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Targeting the Unique Mechanism of Bacterial 1-deoxy-D-xylulose 5-phosphate (DXP) Synthase

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Abstract

The bacterial metabolite 1-deoxy-D-xylulose-5-phosphate (DXP) is essential in bacterial central metabolism feeding into isoprenoid, thiamin diphosphate (ThDP), and pyridoxal phosphate *de novo* biosynthesis. Halting its production through the inhibition of DXP synthase is an attractive strategy for the development of novel antibiotics. Recent work has revealed that DXP synthase utilizes a unique random sequential mechanism which requires ternary complex formation between pyruvate-derived C2 α -lactylthiamin diphosphate (LThDP), D-glyceraldehyde-3-phosphate (D-GAP) and enzyme, setting it apart from all other known ThDP-dependent enzymes. Herein, we describe the development of bisubstrate inhibitors bearing an acetylphosphonate (AP) pyruvate mimic and a distal negative charge mimicking the phosphoryl group of D-GAP, designed to target the unique form of DXP synthase that binds LThDP and D-GAP in a ternary complex. A D-phenylalanine-derived triazole acetylphosphonate (D-PheTrAP) emerged as the most potent inhibitor in this series, displaying slow-tight-binding inhibition with K_i^* of 90 ± 10 nM, forward (k_1) and reverse (k_2) isomerization rates of 1.1 and 0.14 min^{-1} , respectively, and exquisite selectivity ($>15,000$ -fold) for DXP synthase over mammalian pyruvate dehydrogenase. D-PheTrAP is the most potent, selective DXP synthase inhibitor described to date and represents the first inhibitor class designed specifically to exploit the unique E-LThDP-GAP ternary complex in ThDP enzymology.

Graphical Abstract

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Associated Content

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Experimental procedures for the synthesis of **1** – **23** and the characterization of all new compounds. Inhibition plots for inhibitors **3** – **23** against DXP synthase to determine K_i values. Progress curves for analysis of time-dependent inhibition of DXP synthase by D-PheTrAP. Inhibition plots comparing the activity of MAP, **12**, and D-PheTrAP against DXPS^{Wt} and DXPS^{R478A}. Inhibition plots comparing D-PheTrAP and BAP activity against DXP synthase and porcine PDH.

Introduction

Current antibacterial agents target a limited number of processes in bacterial cells, including biosynthesis of proteins, DNA/RNA, folate and cell wall, and the rate of discovery of new inhibitors against these targets is dwindling.^{1,2} Antibacterial agents acting on alternative essential cellular processes are needed to address the rapid emergence of drug-resistant bacterial pathogens. Recent studies point to bacterial central metabolism as a potential target space,^{3–10} although this is generally thought to be difficult due to the conservation of metabolic enzymes in mammals and bacteria. Thus, essential bacterial metabolic enzymes with no mammalian counterpart are being investigated as potential targets. DXP synthase is one such essential enzyme in bacterial central metabolism. It catalyzes the thiamin diphosphate (ThDP) dependent decarboxylation and condensation of pyruvate and D-glyceraldehyde-3-phosphate (D-GAP) to form 1-deoxy-D-xylulose 5-phosphate (DXP). DXP is a branch point metabolite required for the *de novo* synthesis of essential isoprenoid precursors, dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP), and essential cofactors pyridoxal phosphate (PLP) and ThDP, the latter of which DXP synthase itself requires for catalysis (Figure 1). Recent studies have demonstrated that selective inhibition of DXP synthase inhibits growth of a number of clinically important gram-negative pathogens.¹¹

Inhibitors resembling substrate or cofactor have been pursued against DXP synthase.^{11–15} Amongst these are the alkylacetylphosphonates (alkylAPs) which are known to inhibit ThDP-dependent pyruvate decarboxylase enzymes.^{16,17} The acetylphosphonate moiety mimics the natural ketoacid substrate, pyruvate, to form a reversible covalent phosphonolactyl ThDP intermediate (PLThDP, Figure 2).^{16,18,19} While methylacetylphosphonate (MAP) and its structural analog acetylphosphinate (AcPhi) have been useful mechanistic probes in ThDP enzymology, a lack of potency and poor selectivity has limited their usefulness as antimicrobial agents. The rational development of D-GAP competitive inhibitors has been more challenging with both known D-GAP competitive inhibitors emerging from screening approaches.^{20,21}

Until recently, the conserved nature of ThDP-dependent catalytic mechanisms and the ubiquity of pyruvate as a substrate for ThDP enzymes in mammals and pathogens suggested that targeting DXP synthase selectively would be challenging. Fortunately, work by our group^{22–24} and others^{25–27} has shown that DXP synthase is unique among ThDP-dependent enzymes. The active site of DXP synthase is approximately twice the volume of pyruvate dehydrogenase and transketolase active sites and can accommodate sterically demanding acceptor substrates.^{15,28} We have shown that incorporation of steric bulk into the alkylAP scaffold provides some measure of selectivity of inhibition of DXP synthase over PDH or TK.^{11,14,15,17} The mechanism of DXP synthase is also unique amongst ThDP-dependent enzymes as it requires ternary complex formation between the enzyme, donor substrate-cofactor adduct and acceptor substrate (E-LThDP-GAP, Figure 3) to catalyze DXP formation, a finding that is consistent with the observed large active site volume.^{22–25,29} This contrasts the commonly observed ping-pong mechanism of other ThDP-dependent pyruvate decarboxylase enzymes in which the first product, CO₂, is released from the enzyme before acceptor substrate binding. The unique requirement for ternary complex

formation in DXP synthase catalysis suggests that it should be possible to design inhibitors that incorporate mimics of both donor and acceptor substrates to target this enzyme with high potency and selectivity.

Here, we describe the design and synthesis of a series acetylphosphonate inhibitors of DXP synthase. Copper-catalyzed alkyne-azide cycloaddition (CuAAC) was used to introduce diversity into the alkylAP scaffold, addressing instability issues associated with synthetic intermediates en route to alkylacetylphosphonates and extending the SAR beyond the hydrocarbon series previously described.^{11,14} Several triazole-based alkylAP inhibitors emerged with nanomolar inhibitory activity. The most potent of these, D-PheTrAP, is a slow, tight-binding inhibitor with a K_i^* of 90 ± 10 nM and >15,000-fold selectivity for DXP synthase over mammalian pyruvate dehydrogenase (PDH). Comparative kinetic analyses demonstrated that this inhibitor can bind to both the pyruvate and D-GAP binding sites, thus exploiting the unique mechanism of DXP synthase by mimicking the ternary complex DXPS-LThDP-D-GAP and displaying exquisite potency and selectivity. These potent new inhibitors will serve dual roles as interesting new mechanistic probes of DXP synthase and as scaffolds for the design of more effective antimicrobial agents targeting DXP synthase.

Experimental Procedures

Chemical Synthesis of Inhibitors

The chemical synthesis and characterization of compounds **1** and **3 – 23** is outlined in Scheme 1 and detailed in the supporting information.

Inhibition Assays To Determine K_i Values on DXP Synthase

Assays were performed in a 96-well plate at 23 °C. Each reaction was initiated by simultaneous addition of both substrates (pyruvate and D-GAP) at specified concentrations. Each well contained buffer (100 mM HEPES, 2 mM MgCl₂, 5 mM NaCl, 2.5 mM DTT, pH 8), 1 mM thiamin diphosphate, 200 μM NADPH, 2 μM IspC, 100 nM DXP synthase, specified inhibitor (0.001 – 100 μM), pyruvate (50 – 250 μM), and D-GAP (50 – 250 μM).

Mode of inhibition was determined by analyzing IC₅₀ as a function of [substrate]/ K_m . One substrate was held at a sub-saturating concentration ($2 \times K_m$) and the other was varied ($2 \times K_m$ and $10 \times K_m$). A positive correlation between IC₅₀ and [substrate] indicated competitive inhibition with respect to the varied substrate; no correlation indicated noncompetitive inhibition with the varied substrate. Inhibition constants (K_i) were determined by plotting the relative activity of DXP synthase as a function of inhibitor concentration and fitting that curve to the Morrison equation. For these experiments, pyruvate was held at a sub-saturating concentration (50 μM, $2 \times K_m$) and D-GAP was held at a saturating concentration (250 μM, $10 \times K_m$). Most analyses were performed with 12 inhibitor concentrations ranging from 21 nM to 10 μM.

Inhibition Assays Comparing Wildtype and R478A Variant of DXP Synthase

Assays were performed in a 96-well plate at 37 °C. Each reaction was initiated by simultaneous addition of both substrates (pyruvate and D-GAP). Each well contained buffer

(100 mM HEPES, 2 mM MgCl₂, 5 mM NaCl, 2.5 mM DTT, pH 8), 1 mM thiamin diphosphate, 200 μM NADPH, 2 μM IspC, 50 nM DXPS^{wt} or 400 nM DXPS^{R478A}, specified inhibitor (9 concentrations from 0.1 – 200 μM), 50 μM pyruvate, and ~1 × K_m D-GAP (25 μM for DXPS^{wt} and 2500 μM for DXPS^{R478A}).

Inhibition Assays for Porcine Pyruvate Dehydrogenase E1 subunit (PDH)

Assays were performed in a 96-well plate at 23 °C. Each reaction was initiated by addition of pyruvate. Each well contained buffer (100 mM HEPES, 2 mM MgCl₂, 5 mM L-cysteine, 0.3 mM TCEP, pH 8), 1 mM thiamin diphosphate, 2.5 mM NAD⁺, 100 μM Coenzyme A, 0.01 U/mL PDH, specified inhibitor (39 – 1000 μM), and 60 μM (2 × K_m) pyruvate.

Time-dependence of Inhibition Assays with D-PheTrAP

Assays were performed in a 96-well plate at 23 °C. Each reaction was initiated by addition of a solution of pyruvate and D-PheTrAP. Each well contained buffer (100 mM HEPES, 2 mM MgCl₂, 5 mM NaCl, 2.5 mM DTT, pH 8), 1 mM thiamin diphosphate, 200 μM NADPH, 2 μM IspC, 100 nM DXP synthase, D-PheTrAP (5 concentrations from 0.2 – 6.4 μM), 100 μM pyruvate, and 250 μM D-GAP. Samples were initiated simultaneously through the use of a multichannel pipette and the delay between reaction initiation and the first read by the instrument was 18 seconds for all 4 replicates. Thus, 18 seconds was added to all instrument reads.

The curves were fitted to Equation 1³⁰,

$$[\text{NADPH}] = [\text{NADPH}]_0 - \left[v_s t + \frac{(v_i - v_s)(1 - e^{-k_{obs}t})}{k_{obs}} \right]$$

by nonlinear regression analysis using the GraphPad Prism software to determine values for [NADPH]₀, initial rate (v_i), steady-state rate (v_s), and the observed isomerization constant (k_{obs}). These values were then used in Equation 2³⁰

$$k_2 = \frac{k_{obs} v_s}{v_i}$$

to determine a value of 0.14 ± 0.07 min⁻¹ for k₆, which was then, in turn, used in Equation 3³⁰

$$K_i^* = K_i \left(\frac{k_2}{k_1 + k_2} \right)$$

to establish a rate of 1.1 min^{-1} for k_5 . Rates for k_5 and k_6 were also determined by plotting k_{obs} as a function of [D-PheTrAP] and fitting the plot to Equation 4³⁰

$$k_{\text{obs}} = k_2 + \left(\frac{k_1[I]}{K_i^{\text{app}} + [I]} \right)$$

Results

Inhibitor Design and Synthesis

To successfully target the unique ternary complex formed during DXP synthase catalysis, the binding site of each substrate must be considered. Since alkylAPs are well-established pyruvate-competitive inhibitors, the principle challenge of this inhibitor design is targeting the D-GAP binding site. While there is presently no substrate-bound crystal structure of DXP synthase, steady-state kinetic analyses have revealed a key cationic binding pocket formed by R420 and R478A which is crucial for efficient binding of D-GAP via its phosphate.²³ We hypothesized that installing a negative charge on the alkylAP scaffold distal to the AP would result in more potent inhibitors through interactions with the cation binding pocket (Figure 4). For this reason, the inhibitors series was designed to include compounds with functionality that would be charged at physiological pH (Figure 5).

The synthesis of acetylphosphonates bearing simple alkyl substituents is well-established.^{11,14,16} A trialkyl phosphite and excess acetyl chloride are combined at ambient temperature to provide a dialkylAP and the alkyl chloride byproduct. Overnight treatment of the dialkylAP with LiBr results in dealkylation to give the desired alkylAP mono lithium salt. While this synthetic route is quick and reliable for the preparation of simple alkylAPs, the unstable dialkylAP intermediate (**2**) is susceptible to P-C cleavage which results in formation of the unwanted H-phosphonate and acylated side products, and significantly restricts the types of functional groups that can be incorporated into the alkyl substituent. This instability requires the introduction of functional diversity following the formation of the alkylAP mono lithium salt. CuAAC provides an ideal method for late-stage functionalization as it is broadly functional group tolerant and performs well in polar protic solvents required to dissolve the charged alkylAP intermediates.³¹

Homopropargyl acetylphosphonate (**3**, Scheme 1) was selected over azido or propargyl analogs because 1) alkyl azides are incompatible with P(III) species due to the Staudinger reaction, and 2) tripropargyl phosphite is known to spontaneously undergo a [2,3]-sigmatropic rearrangement to give an allene phosphonate.³² Homopropargyl analog **3** was prepared using the standard procedure for alkylAP synthesis (Scheme 1a).

To synthesize the azide coupling partners, we employed nucleophilic displacement of an alkyl bromide with sodium azide (for analogs **4** – **12**), diazotransfer from an imidazolyl sulfonyl azide³³ to D- and L-phenylalanine, alanine, and tyrosine (for analogs **15** – **17**), and nucleophilic aromatic substitution of a diazonium salt prepared from 2-, 3-, or 4-amino phenol (for analogs **18** – **20**, Scheme 1b). The azides corresponding to *N*-acyl sulfonamides

21 and **22** were prepared by carbodiimide coupling of commercially available 2-azidoacetic acid and methane- and *p*-toluenesulfonamide, respectively. The azide derived from L-phenylalanine and *p*-toluenesulfonamide were coupled in an analogous reaction to obtain the precursor for **23**.

AlkylAPs are an underexplored class of inhibitors, limited to simple aliphatic analogs^{11,14} and benzyl AP¹⁵. As such, we sought to explore the steric and electronic constraints of the DXP synthase active site by evaluation of inhibitors including, but not limited to, analogs with negative charge character distal to the AP. Analogs **4** – **12** (Figure 5c) were selected to test the steric preferences of the active site in the absence of charge with both aromatic and non-aromatic substituents. Carboxylate-containing analogs **13**, **15** – **17** were designed to place a negative charge into the putative cation binding pocket. Analogs **15** – **17** are derived from amino acids, with both their D and L enantiomers easily accessible providing additional probes to interrogate the active site architecture. Phenols (**18** – **20**) and *N*-acyl sulfonamides (**21** – **23**) were selected as isosteres of carboxylic acids due to their charged character at physiological pH and differing steric demands.

Negative Charge Distal to the Acetyl Phosphonate is Advantageous for Inhibitor Binding

To evaluate the inhibitory activity of the acetyl phosphonate analogs, we measured the activity of purified DXP synthase following a short preincubation with inhibitor using an IspC-enzyme coupled assay.^{11,14,20} Initial inhibitory activity was assessed by IC₅₀ determination under balanced assay conditions containing $2 \times K_m$ of both substrates. Inhibitors exhibiting potent inhibitory activity (IC₅₀ < 3 μ M) were further evaluated with varying substrates to establish mode of inhibition. The Morrison tight-binding inhibition model was used to obtain inhibition constants (K_i) for these potent inhibitors (Figure S1). This inhibition model is necessary as the concentration of free inhibitor does not remain constant (as assumed by standard steady-state models of inhibition) for K_i values approaching the concentration of enzyme being analyzed.^{30,34} Compounds with IC₅₀ values > 3 μ M were evaluated using a standard competitive inhibition model with varying concentrations of pyruvate (Figure S2).

Analysis of the inhibitory activity of compounds **4** – **23** reveals interesting structure-activity relationships, with the chief finding that incorporation of a negative charge distal to the acetyl group correlates with increased potency. This finding supports our hypothesis that incorporating negative charge in an acetyl phosphonate permits binding to the D-GAP and pyruvate binding sites. Acid **13** is 7-fold more potent than its methyl ester **14**. Likewise, comparison of phenethyl inhibitor **12** to carboxy-containing (*S*)- or (*R*)-**15**, differing only in the presence of carboxyl α to the triazole, reveals potency increases of 16- and 89-fold, respectively. In the absence of a charged group, introducing steric bulk (**8** – **12**) results in diminished inhibitory activity. *R*-Benzyl compound (*R*)-**15** (D-PheTrAP) is the most potent inhibitor in the series, displaying more potent inhibitory activity than its enantiomer (*S*)-**15** and its hydroxylated analog (*R*)-**17**. Surprisingly, the parent alkyne **3** was considerably more potent than its alkane counterpart BAP. The source of this increased potency is not readily apparent, however it is conceivable that the increased electronegativity of the terminal *sp*-hybridized carbon of **3**, may be able to engage in polar interactions unavailable to BAP.

Given the emerging preference for negative charge, we explored other negatively charged functional groups. *N*-acyl sulfonamides are known isosteres of carboxylic acids with approximately equal pK_a s. Interestingly, *N*-acyl sulfonamides **21** and **22** were approximately equal in their inhibitory activity despite the size difference between methyl (**21**) and toluene (**22**). The slightly better inhibitory activity displayed by *N*-acyl sulfonamides compared to carboxylic acid (**13**) suggested it might be possible to combine structural features of the more elaborated carboxylic acids and *N*-acyl sulfonamides to create more potent inhibitors. Unfortunately, the chimeric compound **23** is less potent than either of its parents, (*S*)-**15** or **22**. In addition to *N*-acyl sulfonamides, phenols were synthesized to test another functionality with negative charge character. Phenols **18** – **20** display potent inhibitory activity with *o*-phenolic compound **18** second only to D-PheTrAP in potency.

Time-dependent Inhibition by D-PheTrAP

The most potent inhibitor in the series, D-PheTrAP, was interrogated further to determine whether its inhibition mechanism is time-dependent. Given the putative conformational change that occurs upon binding of D-GAP to the E-LThDP complex,^{23,29,35} we reasoned that bisubstrate analogs could induce a conformation change manifesting as time-dependent inhibition. Kinetic analysis revealed a slow binding mechanism as evidenced by nonlinear reaction progress curves (Figure 6b, S3). From these data, we determined the DXP synthase – inhibitor complex undergoes isomerization to form a high affinity complex with forward and reverse isomerization rates of 1.1 and 0.14 min⁻¹. Furthermore, the slow binding behavior is consistent with a Type C, induced fit mechanism^{30,34} in which a measurable K_i^{app} corresponding to the affinity of the encounter complex and a measurable K_i^* corresponding to the isomerized, high affinity complex can be determined. Viewed in the context of the mechanism of DXP synthase, the time-dependent inhibition observed by D-PheTrAP could arise from inhibitor-induced conformational changes that mimic the natural reaction on DXPS. First, pyruvate binds to form the LThDP intermediate, mimicked by AP binding to form phosphonyl-LThDP intermediate. Subsequently, D-GAP binding induces a conformational change to promote decarboxylation, mimicked by polar interactions between the negatively charged carboxyl and the cationic pocket formed by Arg420 and Arg478. Previously reported alkylAP inhibitors of DXP synthase that lack a negative charge capable of interacting with the D-GAP binding site do not display time-dependent inhibition, suggesting that negative charge of the carboxyl group is responsible for the observed isomerization.

D-PheTrAP is a Selective Bisubstrate Inhibitor

Inhibitors reported here were designed to be bisubstrate inhibitors capable of binding to DXP synthase and mimicking the unique E-LThDP-GAP ternary complex on DXP synthase. To understand bisubstrate inhibitor behavior, the mechanism of DXP synthase must be considered. DXP synthase follows a preferred order random sequential mechanism with pyruvate binding first preferred.²³ K_d^{D-GAP} for free DXP synthase is approximately 40-fold higher than $K_m^{pyruvate}$ and ~ 80-fold higher than K_m^{D-GAP} ; as a result, increasing [pyruvate] results in decreased activity of these inhibitors while varying [D-GAP] has no measurable effect on inhibitor activity, consistent with competitive and noncompetitive modes of inhibition, respectively (Figure S4). The observed noncompetitive inhibition of a bisubstrate

inhibitor with respect to the second substrate in a preferred order random sequential mechanism such as this is well established^{36,37} and demonstrates only that D-GAP is incapable of displacing the inhibitor from DXP active site once it has bound. To establish where these more potent acetylphosphonate inhibitors are binding to DXP synthase and, by extension, whether or not they are bisubstrate inhibitors, simple mode of inhibition experiments are insufficient to fully understand inhibitor binding.

As a first step, we utilized a computational approach to determine if the phosphonolactyl species corresponding to the ThDP-D-PheTrAP covalent adduct (Figure 7) can interact with the cationic pocket in the D-GAP binding site. R420A and R478A DXP synthase variants were previously demonstrated to have greatly reduced affinity for D-GAP while maintaining affinity for pyruvate comparable to wildtype DXP synthase, suggesting a role of these Arg residues in D-GAP binding.²³ A “flexible residues” method³⁸ was applied in which the ThDP cofactor present in the *Deinococcus radiodurans* DXP synthase crystal structure²⁷ was modified to the phosphonoLThDP adduct corresponding to D-PheTrAP; this DXP synthase active site was then subjected to the AutoDock Vina docking algorithm³⁹ to find low energy modes of binding. This analysis revealed several predicted modes of binding that placed the carboxylate of D-PheTrAP in contact with R420 and R478 (Figure 7). Additionally, the phenyl ring was placed into a hydrophobic pocket easily accessed by the *R*-stereochemistry of D-PheTrAP, but not the *S*-stereochemistry of its enantiomer (*S*)-**15**, in line with the observed difference in inhibitory activity. This binding mode similarly explains the diminished activity of D-Tyr-derived (*R*)-**17** compared to D-PheTrAP as its *p*-hydroxyl interrupts the non-polar binding interface. While not definitive, this model clearly shows that D-PheTrAP is capable of simultaneously interacting when the ThDP cofactor and the D-GAP binding site, warranting biochemical validation.

In order to determine if the cationic binding pocket contributes to inhibitor binding, we compared the inhibitory activity of D-PheTrAP ((*R*)-**15**) and its des-carboxy counterpart phenethyl triazole **12** against wildtype *E. coli* DXP synthase and the R478A variant. The R478A variant was selected over the R420A variant due to its higher activity and saturable kinetics.²³ MAP, a known DXP synthase inhibitor possessing only a -CH₃ substituent, is incapable of interacting with the Arg478 after it has formed the covalent phosphonoLThDP adduct on the enzyme and thus offers an appropriate negative control. Our analysis reveals minimal differences in the inhibitory activity for MAP and triazole **12** with 3- and 0.9-fold changes, respectively, between the wildtype and variant enzymes (Table 1, Figure S5). However, the inhibitory activity of D-PheTrAP is 100-fold lower on R478A compared to wildtype, consistent with the 55-fold increase in D-GAP K_m observed on this variant. The requirement of both the negatively charged carboxylate and the positively charged Arg sidechain strongly suggests that D-PheTrAP acts as a bisubstrate inhibitor binding simultaneously in pyruvate and GAP binding sites to target the unique DXP synthase-LThDP-GAP ternary complex formation.

In addition to potent inhibitory activity, this mechanism of inhibition should impart exquisite selectivity for DXP synthase, given that other ThDP enzymes do not adopt this ternary complex. As an initial assessment of selectivity, D-PheTrAP was tested for inhibitory activity against porcine pyruvate dehydrogenase (PDH) E1 subunit. D-PheTrAP (1 mM)

displays only 15% inhibition against PDH (Figure S6). Under comparable conditions ($2 \times K_m$ pyruvate), less than 65 nM of D-PheTrAP is required for 15% inhibition of DXP synthase providing an estimated selectivity ratio of >15,000-fold for DXP synthase over mammalian PDH. For comparison, BAP, our first generation selective alkylAP inhibitor of DXP synthase displays only 60-fold more potent activity against DXP synthase compared to PDH.¹⁴ To our knowledge, D-PheTrAP is the most selective inhibitor of DXP synthase discovered.

Discussion

DXP synthase is vital to bacterial central metabolism. Through a unique mechanism, it catalyzes the synthesis of DXP which is used as a precursor to essential isoprenoids, ThDP, and PLP. Thus, DXP synthase represents a viable target in bacterial metabolism, highly sought for development of new antibacterial strategies. Here we presented a novel series of DXP synthase inhibitors prepared through the CuAAC of homopropargyl AP (**3**) and various organic azides several of which display nanomolar inhibition constants for DXP synthase. The most potent compound, D-PheTrAP, exhibits slow-tight-binding inhibition. Through computational and kinetic experiments, we demonstrated that D-PheTrAP behaves as a bisubstrate inhibitor binding to both the pyruvate and D-GAP binding sites in the DXP synthase active site. D-PheTrAP is the first inhibitor of its type to exploit the unique mechanism of DXP synthase. With an inhibition constant of 90 ± 10 nM, it is the most potent inhibitor to date and it displays a >15,000-fold preference for DXP synthase over PDH, providing yet another validation of the unique mechanistic enzymology of this important bacterial enzyme.

From an enzymological standpoint, the slow-binding behavior of D-PheTrAP is also intriguing. DXP synthase catalyzes the formation of the L-ThDP intermediate which is uniquely stable on DXP synthase and requires the presence of an acceptor substrate, D-GAP, to promote release of CO₂ and subsequent carbologation to form DXP. Mounting evidence suggests that DXP synthase undergoes conformational changes upon binding of substrates.^{23,29,35} The isomerization of DXP-D-PheTrAP to [DXP-D-PheTrAP]* is likely promoted by enzyme dynamics that underlie natural substrate-induced conformational changes, making D-PheTrAP an interesting new probe to study DXP synthase mechanism and guide selective inhibitor design.

The series of inhibitors presented are a clear proof of principle that DXP synthase can be selectively inhibited with bisubstrate analogs. Our modular synthesis utilizing the CuAAC to introduce diversity in the last step will allow the rapid synthesis of new, more potent, selective inhibitors, toward development of a new class of antimicrobial agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. DXP is a Vital Branchpoint Metabolite

DXP synthase catalyzes the condensation of pyruvate and D-GAP to produce DXP which is processed on to form ThDP, PLP, and isoprenoids, which are all essential to cell growth.

Figure 2.
Acetyl Phosphonates Inhibit Pyruvate Decarboxylase Enzymes through the Formation of a Covalent PLThDP Dead-end Intermediate.

Figure 3. The Mechanism of DXP Synthase

Unlike other ThDP-dependent enzymes, DXP synthase forms a long-lived LThDP intermediate. D-GAP binding increases the rate of decarboxylation by 600-fold.²²

a) DXP Synthase Active Site

b) DXP Synthase Active Site

Figure 4. Bisubstrate Inhibitor Design

Depiction of E-LThDP-GAP ternary complex and bisubstrate inhibitor. The active site of DXP synthase is simplified to reflect the binding sites of its substrates pyruvate and D-GAP. a) Pyruvate, in the form of LThDP, and D-GAP in the active site form the ternary complex which is unique to DXP synthase. b) Design of an alkylAP capable of acting as a bisubstrate inhibitor.

Figure 5. Enzyme Inhibitory Activity of Inhibitors 3–23

a) A representative inhibition plot fit to the Morrison K_I equation. b) Inhibition constants for inhibitors 3–23. All inhibitors were preincubated with DXP synthase for 5 minutes prior to reaction initiation with substrates. c) Structures of compounds 4 – 23. Asterisks indicate the location of the chiral center indicated by *R* or *S*.

Figure 6. D-PheTrAP is a Slow Binding Inhibitor of DXP Synthase

a) The mechanism of D-PheTrAP binding is described by the initial binding of the inhibitor to free enzyme to form an initial complex, described by K_i , followed by an isomerization to a higher affinity complex with forward and reverse isomerization rates of k_1 and k_2 , respectively. b) A representative set of progress curves of the DXP synthase reaction with varied concentrations of D-PheTrAP c). Observed isomerization (k_{obs}) rates were determined from progress curves as a function of inhibitor concentration. The square parabolic shape is indicative of slow-binding mechanism described in (a).^{30,34} The method for determining k_1 and k_2 is described in supporting information.

Figure 7. Docking Study with PLThDP Complex of D-PheTrAP

The covalent adduct corresponding to the DXP synthase catalyzed reaction of D-PheTrAP with ThDP was prepared in Pymol and docked into the DXP synthase active site (PDB: 2O1X) using a flexible residues approach in AutoDock Vina. Residue numbers with and without parentheses are shown for *E. coli* and *D. radiodurans* DXP synthase, respectively.

Scheme 1. Synthesis of Homopropargyl AP 3, General Reactions for the Formation of Azides, and the CuAAC To Form Triazole 4–23

a) Synthetic scheme for the synthesis of homopropargyl AP 3. b) General scheme for the synthesis of azide precursors. c) CuAAC to synthesis triazole APs **4 – 23**.

Table 1

Comparative Inhibitory Activity of MAP, Phenethyl Inhibitor 12, and D-PheTrAP with Wildtype and the R478A Variant of DXP Synthase.

	DXPS _{wt}	DXPS _{R478A}	DXPS _{R478A} /DXPS _{wt}
K_m^{D-GAP} (μM)	42 $\pm$ 7	2300 $\pm$ 400	55
IC ₅₀ ^{MAP} (μM)	1.8 $\pm$ 0.3	5.0 $\pm$ 0.6	3
IC ₅₀ ¹² (μM)	80 $\pm$ 30	70 $\pm$ 10	0.9
IC ₅₀ ^{D-PheTrAP} (μM)	0.3 $\pm$ 0.1	33 $\pm$ 4	100