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DXP synthase-catalyzed C-N bond formation: Nitroso substrate specificity studies guide selective inhibitor design

Francine Morris^a, Ryan Vierling^a, Lauren Boucher^b, Jürgen Bosch^b [Professor], and Caren L. Freel Meyers^{*,a} [Professor]

^aDepartment of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine 725 North Wolfe St Baltimore, MD 21205

^bDepartment of Biochemistry and Molecular Biology, The Johns Hopkins Malaria Research Institute, Bloomberg School of Public Health 615 North Wolfe Street Baltimore, MD 21205

Abstract

1-Deoxy-D-xylulose 5-phosphate (DXP) synthase catalyzes the first step in the non-mammalian isoprenoid biosynthetic pathway to form DXP from pyruvate and D-glyceraldehyde 3-phosphate (D-GAP) in a thiamin diphosphate-dependent manner. Its unique structure and mechanism distinguish DXP synthase from its homologs, suggesting it should be pursued as an anti-infective drug target. However, few reports describe development of selective inhibitors of this enzyme. Here, we reveal a function of DXP synthase that catalyzes C-N bond formation and exploit aromatic nitroso substrates as active site probes. Substrate specificity studies reveal high affinity of DXP synthase for aromatic nitroso substrates compared to the related ThDP-dependent enzyme Pyruvate Dehydrogenase (PDH). Results from inhibition and mutagenesis studies indicate nitroso substrates bind to *E. coli* DXP synthase in a manner distinct from D-GAP. Our results suggest that incorporation of aryl acceptor substrate mimics into unnatural bisubstrate analogs will impart selectivity to DXP synthase inhibitors. As proof of concept, we show selective inhibition of DXP synthase by benzylacetylphosphonate (BnAP).

Keywords

enzyme inhibitors; kinetics; isoprenoid biosynthesis; substrate specificity; 1-Deoxy-D-xylulose 5-phosphate (DXP) synthase

Introduction

The isoprenoids are a vast and structurally diverse class of natural products derived from two simple bioprecursors, isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP). Essential in all living organisms, isoprenoids are biosynthesized via two distinct pathways. The mevalonate pathway for IDP and DMADP biosynthesis is found in mammals and fungi. In contrast, most human pathogens, including many bacterial pathogens ^[1] and the malaria parasite, *P. falciparum*, ^[2] are known to use the methylerythritol phosphate (MEP) pathway (Scheme 1) for the generation of IDP and DMADP. ^[3] Its essentiality and

*Tel 410-502-4807; FAX: 410-955-3023 cmeyers@jhmi.edu.

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Put a ring on it: Selective inhibition of DXP synthase is a challenge in developing anti-infectives targeting isoprenoid biosynthesis. DXP synthase preferentially turns over sterically demanding aryl nitroso substrates to form C-N bonds, suggesting a new design for unnatural bisubstrate analogs as selective inhibitors of isoprenoid biosynthesis.

prevalence in human pathogens, and absence in mammals, renders the MEP pathway a target for the development of new anti-infective agents which are desperately needed to combat the emergence and re-emergence of drug resistance.

Seven biosynthetic steps comprise the MEP pathway, beginning with the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) from pyruvate (donor substrate) and D-glyceraldehyde 3-phosphate (D-GAP, acceptor substrate). This first transformation is catalyzed by DXP synthase in a thiamin diphosphate (ThDP)-dependent manner,^[4] and is believed to play a regulatory role in isoprenoid biosynthesis.^{[5] [6] [7] [8]} In addition, DXP synthase is a branch point in pathogen metabolism.^{[4] [9] [10]} Its product, DXP, is required for IDP/DMADP biosynthesis and is also a precursor in pyridoxal biosynthesis and, notably, thiamin diphosphate (ThDP) biosynthesis which is required for the formation of DXP itself.

The importance of DXP synthase in pathogen metabolism highlights this enzyme as a particularly interesting new drug target. Additionally, DXP synthase is mechanistically distinct from other ThDP-dependent enzymes. The enzyme combines decarboxylase and carboligase chemistry in a ThDP-dependent condensation of pyruvate and D-GAP (Scheme 1). A report by Eubanks, *et al.*^[11] provided compelling evidence for a unique catalytic mechanism in which binding of both acceptor and donor substrates is required to induce decarboxylation of pyruvate in the formation of a kinetically competent ternary complex. In subsequent studies,^{[12] [13]} we have provided further support for ternary complex formation during DXP synthase catalysis and D-GAP-promoted decarboxylation of the C2 - lactylthiamin diphosphate (LThDP) intermediate, the pre-decarboxylation intermediate formed by reaction of pyruvate and ThDP. In contrast, all other ThDP-dependent enzymes are believed to follow classical ping-pong kinetics in which activation of pyruvate and release of carbon dioxide precedes binding of the acceptor substrate.^{[14] [15]} In addition, structural analysis indicates DXP synthase bears a unique domain arrangement placing the active site between domains of the same monomer of the homodimer.^[16] This is in contrast to its homologs, where the active site is at the dimer interface.^{[14] [16]} Taken together, these observations highlight unique aspects of DXP synthase catalysis and structure that distinguish it from its mammalian homologs, and suggest that it should be possible to selectively target this enzyme toward the development of new anti-infective agents.. However, reports describing the development of selective DXP synthase inhibitors are scarce,^{[17] [18]} likely due to a perception that selective inhibition of DXP synthase over mammalian ThDP-dependent enzymes will be difficult. We have pursued substrate specificity studies of DXP synthase with the expectation that important substrate binding determinants will be revealed that could guide selective inhibitor design. Previously, we have shown that aliphatic aldehydes are accepted as alternative substrates to generate the corresponding α -hydroxy ketones,^[19] suggesting DXP synthase displays some flexibility toward nonphosphorylated acceptor substrates. A subsequent study revealed the selective inhibitory activity of a series of alkylacetylphosphonates designed to act as unnatural bisubstrate analogs targeting a conformation of DXP synthase that uniquely accommodates both a donor and acceptor substrate in the formation of a ternary complex.^[18] The largest alkylacetylphosphonate, butyl- acetylphosphonate, exhibited greater selectivity of inhibition compared to ethyl- and methyl-acetylphosphonates, indicating selective targeting of DXP synthase is possible.

In the present study, we explore the capacity of DXP synthase to bind sterically demanding scaffolds by evaluating its usage of aromatic acceptor substrates. We demonstrate the capacity of DXP synthase to catalyze the formation of C-N bonds to generate aromatic hydroxamic acids or amides from nitroso substrates. The intrinsically higher reactivity of nitroso substrate analogs compared to their aldehyde counterparts has permitted a substrate specificity study revealing aromatic substrates with high affinity for the enzyme. Further,

our results suggest aromatic substrates may adopt a different binding mode from D-GAP in a relatively large active site compared to PDH or TK. These results have prompted the design and synthesis of a DXP synthase inhibitor bearing an aromatic component to impart selectivity.

Results

Aromatic aldehydes as DXP synthase substrates

Some ThDP-dependent enzymes are known to catalyze C-C bond formation using aromatic substrates with varying turnover efficiencies; [20] [21] [22] however, there are no reports describing DXP synthase usage of aromatic substrates. As a starting point, we tested several aromatic aldehydes as acceptor substrates. 2-Hydroxy-4,6-dinitrobenzaldehyde appeared to be amongst the best of those tested, and was therefore fully characterized as a substrate for DXP synthase (Figure S1). In this case, the K_m is $512 \pm 20 \mu\text{M}$, ~18-fold higher than the natural substrate, D-GAP, and the k_{cat} is low ($k_{cat} = 0.35 \pm 0.05 \text{ min}^{-1}$). The aromatic aldehyde study suggested that there may be flexibility in the active site of DXP synthase toward aromatic acceptor substrates (data not shown). However, a significant number of aromatic aldehydes are not turned over by DXP synthase, suggesting the low intrinsic reactivity of aromatic aldehydes as a limiting factor in substrate specificity studies to probe the enzyme active site.

DXP synthase-catalyzed C-N bond formation

The nitroso group is a functional isostere of the aldehyde group and is known to possess higher reactivity toward nucleophiles. In fact, ThDP-utilizing enzymes transketolase (TK), pyruvate decarboxylase (PDC), benzaldehyde lyase (BAL) and pyruvate dehydrogenase (PDH) have been shown to use aromatic nitroso analogs as acceptor substrates in the formation of hydroxamic acids. [23] [24] [25] [26] [27a,b] We hypothesized that a substrate specificity study of DXP synthase using the intrinsically more reactive aromatic nitroso compound class would better inform us about key binding elements of aromatic substrates. In addition, we postulated that such a study could reveal a new application of DXP synthase as a biocatalyst for the generation of the medicinally-important hydroxamic acid class.

Thus, a series of aromatic nitroso analogs was tested as substrates for DXP synthase. Notably, DXP synthase turns over a range of structurally diverse nitroso substrates (**1**, **3-9**, Scheme 2); most aldehyde counterparts for the nitroso analogs tested are not substrates for the enzyme, consistent with the idea that the nitroso isostere is more reactive. A representative HPLC stackplot, illustrating DXP synthase-catalyzed conversion of the simplest aromatic nitroso analog, nitrosobenzene (**1**), to the corresponding hydroxamic acid (**2**) is shown in Figure 1 (Figure S2). A single *C*-nitroso analog **10** did not act as substrate for the enzyme. Similarly, *N*-nitroso compounds are not substrates. The electron rich *p*-dimethylamino nitroso analog **4** is a substrate for the enzyme (Figure S4), whereas it is not a substrate for yeast TK. [26] Interestingly, the corresponding amides, presumably produced via a mechanism involving the unstable hydroxamic acid as an intermediate, [25] were detected as the major products of several electron rich substrates (**4-8**, Scheme 2, Figure S4-8)). This result has been reported in the study that examined the turnover of **4** by PDC. [25] However, the observation that the amide is also isolated from naphthol substrates was unexpected. In order to rule out the possibility that BSA added to enzymatic reaction mixtures catalyzes formation of amide products, control reactions were performed on **4** and **5** in the absence of BSA. In both cases, only the corresponding amides were detected.

Kinetic parameters were measured spectrophotometrically for the alternative substrates shown in Table 1. Initially, specificity constants (k_{cat}/K_m) were measured, revealing a $k_{cat}/$

K_m for nitrosobenzene that is comparable to the natural acceptor substrate, D-GAP. Reduced specificity constants were measured for larger naphthol-containing substrates (**5-8**, Table 1), an observation that is consistent with the idea that sterically demanding naphthol substrates could exhibit a reduction in efficiency of turnover as a consequence of reduced affinity for the enzyme. However, detailed kinetic analysis of nitroso substrate turnover suggests this is not the case. Small nitrosobenzene analogs display higher reactivity (high k_{cat}) but lower affinity (higher K_m) relative to D-GAP (**1** and **3**, Table 1). Contrary to our expectations, several sterically demanding alternative substrates exhibit high affinities for DXP synthase, with nitrosonaphthols **5-8** showing comparable affinity to the natural substrate. In these cases, a reduced k_{cat} accounts for lower turnover efficiency, in line with previous reports on the sensitivity of nitroso turnover to substituent effects.^[26] The remarkably high affinities measured for sterically demanding substrates on DXP synthase is in stark contrast to previously reported trends in nitroso turnover by ThDP-dependent enzymes,^[27] where increasing steric bulk of the substrate correlates with decreased affinity.

Aromatic nitroso substrates exhibit low affinity for a smaller PDH active site

As a basis for selective inhibitor design, we sought to determine whether DXP synthase displays higher affinity for sterically demanding substrates relative to pyruvate dehydrogenase (PDH). Thus, nitroso analogs **1**, **4** and **6** were evaluated as substrates for porcine PDH (Figure S10, Figure S11). Our results indicate these aromatic substrates exhibit significantly lower affinities for PDH compared to DXP synthase (Table 2) in contrast to the trend observed for DXP synthase. Nitrosobenzene displays a 2.7-fold increase in K_m for PDH compared to DXP synthase, whereas the largest of the nitroso substrates tested, nitrosonaphthol **6**, displays ~19-fold increase in K_m for PDH compared to DXP synthase. We hypothesized the DXP synthase active site may be comparatively larger to accommodate ternary complex formation during catalysis. Indeed, a comparison of active site volumes (calculated by crystal structure coordinates that were aligned in Coot^[28] and then analyzed using Pocket-Finder^[29]) suggests the DXP synthase active site is significantly larger than the ThDP-dependent enzymes, PDH or transketolase (Figure 2, Figure S12). The hydrophobic nature of alternative nitroso substrates tested could potentially drive selectivity of turnover by DXP synthase. However, when the active site pockets of DXP synthase, PDH and TK were analyzed using fpocket,^[30] the computed hydrophobicity score (based on the hydrophobicity scale published by Monera et al.)^[31] indicates that the PDH pocket is more hydrophobic than DXP synthase, while TK has the least hydrophobic pocket (Table S1). Taken together, these results suggest that incorporation of sterically demanding fragments into inhibitor scaffolds may drive selective inhibition and is facilitated mostly by the larger cavity of DXP synthase.

Inhibition of DXP formation by nitroso alternative substrates

The low K_m values measured for aromatic nitroso substrates suggest these analogs bind with reasonable affinity in the enzyme active site. Thus, we hypothesized that alternative substrates bearing aromatic scaffolds could also act as inhibitors of the natural reaction. Compounds **1** and **3-9** were evaluated as inhibitors of DXP synthase using an HPLC-based assay previously reported.^[18] Interestingly, all nitroso compounds exhibited weak inhibitory activity with IC_{50} values ranging from 208 μM to > 2 mM and with no apparent trend with measured K_m values (Table 3). As one of the higher affinity substrates, the readily available nitrosonaphthol **5** was selected for further evaluation in an effort to understand the mechanism of inhibition. This inhibitor was found to exhibit a competitive inhibition pattern with respect to D-GAP (apparent $K_i = 422 \mu M \pm 80 \mu M$, Figure S14). The >10-fold difference between the K_m ($41 \pm 10 \mu M$) and K_i suggests that nitrosonaphthols could adopt a binding mode for turnover that is distinct from the binding mode for inhibition. Alternatively, K_i may reflect the affinity of the Michaelis-Menten complex between enzyme

and nitrosonaphthol, whereas the K_m for this substrate may be indicative of a higher affinity ternary complex further along the reaction coordinate in this two substrate system.

Nitrosonaphthols and D -GAP adopt distinct binding modes during turnover

R478 and R420 are known to be essential for binding of D -GAP, presumably by anchoring the phosphate group (results to be published elsewhere). Two DXP synthase variants (R478A and R420A) were evaluated as catalysts for C-N bond formation using nitrosonaphthols **5-7**. While both of these variants adversely affect the binding of D -GAP, they have no apparent effect on the affinities of nitroso substrates in C-N bond formation, as indicated by comparable K_m values measured for nitroso substrates by both variants and wild type enzyme (Table 4, Figure S15). This is consistent with the notion that nitrosonaphthols adopt a binding mode for turnover that is distinct from D -GAP.

Selective inhibition of DXP synthase by benzyl acetylphosphonate (BnAP)

Our results suggest that the comparatively large active site of DXP synthase can accommodate sterically demanding scaffolds, but in a manner that does not interfere with DXP formation. On this basis, we hypothesized that aromatic components could be incorporated into unnatural bisubstrate analogs to impart selectivity of inhibition against DXP synthase. To demonstrate this concept, we prepared benzyl acetylphosphonate (BnAP) as a potential selective inhibitor of DXP synthase. BnAP incorporates the acetyl phosphonate moiety as a pyruvate mimic and a benzyl group to mimic the alternative acceptor substrate, nitrosobenzene (Figure 3a). As expected, BnAP is a competitive inhibitor with respect to pyruvate with reasonable potency against DXP synthase ($K_i = 10.4 \pm 1.3$ μ M, Figure 3), and exhibits ~85-fold higher inhibitory activity against DXP synthase compared to PDH (Figure 3). Additionally, BnAP exhibits an uncompetitive inhibition pattern with respect to D -GAP ($K_i = 70 \pm 8$ μ M, Figure 3d). The requirement for D -GAP binding is consistent with the idea that aromatic scaffolds adopt a binding mode that is distinct from D -GAP.

Discussion

DXP synthase represents an attractive drug target for the development of new anti-infective agents, and selective inhibitors of this enzyme are sought. The present study highlights C-N bond formation as a new reaction catalyzed by DXP synthase and demonstrates nitroso substrates as useful tools for probing the active site of this potential drug target. Our study shows that DXP synthase-catalyzed C-N bond formation can lead to the generation of hydroxamic acids and amides, with electron rich nitroso substrates giving predominantly amide products. Although the mechanism for this transformation is not elucidated, it is thought to occur via a hydroxamic acid intermediate.^[25] Notably, we have demonstrated that nitroso substrate analogs bearing a naphthol scaffold exhibit exceptional affinity for DXP synthase that is comparable to the natural acceptor substrate, D -GAP. Further, sterically demanding substrates are selectively turned over by DXP synthase and show considerably lower affinity for the ThDP-dependent enzyme PDH. Consistent with this finding, active site volume calculations indicate the DXP synthase active site is significantly larger compared to PDH or transketolase and can uniquely accommodate sterically demanding alternative substrates. The alternative acceptor substrates tested in this study are surprisingly weak inhibitors of DXP formation with nitrosonaphthol **5** acting as a weak competitive inhibitor against D -GAP. The >10-fold discrepancy between K_m and K_i for this compound could suggest multiple binding modes are possible for **5**, or could reflect a lower affinity complex *en route* to a higher affinity ternary complex (described by $K_m^{\text{nitrosonaphthol}}$). Evidence that nitrosonaphthols adopt a distinct binding mode to D -GAP during turnover was obtained through substitution of R478 and R420, active site residues essential for D -GAP binding.

R478A and R420A variants display efficient turnover and comparable affinity for nitrosonaphthols compared to wild type DXP synthase. Further studies are needed to define critical residues for C-N bond formation, and especially those that are important for aromatic substrate binding.

Taken together, the data suggest that incorporation of an aromatic group into an unnatural bisubstrate analog scaffold should serve to impart selectivity of inhibition against DXP synthase over other ThDP-dependent enzymes. Indeed, benzylacetylphosphonate selectively inhibits DXP synthase with a K_i of $10.4 \pm 1.3 \mu\text{M}$ and $K_i^{\text{PDH}}/K_i^{\text{DXPS}} \sim 85$. Although comparable in inhibitory activity to butylacetylphosphonate,^[18] an increase in $K_i^{\text{PDH}}/K_i^{\text{DXPS}}$ is observed with BnAP, suggesting sterically demanding aromatic acetylphosphonates as a promising new class of selective DXP synthase inhibitors.

Experimental Section

General

Unless otherwise noted, all reagents were obtained from commercial sources. HPLC analyses were performed on a Beckman Gold Nouveau System with a Grace Alltima 3 μm C18 analytical Rocket® column (53 mm $\times$ 7 mm). Spectrophotometric analyses were carried out on a Beckman DU 800 UV/Vis spectrophotometer. Mass spectrometric analyses were either performed on Shimadzu LC-MS IT-TOF, Thermo Fisher Finnigan LCQ Classic or obtained through the University of Illinois at Urbana-Champaign Mass Spectrometry Lab. All enzymatic reactions were carried out in low-retention microcentrifuge tubes to prevent adsorption of hydrophobic substrates. All enzyme reactions contain 10% DMSO to solubilize hydrophobic substrates. The natural reaction is minimally impacted under these conditions. Purification of recombinant DXP synthase was performed as previously described.^[19] Protein concentration was determined using the Bradford assay. Porcine pyruvate dehydrogenase was obtained from a commercial source and specificity activity was determined by the manufacturer. For chemical synthesis, dichloromethane was distilled over calcium hydride. Anhydrous acetonitrile was packed in Sure-Seal bottles. All reactions were carried out under an inert argon atmosphere. NMR spectra were taken on a Varian 500 MHz spectrometer. Reaction progress was monitored via ^{31}P NMR with triphenylphosphine oxide (TPPO, $\delta = 0$ ppm) dissolved in deuterated benzene as an external standard. Chemical shifts are reported in units of parts per million (ppm), relative to a standard reference point. ^1H NMR chemical shifts are reported relative to tetramethylsilane (TMS, $\delta = 0$ ppm) as internal reference. Preparative HPLC was performed on a Beckman Gold Nouveau system with a Varian Dynamax 250 $\times$ 21.4 mm Microsorb C18 column.

HPLC analysis of DXP synthase-catalyzed C-N bond formation and product characterization

Reaction mixtures containing 100 mM HEPES, pH 8.0, 2 mM MgCl_2 , 5 mM NaCl, 1 mM ThDP, 1 mg/mL BSA, 10-20 mM pyruvate, 10% DMSO, 0.5 mM – 5 mM nitroso substrate were pre-incubated at 37 °C for 5 min. Reactions were initiated with 1-5 μM enzyme. Aliquots of enzymatic mixture were removed at various time intervals and quenched into an equal volume of cold methanol. Quenched mixtures were incubated on ice for 20 minutes. Precipitated proteins were removed by centrifugation, and the supernatant was analyzed by HPLC with UV detection using the following conditions: Flow rate = 3 mL/min; Solvent A: 100 mM NH_4OAc , pH 4.6; Solvent B: acetonitrile; Method: 0-100% B over 10 min. New products formed were extracted from the supernatant using ethyl acetate (3 $\times$). Combined organic extracts were concentrated, and the resulting samples were dissolved in MeOH and re-subjected to HPLC analysis to confirm that product degradation does not take place

during the extraction procedure. Products were subsequently characterized by mass spectrometry.

Determination of kinetic parameters for nitroso substrates

Reaction mixtures containing 100 mM HEPES, pH 8.0, 2 mM MgCl₂, 5 mM NaCl, 1 mM ThDP, 1 mg/mL BSA, 10 – 20 mM pyruvate, 10% DMSO v/v, and 10 – 300 μ M nitroso substrate were pre-incubated at 37 °C for 5 min. Enzymatic reactions were initiated by addition of 0.5 – 2 μ M DXP synthase (or 0.1 units/ml PDH) and monitored spectrophotometrically by measuring the rate of disappearance of the nitroso substrate at its corresponding λ_{max} . Substrate concentration as a function of time was determined from absorbance values using Beer's Law. Initial reaction rates were determined from the linear range of the reaction progress curve, usually within 1-3 min. Data analysis to determine k_{cat} and K_{m} for each alternative substrate was carried out using GraFit version 7 from Erithacus Software.

Evaluation of nitroso substrates as inhibitors of DXP formation

Reaction mixtures containing 100 mM HEPES, pH 8.0, 2 mM MgCl₂, 5 mM NaCl, 1 mM ThDP, 1 mg/mL BSA, 10% DMSO v/v, 30 μ M D-GAP, 80 μ M pyruvate, and varying concentrations of nitroso inhibitor were pre-incubated at 37 °C for 5 min. Enzyme reactions were initiated by addition of 0.1 μ M DXP synthase. Aliquots (150 μ L) of the enzymatic mixture were removed between 0.5 and 3 minutes and quenched into ice-cold methanol (150 μ L). Precipitated protein was removed by centrifugation, and the supernatant was diluted in an equal volume of water. The nitroso substrate was removed by extraction into acetonitrile (3 $\times$), using a previously described freeze-extraction technique. [32] The aqueous layer maintained a constant ratio of D-GAP and DXP during the extraction, and was subjected to derivatization conditions to produce the corresponding hydrazones, using 5-fold excess 2,4-dinitrophenylhydrazine [19] for 20 min to ensure complete derivatization of substrates and product at low concentration. The derivatization mixtures were analyzed by HPLC as previously described. [19] To determine initial reaction rates in the presence of varying inhibitor concentration, the D,L-GAP and DXP hydrazone HPLC peak areas were measured, and the product concentration was determined as a percent of total peak area and plotted against reaction time. Initial rates GraFit version 7 from Erithacus Software was utilized to generate IC₅₀ curves.

Active site volume calculations

Coordinates for ThDP-dependent enzymes, *D. radiodurans* DXS (2O1X), [16] human PDHE1p (3EXE) [33] and transketolase (3MOS) [34] were structurally aligned in Coot [28] using LSQ Superpose and residue ranges A:151-164 (2O1X), E:164-177 (3EXE) and A:152-165 (3MOS). The choice of residues was based on the close proximity to ThDP in order to maximize a similar orientation of the active site region of interest. The r.m.s. deviation, calculated with VMD [35] between residues lining the ThDP binding site was 1.54 Å (2O1X:3EXE) and 1.01 Å (2O1X:3MOS) for 16 C $_{\alpha}$ backbone atoms. The biological assembly of transketolase (3MOS) was determined using the PISA [36] web-server. Aligned structures were uploaded to the Pocket-Finder [29] web-server to determine active site pocket volumes. Co-factors ThDP or ThDP and metal ions were treated as part of the protein and all other molecules discarded for purposes of defining the protein surface for pocket detection. Pocket-Finder reported volumes and generated space-filling models for the active site pocket in each structure corresponding to the pocket adjacent to TDP in chain A of 2O1X. An overlay of the mesh representations with respect to the active site co-factor and metal ion was rendered in PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0, Schrödinger, LLC).

Active site pocket hydrophobicity calculations using fpocket

Fpocket^[30] (Table S1) was run to detect and analyze pockets in DXP synthase (2O1X), PDH (3EXE) and TK (3MOS). The complete coordinate file for DXP synthase and PDH, and the biological assembly for TK, were used as inputs for fpocket. The default cofactor list for fpocket was modified to include TDP and TPP prior to program compilation so that the ThDP cofactor would be treated as a part of the protein as opposed to a removable ligand. The pockets corresponding to the active sites used for the volume calculations using Pocket Finder were determined visually and the parameters recorded.

Synthesis of BnAP (Figure S16)

Benzylacetylphosphonate was prepared from phosphorus trichloride using standard procedures. Tribenzyl phosphite was generated from benzyl alcohol, diisopropylethylamine and phosphorous trichloride according to Saady *et al.*^[37] The spectral properties of the compound are identical to published values. For the preparation of benzylacetylphosphonate (BnAP), a flame-dried flask, cooled under argon, was charged with 0.32 mL (4.5 mmol) acetyl chloride. Tribenzyl phosphite (0.46 g, 1.3 mmol) was dissolved in 13 mL of anhydrous dichloromethane, and the resulting mixture was added dropwise to acetyl chloride. Reaction progress was monitored via ³¹P NMR, and complete conversion of tribenzyl phosphite (−113 ppm) to dibenzylacetyl-phosphonate (−26 ppm) was observed within one hour. Volatiles were removed *in vacuo*, and the crude material was used without further purification. Dibenzylacetylphosphonate was dissolved in 2.2 mL of anhydrous acetonitrile, and lithium bromide (0.17 g, 0.95 mmol) was added in one portion. The reaction mixture was heated to 50 °C for ~ 4 hours. The lithium salt of benzylacetylphosphonate precipitated from solution and was removed by filtration. The filter cake was washed successively with 20 mL portions of cold acetonitrile and diethyl ether. The crude product was purified by reversed-phase preparative HPLC. Flow rate = 10 mL/min; Solvent A: 50 mM HNet₃OAc, pH 6.0; Solvent B: Methanol; Method 5-80% B over 75 minutes. The purity of fractions was determined by analytical reverse-phase HPLC. Flow rate = 3 mL/min; Solvent A: 50 mM HNet₃OAc, pH 6.0; Solvent B: Methanol; Method 5-80% B over 12 minutes. Combined fractions were lyophilized to yield a final mass of 0.0975 grams BnAP as the triethylammonium salt (24% over two steps). ³¹P NMR (D₂O): −27.43 (s) ¹H-NMR (D₂O): 1.20 (t, 9H), 2.31 (d, 3H), 3.11 (m, 6H), 4.91 (d, 2H), 7.35 (m, 5H). HRMS (ESI), calculated m/z for C₁₅H₂₇NO₄P (triethylammonium salt), [M + H]⁺ = 316.1678; observed: 316.1673.

Inhibition of DXP synthase by BnAP

In order to evaluate the inhibitory activity of BnAP against DXP synthase, a continuous spectrophotometric coupled assay was used to measure formation of DXP by monitoring IspC consumption of NADPH (340 nm).^[2] DXP synthase reaction mixtures (previously described) including BnAP (15, 30, and 60 μM), IspC (1 μM) and NADPH (100 μM) were pre-incubated at 37 °C for 5 minutes. Initial rates were measured after the reaction was initiated by the addition of DXP synthase. Inhibition of the coupling enzyme (IspC) by BnAP was not observed up to 1.5 mM. Experiments were performed in triplicate. Double reciprocal analysis of data was carried out using GraFit version 7 from Erithacus Software.

Inhibition of PDH by BnAP

Pyruvate dehydrogenase activity was measured spectrophotometrically as previously reported^[38] by monitoring absorbance changes at 340 nm due to reduction of NAD⁺ by PDH. Reaction mixtures contained 100 mM HEPES (pH 8.0), 1 mg/mL BSA, 0.2 mM ThDP, 0.1 mM coenzyme A, 1 mM MgCl₂, 2 mM cysteine, 0.3 mM *tris*(2-carboxyethyl)phosphine (TCEP). The reaction was initiated with enzyme (0.01 units/ml) and

activity was monitored at 30 °C. For inhibition studies, reaction mixtures (described above) including BnAP (0.5, 1, 2.25 mM) were pre-incubated at 30°C for 5 minutes. Initial rates were measured immediately after reactions were initiated by addition of PDH (0.01units/ml). Double reciprocal analysis of data was carried out using GraFit version 7 from Erithacus Software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HPLC analysis of the DXP synthase-dependent conversion of nitrosobenzene to hydroxamic acid 2.

Figure 2. Overlay of DXP synthase, transketolase (TK) and PDH E1 subunit active site pockets. The DXP synthase active site (cyan) is predicted to be larger than TK (purple) or PDH E1 subunit (brown) active sites.

Figure 3.

BnAP is a selective inhibitor of DXP synthase. Representative double reciprocal plots are shown. A) Design of BnAP as a selective inhibitor of DXP synthase. B) BnAP is a competitive inhibitor of DXP synthase with respect to pyruvate ($K_i = 10.4 \pm 1.3 \mu\text{M}$). The concentration of pyruvate was varied (20-200 μM) at several fixed concentrations of BnAP (0 (), 15 (), 30 () and 60 () μM) and 100 μM D-GAP; C) BnAP is an uncompetitive inhibitor of DXP synthase with respect to D-GAP ($K_i = 70 \pm 8 \mu\text{M}$). The concentration of D-GAP was varied (10-120 μM) at fixed concentrations of BnAP (0 (), 25 (), 50 () and 75 () μM) and 200 μM pyruvate; D) BnAP is a competitive inhibitor of PDH with respect to pyruvate and exhibits selective inhibition against DXP synthase compared to PDH ($K_i^{\text{PDH}} = 882 \pm 78 \mu\text{M}$, $K_i^{\text{PDH}}/K_i^{\text{DXPS}} \sim 85$). The concentration of pyruvate was varied (20-200 μM) at several fixed concentrations of BnAP (0 (), 0.5 (), 1 () and 2.25 () mM).

Scheme 1.
Biosynthesis of isoprenoids IDP and DMADP via the methylerythritol phosphate (MEP) pathway.

Accepted as substrates Not accepted as substrates

Scheme 2.
Nitroso substrate usage by DXP synthase.

Table 1Substrate specificity of nitroso substrates.^a

Substrate	k_{cat} (min ⁻¹) ^b	K_M (μM) ^b	$k_{cat}/K_M, \times 10^4$ (M ⁻¹ •min ⁻¹)
D-GAP	102 ± 7	28 ± 4	364 ± 60
1	175 ± 19	133 ± 18	132 ± 20
3	36 ± 7	99 ± 16	36 ± 9
4	0.9 ± 0.1	54 ± 13	1.7 ± 0.5
5	1.1 ± 0.2	41 ± 10	2.7 ± 0.8
6	2.0 ± 0.2	24 ± 6	8 ± 2
7	1.18 ± 0.04	18 ± 4	6.6 ± 1.5
8	1.3 ± 0.2	63 ± 7	2.1 ± 0.4
9	1.4 ± 0.2	387 ± 18	0.36 ± 0.05

^aReaction conditions: 100 mM HEPES, pH 8.0, 2 mM MgCl₂, 5 mM NaCl, 1 mM ThDP, 1 mg/mL BSA, 10 – 20 mM pyruvate, 10% DMSO v/v, 37 °C

^bPerformed in triplicate. Values shown are the average ± SEM.

Table 2

Determination of K_m for nitroso substrates against *E. coli* DXP synthase (DXPS) compared to Porcine PDH E1 subunit

Substrate	PDH K_m , μM ^[a]	$K_m^{\text{PDH}}/K_m^{\text{DXPS}}$
1	350 ± 30	2.7
4	408 ± 60	7.5
6	450 ± 16	19.3

^aPerformed in triplicate. Values shown are the average ± SEM.

Reaction conditions: 100 mM HEPES, pH 8.0, 2 mM MgCl₂, 5 mM NaCl, 1 mM ThDP, 1 mg/mL BSA, 10 – 20 mM pyruvate, 10% DMSO v/v, 37 °C

Table 3Inhibition of DXP formation by nitroso substrates.^a

Substrate	IC ₅₀ (μM) ^b
1	208 ± 20
3	291 ± 11
4	844 ± 170
5	1065 ± 190 (K _i = 422 ± 80 μM)
6	522 ± 60
7	354 ± 90
8	> 2000
9	> 2000

^aReaction conditions: 100 mM HEPES, pH 8.0, 2 mM MgCl₂, 5 mM NaCl, 1 mM ThDP, 1 mg/mL BSA, 10% DMSO v/v, 30 μM D-GAP, 80 μM pyruvate, 37 °C.

^bPerformed in triplicate. Values shown are the average ± SEM.

Table 4WT, R478A and R420A catalyze comparable turnover of nitrosonaphthols.^a

Alternative Substrate	WT DXP synthase K_m , μM	R478A K_m , μM^b	R420A K_m , μM^b
5	41 $\pm$ 10	23 $\pm$ 4	32 $\pm$ 5
6	24 $\pm$ 6	20 $\pm$ 3	16 $\pm$ 1
7	18 $\pm$ 4	13 $\pm$ 1	14 $\pm$ 2

^aReaction conditions: 100 mM HEPES, pH 8.0, 2 mM MgCl_2 , 5 mM NaCl, 1 mM ThDP, 1 mg/mL BSA, 10 – 20 mM pyruvate, 10% DMSO v/v, 37 °C

^bPerformed in triplicate. Values shown are the average $\pm$ SEM.