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Challenges and Hallmarks of Establishing Alkylacetylphosphonates as Probes of Bacterial 1-Deoxy-D-xylulose 5-Phosphate Synthase

Sara Sanders[†], Ryan J. Vierling^{†,§}, David Bartee[†], Alicia A. DeColli[†], Mackenzie J. Harrison[‡], Joseph L. Aklinski[‡], Andrew T. Koppisch[‡], and Caren L. Freel Meyers^{†,*}

[†]Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, United States

[‡]Department of Chemistry, Northern Arizona University, Flagstaff, Arizona 86011, United States

Abstract

1-Deoxy-D-xylulose 5-phosphate (DXP) synthase catalyzes the thiamin diphosphate (ThDP)-dependent formation of DXP from pyruvate and D-glyceraldehyde 3-phosphate. DXP is at a metabolic branch point in bacteria, feeding into the methylerythritol phosphate pathway to indispensable isoprenoids and acting as a precursor for biosynthesis of essential cofactors in central metabolism, pyridoxal phosphate and ThDP, the latter of which is also required for DXP synthase catalysis. DXP synthase follows a unique random sequential mechanism and possesses an unusually large active site. These features have guided the design of sterically demanding alkylacetylphosphonates (alkylAPs) toward the development of selective DXP synthase inhibitors. alkylAPs studied here display selective, low μM inhibitory activity against DXP synthase. They are weak inhibitors of bacterial growth in standard nutrient rich conditions. However, bacteria are significantly sensitized to most alkylAPs in defined minimal growth medium, with minimal inhibitory concentrations (MICs) ranging from low μM to low mM and influenced by alkyl-chain length. The longest analog (**C₈**) displays the weakest antimicrobial activity and is a substrate for efflux via AcrAB-TolC. The dependence of inhibitor potency on growth environment emphasizes the need for antimicrobial screening conditions that are relevant to the *in vivo* microbial microenvironment during infection. DXP synthase expression and thiamin supplementation

*Corresponding Author Tel: 410-502-4807. Fax: 410-955-3023. cmeyers@jhmi.edu.

§Present Address

R.J.V.: Pipeline and Hazardous Materials Safety Administration, US Department of Transportation, Washington, DC, 20590, United States.

ORCID

Caren L. Freel Meyers: 0000-0003-1458-0897

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.6b00168. Synthesis of **3**, **5**, **6**, **7**, and **8**; *hPrAP* inhibitory activity against DXP synthase; reversible inhibition of DXP synthase and PDH by alkylAPs; competitive inhibition of DXP synthase by **3**, **5**, **6**, and **7**; competitive inhibition of porcine PDH E1 by **3**, **5**, **6**, and **7**; antimicrobial activity of **1–7** against *E. coli* MG1655 in CAMHB or M9 minimal medium; antimicrobial activity of acetylphosphonates against *E. coli* BW25113 and *tolCBW25113*; DXP synthase overexpression in *E. coli* BL21 (DE3); interdependence of ThDP and DXP synthase activity; effects of exogenous thiamin on *E. coli* cells treated with alkylAPs; thiamin and ThDP-mediated rescue of BAP-treated *E. coli* growth; NMR characterizations of **3**, tris(*n*-pentyl) phosphite, **5**, **6**, tris(*n*-octyl) phosphite, **7**, and **8** (PDF)

studies offer support for DXP synthase as an intracellular target for some alkylAPs and reveal both the challenges and intriguing aspects of these approaches to study target engagement.

Graphical Abstract

Keywords

1-deoxy-D-xylulose 5-phosphate synthase; bacterial metabolic branch point; isoprenoid biosynthesis; PLP biosynthesis; thiamin biosynthesis; growth medium effect

The bacterial enzyme 1-deoxy-D-xylulose 5-phosphate (DXP) synthase catalyzes the condensation of D-glyceraldehyde 3-phosphate (D-GAP) and pyruvate in a thiamin diphosphate (ThDP)-dependent process to produce DXP and CO₂. DXP is then proportioned into three biosynthetic pathways: isoprenoid biosynthesis,¹ thiamin diphosphate (ThDP or Vitamin B₁) biosynthesis,^{2–4} and pyridoxal phosphate (PLP or Vitamin B₆) biosynthesis^{5,6} (Figure 1a). Together, these three pathways play roles in many facets of central metabolism. The cofactors ThDP and PLP are utilized in a myriad of processes (e.g., TCA cycle, pentose phosphate pathway, amino acid biosynthesis, metabolism, etc.).^{7–9} Isoprenoid-derived small molecules that are critical to the growth and survival of bacteria include ubiquinone/menaquinone for electron transport, Lipid II for cell wall biosynthesis, and various terpene-based virulence factors.^{10,11}

While its role in central metabolism highlights DXP synthase as a potentially attractive target for the development of antibiotic agents, few inhibitors of DXP synthase are reported.^{1–3,12–16} In part, this is due to the challenge of selectively targeting a bacterial ThDP-dependent enzyme. However, there is mounting evidence to suggest DXP synthase is unique among ThDP-dependent enzymes. Structural studies of DXP synthase indicate the enzyme possesses unique domain architecture¹⁷ and an unusually large active site volume;¹⁸ these observations are consistent with mechanistic studies offering support for ternary complex formation in DXP synthase catalysis.^{19–22} Our mechanistic studies suggest GAP serves two distinct roles in DXP formation, as a trigger for lactyl thiamin diphosphate (LThDP) decarboxylation and as an acceptor substrate during carbonylation.^{21,22} As new insights emerge about the distinctive characteristics of DXP synthase, so will new

opportunities for the development of selective inhibitors. Our previous work has established that sterically demanding alkylacetylphosphonates (alkylAPs) can selectively inhibit DXP synthase¹⁵ (Figure 1b) and butylacetylphosphonate (BAP) possesses antimicrobial activity by a mechanism likely involving reversible inhibition of DXP synthase.²³

The goal of the present study was to identify determinants for alkylAP selectivity and antimicrobial activity toward the development of selective inhibitors of DXP synthase as novel antimicrobial agents. Our results demonstrate the capacity of DXP synthase to accommodate extension of the alkyl chain but not alkyl branching adjacent to the acetylphosphonate scaffold. We also show that the alkylAPs in this series are more potent inhibitors of DXP synthase than mammalian ThDP-dependent pyruvate dehydrogenase (PDH). Antimicrobial studies reveal a striking dependence of inhibitor potency on the growth environment, in which most alkylAPs exhibit significantly enhanced antimicrobial activity under conditions of nutrient restriction. In this series, only the long-chain octylacetylphosphonate is a substrate for efflux under either growth condition tested here. The challenges underlying the confirmation of DXP synthase as an intracellular target of alkylAPs are considered in the context of this metabolic branch point and alkylAP inhibition mechanism. We show that growth of alkylAP-treated *E. coli* cells is rescued by overexpression of DXP synthase and by exogenous thiamin to varying extents across the series, supporting DXP synthase as an intracellular target of some alkylAPs. Overall, these results underscore the importance of considering growth environment for discovery of antimicrobial agents targeting essential metabolism pathways and highlight the challenges and progress toward establishing alkylAPs as new probes to investigate the role of DXP synthase in bacterial cells and the mechanisms underlying alkylAP antimicrobial activity.

RESULTS

Linear Alkylacetylphosphonates Inhibit *E. coli* DXP Synthase

Our previous work has shown that pathogenic DXP synthase enzymes are selectively inhibited by the sterically demanding alkylAPs, butylacetylphosphonate (BAP)^{15,23} and benzylacetylphosphonate (BnAP),¹⁸ and these are more potent inhibitors of DXP synthase than the related ThDP-dependent enzymes, pyruvate dehydrogenase (PDH)^{15,18} and transketolase (TK).¹⁵ BAP and BnAP exhibit 60-fold and 85-fold more potent inhibitory activity, respectively, against DXP synthase compared to PDH, and so far, the alkylAP class is shown to be inactive against TK up to 1 mM.¹⁵ We theorized selectivity is achieved as a result of the comparatively large active site of DXP synthase and its unique mechanism requiring ternary complex formation during catalysis.^{15,18} Here, we report the synthesis and evaluation of a series of alkylAPs toward defining steric constraints of this inhibitor class against *E. coli* DXP synthase.

We prepared alkylAPs bearing alkyl chains up to C₈ (**1–7**) and the branched alkylAP isopropylacetylphosphonate (**8**, Figures 1b, S1, and S2) and compared their DXP synthase inhibitory activities. Isopropylacetylphosphonate (**8**, Figure 1b) exhibits weak inhibition of *E. coli* DXP synthase with an IC₅₀ an order of magnitude higher than that of **4** (Figure S3); thus, full characterization of this analog was not pursued further. In contrast, straight-chain alkylAPs display apparent *K_i* values in the low micromolar range (Table 1) and are

reversible, competitive inhibitors with respect to pyruvate (Figures S4 and S5). The results indicate that, while alkylAPs bearing long alkyl chains are readily accommodated in the DXP synthase active site, an analog bearing a branched alkyl group adjacent to the acetylphosphonate moiety exhibits lower affinity for DXP synthase. This could be a result of unfavorable steric interactions near the cofactor binding site.

alkylAPs Exhibit More Potent Inhibition of *E. coli* DXP Synthase Compared to Mammalian PDH

A comparison of inhibitor potency against the ThDP-dependent mammalian pyruvate dehydrogenase (PDH) and DXP synthase can be used as one measure of selectivity of DXP synthase inhibition by alkylAPs.^{15,18} We hypothesized that increasing the alkyl-chain length on the AP scaffold would also lead to increased selectivity of inhibition against DXP synthase, as this enzyme possesses a larger active site compared to PDH and other related ThDP-dependent enzymes.¹⁸ Thus, the inhibitory activities of **1–7** against PDH were compared. Compounds **1–7** are reversible competitive inhibitors with respect to pyruvate (Figures S4 and S6) and exhibit significantly weaker inhibitory activity against mammalian PDH compared to DXP synthase (Table 1). As expected, alkylAP potency against PDH decreases with increasing alkyl-chain length up to C₄ (**4**). However, compound **4** is the weakest PDH inhibitor in this series, and alkylAPs bearing longer-chain lengths display increasing, albeit weak, inhibitory activity against PDH as the carbon-chain length increases from 5 to 8 carbons.

Alkylacetylphosphonates Exhibit Weak Antimicrobial Activity against *E. coli* MG1655 in Nutrient Rich Growth Medium

BAP (**4**) was previously shown to have very modest (low millimolar) antimicrobial activity against *E. coli* under standardized broth dilution assay conditions²⁴ by a mechanism involving inhibition of DXP synthase.²³ Here, the antimicrobial activities of alkylAPs **1–7** were evaluated against wild-type *E. coli* MG1655 under standard growth conditions (CAMHB growth medium).²⁴ Despite the observation that these alkylAPs exhibit comparable low-micromolar inhibitory activity against DXP synthase (Table 1), only **4**, **5**, and **6** exert weak, dose-dependent inhibition of *E. coli* growth in CAMHB medium (Table 2, Figure S7); under these conditions, **1**, **2**, **3**, and **7** are inactive against *E. coli* up to 5000 μ M.

Antimicrobial Activity of Alkylacetylphosphonates against *E. coli* MG1655 Is Dramatically Enhanced in Defined Minimal Growth Medium

Antibacterial discovery has traditionally emphasized nutrient-rich growth conditions²⁵ for clinical evaluation. However, assessing inhibitor activity in nutrient rich culture is often poorly predictive of antibacterial activity in the host environment, which typically restricts nutrient access to the pathogen.^{25–29} While effects of culture medium are not usually pronounced in known antibacterial classes such as those interfering with replication or protein synthesis,²⁶ they are especially relevant to discovery of compounds targeting essential metabolism processes whose functions may be conditional or contextual.^{25,26} Thus, we evaluated antimicrobial activities of **1–7** against *E. coli* MG1655 in M9 minimal growth medium lacking vitamins, peptides, amino acids, and other nutrients that could suppress

antimicrobial activity of DXP synthase inhibitors. Notably, the majority of alkylAPs (**1–5**) display significantly increased activity under these conditions (Table 2, Figure S7). Alkyl-chain length appears to influence antimicrobial potency under nutrient limitation, with the C₄ analog (**4**) displaying the most potent antimicrobial activity in the series and longer-chain alkylAPs displaying significantly weaker activity. The micromolar minimal inhibitory concentrations (MICs) measured for alkylAPs **1–5** suggests they are readily taken up by *E. coli* in minimal medium and have the capacity to exert potent antimicrobial effects under nutrient limitation, presumably through inhibition of three essential pathways simultaneously. Although the intracellular levels of alkylAP achieved and degree of DXP synthase inhibition under these conditions are not yet known, there may be a distinct benefit to inhibition of DXP synthase in bacterial cells, given the potentially cooperative effects of targeting three essential metabolic pathways.

Antimicrobial Activity of Butylacetylphosphonate (**4**) Is Enhanced against Other Bacterial Pathogens in M9 Minimal Medium

Given that butylacetylphosphonate (**4**, BAP) exhibits the most potent antimicrobial activity in M9 minimal growth medium, we evaluated this analog against a number of other clinically relevant Gram-negative pathogens, including a clinical isolate of *Escherichia coli*, *Salmonella enterica* LT2, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca*, and the Gram-positive environmental isolate *Bacillus thuringiensis* HD (Table 3). As shown for *E. coli* MG1655, the antimicrobial activity of **4** is also dramatically enhanced against this panel of bacterial strains grown under nutrient-limited conditions. *E. coli* fsr-pET28a, which is resistant to another MEP pathway inhibitor (fosmidomycin), is susceptible to **4**, demonstrating the activity of **4** against an antibiotic-resistant strain and indicating there is no cross-resistance with fosmidomycin.³⁰ These data indicate that a broad spectrum of bacterial pathogens is penetrable and susceptible to this inhibitor class.

Octylacetylphosphonate (**7**) Is Susceptible to Efflux by *E. coli* AcrAB-TolC

The modest antimicrobial activity observed for **6** and **7**, despite low-micromolar enzyme inhibitory activities, may indicate that a high intracellular inhibitor concentration is not achieved. Low cell permeability and/or susceptibility to efflux could contribute to this problem. We tested the hypothesis that efflux via the AcrAB-TolC transporter confers resistance to **6** and **7** as a starting point to explain the weak antimicrobial activities of these alkylAPs.

The AcrAB-TolC transporter is a tripartite pump and member of the resistance-nodulation-division (RND) family³¹ of multidrug resistance efflux pumps in Gram-negative bacteria. It is composed of AcrB, a periplasmic membrane fusion protein (MFP), AcrA, a periplasmic adapter protein, and the porin TolC, an outer membrane factor (OMF). While there are multiple component combinations that can be assembled,^{32–34} TolC is the outer OMF common to almost all *E. coli* RND pumps responsible for resistance to endogenous toxins and clinically used agents.³⁵ We evaluated antimicrobial activities of **6** and **7** against a genetic variant of *E. coli* lacking the *tolC* gene (*tolC*) and the corresponding parent strain (BW25113) in both rich growth medium (CAMHB) and M9 minimal growth medium. Under both growth conditions, the *tolC* *E. coli* variant exhibits significantly increased

susceptibility only to octylacetylphosphonate **7** (Figure 2), indicating **7** is a substrate for efflux via the AcrAB-TolC transporter. Interestingly, the antimicrobial activity of **7** against the *tolC* *E. coli* is not fully restored to that of the most potent analog, **4**, despite comparable low-micromolar enzyme inhibitory activity. In contrast to **7**, alkylAP **6** displays comparable antimicrobial activity against both strains in M9 minimal medium (Figure S8). The antimicrobial activities of alkylAPs **1–5** are minimally impacted by deletion of TolC (Figure S8) in CAMHB or M9 minimal medium and thus do not appear to be substrates for efflux by the AcrAB-TolC transporter. These results could suggest that longer alkylAPs exhibiting weak antimicrobial activity that is not associated with efflux may have lower permeability compared to **4**.

DXP Synthase Overexpression Confers Resistance to Some alkylAPs

Associating antibacterial agents with their molecular targets in whole cells is a major challenge in drug development. Target overexpression often increases drug resistance and can provide a tool for target identification. However, overexpression-based resistance to a given drug is not always observable, as expression of a target gene can have myriad effects on the perceived resistance of the cells to the antibiotic. The observed resistance can be influenced both by the drug's mechanism of action and the potential fitness cost of overexpressing the target gene.³⁶ The challenges surrounding DXP synthase aggregation/insolubility^{37,38} and toxicity induced by DXP synthase overexpression^{39–43} or accumulation of prenyl pyrophosphates⁴⁴ are well documented. Consistent with these reports, we have found that, under conditions where DXP synthase expression is strictly inducible by IPTG⁴⁵ or L-arabinose (unpublished work), target expression does not confer resistance to BAP (**4**), and a significant IPTG/arabinose-induced toxicity is observed. However, basal expression of DXP synthase in BL21 cells harboring the *dxs*-pET37b plasmid is sufficient to confer resistance to BAP (**4**).²³ Thus, we evaluated **1–7** against *E. coli* BL21 cells harboring the *dxs*-pET37b plasmid grown in LB medium where basal DXP synthase expression is evident relative to the pET37b control (Figure S9). Increasing intracellular levels of DXP synthase confers resistance to alkylAPs **3–6** relative to the pET37b control (Figure 3), despite the potentially opposing effects on cell growth caused by aggregation and toxicity in this system. In contrast, BL21 cells overexpressing an inactive DXP synthase variant (E370A*dxs*-pET37b)^{17,23} are not resistant to the effects of alkylAPs. Suppression of the activities of **3** and **5** by DXP synthase overexpression is less pronounced than for **4** and **6**. Further, although alkylAP **2** is only weakly active at 5000 μ M, DXP synthase overexpression does not appear to confer resistance (data not shown), suggesting these smaller alkylAPs may be less selective for DXP synthase in cells. alkylAPs **1** and **7** are inactive under the growth conditions required for target overexpression (data not shown). These results support DXP synthase as an intracellular target of some alkylAPs.

Thiamin Supplementation Confers Resistance to alkylAPs

As noted, DXP acts as a precursor in isoprenoid biosynthesis and vitamin biosynthesis pathways to ThDP and PLP, both of which are essential in a myriad of central metabolic processes. Supplementation of the growth medium with 1-deoxy-D-xylulose (DX) could suppress the activity of BAP by providing a precursor to this enzyme product.^{12,13}

Interestingly, the growth inhibitory activity of BAP in M9 minimal medium cannot be suppressed by supplementation with DX (data not shown). It is likely that catabolite repression induced by glucose^{46–48} prevents efficient uptake and phosphorylation of DX under these conditions.

The interdependence of ThDP and DXP synthase and the interconnection of ThDP with converging steps of its own biosynthesis and the biosynthesis of PLP (Figure S10) suggest that thiamin supplementation in minimal medium should eliminate the numerous bottlenecks in metabolism that are imposed by inhibition of DXP synthase. However, it is possible that, even under thiamin supplementation, the time required to overcome DXP synthase inhibition and restore activity to all of the ThDP-dependent metabolic processes required to re-establish cell growth could impart a significant delay in outgrowth, as we have previously proposed.²³ Shown in Figure 4a, exogenous thiamin confers resistance of *E. coli* to the potent effects of **4** (BAP) to some degree, under growth conditions used to determine MIC^{BAP} (starting inoculum of 10⁵ CFU/mL in M9-glucose). However, at BAP concentrations above the MIC, thiamin-mediated suppression of BAP activity appears diminished. Further analysis of *E. coli* growth in the presence of BAP at the MIC confirms outgrowth in the presence of 100 nM thiamin at later time points (>16 h), but pronounced outgrowth is not detectable within the time frame for MIC determination (Figure 4b).

Redesign of experimental conditions to enable the detection of thiamin-dependent suppression of alkylAP activity could entail either MIC determination at later time points or use of a higher starting inoculum density to guarantee faster outgrowth. To avoid issues with evaporation of small culture volumes over longer incubation times, we pursued the latter experimental design, using a higher starting inoculum density (e.g., 10⁷ CFU/mL). Indeed, thiamin-dependent suppression of the activity of **4** is unmistakable despite the apparent delay in outgrowth under thiamin supplementation (Figure 4c). The MIC of BAP, albeit elevated at higher inoculum density under thiamin-free growth conditions, is dramatically increased under thiamin supplementation (Figure 4d).

Exogenous thiamin also suppresses the activities of alkylAPs **1–7** to varying degrees (Table 4 and Figure S11). Marked suppression of alkylAP activity by exogenous thiamin is evident for alkylAPs **2**, **4**, and **5**, whereas the effects are less pronounced for short (**1**) and long (**6** and **7**) alkylAPs (Table 4 and Figure S11). Curiously, exogenous thiamin confers resistance to alkylAP **3** under these conditions, but this effect diminishes at higher concentrations of **3** in which no inhibition is observed by this analog (Figure S11c).

To determine what drives the delay in outgrowth, we measured the growth of alkylAP-treated *E. coli* over a range of thiamin concentrations (0–10 μ M). The results show that the maximum attainable outgrowth of alkylAP-treated *E. coli* is achieved by 16 h in the presence of 100 nM thiamin over a range of alkylAP concentrations (Figure 5a). The time at which outgrowth is observed appears to be independent of thiamin concentration above 100 nM (Figure 5b). Rather, the duration of the delay is dependent upon the concentration of alkylAP, with higher alkylAP causing a longer delay to outgrowth. In theory, supplementation with 100 nM thiamin could provide sufficient ThDP to convert the available apo enzyme pool to E-ThDP as new DXP synthase is produced (Figure 6). alkylAP

competes with pyruvate binding to E-ThDP, reducing the fraction of E-ThDP converted to the active E-LThDP complex required for DXP formation and cell growth. In the presence of alkylAP, time is required to produce E-LThDP levels needed to support growth. A higher alkylAP concentration results in a lower fraction of E-ThDP converted to active E-LThDP and a longer delay to achieve DXP levels required for outgrowth.

To determine whether the duration of the delay to outgrowth at a given alkylAP concentration reflects the time required for phosphorylation of thiamin to its active form, thiamin diphosphate (ThDP), we tested the capacity of ThDP to suppress the activity of compound **4**. Under the nutrient restriction conditions used here (M9-glucose), efficient transport of both thiamin and ThDP is expected. Thiamin and its two phosphorylated forms (thiamin monophosphate and thiamin diphosphate) are known to be actively transported into *E. coli* and *Salmonella enterica* by the ABC transport system encoded by *thiBPQ*.^{4,49,50} Transporter expression is mediated by the thiamin riboswitch (*thi-box*)^{51–53} and negatively regulated by ThDP. Thus, at low intracellular thiamin concentration, expression of the transporter is upregulated. As shown in Figure S12, exogenous ThDP clearly suppresses the activity of **4** in a time-dependent manner similar to exogenous thiamin, suggesting the time required to phosphorylate thiamin and accumulate ThDP does not contribute to the growth delay of alkylAP-treated cells under thiamin supplementation.

DISCUSSION

Inhibition of DXP synthase has the potential to negatively impact multiple essential metabolic pathways to produce potent antimicrobial effects. Despite the formidable challenge associated with selective inhibition of ThDP-dependent enzymes, we have previously demonstrated that selective inhibition of this ThDP-dependent enzyme is feasible with alkylacetylphosphonates.^{15,18,23} Here, we have synthesized and evaluated a series of alkylacetylphosphonates to define the requirements for potent enzyme inhibition and antimicrobial activity of straight-chain alkylAPs toward the goal to develop selective agents targeting this metabolic branch point in bacterial pathogens.

Biochemical inhibition studies show that extending the alkyl-chain length on the acetylphosphonate scaffold is well-tolerated by DXP synthase, with inhibitory constants in the low micromolar range and competitive inhibition modes with respect to pyruvate. Introducing an isopropyl group adjacent to the acetylphosphonate scaffold dramatically reduces inhibitory activity; unfavorable steric interactions could arise between bulky acetylphosphonates and the region of the active site in close proximity to the C₂ of ThDP. Overall, alkylAPs are significantly weaker competitive inhibitors of PDH compared to DXP synthase, as predicted. However, increasing the alkyl-chain length beyond four carbons does not further reduce the inhibitory activity against PDH, contrary to our expectations. It is possible that increasing alkyl-chain length beyond four carbons increases inhibitor affinity through favorable hydrophobic interactions on PDH.

For the majority of alkylAPs studied here, antimicrobial activity is markedly increased in defined M9 minimal medium compared to nutrient rich CAMHB growth medium. alkylAP potency in M9-glucose varies from low micromolar to low millimolar across this compound

series, with medium-chain butylacetylphosphonate, **4**, emerging as the most potent growth inhibitor. The variability in antimicrobial activity, despite similar enzyme inhibitory activities, indicates that alkyl-chain length influences antimicrobial activity under nutrient limitation. This finding suggests it may be possible to optimize alkylAP potency through modification at this position. The lower potency of long-chain alkylAPs **6** and **7** compared to short- and medium-chain alkylAPs suggests they may accumulate to lower levels in cells as a result of poor permeability and/or susceptibility to efflux. However, only octylacetylphosphonate **7** is a substrate for the AcrAB-TolC efflux pump. It is possible that a distinct OMF is at play in the efflux of **6**. The differences in growth inhibitory activities of these alkylAPs could also reflect differences in cellular uptake. It is unknown if bacteria are penetrable to alkylAPs by mechanisms involving active transport or by diffusion through porins. More detailed studies are required to understand the mechanisms of alkylAP entry into bacterial cells.

The essentiality of DXP synthase in *E. coli* grown under either nutrient rich or nutrient limited growth conditions^{54–56} demands the development of new probes to study this target and, thus, underscores the importance of understanding the factors driving the remarkable dependence of alkylAP potency on growth environment. We have considered three potential explanations for the observed decrease in alkylAP potency in CAMHB, an undefined nutrient rich medium containing acid hydrolysate of casein, beef extract, and starch. First, cells may overcome DXP synthase inhibition more efficiently in this nutrient rich medium containing metabolites, amino acids, and cofactors (including ThDP and PLP) that are used immediately in cellular processes that negate alkylAP-mediated DXP synthase inhibition, e.g., by increasing pyruvate levels or by accumulating ThDP-bound DXP synthase. ThDP is required for alkylAP-mediated inhibition. A steady supply of this cofactor with other nutrients from the growth medium could ensure rapid alkylAP “processing” with concomitant replenishment of active holoenzyme for DXP production, alleviating the multiple metabolic blockades imposed by inhibiting this enzyme. The delayed outgrowth observed under thiamin supplementation in M9 minimal medium indicates that the effects of introducing *only* this cofactor into metabolism are not immediate, and time is required for ThDP-dependent restoration of normal growth. Second, DXP synthase plays a potentially complex and poorly understood role in bacterial metabolism. The significant shift in cellular metabolism that accompanies the change in growth environment^{54,57,58} may increase the demand for DXP biosynthesis to support production of amino acids, cofactors, and precursor metabolites under nutrient limitation. Thus, inhibition of this step could have more pronounced growth inhibitory effects under nutrient restriction in M9 minimal medium which contains only inorganic salts and glucose as a carbon source. Finally, it is conceivable that the uptake efficiency for the majority of alkylAPs studies here is enhanced under nutrient limitation in M9 minimal medium, leading to higher intracellular concentrations and increased antimicrobial activities. These polar small molecules are unlikely to enter cells by passive diffusion mechanisms common to large lipophilic antibiotics.⁵⁹ alkylAPs may enter cells by diffusion through porins⁵⁹ or by active nutrient transporters as in the cases of fosmidomycin,⁶⁰ fosfomicin,⁶¹ and D-cycloserine.⁶² Nutrient limitation itself may alter expression of transporters that aid cellular uptake for the alkylAP analogs⁶³ or lacks compounds found in rich medium that may otherwise serve to interfere with transport of the

analogs (akin to the inhibited uptake of the antibiotic D-cycloserine by D-alanine through the D-alanine–glycine transport system).⁶² Finally, some combination of effects altering cellular metabolism and alkylAP uptake could certainly be at play to cause this striking difference in alkylAP activity accompanying the change in growth environment. The dramatic influence of growth environment has important implications for the development of DXP synthase probes and for the discovery of new agents targeting this underexplored metabolic branch point. Similar to other antibacterial classes targeting primary metabolism²⁶ (e.g., sulfonamide inhibitors of bacterial folate biosynthesis⁶⁴ and antifolate inhibitors of bacterial dihydrofolate reductase⁶⁵), DXP synthase inhibitor activity in rich medium could be poorly predictive of inhibitor activity *in vivo*. Nutrient availability in the host is not uniform. Nutrients (including vitamins) available to the pathogen are variable and dependent upon the *in vivo* microenvironment of the infectious pathogen. Host mechanisms to induce nutrient starvation during infection are a key defense against bacterial pathogens, and successful pathogens have evolved mechanisms to counter these defenses. The fact that vitamin biosynthesis machinery is found in most pathogenic bacteria suggests a requirement for *de novo* vitamin biosynthesis for pathogen adaptation in the host during infection. While it is difficult to mimic the dynamic *in vivo* environment in bacterial cell culture, antimicrobial screening conditions that more closely mimic the nutrient-limiting microenvironments of microbes during infection^{26,27} should be carefully considered in DXP synthase inhibitor discovery.

The novel DXP synthase mechanism combined with its essential cellular function underscore its appeal as a potential new drug target. At the same time, the mechanistic and functional complexities of DXP synthase pose formidable challenges in studies of this target in its cellular context. We have undertaken two approaches to gain evidence supporting DXP synthase as an intracellular target of alkylAPs, including target overexpression²⁷ and metabolite suppression²⁶ experiments. Overexpression of this metabolic branch point enzyme may have deleterious effects on metabolism. There is evidence that DXP synthase aggregates upon overexpression,^{37,38,66} and its overexpression causes toxicity to bacterial cells.^{39–41,43,45,67} Despite this, we have successfully shown that basal expression of this enzyme confers resistance to some alkylAPs, albeit to different degrees. The unknown extent to which the opposing effects of aggregation and toxicity may contribute to the degree of resistance observed makes it difficult to assess target selectivity for the different alkylAPs.

Rescuing growth of alkylAP-treated *E. coli* by supplementation with key metabolites highlights some specific challenges of using this approach to confirm target engagement of alkylAPs. Despite measurable incorporation of labeled DX into ubiquinone in wild-type *E. coli* grown in glucose minimal medium after 24 h,⁶⁸ DX-mediated growth rescue of alkylAP-treated *E. coli* in M9-glucose is not observed. This is not surprising, given that glucose-induced catabolite repression takes place in this medium over the shorter time scale.^{46–48} Further, DX-dependent growth of bacterial strains lacking the *dxs* gene under nutrient-rich growth conditions^{69,70} is supplemented by vitamins and other nutrients that likely rescue two of the three pathways. Under nutrient limitation, however, the metabolic burden of DXP synthase inhibition may be more difficult to overcome by provision of DXP, given the underlying requirement for ThDP and PLP to support flux of DXP into all three pathways.

In contrast, exogenous thiamin should eliminate the many bottlenecks in metabolism introduced by DXP synthase inhibition, given its interdependence with DXP synthase and its interconnection with converging steps of ThDP and PLP biosynthesis. Indeed, addition of thiamin at a minimal concentration of 100 nM appears to substantially reduce the susceptibility of *E. coli* toward alkylAPs, and this effect is most pronounced for medium-chain analogs **4** and **5**. An intriguing characteristic of growth rescue by thiamin supplementation is the delayed outgrowth of alkylAP-treated *E. coli* under these conditions, the timing of which appears to be dependent upon the concentration of alkylAP. This is consistent with the hypothesis that time is required for ThDP-dependent biosynthesis of nucleotides, amino acids, cofactors and other metabolites that are required to overcome DXP synthase inhibition and restore normal growth. Also pertinent is the mechanism of alkylAP-mediated inhibition in which ThDP is itself required for formation of the inhibition complex (PLThDP). In this model, alkylAP and pyruvate compete for newly synthesized holo DXP synthase (E-ThDP). The effect of inhibition through E-PLThDP formation is eventually overcome as new E-ThDP is produced and the productive E-LThDP complex is formed at sufficient levels to support growth (Figure 6). Perhaps the time required for cells to overcome alkylAP-mediated inhibition by this mechanism contributes to the delay in outgrowth. This could have important implications for discovery of new agents targeting DXP synthase, as it is conceivable that delayed outgrowth under thiamin supplementation is a novel and distinct hallmark of ThDP-dependent inhibition of DXP synthase in bacteria. As the most potent alkylAP in this series, butylacetylphosphonate (**4**) could emerge as a useful new probe to study this phenomenon.

Taken together, these results reveal intriguing aspects of alkylAP biochemical and antimicrobial activity and offer insights into the challenges associated with the study of this target. More in depth studies are required to fully understand the context and mechanisms underlying alkylAP antimicrobial activity and the impact of DXP synthase inhibition on cellular metabolism in clinically relevant pathogens.

METHODS

General Methods

Unless otherwise noted, all reagents were obtained from commercial sources. Spectrophotometric analyses were performed on a Beckman DU800 UV/Visible spectrophotometer (Brea, CA, USA). *E. coli* wild-type DXP synthase and *E. coli* MEP synthase (IspC) were overexpressed and purified as reported previously.^{20,71} Pyruvate dehydrogenase from porcine heart was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antimicrobial data were collected on a Molecular Devices Spectramax Plus 384 plate reader or a Tecan Infinite M200 Nanoquant plate reader by measuring OD600 over time. The *E. coli* *tolC* efflux transporter deletion mutant (JW5503-1) and parent BW25113 strains were obtained from the Yale Coli Genetic Stock Center (New Haven, CT, USA). The *E. coli* Fsr overexpression strain was constructed via transformation of a pET-28a-based plasmid (pJLA-Fsr; Kan^R) containing the *fsr* gene from *E. coli* under an IPTG inducible promoter into *E. coli* BL21 (DE3). Clinical isolates of all pathogens were from an in-house strain library maintained at NAU. All microbial manipulation of pathogenic bacteria was

conducted in a certified biosafety level 2 laboratory while following all associated safety protocols.

Synthesis of Acetylphosphonates

Compounds **1** (MAP), **2** (EAP), and **4** (BAP) were synthesized as reported previously.^{15,72} Specific experimental details for **3** and **5–8** are reported in the Supporting Information.

Kinetic Analyses

Enzyme kinetic analyses were performed using *E. coli* DXP synthase as all previous mechanistic studies have been performed on this enzyme. Kinetics were performed on a Beckmann-Coulter DU800 spectrophotometer with temperature control. Michaelis-Menten kinetic analyses of DXP synthase were performed spectrophotometrically using IspC as a coupling enzyme and measuring consumption of NADPH at 340 nm as described previously.^{15,18,20} To measure inhibition of DXP synthase by alkylacetylphosphonates, the assay buffer was incubated at 37 °C and consisted of 100 mM HEPES (pH = 8.0), 0.1 mg/mL bovine serum albumin (BSA), 5 mM NaCl, 2 mM MgCl₂, 2.5 mM tris(2-carboxyethyl)-phosphine (TCEP), 1 mM ThDP, 100 μM NADPH, 1 μM IspC, and 140 μM D-GAP ($5 \times K_M$). The concentration of pyruvate was varied from 12 to 240 μM, and inhibitor concentrations varied from 5 to 50 μM. In all cases, reactions were initiated by the addition of DXP synthase (50 or 100 nM). Experiments were performed in triplicate. To measure inhibition of porcine pyruvate dehydrogenase E1 subunit, enzyme activity was measured spectrophotometrically as previously reported^{15,18,73} by measuring formation of NADH at 340 nm. The assay buffer was incubated at 30 °C and consisted of 100 mM HEPES (pH = 8.0), 0.1 mg/mL BSA, 0.1 mM coenzyme A, 2 mM MgCl₂, 0.3 mM TCEP, 0.2 mM ThDP, 2 mM L-cysteine, and 2.5 mM nicotinamide adenine dinucleotide (NAD⁺). The concentration of pyruvate was varied from 12.5 to 250 μM, and inhibitor concentrations were varied from 50 to 1000 μM. The reaction was initiated by addition of enzyme. Data were subjected to nonlinear regression analysis to determine K_i (GraphPad Prism). Data sets for compounds **1**, **2**, and **4**, previously subjected to Lineweaver-Burke analysis,¹⁵ were reanalyzed with GraphPad Prism for uniformity.

Antimicrobial Susceptibility of *E. coli* MG1655 to alkylAPs in CAMHB Medium

Using aseptic techniques, 3 isolated colonies were selected from a plate containing ATCC MG 1655 *E. coli* K-12 and inoculated into 5 mL of Mueller Hinton Broth 2 (CAMHB, containing acid hydrolysate of casein, beef extract, and starch, pH 7.3; Sigma, St. Louis, MO, USA) at 37 °C. The inoculated culture was incubated with shaking until the turbidity matched a MacFarland standard of 0.5 ($\sim OD_{600} = 0.1$).^{24,74} Colony counts of the experimental inoculum were verified by incubation on CAMHB agar plates for 16 h at 37 °C to confirm consistency between experiments. The standardized inocula (MacFarland = 0.5) contained approximately $1-2 \times 10^8$ CFU/mL and were diluted 1:100 into CAMHB to yield the experimental inoculum which was mixed 1:1 with CAMHB containing the antimicrobial agent at double the desired concentration. The final concentration per well was therefore approximately 10^5 CFU/mL in a volume of 200 μL. The 96-well plates were incubated at

37 °C for 16 h with intermittent shaking. Fractional growth was determined at 16 h relative to the no drug control. Experiments were performed in triplicate.

Antimicrobial Susceptibility of *E. coli* MG1655 to alkylAPs in M9 Minimal Medium

Using aseptic techniques, 3 isolated colonies were selected from a plate containing ATCC MG 1655 *E. coli* K-12, inoculated into 5 mL of M9-glucose minimal medium (containing potassium phosphate, sodium phosphate, sodium chloride, ammonium chloride, magnesium sulfate, calcium chloride, and 0.4% w/v glucose),⁷⁵ and grown to saturation overnight with shaking at 37 °C. The saturated culture was then subcultured (1:50 dilution) into fresh M9-glucose and grown to exponential phase as measured by absorbance ($OD_{600} = 0.4$).²⁶ Colony counts of the experimental inoculum were verified by dilution and enumeration on CAMHB agar for 16 h at 37 °C to confirm consistency between experiments. The exponential phase cell culture was diluted 1:1000 into M9-glucose to yield the experimental inoculum which was mixed 1:1 with M9-glucose containing the antimicrobial agent at 2× the desired concentration. The final concentration of bacteria in each well was approximately 10^5 CFU/mL in a final volume of 200 μ L. The 96-well plates were incubated at 37 °C for 16 h with intermittent shaking. Fractional growth was determined at 16 h relative to the no drug control. Experiments were performed in triplicate.

Antimicrobial Susceptibility of Gram-Negative Bacteria to Butylacetylphosphonate (4)

All clinical isolates were streaked onto CAMHB agar and allowed to grow for 12 h at 37 °C. Colonies were inoculated via aseptic technique into CAMHB (5 mL) and grown to saturation overnight. At this time, organisms were subcultured into M9-glucose (approximately 2 μ L 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 similarly verified to be approximately 2×10^8 CFU/mL via dilution and enumeration. Experiments involving the *E. coli*-Fsr expression strain were performed in the presence of kanamycin (70 μ g/mL) to maintain retention of the pJLA-Fsr plasmid by the cells. At a minimum, experiments were performed in triplicate.

Antimicrobial Susceptibility of *E. coli* BW25113- *tolC* to alkylAPs

Experiments were performed following the procedure described above for the appropriate medium, but starter colonies were picked from plates of either *E. coli* BW25113 (parent) or BW25113- *tolC* (JW5503-1). Cultures grown in M9-glucose medium were supplemented with 20 μ M FeSO₄. Fractional growth was determined at 16 h relative to the no drug control in that cell line. Experiments were performed in triplicate.

Target Identification by DXP Synthase Overexpression in *E. coli*

Using aseptic techniques, 3 isolated colonies were selected from plates of BL21(DE3) *E. coli* containing pET37b, *dxp*ET37b, or E370A*dxp*ET37b expression plasmids, inoculated into 5 mL of Miller LB Broth (LB, Fisher Scientific) containing 50 μ g/mL kanamycin (Kan, Fisher Scientific), and grown to saturation overnight with shaking at 37 °C. The saturated culture was then subcultured (1:50 dilution) into fresh LB containing Kan and grown to

exponential phase as measured by absorbance ($OD_{600} = 0.4$). The exponential phase cell culture was diluted 1:100 000 into LB to yield the experimental inoculum which was mixed 1:1 with LB containing both Kan and the antimicrobial agent at double the desired concentration. Due to the apparent toxicity of IPTG in the presence of alkylAPs, IPTG was omitted.²³ Sufficient DXP synthase production is achieved from leaky expression from this promoter in LB (verified by SDS-PAGE, Figure S9). Colony counts of the experimental inoculum were verified by incubation on LB agar plates for 16 h at 37 °C to confirm consistency between experiments. The final bacterial cell density in each well was approximately 2×10^3 CFU/mL in a final volume of 200 μ L. The 96-well plates were incubated at 37 °C for 13 h with intermittent shaking. Fractional growth was determined at 13 h relative to the no drug control. Experiments were performed in triplicate.

Thiamin Suppression of alkylAP Activity against *E. coli*

Experiments were performed following the procedure described above for antimicrobial susceptibility of *E. coli* MG1655 to alkylAPs in M9-glucose minimal medium, except that the exponential phase cell culture was diluted 1:10 into M9-glucose or M9-glucose containing 200 nM thiamin (2-fold the desired thiamin concentration). This alternate dilution scheme yielded a final concentration of approximately 10^7 CFU/mL *E. coli* and 100 nM thiamin per well. Optimal thiamin concentration for thiamin-mediated growth rescue was determined by evaluating alkylAP antimicrobial activity over a range of thiamin concentrations (0–10 μ M, Figure 5). Similar robust rescue of growth is observed between 100 nM and 10 μ M thiamin. Experiments to measure thiamin-mediated growth rescue for alkylAPs were performed in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) DXP synthase catalyzes the formation of an essential branch point metabolite, DXP, which feeds into isoprenoid, thiamin diphosphate (ThDP), and pyridoxal phosphate (PLP) biosynthesis in bacterial pathogens. (b) DXP synthase is inhibited by alkylacetylphosphonates (alkylAPs) via formation of a phosphonolactyl thiamin diphosphate (PLThDP) adduct. Compounds **1–8** are the focus of this work.

Figure 2.

Octylacetylphosphonate (**7**) is susceptible to efflux by the AcrAB-TolC pump. MIC₉₀ values (a) were measured against *E. coli* BW21153 parent (●) and *tolC* deletion mutant (▲) strains in CAMHB (b) and M9-glucose (c) growth media.

Figure 3.

DXP synthase overexpression analysis for **3**, **4**, **5**, and **6**. Fractional growth inhibition is shown for **3** (a), **4** (b), **5** (c), and **6** (d), with the following legend: pET37b BL21 (DE3) (black), *dxs*-pET37b BL21(DE3) (dark gray), and E370A-*dxs*-pET37b BL21(DE3) (light gray).

Figure 4.

Time dependence of thiamin rescue in BAP-treated *E. coli*. (a) Representative dose response showing suppression of BAP activity by thiamin (Th, 100 nM) is subtle at a starting inoculum density of 10^5 CFU/mL. (b) Evidence for a delay in thiamin-mediated rescue of BAP inhibitory effects at MIC (5 μM) after 16 h (blue diamonds). (c) Suppression of BAP activity at its MIC (20 μM) by thiamin is unambiguous by 16 h when using a starting inoculum density of 10^7 CFU/mL, conditions under which *E. coli* outgrowth is faster. (d) Representative dose response showing exogenous thiamin significantly increases MIC^{BAP} when the starting inoculum density is 10^7 CFU/mL.

Figure 5.

Time dependence of thiamin rescue depends upon alkylAP concentration. (a) Representative dose response showing suppression of the activity of **4** (5–1250 μM) in the presence of thiamin (1 nM–10 μM). Thiamin added to a final concentration of 100 nM is sufficient to achieve maximum outgrowth within 16 h. (b) Representative growth curves showing the time to outgrowth is independent of thiamin concentration above 100 nM but dependent upon alkylAP concentration. Thiamin was added to a final concentration of 100 nM (triangles), 1 μM (circles), or 10 μM (squares) in the presence of low (20 μM **4**, blue) or high (625 μM , green) concentrations of **4** and in the absence of inhibitor (black/gray).

Figure 6.
Model for alkylAP-dependent delay to outgrowth during thiamin-mediated growth rescue.

Table 1

Inhibitory Activities of alkylAPs against DXP Synthase (DXPS) and PDH

cpd	R =	K_i^{DXPS} (μM)	K_i^{PDH} (μM)
1^a	CH ₃	1.0 $\pm$ 0.3	30 $\pm$ 10
2^a	CH ₂ CH ₃	6.71 $\pm$ 0.02	47 $\pm$ 2
3	(CH ₂) ₂ CH ₃	7.1 $\pm$ 0.8	62 $\pm$ 8
4^a	(CH ₂) ₃ CH ₃	5.6 $\pm$ 0.8	335 $\pm$ 8
5	(CH ₂) ₄ CH ₃	1.7 $\pm$ 0.3	240 $\pm$ 40
6	(CH ₂) ₅ CH ₃	8.9 $\pm$ 0.5	117 $\pm$ 9
7	(CH ₂) ₇ CH ₃	1.4 $\pm$ 0.2	57 $\pm$ 5

^aPreviously characterized.¹⁵

Table 2Minimum Inhibitory Concentration (MIC) of alkylAPs in CAMHB and M9 Minimal Medium^a

cpd	MIC ₉₀ (μ M)	
	CAMHB	M9
1	>5000	78
2	>5000	20
3	>5000	10
4	2500	5
5	>5000 ^b	156
6	5000	1250
7	>5000	5000

^aThe highest concentration tested in each case is 5000 μ M. For some compounds tested in CAMHB, MICs could not be determined within this concentration range and are designated by an MIC₉₀ > 5000 μ M.

^bFractional growth = 0.23.

Table 3

Minimum Inhibitory Concentration (MIC) of 4 against Pathogenic Bacteria Grown in CAMHB or M9 Minimal Medium^a

strain	BAP MIC ₉₀ (μM)	
	CAMHB	M9
<i>Salmonella enterica</i> LT2	21505	11
<i>Pseudomonas fluorescens</i>	>21505	43
<i>Rhizobium radiobacter</i>	>21505	22
<i>Micrococcus</i> sp.	10753	86
<i>Klebsiella pneumoniae</i>	>21505	1376
<i>Enterobacter cloacae</i>	>21505	688
<i>Escherichia coli</i> fsr-pET28a	10753	43
<i>Escherichia coli</i> clinical	>21505	11
<i>Bacillus thuringiensis</i> HD 34	>21505	86
<i>Klebsiella oxytoca</i>	>21505	43

^aThe highest concentration tested is 21 505 μM. For some strains tested in CAMHB, MICs could not be determined within this concentration range and are designated by an MIC₉₀ > 21 505 μM.

Table 4

Minimum Inhibitory Concentration (MIC) of alkylAPs in the Presence or Absence of Exogenous Thiamin (Th)^a

cpd	MIC ₈₀ (μM)	
	M9	M9 + Th
1	2500	5000
2	1250	>5000
4	20	>5000
5	313	>5000
6	5000	>5000 ^b

^aThe highest concentration tested was 5000 μM. For some compounds tested under exogenous thiamin, MICs could not be determined within this concentration range.

^bGrowth is fully restored under exogenous Th.