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Enamide Prodrugs of Acetyl Phosphonate Deoxy-D-xylulose-5-phosphate Synthase Inhibitors as Potent Antibacterial Agents

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Abstract

To fight the growing threat of antibiotic resistance, new antibiotics are required that target essential bacterial processes other than protein, DNA/RNA, and cell wall synthesis which constitute the majority of currently used antibiotics. 1-Deoxy-D-xylulose-5-phosphate (DXP) synthase is a vital enzyme in bacterial central metabolism feeding into the *de novo* synthesis of thiamine diphosphate (ThDP), pyridoxal phosphate (PLP), and essential isoprenoid precursors, isopentenyl diphosphate and dimethylallyl diphosphate. While potent and selective inhibitors of DXP synthase *in vitro* activity have been discovered, their antibacterial activity is modest. To improve the antibacterial activity of selective alkyl acetylphosphonate (alkylAP) inhibitors of DXP synthase, we have synthesized peptidic enamide prodrugs of alkylAPs inspired by the natural product dehydrophos, a prodrug of methyl acetylphosphonate. This prodrug strategy achieves dramatic increases in activity against Gram-negative pathogens for two alkylAPs, butyl acetylphosphonate and homopropargyl acetylphosphonate, decreasing MICs against *E. coli* by 33- and nearly 2000-fold, respectively. Antimicrobial studies and LC-MS/MS analysis of alkylAP-treated *E. coli* establish that the increased potency of prodrugs is due to increased accumulation of alkylAP inhibitors of DXP synthase via transport of the prodrug through the OppA peptide permease and subsequent amide hydrolysis. This work demonstrates the promise of targeting DXP synthase for the development of novel antibacterial agents.

Graphical Abstract

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Evaluation of antibacterial activity of **30** and **31** in the presence of exogenous thiamin, phylogenetic analysis of peptide transporters of Gram-negative bacteria tested for antibacterial activity, LC-MS/MS standard curves and representative ion chromatograms, and NMR spectra for compounds **22** – **33**.

Keywords

bacterial central metabolism; phosphonate prodrug; 1-deoxy-D-xylulose 5-phosphate synthase; bacterial metabolic branch point; OppA peptide permease; Dehydrophos

Introduction

1-Deoxy-D-xylulose-5-phosphate (DXP) synthase is an essential enzyme in bacterial central metabolism and a potential new target under investigation in our laboratory for the development of novel antibacterial strategies to combat antibiotic resistance. It catalyzes the thiamin diphosphate (ThDP)-dependent decarboxylative condensation of pyruvate and D-glyceraldehyde-3-phosphate (D-GAP) to form DXP, a branchpoint intermediate that feeds into the *de novo* biosynthesis of thiamin diphosphate (ThDP), pyridoxal phosphate (PLP), and the methyl erythritol phosphate (MEP) pathway to essential isoprenoids. DXP synthase is present in most pathogenic bacteria, the chloroplast of plants, and apicomplexan parasites, but is absent in animals.^{1–5} Its structure and mechanism are unique when compared to other ThDP-dependent enzymes.^{6–9} Its active site is nearly twice the volume of mammalian pyruvate dehydrogenase (PDH) and transketolase (TK), and its domain arrangement places the active site at the interface of two domains within the same monomer rather than at the dimer interface as in PDH and TK.¹⁰ Additionally, unlike all other known ThDP-dependent enzymes which display ping-pong kinetics, DXP synthase uses a preferred order random sequential mechanism requiring the formation of a unique Enzyme-LactylThDP-(D-GAP) ternary complex (Scheme 1) prior to release of the first product, CO₂.^{7–9} These mechanistic findings evoke questions about DXP synthase function in bacteria⁶, and highlight targetable features of DXP synthase that establish a basis for the development of selective chemical probes to study DXP synthase function and to serve as starting points for new antimicrobial agents.¹¹

We have developed alkylAPs as selective mechanism-based inhibitors of DXP synthase bearing an acetylphosphonate group that mimics pyruvate and a sterically demanding alkyl group that is readily accommodated by the large active site. These inhibitors interact with the ThDP cofactor to form reversible covalent intermediates (phosphono-LThDP).^{11–13} Our early studies identified butyl acetylphosphonate (BAP) as a promising selective inhibitor with micromolar inhibitory activity and 60-fold selectivity for DXP synthase over PDH and no inhibitory activity against TK.¹² Additional studies demonstrated that BAP exhibits growth medium-dependent antibacterial activity against a number of clinically-important bacterial pathogens, with its activity being markedly increased in defined, minimal medium compared to rich medium.^{13,14} Second generation alkylAPs¹¹ (Scheme 1) were recently developed as bisubstrate analog inhibitors capable of interacting with both the ThDP

cofactor and D-GAP binding site. *In vitro* analyses reveal potent nanomolar inhibition and exquisite selectivity for DXP synthase (>15,000-fold selectivity over PDH) in the best case, further confirming the unique mechanism of this interesting bacterial enzyme and opening new avenues for inhibitor development.

Inhibitor uptake into bacterial cells has emerged as a key barrier in the development of selective DXP synthase inhibitors as potential antibacterial agents.¹⁵ Thus, we have sought a strategy to increase the intracellular accumulation of DXP synthase inhibitors to improve their antibacterial activity. Herein, we show that homopropargyl acetylphosphonate (hpAP $K_i = 470 \pm 40$ nM)¹¹, a precursor to second generation bisubstrate analog inhibitors and a more potent inhibitor of DXP synthase than BAP, displays growth medium-dependent activity against *E. coli* that is similar to BAP. Second generation DXP synthase inhibitors exhibit variable, growth medium-dependent activity and poor correlation to enzyme inhibitory activity with the most potent enzyme inhibitors lacking antibacterial activity altogether. A prodrug strategy inspired by the natural product dehydrophos (Figure 1) was applied to alkylAPs in an effort to increase the efficiency of inhibitor uptake. The peptidic prodrugs take advantage of active transport through the OppA peptide transporter, which is known to be upregulated under nutrient-limited conditions associated with infection¹⁶ and has been demonstrated to be required for host colonization, specifically, in uropathogenic *E. coli* (UPEC).¹⁷ Prodrugs were synthesized and evaluated against several Gram-negative bacteria revealing a dramatic increase in antibacterial activity for some DXP synthase inhibitors against several clinically-relevant pathogens. Active prodrugs were shown to behave similarly to the parent alkylAPs with regard to growth medium dependence and ablation of activity by exogenous thiamin (Figure S1), supporting a mechanism of antimicrobial activity involving conversion of enamide prodrugs to active alkylAPs. LC-MS/MS analysis of *E. coli* cell following prodrug treatment of and use of permease deletion mutants provide direct evidence for the conversion of the enamide prodrug to alkylAP following prodrug uptake by the OppA peptide transporter.

Results

Potent alkylAP-mediated inhibition of DXP synthase *in vitro* does not correlate with antibacterial activity.

BAP was previously identified as a selective DXP synthase inhibitor which possesses antibacterial activity against several Gram-negative pathogens.^{13,14} Work in our lab has recently demonstrated the application of CuAAC chemistry to expand SAR around the alkylAP scaffold toward enhancing potency and selectivity of inhibition of DXP synthase (Figure 2).¹¹ This new series of alkylAP DXP synthase inhibitors includes bisubstrate analog inhibitors which effectively target the unique Enzyme-LThDP-(D-GAP) ternary complex, resulting in high potency and exquisite selectivity of inhibition of DXP synthase.¹¹ Here, we sought to learn whether this increased inhibition potency leads to enhanced antimicrobial activity. Thus, inhibitors (**1** – **21**, Figure 2) were evaluated against *E. coli* MG1655 in liquid culture. Each compound was tested for its ability to halt cell growth and compared to a no inhibitor control to determine fractional growth.

The alkyne precursor to second generation bisubstrate analog inhibitors, homopropargyl acetylphosphonate (hpAP **1**), and alkylAPs **2** – **10** linking uncharged alkyl or aryl groups to the acetylphosphonate through a triazole linker generally exhibit more potent antibacterial activity against *E. coli* than the charged bisubstrate mimics **11**, **13** – **21**, despite their generally inferior biochemical inhibitory activity (Figure 2).¹³ Aromatic inhibitors **7**, **8**, and **10** exhibit the most potent antibacterial activity with MICs of 4.9, 2.4, and 2.4 μ M, respectively. Curiously, the closely related benzyl triazole alkylAP **6** is inactive as an antimicrobial agent under these conditions (MIC > 1250 μ M) despite having similar enzyme inhibitory activity to **7**, **8**, and **10**.

Dianionic bisubstrate analogs **11**, **13** – **21** generally display weak antibacterial activity or fail to inhibit bacterial growth altogether. The simplest bisubstrate inhibitor **11** and its methylated analogs (*S*)-**13** and (*R*)-**13** exhibit modest growth inhibition (MIC = 313, 156, and 625 μ M) while the most potent enzyme inhibitors (*R*)-**14**, **16**, and **17** (K_i = 90, 110, and 320 nM, respectively) are inactive against *E. coli* up to 1.25 mM. The *N*-acyl sulfonamide inhibitors **19** and **20**, derived from carboxylate **11**, display modest antibacterial activity. Masking the negative charge of carboxylate **11** in the form of methyl ester **12** results in a loss of antibacterial activity suggesting a simple charge-masking prodrug strategy will be ineffective for alkylAPs.

Antibacterial activity does not correlate with enzyme inhibitor activity for these inhibitors (Figure 2). For example, hpAP exhibits 6-fold higher inhibition potency against DXP synthase compared to BAP, but 8-fold lower antibacterial activity against *E. coli* in liquid culture, and the most potent inhibitors of DXP synthase, (*R*)-**14** and **16**, lack antibacterial activity altogether.

Design and synthesis of enamide prodrugs of alkylAPs.—We hypothesized that the lack of a correlation between enzyme inhibitory activity and antimicrobial activity is due to variable inhibitor uptake and access to the DXP synthase target in bacterial cells. Given the potential importance of these probes to validate DXP synthase as a target and study its function in bacterial pathogens, we sought a strategy to enhance uptake and, thus, antimicrobial activity of our alkylAP inhibitors. Methyl acetylphosphonate (MAP), an indiscriminate inhibitor of ThDP-dependent enzymes, has been known to biochemists for decades¹⁹ and to Nature for much longer; *Streptomyces luridus* produces the natural product dehydrophos, a peptidic prodrug of MAP (Figure 1).²⁰ As a tripeptide mimic, dehydrophos enters bacterial cells through the OppA peptide transporter where it is then hydrolyzed by several intracellular peptidases to yield a phosphono-enamine intermediate which can spontaneously tautomerize to an imine and undergo hydrolysis to release MAP (Figure 1).¹⁸ Dehydrophos exhibits growth inhibitory activity against bacteria, including *Escherichia coli*, *Bacillus subtilis*, and *Salmonella enterica*, while MAP was shown to be inactive.^{18,21}

The OppA peptide transporter is of particular interest as it has been implicated in bacterial virulence.¹⁶ OppA is the substrate recognition portion of the oligopeptide permease (Opp) transporter complex, an ABC transporter, which spans the inner membrane of many bacteria. Important for the import of short peptides, the Opp transport system is required for colonization by UPEC due to a reliance on amino acids and short peptides present in the

bladder and urine to fulfill metabolic needs during infection.¹⁷ Exploiting the reliance of UPEC on the Opp system could provide selectivity for pathogenic bacteria over the gut microbiota.

In this work, we have applied a dehydrophos-inspired prodrug strategy to the well-studied, selective DXP synthase inhibitor BAP and the structurally similar hpAP (**1**), which shows more potent enzyme inhibitory activity against DXP synthase compared to BAP, but less potent antimicrobial activity against *E. coli*. In addition, we applied this prodrug strategy to the highly potent and selective bisubstrate analogs, (*R*)-**14** and **16**, both of which are inactive against *E. coli*.

The prodrug synthesis reported by Kuemin and van der Donk²¹ provided an excellent starting point for the development of new enamide prodrugs. Commercially available *N*-Cbz-L-serine was converted to the *N*-Boc-Gly-(L-Leu)-phosphonoserine intermediate (**22**) over several steps, as previously described.²¹ Phosphonate **22** was then selectively demethylated to produce the monoester triethylammonium salt (**23**) after purification (Scheme 2). The homopropargyl phosphonoester was installed via the Mitsunobu reaction²² to produce the mixed diester which was immediately subjected to TBAF-mediated desilylation to yield **24**. Attempts to synthesize the mixed ester via electrophilic activation of the phosphorus center (e.g., by carbodiimide coupling or by production of the phosphonochloridate via oxalyl chloride) failed, possibly due to steric hinderance of the phosphonic acid. The free alcohol of **24** was activated by reaction with mesyl chloride to give the corresponding mesylate which underwent elimination under basic conditions to yield the enamide **26**. Subsequent acid-promoted cleavage of Boc provided the penultimate product (**28**) which was subjected to another thiophenol-mediated deprotection to selectively remove the methyl ester. The resulting desired zwitterionic prodrug **30** was obtained following C₁₈ reversed phase chromatography. Synthesis of triazole-containing prodrugs of bisubstrate analogs **32** and **33** was achieved through CuAAC²³ of alkyne **30** with the corresponding azides (Scheme 3).

The synthesis of the BAP enamide prodrug **31** was easily accomplished by subjecting **24** to catalytic hydrogenation to generate the butyl ester **25** prior to the generation of the corresponding olefin **27** (Scheme 2). BAP prodrug **31** was accessed from **27** as described above for the synthesis of alkyne **30**.

Antimicrobial activity of alkylAP enamide prodrugs.—Prodrugs **30** – **33** were evaluated for bacterial growth inhibitory activity against *E. coli* grown in M9-glucose. Prodrugs **30** and **31** display a remarkable increase in potency compared to their acetyl phosphonate parent drugs. The MIC decreased by nearly 2000-fold for the hpAP prodrug (**30**) compared to the parent inhibitor hpAP (**1**, Table 1). Similarly, a 33-fold increase in potency was observed for the BAP prodrug **31**. Interestingly, both of these analogs outperform the natural product dehydrophos (MIC = 680 nM). However, neither prodrugs **32** nor **33** displays enhanced potency compared to bisubstrate analog inhibitors (*R*)-**14** and **16**, and thus were not studied further. The additional negative charge and substantial increase in size of (*R*)-**14** and **16** compared to dehydrophos and prodrugs **30** and **31** may reduce affinity for OppA to prevent transport.

To understand how the antibacterial activity of **30** and **31** compare to that of their parent alkylAPs, the prodrugs were further evaluated under exogenous thiamin (100 nM), a condition known to suppress the activity of alkylAPs targeting DXP synthase.¹³ As expected, supplementation with thiamin rescues the growth of cells treated with **30** or **31**, consistent with the behavior of the parent alkylAPs and suggesting that the prodrugs are being processed intracellularly to active alkylAP inhibitors of DXP synthase to exert their antimicrobial activity.

Encouraged by the potent activity observed for **30** and **31** against *E. coli* MG1655, we sought to examine the potency of these prodrugs against more clinically-relevant Gram-negative bacteria. Similar to the lab strain of *E. coli*, other bacterial pathogens including *Salmonella typhimurium*, *Enterobacter cloacae*, and a clinical strain of *E. coli* are all susceptible to growth inhibition by the homopropargyl prodrug **30** at sub-micromolar concentrations (Table 2). Notably, only modest antibacterial activity was observed against *Klebsiella oxytoca* in the presence of **30**, and both *Pseudomonas* species tested were resistant to these prodrugs up to 40 μ M. The lack of activity against the more distantly-related *Pseudomonas* species suggests that differences in active uptake and/or activation likely arising from differences in affinity of prodrugs for OppA or hydrolases, will likely dictate microbial susceptibility to the prodrugs (Figure S2). This suggests that future iterations of enamide alkylAP prodrugs may be tuned for selective uptake by other peptide transporters toward the development of narrow spectrum agents.

OppA is required for transport of alkylAP enamide prodrugs in *E. coli*.—Peptide uptake is an important and well-studied mechanism used by bacteria to acquire amino acids for cell anabolism and to recycle cell wall components.²⁴ The oligopeptide permease (Opp) system functions to transport peptides, preferentially tri- and tetra-peptides, into the cell in an ATP-dependent manner.^{25,26} Studies by Cierllo et al.¹⁸ demonstrated that in *Salmonella enterica*, OppA is required for the uptake of dehydrophos and that its intracellular activation to provide MAP is catalyzed by several different peptidases (PepA, B, D, N). To determine which proteins are necessary for the uptake and activation of prodrugs **30** and **31** in *E. coli*, we measured prodrug activity in several different deletion mutants obtained from the Keio collection²⁷ (Figure 3). Our results show that the *oppA* deletion mutant is resistant to prodrugs **30** and **31**, indicating that OppA is required for transport of **30** and **31** in *E. coli*, similar to its requirement for dehydrophos transport in *S. enterica*.¹⁸ The deletion of other uptake systems does not affect the growth inhibitory activity of **30** and **31**. The activities of the parent acetyl phosphonates (BAP and hpAP) are also unchanged against the *dpp*, *opp*, or *tpp* peptide permease deletion mutants, indicating these transporters are not required for alkylAP uptake. Single knockout mutants of peptidases PepA, PepB, and PepC remain susceptible to prodrugs **30** and **31**, suggesting that there is likely redundancy among peptidases that can activate the enamide prodrugs once imported to the cytoplasm, similar to what is observed for activation of dehydrophos in *S. enterica*.¹⁸

Enamide prodrugs are rapidly converted to alkylAP inhibitors of DXP synthase in *E. coli*.—Toward establishing a mechanism for the dramatic increase in potency of the enamide prodrugs compared to the parent alkylAPs, an LC-MS/MS method

was developed to directly observe the intracellular concentrations of both enamide prodrugs and parent alkylAPs. The treatment of *E. coli* with prodrug results in an immediate observable accumulation of the parent alkylAP and a steady, low concentration of enamide prodrug (Figure 4), suggesting prodrugs are efficiently converted to parent inhibitors upon transport into *E. coli*. Treatment of *E. coli* with 5 μ M of either parent alkylAP or enamide prodrug reveals a significant increase in the intracellular accumulation of alkylAP with the enamide prodrug compared to direct treatment with BAP or **1** (Figure 5).

Discussion

The ThDP-dependent enzyme DXP synthase is an essential bacterial enzyme key to the *de novo* biosynthesis of isoprenoids, ThDP, and PLP. Its involvement in three separate and vital processes in bacterial central metabolism suggest a key role for DXP synthase in bacterial pathogen metabolism during infection. Our insights from mechanistic studies^{28–30} raise additional questions about other potential functions of this target that require further investigation. To address these interesting biological questions, DXP synthase probes displaying higher penetration into bacterial cells are required. Here, we present the synthesis and evaluation of peptidic prodrugs of alkylAP DXP synthase inhibitors, designed to display enhanced uptake by the OppA permease. The alkylAP prodrugs **30** and **31** exhibit excellent antibacterial activity by a mechanism involving transport and activation of alkylAP prodrugs to the parent alkylAPs with enhanced activity due to increased intracellular accumulation of the active alkylAPs, BAP and hpAP (**1**). Furthermore, the relative activities of the prodrugs **30** and **31** correlate with the enzyme inhibitory activities of their parent alkylAPs suggesting DXP synthase inhibitor activity is predictive of antibacterial activity if cellular uptake is similar.

Interestingly, extension of the dehydrophos-inspired prodrug strategy to the most potent bisubstrate inhibitors of DXP synthase, **32** and **33**, does not afford the same enhancement in antibacterial activity. Dehydrophos and alkylAP analogs studied here enter bacterial cells through the OppA transporter. Early work to determine substrate preferences revealed that most tri- and tetrapeptides are substrates for transport.^{25,26} OppA is also capable of transporting carboxylic acid esters²⁵, consistent with its transport of phosphonyl monoesters which contain both carboxylate and ester character. Prodrugs **32** and **33** may be too large to be recognized by OppA and likely more closely resemble a penta- or hexapeptide than the preferred tripeptide substrate. Alternatively, the added negative charge distal to the amino terminus could be detrimental to transporter binding. Given the redundancy and broad substrate specificity of bacterial peptidases, it seems unlikely that added bulk and charge introduced with prodrugs **32** and **33** precluded their intracellular activation, however, we cannot exclude this possibility at this time. Future work will explore new peptidic enamide prodrugs of alkylAPs in search of scaffolds which will permit the most potent alkylAP DXP synthase inhibitors to accumulate in bacteria.

The molecular determinants of the transport and accumulation of parent alkylAPs are not fully understood. While simple first generation alkylAPs display similar low micromolar inhibition constants for DXP synthase¹³, BAP possesses the most potent antibacterial activity. Both longer and shorter chain alkylAPs are apparently unable to reach the same

intracellular concentrations resulting in reduced activity. Second generation alkylAPs examined here reveal an apparent preference for uncharged aromatic functional groups as observed with **7** – **8** and **10** however the addition of a charged functional group, present in the most potent DXP synthase inhibitors, appears to abolish antibacterial activity in some cases. In other cases, the presence of a negative charge without added substituents at the bridging methylene to the triazole moiety appears to be tolerated to some extent as evidenced by **11**, **13**, **19**, and **20**.

The development of antibacterial agents through the targeted inhibition of DXP synthase has been a goal since the discovery of the enzyme over 20 years ago. Even with the development of selective inhibitors of the enzyme, achieving potent antibacterial activity remains a challenge. While the dehydrophos-inspired strategy reported herein may have limited flexibility for significant structural modifications to the prodrug scaffold, its application to simple alkylAPs can dramatically enhance alkylAP potency. Our results demonstrate the advantage of such strategies for functional studies of this target and the potential of DXP synthase inhibitors to be potent antibacterial agents when intracellular accumulation is achieved. New delivery strategies that are amenable to the most potent DXP synthase inhibitors in the alkylAP class promise to produce extremely potent antibacterial agents.

Experimental

General.

Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. Diisopropylethylamine (*i*Pr₂NEt) and dichloromethane (DCM) were distilled after drying over 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 (Millipore) were used for thin layer chromatography (TLC) and developed with Ninhydrin except where otherwise noted. ¹H and ³¹P NMR spectra were acquired on a Bruker Avance III 500 spectrometer operating at 500 MHz for ¹H and 202 MHz for ³¹P. For ¹H NMR, chemical shift values are reported as δ (ppm) relative to CHCl₃ at δ 7.27 ppm, MeOH at δ 3.31 ppm, or DMSO at δ 2.50 ppm. ³¹P chemical shifts are reported relative to triphenylphosphine oxide (TPPO) 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.

Boc-*N*-Gly-Leu-*N*-(*O*-tertbutyldimethylsilyl ether)phosphonoserine dimethyl diester (**22**).

N-Boc protected glycine (0.689 g, 3.93 mmol) and HATU were suspended in DMF (11 mL) and *i*Pr₂NEt (1.15 mL, 6.56 mmol) was added. A solution of H₂N- Leu-*N*-(*O*-tertbutyldimethylsilyl ether)phosphonoserine dimethyl diester (1.30 g, 3.28 mmol) in DMF (5 mL) was added to the glycine/HATU mixture, and the mixture was stirred at ambient temperature for 16 h. The reaction mixture was diluted with DCM (20 mL) and washed with aqueous citric acid (5% w/v, 30 mL), saturated aqueous NaHCO₃ (30 mL), and saturated aqueous NaCl (30 mL). The organic layer was dried over MgSO₄, then filtered, and

condensed under reduced pressure. The resultant residue was purified by silica flash chromatography (1:0 to 9:1 DCM/MeOH) to yield **22** as a colorless oil (3:2 mixture of diastereomers, 0.984 g, 54% yield). ^1H NMR (500 MHz, CDCl_3) δ ppm 6.39 – 6.67 (m, 2 H), 5.04 – 5.30 (m, 1 H), 4.49 (br. s., 2 H), 3.94 – 4.09 (m, 2 H), 3.79 – 3.89 (m, 2 H), 3.76 (d, $J=10.7$ Hz, 6 H), 2.81 (br. s., 6 H), 1.69 – 1.79 (m, 3 H), 1.50 – 1.60 (m, 1 H), 1.46 (s, 9 H), 0.94 (br. s., 6 H), 0.91 (br. s., 9 H), 0.08 (br. s., 6 H). ^{31}P NMR (202 MHz, CDCl_3) δ ppm 0.04 (s), –0.08 (s). HRMS (ESI) calcd for $\text{C}_{23}\text{H}_{49}\text{N}_3\text{O}_8\text{PSi}$ $[\text{M} + \text{H}]^+$ 554.3021, found 554.3026.

Boc-*N*-Gly-Leu-*N*-(*O*-tertbutyldimethylsilyl ether)phosphoserine methyl monoester (23).

To a solution of dimethyl phosphonate **22** (3.00 g, 5.42 mmol) in THF (13.5 mL) was added $i\text{Pr}_2\text{NEt}$ (2.84 mL, 16.3 mmol) followed by thiophenol (0.83 mL, 8.13 mmol). The solution was stirred at ambient temperature for 5 days at which point additional $i\text{Pr}_2\text{NEt}$ (2.84 mL, 16.3 mmol) and thiophenol (0.83 mL, 8.13 mmol) were added. After overnight stirring, the reaction had reached completion as observed by ^{31}P NMR. The reaction mixture was transferred to a silica plug which was washed sequentially with THF (30 mL), DCM (30 mL), and DCM/MeOH/triethylamine (8:1.9:0.1, 100 mL). The desired product was collected in the DCM/MeOH/TEA elution which was condensed under reduced pressure and then further dried *in vacuo* to yield monoester **23** as a colorless oil (3:2 mixture of diastereomers, 2.974 g, 86% yield). ^1H NMR (500 MHz, $\text{MeOD}-d_4$) δ ppm 4.48 – 4.65 (m, 1 H), 4.25 – 4.41 (m, 1 H), 4.03 – 4.14 (m, 1 H), 3.65 – 3.82 (m, 2 H), 3.55 (t, $J=10.5$ Hz, 3 H), 3.04 – 3.17 (m, 6 H), 1.54 – 1.76 (m, 3 H), 1.45 (s, 9 H), 1.27 (t, $J=7.2$ Hz, 9 H), 0.93 (t, $J=5.7$ Hz, 6 H), 0.88 (d, $J=5.2$ Hz, 9 H), 0.01 – 0.11 (m, 6 H). ^{31}P NMR (202 MHz, $\text{METHANOL}-d_4$) δ ppm –7.94 (s), –8.08 (s). HRMS (ESI) calcd for $\text{C}_{22}\text{H}_{47}\text{N}_3\text{O}_8\text{PSi}$ $[\text{M} + \text{H}]^+$ 540.2865, found 540.2869.

Boc-*N*-Gly-Leu-*N*-phosphoserine homopropargyl methyl diester (24).

To a stirred solution of phosphonate monoester **23** (2.97 g, 4.64 mmol) in THF (40 mL), triphenylphosphine (2.43 g, 9.28 mmol) and homopropargyl alcohol (0.70 mL, 9.28 mmol) were added followed by dropwise addition of diisopropyl azodicarboxylate (DIAD, 1.82 mL, 9.28 mmol). After 30 min at ambient temperature, the starting material was consumed as determined by ^{31}P NMR. Solvent was removed under reduced pressure and the resulting residue was purified by silica flash chromatography (1:0 to 9:1 DCM/MeOH) to provide a mixture of mixed diester and TPPO.

The mixture of mixed diester and TPPO was dissolved in THF (30 mL) and treated with tetrabutylammonium fluoride (TBAF, 1.0 M in THF, 9.3 mL, 9.3 mmol). The reaction mixture was stirred at ambient temperature for 30 min, at which point the reaction was quenched by the addition of silica (10 g). Solvent was removed under reduced pressure and the resulting silica mixture was loaded onto a silica flash column (1:0 to 9:1 DCM/MeOH) and purified to yield **24** as a white solid (mixture of diastereomers, 1.76 g, 85% yield). ^1H NMR (500 MHz, CDCl_3) δ ppm 7.21 (br. s., 1 H), 6.75 – 6.98 (m, 1 H), 5.35 (br. s., 1 H), 4.52 (br. s., 2 H), 4.09 – 4.26 (m, 2 H), 3.68 – 4.08 (m, 8 H), 2.59 (br. s., 2 H), 2.01 – 2.11 (m, 1 H), 1.69 (br. s., 2 H), 1.59 (d, $J=7.7$ Hz, 1 H), 1.46 (br. s., 9 H), 0.94 (br. s., 6

H). ^{31}P NMR (202 MHz, CDCl_3) δ ppm -1.06 - -1.00 (m), -1.08 (br. s.), -1.18 (s). HRMS (ESI) calcd for $\text{C}_{20}\text{H}_{37}\text{N}_3\text{O}_8\text{P}$ $[\text{M} + \text{H}]^+$ 478.2313, found 478.2314.

Boc-*N*-Gly-Leu-*N*-phosphoserine butyl methyl diester (25).

To a solution of alkyne **24** (0.382 g, 0.8 mmol) in MeOH (10 mL) was added Pd/C (10% w/w, 40 mg). Hydrogen was bubbled through the solution for 2 min to saturate the solution and then the reaction mixture was stirred vigorously overnight at ambient temperature under an atmosphere of H_2 maintained by a balloon of H_2 . After 16 h, the reaction mixture was filtered to remove solids and volatiles were removed under reduced pressure. The resulting residue was purified by silica flash chromatography (1:0 to 9:1 DCM/MeOH) yielding butyl methyl diester **25** as a white solid (1:1:2:2 mixture of diastereomers, 0.219 g, 57% yield). ^1H NMR (500 MHz, $\text{MeOD}-d_4$) δ ppm 4.44 – 4.58 (m, 2 H), 4.10 (q, $J=6.5$ Hz, 2 H), 3.82 – 3.90 (m, 1 H), 3.79 (d, $J=10.7$ Hz, 3 H), 3.74 (br. s., 1 H), 1.57 – 1.78 (m, 5 H), 1.47 (s, 9 H), 1.40 – 1.45 (m, 2 H), 1.19 (m, $J=6.1$ Hz, 1 H), 0.91 – 1.02 (m, 9 H). ^{31}P NMR (202 MHz, $\text{MeOD}-d_4$) δ ppm -0.46 (br. s.), -0.49 (br. s.), -0.70 (s), -0.77 (s). HRMS (ESI) calcd for $\text{C}_{20}\text{H}_{41}\text{N}_3\text{O}_8\text{P}$ $[\text{M} + \text{H}]^+$ 482.2626, found 482.2629.

Boc-*N*-Gly-Leu-*N*-phosphonodehydroalanine homopropargyl methyl diester (26).

Alcohol **3** (1.38 g, 2.88 mmol) was dissolved in DCM (29 mL) and cooled to 0°C . To the solution was added $i\text{Pr}_2\text{NEt}$ (2.0 mL, 11.5 mmol) followed by mesyl chloride (0.89 mL, 11.5 mmol). After 10 min at 0°C , the reaction was quenched by the addition of silica (3.65 g) and volatiles were removed under reduced pressure. The resulting silica mixture was then loaded onto a silica flash column and purified (EtOAc to 9:1 EtOAc/MeOH) to yield the corresponding mesylate which was used immediately in the next reaction.

DBU (0.86 mL, 5.8 mmol) was added to a solution of the corresponding mesylate in anhydrous DCM (12 mL). The reaction mixture was stirred at ambient temperature for 1 h at which point it was quenched by the addition of silica (3.5 g). Volatiles were removed under reduced pressure, and the resulting silica mixture was loaded onto silica flash column and purified (2:8:0 to 0:10:0 to 0:9:1 Hexanes/EtOAc/MeOH) to yield alkene **26** as a white solid (1:1 mixture of diastereomers, 714 mg, 54% yield). ^1H NMR (500 MHz, CDCl_3) δ ppm 7.84 (br. s., 1 H), 6.61 (br. s., 1 H), 6.64 (d, $J=53.0$ Hz, 1 H), 5.70 (dd, $J=19.5$, 7.4 Hz, 1 H), 5.19 (br. s., 1 H), 4.49 (br. s., 1 H), 4.08 – 4.24 (m, 2 H), 3.79 (d, $J=11.5$ Hz, 3 H), 3.69 – 3.91 (m, 2 H), 2.62 (br. s., 2 H), 2.09 (d, $J=13.4$ Hz, 1 H), 1.54 – 1.79 (m, 3 H), 1.46 (br. s., 9 H), 0.95 (br. s., 6 H). ^{31}P NMR (202 MHz, CDCl_3) δ ppm -12.10 (s), -12.16 (s). HRMS (ESI) calcd for $\text{C}_{20}\text{H}_{35}\text{N}_3\text{O}_7\text{P}$ $[\text{M} + \text{H}]^+$ 460.2207, found 460.2211.

Boc-*N*-Gly-Leu-*N*-phosphonodehydroalanine butyl methyl diester (27).

Alcohol **25** (0.219 g, 0.46 mmol) was dissolved in anhydrous DCM (4.6 mL) and cooled to 0°C . To the solution was added $i\text{Pr}_2\text{NEt}$ (0.32 mL, 1.82 mmol) followed by mesyl chloride (0.14 mL, 1.82 mmol). After 20 min at 0°C , the reaction was quenched by the addition of silica (2 g) and volatiles were removed under reduced pressure. The resulting silica mixture was then loaded onto a silica flash column and purified (EtOAc to 9:1 EtOAc/MeOH) to yield the corresponding mesylate which was used immediately in the next reaction.

DBU (0.136 mL, 0.91 mmol) was added to a solution of corresponding mesylate in anhydrous DCM (2 mL). The reaction mixture was stirred at ambient temperature for 1 h at which point it was quenched by the addition of silica (1 g). Volatiles were removed under reduced pressure and the resulting silica mixture was loaded onto silica flash column and purified (2:8:0 to 0:10:0 to 0:9:1 Hexanes/EtOAc/MeOH) to yield alkene **27** as a white solid (1:1 mixture of diastereomers, 162 mg, 77% yield). ¹H NMR (500 MHz, CDCl₃) δ ppm 7.83 (br. s., 1 H), 6.68 (br. s., 1 H), 6.60 (br. s., 1 H), 5.61 (dd, *J*=18.7, 6.4 Hz, 1 H), 5.20 (br. s., 1 H), 4.49 (br. s., 1 H), 3.99 – 4.17 (m, 2 H), 3.83 (q, *J*=18.1 Hz, 2 H), 3.76 (d, *J*=11.2 Hz, 3 H), 1.62 – 1.78 (m, 4 H), 1.46 (br. s., 10 H), 1.41 (q, *J*=7.1 Hz, 2 H), 0.95 (br. s., 9 H). ³¹P NMR (202 MHz, CDCl₃) δ ppm –12.09 (s), –12.12 (s). HRMS (ESI) calcd for C₂₀H₃₉N₃O₇P [M + H]⁺ 464.2520, found 464.2523.

H₂N-Gly-Leu-*N*-phosphonodehydroalanine homopropargyl methyl diester -TFA salt (**28**).

Anisole (0.24 mL, 2.22 mmol) was added to a solution of Boc-protected amine **26** (0.600 g, 1.31 mmol) in DCM (13 mL). TFA (1.42 mL, 18.54 mmol) was added and the reaction mixture was stirred at ambient temperature for 4 h at which point the starting material was consumed. Toluene (20 mL) was added to the reaction mixture and all volatiles were then removed under reduced pressure. The resulting residue was purified by silica flash chromatography (9:1 DCM/MeOH) yielding the TFA salt amine **28** as a white solid (0.538 g, 87% yield). ¹H NMR (500 MHz, MeOD-*d*₄) δ ppm 6.43 (d, *J*=42.0 Hz, 1 H), 5.82 (d, *J*=17.9 Hz, 1 H), 4.57 (t, *J*=7.5 Hz, 1 H), 4.15 (quin, *J*=7.3 Hz, 2 H), 3.81 (d, *J*=11.3 Hz, 3 H), 3.70 (s, 2 H), 2.61 (q, *J*=6.3 Hz, 2 H), 2.37 – 2.43 (m, 1 H), 1.71 (spt, *J*=6.7 Hz, 1 H), 1.57 – 1.64 (m, 2 H), 0.98 (d, *J*=6.6 Hz, 3 H), 0.95 (d, *J*=6.4 Hz, 3 H). ³¹P NMR (202 MHz, MeOD-*d*₄) δ ppm –11.44 (s). HRMS (ESI) calcd for C₁₅H₂₇N₃O₅P [M + H]⁺ 360.1683, found 360.1682.

H₂N-Gly-Leu-*N*-phosphonodehydroalanine butyl methyl diester (**29**).

A stirred solution of Boc-protected amine **27** (0.160 g, 0.35 mmol) was treated with anisole (63 μL, 0.59 mmol) and TFA (0.38 mL, 11.9 mmol). After stirring at ambient temperature for 4 h, toluene (20 mL) was added to the reaction mixture and all volatiles were removed under reduced pressure. Purification of the resulting residue by silica flash chromatography (9:1 DCM/MeOH) yielded the TFA salt of amine **29** as a white solid (1:1 mixture of diastereomers, 0.133 g, 81% yield). ¹H NMR (500 MHz, MeOD-*d*₄) δ ppm 6.45 (dd, *J*=41.5, 6.9 Hz, 1 H), 5.78 (dd, *J*=18.0, 8.1 Hz, 1 H), 4.53 – 4.61 (m, 1 H), 4.09 (q, *J*=6.7 Hz, 2 H), 3.77 (d, *J*=11.2 Hz, 3 H), 3.66 (s, 2 H), 1.69 (quin, *J*=7.7 Hz, 4 H), 1.62 (dd, *J*=14.0, 8.6 Hz, 2 H), 1.43 (sxt, *J*=7.4 Hz, 2 H), 0.97 (d, *J*=5.7 Hz, 3 H), 0.95 (d, *J*=7.1 Hz, 3 H). ³¹P NMR (202 MHz, MeOD-*d*₄) δ ppm –11.49 (s), –11.53 (s). HRMS (ESI) calcd for C₁₅H₃₁N₃O₅P [M + H]⁺ 364.1996, found 364.1994.

H₂N-Gly-Leu-*N*-phosphonodehydroalanine homopropargyl monoester (**30**).

To a solution of Diester **28** (0.537 g, 1.13 mmol) in DMF (2.2 mL), was added *i*Pr₂NEt (1.19 mL, 6.81 mmol) followed by thiophenol (0.35 mL, 3.40 mmol). The solution was stirred at ambient temperature overnight. After 17 h, the reaction appeared to be complete by ³¹P NMR. Aqueous TFA (0.1% v/v, 1 mL) was added to the reaction mixture resulting in

precipitation. The mixture was transferred to Eppendorf tubes and centrifuged to pellet the solid. Supernatant was collected and purified by RP-flash chromatography (solvent A: water, solvent B: acetonitrile; gradient 0–1 CV, 0% B; 1–8 CV, 0 – 60% B; 8 – 11 CV, 100 % B) to yield hpAP prodrug **30** as a white powder (191 mg, 49% yield). ^1H NMR (500 MHz, D_2O) δ ppm 6.13 (d, $J=36.6$ Hz, 1 H), 5.66 (d, $J=16.2$ Hz, 1 H), 4.37 – 4.45 (m, 1 H), 3.83 (s, 2 H), 3.85 (q, $J=6.8$ Hz, 2 H), 2.47 (td, $J=6.2, 2.5$ Hz, 2 H), 2.34 (br. s., 1 H), 1.63 (m, $J=4.4$ Hz, 2 H), 0.91 (d, $J=4.2$ Hz, 3 H), 0.87 (d, $J=4.4$ Hz, 3 H). ^{13}C NMR (126 MHz, D_2O) δ ppm 173.5 (d, $J=7.3$ Hz), 167.2 (s), 135.0 (d, $J=191.6$ Hz), 116.7 (d, $J=10.9$ Hz), 81.8 (s), 70.6 (s), 63.2 (d, $J=5.5$ Hz), 53.3 (s), 40.3 (s), 39.7 (s), 24.3 (s), 22.1 (s), 20.6 (s), 20.2 (d, $J=7.3$ Hz). ^{31}P NMR (202 MHz, D_2O) δ ppm –17.63 (s). HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{25}\text{N}_3\text{O}_5\text{P}$ [$\text{M} + \text{H}$] $^+$ 346.1526, found 346.1525.

H₂N-Gly-Leu-*N*-phosphonodehydroalanine butyl monoester (31).

To a solution of diester **29** (0.133 g, 0.28 mmol) in DMF (1 mL), was added $i\text{Pr}_2\text{NEt}$ (0.29 mL, 1.7 mmol) followed by thiophenol (86 μL , 0.84 mmol). The solution was stirred at ambient temperature overnight. After 17 h, the reaction appeared to be complete by ^{31}P NMR. Aqueous TFA (0.1% v/v, 1 mL) was added to the reaction mixture resulting in precipitation. The mixture was transferred to Eppendorf tubes and centrifuged to pellet the solid. Supernatant was collected and purified by RP-flash chromatography (solvent A: water, solvent B: acetonitrile; gradient 0–1 CV, 0% B; 1–8 CV, 0 – 60% B; 8 – 11 CV, 100 % B) to yield BAP prodrug **31** as a white powder (49 mg, 51% yield). ^1H NMR (500 MHz, D_2O) δ ppm 6.13 (d, $J=35.5$ Hz, 1 H), 5.63 (d, $J=15.9$ Hz, 1 H), 4.34 – 4.48 (m, 1 H), 3.81 (s, 2 H), 3.75 (q, $J=6.4$ Hz, 2 H), 1.63 (m, $J=4.9$ Hz, 3 H), 1.52 (quin, $J=7.0$ Hz, 2 H), 1.25 – 1.34 (m, 2 H), 0.91 (d, $J=4.6$ Hz, 3 H), 0.87 (d, $J=4.4$ Hz, 3 H), 0.84 (t, $J=7.4$ Hz, 3 H). ^{13}C NMR (126 MHz, D_2O) δ ppm 173.5 (d, $J=7.3$ Hz), 167.4 (s), 135.1 (d, $J=188.0$ Hz), 116.2 (d, $J=10.9$ Hz), 65.4 (d, $J=5.4$ Hz), 53.4 (s), 40.4 (s), 39.8 (s), 31.9 (d, $J=6.4$ Hz), 24.3 (s), 22.1 (s), 20.6 (s), 18.3 (s), 12.9 (s). ^{31}P NMR (202 MHz, D_2O) δ ppm –17.39 (s). HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{29}\text{N}_3\text{O}_5\text{P}$ [$\text{M} + \text{H}$] $^+$ 350.1839, found 350.1842.

D-PheTrAP Prodrug (32).

To a suspension of alkyne **30** (20 mg, 58 μmol) in *t*-butanol (0.5 mL) and water (0.2 mL) was added (*R*)-2-azido-2-benzyl acetic acid (22 mg, 0.116 mmol), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 29 μL , 0.1 M in DMSO, 2.9 μmol), and CuSO_4 (5.8 μmol , 0.5 M in water, 2.9 μmol) followed by sodium ascorbate (23.2 μL , 0.5 M, 11.6 μmol). The reaction mixture was stirred at ambient temperature overnight. Volatiles were removed under reduced pressure and the resulting residue was dissolved in water (0.5 mL) and purified by RP-flash chromatography (solvent A: water, solvent B: acetonitrile; gradient 0–1 CV, 0% B; 1–8 CV, 0 – 60% B; 8 – 11 CV, 100 % B) to yield D-PheTrAP prodrug **32** as a pale yellow foam (14 mg, 46% yield). ^1H NMR (500 MHz, $\text{MeOD}-d_4$) δ ppm 7.98 (br. s., 1 H), 7.13 – 7.25 (m, 3 H), 7.09 (d, $J=7.1$ Hz, 2 H), 6.27 (d, $J=35.2$ Hz, 1 H), 5.54 (s, 1 H), 5.47 (d, $J=13.4$ Hz, 1 H), 4.41 (dd, $J=9.9, 3.8$ Hz, 2 H), 3.95 (s, 2 H), 3.80 (dd, $J=28.8, 16.0$ Hz, 2 H), 3.62 (d, $J=11.6$ Hz, 1 H), 3.47 (t, $J=10.4$ Hz, 1 H), 2.94 (s, 2 H), 1.61 – 1.75 (m, 2 H), 1.52 – 1.61 (m, 1 H), 0.94 (d, $J=5.8$ Hz, 3 H), 0.89 (d, $J=5.7$ Hz, 3 H). ^{31}P NMR (202 MHz, D_2O :pyridine- d_5 , 9:1) δ ppm –17.54 (s). HRMS (ESI) calcd for $\text{C}_{23}\text{H}_{34}\text{N}_6\text{O}_7\text{P}$ [$\text{M} + \text{H}$] $^+$ 537.2221, found 537.2224.

***o*-phenol-TrAP Prodrug (33).**

To a suspension of alkyne **30** (20 mg, 58 μ mol) in *t*-butanol (0.5 mL) and water (0.2 mL) was added 2-azidophenol (39 mg, 0.29 mmol), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 29 μ L, 0.1 M in DMSO, 2.9 μ mol), and CuSO₄ (5.8 μ mol, 0.5 M in water, 2.9 μ mol) followed by sodium ascorbate (23.2 μ L, 0.5 M, 11.6 μ mol). The reaction mixture was stirred at ambient temperature overnight. Volatiles were removed under reduced pressured and the resulting residue was dissolved in water (0.5 mL) and purified by RP-flash chromatography (solvent A: water, solvent B: acetonitrile; gradient 0–1 CV, 0% B; 1–8 CV, 0 – 60% B; 8 – 11 CV, 100 % B) to yield *o*-phenol prodrug **33** as a pale yellow foam (19 mg, 65% yield). ¹H NMR (500 MHz, D₂O) δ ppm 8.16 (s, 1 H), 7.48 (d, *J*=7.9 Hz, 1 H), 7.41 (t, *J*=7.8 Hz, 1 H), 7.11 (d, *J*=8.3 Hz, 1 H), 7.07 (t, *J*=7.7 Hz, 1 H), 6.08 (d, *J*=36.3 Hz, 1 H), 5.55 (d, *J*=16.3 Hz, 1 H), 4.28 (dd, *J*=9.5, 5.0 Hz, 1 H), 4.08 (q, *J*=5.7 Hz, 2 H), 3.79 (s, 2 H), 3.06 (t, *J*=5.9 Hz, 2 H), 1.46 – 1.59 (m, 2 H), 1.37 – 1.45 (m, 1 H), 0.79 (d, *J*=6.3 Hz, 3 H), 0.76 (d, *J*=6.1 Hz, 3 H). ³¹P NMR (202 MHz, D₂O) δ ppm –17.41 (s). HRMS (ESI) calcd for C₂₀H₃₀N₆O₆P [M + H]⁺ 481.1959, found 481.1965.

Evaluation of antibacterial activity of DXP synthase inhibitors and prodrugs.

MG1655 *E. coli* K-12

Inhibitors were evaluated as previously described by Sanders et al.¹³ Briefly, using aseptic techniques, 3 isolated colonies were selected from a plate containing *ATCC MG1655 E. coli* K-12, inoculated into 5 mL of M9-glucose minimal medium and grown to saturation overnight with shaking at 37 °C. The saturated culture was diluted 50-fold into fresh M9-glucose medium and grown to exponential phase (OD₆₀₀ = 0.4). The exponential phase cell culture was diluted 1:1000 into M9-glucose medium to yield the experimental inoculum which was mixed 1:1 with M9-glucose medium containing the antimicrobial agent at 2× the desired concentration. To confirm consistency between experiments, colony counts of the experimental inoculum were verified by dilution and enumeration on CAMHB agar for 16 h at 37 °C. The final concentration of bacteria in each well was approximately 10⁵ CFU/mL in a final volume of 200 μ L. The 96-well plates were incubated at 37 °C for 16 h with periodic shaking. Fractional growth was determined at 16 h relative to a no drug control. Experiments were performed in triplicate.

For thiamin rescue experiments, antibacterial activity was determined as above with a key exception. Rather than diluting the exponential culture 1000-fold into fresh M9-glucose medium, it was diluted 10-fold into fresh M9-glucose medium prior to addition to M9-glucose medium containing 200 nM thiamin (2× the desired concentration). This results in a 10⁷ CFU/mL starting inoculum and 100 nM thiamin in each well.

Gram-negative bacteria clinical isolates

Inhibitors were evaluated as previously described by Sanders et al.¹³ Clinical isolates of all pathogens were from an in-house strain library maintained at Northern Arizona University.³¹ All microbial manipulation of pathogenic bacteria was conducted in a certified biosafety

level 2 laboratory while following all associated safety protocols. All clinical isolates were streaked onto CAMHB agar and allowed to grow for 12 h at 37 °C. Using aseptic technique, colonies were inoculated into CAMHB (5 mL) and grown to saturation overnight. Following overnight growth, bacteria were subcultured into M9-glucose medium (approximately 5 µL per 5 mL of fresh medium), grown to saturation at 37 °C, and then used in subsequent broth microdilution assays. Experiments were performed essentially as described above, and experimental inoculum for all strains was verified by dilution and enumeration to be approximately 2×10^8 CFU/mL. To maintain retention of the pJLA-Fsr plasmid by the cells involving the *E. coli*-Fsr expression strain, experiments were performed in the presence of kanamycin (70 µg/mL). At a minimum, experiments were performed in triplicate.

LC-MS/MS method to determine intracellular inhibitor concentrations.

E. coli samples were treated with inhibitor and prepared for LC-MS/MS analysis as described by Sanders et al.¹⁵ Samples were analyzed with an LC-MS/MS system comprised of a Waters NanoAcquity UPLC and a TSQ Vantage Triple Quadripole (Thermo Scientific). The liquid chromatography separation was performed on a Waters Acquity UPLC HSS C18 column (1.0 × 50 mm, 1.8 µm) with mobile phase A (1 mM acetic acid in water or 5 mM dimethylhexylammonium acetate) and mobile phase B (acetonitrile). The flow rate was 50 µL min⁻¹. The autosampler temperature was set at 10 °C. The injection volume was 3 µL. The LC gradient was as follows: 0 – 1 min, 2% B; 1 – 7 min, 0 to 100% B; 7 – 9 min, 100% B; 9 – 10.1 min, 100 to 2% B; 10.1 – 12 min, 2% B.

BAP was detected using selective reaction monitoring (SRM) follow the transition of 179 m/z to 63 m/z. The instrument settings were as follows: negative ion mode; scan width – 0.5 m/z; scan time – 1.000 s; collision energy - 18 V; collision gas pressure – 1.6 mTorr; Spray Voltage –4000 V; Sheath Gas 30; Ion Sweep Gas – 0; Aux Gas Pressure – 0; Capillary Temperature 237 °C; S-Lens RF Amplitude – 133; Declustering Voltage – 0.

hpAP (1) was detected using selective reaction monitoring (SRM) follow the transition of 175 m/z to 63 m/z. The instrument settings were as follows: negative ion mode; scan width – 0.5 m/z; scan time – 1.000 s; collision energy - 48 V; collision gas pressure – 1.6 mTorr; Spray Voltage –2000 V; Sheath Gas 40; Ion Sweep Gas – 0; Aux Gas Pressure – 0; Capillary Temperature 240 °C; S-Lens RF Amplitude – 133; Declustering Voltage – 0.

Prodrug 30 was detected using selective reaction monitoring (SRM) follow the transition of 346.15 m/z to 143.12 m/z. The instrument settings were as follows: positive ion mode; scan width – 0.1 m/z; scan time – 1.000 s; collision energy - 20 V; collision gas pressure – 1.0 mTorr; Spray Voltage 3000 V; Sheath Gas 0; Ion Sweep Gas – 0; Aux Gas Pressure – 0; Capillary Temperature 270 °C; S-Lens RF Amplitude – 133; Declustering Voltage – 10.

Prodrug 31 was detected using selective reaction monitoring (SRM) follow the transition of 350.18 m/z to 143.12 m/z. The instrument settings were as follows: positive ion mode scan width – 0.1 m/z; scan time – 1.000 s; collision energy - 20 V; collision gas pressure – 1.0 mTorr; Spray Voltage 3000 V; Sheath Gas 0; Ion Sweep Gas – 0; Aux Gas Pressure – 0; Capillary Temperature 270 °C; S-Lens RF Amplitude – 133; Declustering Voltage – 10.

Supplementary Material

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Figure 1. Transport and activation of dehydrophos and alkylAP prodrugs.

The natural product dehydrophos travels to the cytoplasm via the peptide transporter, OppA.

¹⁸ Once in the cytoplasm it is hydrolyzed an enamine product which then undergoes spontaneous tautomerization and hydrolysis to release MAP. The alkylAP prodrugs are expected to undergo the same transport and activation to reveal potent and selective DXP synthase inhibitors.

Compound	R	R'	MIC (μM)	K_i (μM) ¹¹
BAP	na		4.9	2.6 ± 0.6
1 (hpAP)	na		39	0.47 ± 0.04
2	allyl		625	6 ± 1
3	cyclohexyl		313	0.44 ± 0.09
4	2-hydroxyethyl		78	1.0 ± 0.1
5	pentanyl		313	3 ± 2
6	benzyl		>1250	8 ± 4
7	<i>o</i> -nitrobenzyl		4.9	5.4 ± 0.6
8	<i>p</i> -nitrobenzyl		2.4	2.7 ± 0.6
9	<i>p</i> -(methyl carboxy) benzyl		156	8 ± 5
10	phenethyl		2.4	8 ± 2
11	H	H	313	0.84 ± 0.08
12	H	Me	>1250	1.3 ± 0.3
(S)- 13	S-methyl	H	156	1.3 ± 0.2
(R)- 13	R-methyl	H	625	2.9 ± 0.2
(S)- 14	S-benzyl	H	>1250	2.1 ± 0.3
(R)- 14	R-benzyl	H	>1250	0.09 ± 0.01
(S)- 15	S-(<i>p</i> -hydroxyl)benzyl	H	>1250	1.00 ± 0.06
(R)- 15	R-(<i>p</i> -hydroxyl)benzyl	H	1250	0.33 ± 0.04
16	<i>o</i> -phenol		>1250	0.11 ± 0.02
17	<i>m</i> -phenol		>1250	0.32 ± 0.03
18	<i>p</i> -phenol		>1250	6.0 ± 0.8
19	H	CH ₃	625	0.52 ± 0.06
20	H	<i>p</i> -toluene	156	0.47 ± 0.05
21	S-benzyl	<i>p</i> -toluene	>1250	0.83 ± 0.09

Figure 2. Activity in growth inhibitory assay against MG1655 *E. coli*.

Antibacterial activity was evaluated in M9-glucose minimal medium. Enzyme inhibition constants (K_i) were previously reported by Bartee and Freely Meyers¹¹. Minimal inhibitory concentration (MIC) is defined as the concentration of inhibitor required to inhibit bacterial growth by 90%.

Figure 3.

Evaluation of antibacterial activity of BAP, hpAP (**1**), Pro-hpAP (**30**), and Pro-BAP (**31**) in peptide transporter deletion strains. Only the deletion of OppA desensitizes *E. coli* to prodrugs **30** and **31**. The activity of parent alkylAPs (BAP and **1**) is not affected by the deletion of peptide transporter tested. Experiments were performed in triplicate and error bars indicate standard error.

Figure 4.

Intracellular concentrations of BAP, hpAP (**1**), Pro-hpAP (**30**), and Pro-BAP (**31**) as measured by LC/MS-MS. *MG1655 E. coli* were treated with 40 μ M of a) **30** or b) **31** and collected over the course of 1h. Cell lysates were analyzed and concentrations of prodrug and corresponding alkylAP were determined using a standard curve (Figure S3, S4). Experiments were performed in duplicate and error bars indicate standard deviation.

Figure 5.

Intracellular concentrations of BAP, hpAP (**1**), Pro-hpAP (**30**), and Pro-BAP (**31**) as measured by LC/MS-MS. *MG1655 E. coli* were treated with 5 μ M of BAP, hpAP (**1**), Pro-hpAP (**30**), and Pro-BAP (**31**) incubated at 37 °C for 5 minutes. Cell lysates were analyzed and concentrations of the corresponding alkylAP (BAP for BAP and Pro-BAP, dark grey, and hpAP for hpAP and Pro-hpAP, light grey) were determined using a standard curve (Figure S5, S6). Experiments were performed in duplicate and error bars indicate standard deviation.

Scheme 1.

DXP synthase is an essential ThDP-dependent enzyme for bacterial central metabolism. Unique amongst ThDP-dependent enzymes, DXP synthase forms a stable lactyl-ThDP (LThDP) intermediate which requires D-GAP to bind forming a ternary complex before decarboxylation occurs. AlkylAP inhibitors, such as BAP and (*R*)-**14**, mimic pyruvate to form reversible covalent phosphono-LThDP intermediates to elicit their inhibitory activity.

Scheme 2.
Synthesis of prodrugs **30** and **31**

Scheme 3.
Synthesis of triazole-containing prodrugs

Table 1.Evaluation of Prodrugs against *E. coli* MG1655.

Prodrug	MIC _{M9} (μM)	Parenal alkylIAP	MIC _{M9} (μM)	Fold-increase in potency
Dehydrophos	0.61	MAP	78	128
Pro-hpAP (30)	0.02	hpAP (1)	39	1950
Pro-BAP (31)	0.15	BAP	5	33
32	>1250	(<i>R</i>)- 14	>1250	n/a
33	1250	16	>1250	n/a

Table 2.

Evaluation of hpAP (**1**), BAP, and their respective prodrugs, **30** and **31**, against clinical isolates of Gram-negative bacterial pathogens.

Starin	MIC (μM)			
	BAP	hpAP (1)	Pro-hpAP (30)	Pro-BAP (31)
<i>Escherichia coli</i> (clinical)	11	20	0.0375	0.3
<i>Escherichia coli</i> (FSR)	43	80	0.0375	0.15
<i>Salmonella typhimurium</i>	11	20	0.01875	0.075
<i>Klebsiella oxytoca</i>	43	>160	20	>40
<i>Pseudomonas aeruginosa</i>	n/a	>160	>40	>40
<i>Pseudomonas fluorescens</i>	43	80	>40	>40
<i>Enterobacter cloacae</i>	688	>160	0.625	20