PubMed: 18063720]
6. Nowicka B, Kruk J. Occurrence, biosynthesis and function of isoprenoid quinones. *Biochim Biophys Acta - Bioenerg.* 2010; 1797(9):1587–1605. DOI: 10.1016/J.BBABIO.2010.06.007
7. Chan H-C, Feng X, Ko T-P, et al. Structure and Inhibition of Tuberculosinol Synthase and Decaprenyl Diphosphate Synthase from *Mycobacterium tuberculosis*. *J Am Chem Soc.* 2014; 136(7):2892–2896. DOI: 10.1021/ja413127v [PubMed: 24475925]
8. Edwards RL, Brothers RC, Wang X, et al. MEPicides: potent antimalarial prodrugs targeting isoprenoid biosynthesis. *Sci Rep.* 2017; 7(1):8400.doi: 10.1038/s41598-017-07159-y [PubMed: 28827774]
9. Masini T, Hirsch AKH. Development of Inhibitors of the 2 C -Methyl-D-erythritol 4-Phosphate (MEP) Pathway Enzymes as Potential Anti-Infective Agents. *J Med Chem.* 2014; 57(23):9740–9763. DOI: 10.1021/jm5010978 [PubMed: 25210872]
10. Chofor R, Sooriyaarachchi S, Risseuw MDP, et al. Synthesis and Bioactivity of β -Substituted Fosmidomycin Analogues Targeting 1-Deoxy- d -xylulose-5-phosphate Reductoisomerase. *J Med Chem.* 2015; 58(7):2988–3001. DOI: 10.1021/jm5014264 [PubMed: 25781377]
11. Konzuch S, Umeda T, Held J, et al. Binding Modes of Reverse Fosmidomycin Analogs toward the Antimalarial Target IspC. *J Med Chem.* 2014; 57(21):8827–8838. DOI: 10.1021/jm500850y [PubMed: 25254502]
12. Freiberg C, Wieland B, Spaltmann F, Ehlert K, Brötz H, Labischinski H. Identification of novel essential Escherichia coli genes conserved among pathogenic bacteria. *J Mol Microbiol Biotechnol.* 2001; 3(3):483–489. [PubMed: 11361082]
13. Spangenberg T, Burrows JN, Kowalczyk P, McDonald S, Wells TNC, Willis P. The Open Access Malaria Box: A Drug Discovery Catalyst for Neglected Diseases. In: Russell B, editor PLoS One. Vol. 8. 2013. e62906
14. Imlay LS, Armstrong CM, Masters MC, et al. Plasmodium IspD (2-C-Methyl-D-erythritol 4-Phosphate Cytidyltransferase), an Essential and Druggable Antimalarial Target. *ACS Infect Dis.* 2015; 1(4):157–167. DOI: 10.1021/id500047s [PubMed: 26783558]
15. Bowman JD, Merino EF, Brooks CF, Striepen B, Carlier PR, Cassera MB. Antiapicoplast and gametocytocidal screening to identify the mechanisms of action of compounds within the malaria box. *Antimicrob Agents Chemother.* 2014; 58(2):811–819. DOI: 10.1128/AAC.01500-13 [PubMed: 24247137]

16. Wu W, Herrera Z, Ebert D, et al. A chemical rescue screen identifies a *Plasmodium falciparum* apicoplast inhibitor targeting MEP isoprenoid precursor biosynthesis. *Antimicrob Agents Chemother*. 2015; 59(1):356–364. DOI: 10.1128/AAC.03342-14 [PubMed: 25367906]
17. Masini T, Kroezen BS, Hirsch AKH. Druggability of the enzymes of the non-mevalonate-pathway. *Drug Discov Today*. 2013; 18(23–24):1256–1262. DOI: 10.1016/j.drudis.2013.07.003 [PubMed: 23856326]
18. Richard SB, Lillo AM, Tetzlaff CN, Bowman ME, Noel JP, Cane DE. Kinetic analysis of *Escherichia coli* 2-C-methyl-D-erythritol-4-phosphate cytidyltransferase, wild type and mutants, reveals roles of active site amino acids. *Biochemistry*. 2004; 43(38):12189–12197. DOI: 10.1021/bi0487241 [PubMed: 15379557]
19. Urbansky M, Davis CE, Surjan JD, Coates RM. Synthesis of Enantiopure 2-C-Methyl-D-erythritol 4-Phosphate and 2,4-Cyclodiphosphate from D-Arabitol. *Org Lett*. 2004; 6(1):135–138. DOI: 10.1021/OL0362562 [PubMed: 14703369]
20. De Luca L, Giacomelli G, Porcheddu A. A Very Mild and Chemoselective Oxidation of Alcohols to Carbonyl Compounds. *Org Lett*. 2001; 3(19):3041–3043. DOI: 10.1021/OL016501M [PubMed: 11554838]
21. Burton DJ, Sprague LG. Allylations of [(diethoxyphosphinyl)difluoromethyl]zinc bromide as a convenient route to 1,1-difluoro-3-alkenephosphonates. *J Org Chem*. 1989; 54(3):613–617. DOI: 10.1021/jo00264a021
22. Gao Y, Klunder JM, Hanson RM, Masamune H, Ko SY, Sharpless KB. Catalytic asymmetric epoxidation and kinetic resolution: modified procedures including in situ derivatization. *J Am Chem Soc*. 1987; 109(19):5765–5780. DOI: 10.1021/ja00253a032
23. Giner J-L, Ferris WV, Mullins JJ. Synthesis of 2-Methyl-D-erythritol via Epoxy Ester–Orthoester Rearrangement. *J Org Chem*. 2002; 67(14):4856–4859. DOI: 10.1021/jo020168y [PubMed: 12098298]
24. Webb MR. A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. *Proc Natl Acad Sci*. 1992; 89(11):4884–4887. [PubMed: 1534409]
25. Krasutsky SG, Urbansky M, Davis CE, Lherbet C, Coates RM, Poulter CD. Synthesis of Methylerythritol Phosphate Analogues and Their Evaluation as Alternate Substrates for IspDF and IspE from *Agrobacterium tumefaciens*. *J Org Chem*. 2014; 79(19):9170–9178. DOI: 10.1021/jo501529k [PubMed: 25184438]
26. Blackburn GM, Bowler MW, Jin Y, Waltho JP. Reflections on biocatalysis involving phosphorus. *Biochem*. 2012; 77(10):1083–1096. DOI: 10.1134/S000629791210001X [PubMed: 23157289]
27. Storm DR, Koshland DE. A Source for the Special Catalytic Power of Enzymes: Orbital Steering. *Proc Natl Acad Sci*. 1970; 66(2):445–452. [PubMed: 16591843]
28. Griffin JL, Bowler MW, Baxter NJ, et al. Near attack conformers dominate β -phosphoglucomutase complexes where geometry and charge distribution reflect those of substrate. *Proc Natl Acad Sci U S A*. 2012; 109(18):6910–6915. DOI: 10.1073/pnas.1116855109 [PubMed: 22505741]
29. Majumdar A, Shah MH, Bitok JK, Hassis-LeBeau ME, Freel Meyers CL. Probing phosphorylation by non-mammalian isoprenoid biosynthetic enzymes using ^1H – ^{31}P – ^{31}P correlation NMR spectroscopy. *Mol Biosyst*. 2009; 5(9):935. doi: 10.1039/b903513c [PubMed: 19668858]

Figure 1.

a) Analogs of MEP incorporating a phosphonyl group at C₄. b) A presentative MEP analog scaffold with carbon numbering

Figure 2.

^1H - ^1H NOESY spectra of **21a** and **21b**. Using the acetal proton (H_a) as a guide, proton assignments to each face of the bicycle is possible. For **21a**, H₅ and H₃ show a clear NOESY cross-peak indicating they are 1,3 diaxial. For **21b**, there is no cross-peak observed between H₅ and H₃ reinforcing the stereochemical assignments. The full spectra are presented in the SI (Figures S2 and S3).

Figure 3. Analogs of MEP act as substrates for IspD

HPLC stackplot of **5b** being utilized by *E. coli* IspD to produce the corresponding CDPME analog **CDP-5b**. The cytidine chromophore is detected at 272 nm. Assay conditions: Tris (100 mM, pH 7.4), MgCl₂ (5 mM), DTT (1 mM), CTP (150 μ M), analog **5b** (500 μ M), inorganic pyrophosphatase (0.1 U/mL) and *E. coli* IspD (4 μ M), 37 $^{\circ}$ C.

Figure 4. Analog 5b modeled into the *E. coli* IspD active site¹⁸ (PDB: 1INI)

The crystal structure was acquired with CDPME bound. The MEP portion of CDPME was used to model the analog **5b** into the active site. The close proximity of the fluorine and the ε-amino of lysine 213 suggest a possible hydrogen bond. Threonine 189 appears to make hydrogen bonding contacts with the C₃-OH of the MEP scaffold.

Scheme 1.

The MEP pathway for the synthesis of essential 5-carbon isoprenoid precursors, DMADP and IDP.

Scheme 2. Synthetic route to access analogs **1** – **3**.

Scheme 3.
Synthetic route for analog **4**.

Scheme 4.

Synthetic routes to analogs **5a,b** and compounds **21a,b** for stereochemical characterization

Table 1

Michaelis-Menten constants for MEP and MEP analogs (**1** – **5a,b**). The turnover of substrates was monitored using the MESG EnzCheck assay for the detection of pyrophosphate.²⁴ All experiments were performed in duplicate.

Substrates for <i>E. coli</i> IspD			
	K_m (mM)	K_{cat} (min ⁻¹)	K_{cat}/K_m (M ⁻¹ s ⁻¹)
MEP	0.10 ± 0.02	1090 ± 60	1.8 × 10 ⁵
1	1.3 ± 0.2	39 ± 2	5.0 × 10 ⁵
2	4 ± 1	5.4 ± 0.6	2 × 10
3	0.7 ± 0.5	1.7 ± 0.5	4.0 × 10 ²
4	1.9 ± 0.7	12 ± 2	1.1 × 10 ²
5a	1.0 ± 0.1	13.0 ± 0.6	2.2 × 10 ²
5b	0.24 ± 0.05	10.5 ± 0.5	7.29 × 10 ²

Table 2
MEP analogs as inhibitors of IspD from *E. coli*, *P. falciparum*, and *M. tuberculosis*

Each analog was tested for inhibitory activity against each IspD ortholog at 1 mM. Only **5b** showed significant inhibitory activity. Control experiments were performed in the absence of MEP to ensure inhibitory activity was not masked by analog turnover (Figure S19).

	Relative Activity		
	EcIspD	PfIspD	MtbIspD
MEP	1.00 ± 0.03	1.00 ± 0.06	1.00 ± 0.07
1	0.99 ± 0.03	1.19 ± 0.02	1.0 ± 0.2
2	0.916 ± 0.003	0.8 ± 0.2	1.0 ± 0.1
3	0.95 ± 0.05	1.0 ± 0.2	1.0 ± 0.2
4	0.91 ± 0.03	0.8 ± 0.1	1.1 ± 0.1
5a	0.88 ± 0.02	0.83 ± 0.02	1.1 ± 0.2
5b	0.56 ± 0.04	0.47 ± 0.05	0.9 ± 0.1