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Characterization of Two Late-Stage Enzymes Involved in Fosfomycin Biosynthesis in Pseudomonads

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Abstract

The broad-spectrum phosphonate antibiotic fosfomycin is currently in use for clinical treatment of infections caused by both Gram-positive and Gram-negative uropathogens. The antibiotic is biosynthesized by various streptomycetes, as well as by pseudomonads. Notably, the biosynthetic strategies used by the two genera share only two steps: the first step in which the primary metabolite phosphoenolpyruvate (PEP) is converted to phosphonopyruvate (PnPy), and the terminal step in which 2-hydroxypropylphosphonate (2-HPP) is converted to fosfomycin. Otherwise, distinct enzymatic paths are employed. Here, we biochemically confirm the last two steps in the fosfomycin biosynthetic pathway of *Pseudomonas syringae* PB-5123, showing that Psf3 carries out the reduction of 2-oxopropylphosphonate (2-OPP) to (S)-2-HPP, followed by the Psf4-catalyzed epoxidation of (S)-2-HPP to fosfomycin. Psf4 can also accept (R)-2-HPP as a substrate, but instead performs an oxidation to make 2-OPP. We show that the combined activities of Psf3 and Psf4 can be used to convert racemic 2-HPP to fosfomycin in an enantioconvergent process. X-ray structures of each enzyme with bound substrates provide insights into the stereospecificity of each conversion. These studies shed light into the reaction mechanisms of the terminal two enzymes in a distinct pathway employed by pseudomonads for the production of a potent antimicrobial agent.

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SUPPORTING INFORMATION

Additional experimental procedures, structural characterization details, and supporting figures as mentioned in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Atomic coordinates have been deposited in the Protein Data Bank (www.rcsb.org) with the following accession codes: 5U55 (Psf4-Mn²⁺-(S)-2-HPP), 5U5D (Psf4-Mn²⁺-(R)-2-HPP), 5U57 (Psf4-Fe²⁺-(S)-2-HPP), 5U58 (Psf4-Fe²⁺-(R)-2-HPP), and XXX (Psf3-NADP⁺-2OPP).

INTRODUCTION

Fosfomycin (trade name Monurol), is a clinically approved broad-spectrum natural product antibiotic used for the treatment of a variety of infections including cystitis.¹ The compound contains two notable chemical groups, namely a phosphonate that likely facilitates entry into target cells via the α -glycerophosphate transporter, and an epoxide that acts as a chemical warhead for inactivation of the target UDP N-acetylglucosamine-3-enolpyruvyltransferase that catalyzes the committed step in bacterial peptidoglycan biosynthesis.^{2–5} Due to the unique mode of action, it is often administered in combination with other antimicrobial agents, most notably in combination therapy against lung infections in cystic fibrosis patients.⁶ The antibacterial spectrum of fosfomycin includes several enteric Gram-negative bacteria, but the compound is also effective against several Gram-positive drug-resistant cocci including methicillin-resistant staphylococci and vancomycin-resistant enterococci.^{7,8}

Seto and colleagues identified a putative gene cluster encoding the fosfomycin biosynthetic operon from *Streptomyces wedmorensis* NP-7 and verified the integrity of the pathway through heterologous expression.⁹ Subsequently, gene disruption analysis of the biosynthetic cluster from *Streptomyces fradiae* identified a minimal cluster containing five enzymatic activities.¹⁰ As in the biosynthesis of other phosphonate natural products, the first step of this pathway consists of the thermodynamically unfavorable conversion of phosphoenolpyruvate (PEP) to phosphonopyruvate (PnPy) by PEP mutase.^{11,12} Catalysis of the irreversible decarboxylation of PnPy by a thiamine-dependent decarboxylase drives the reaction forward to yield phosphonoacetaldehyde (PnAA). Reduction of PnAA to hydroxyethylphosphonate,^{13,14} followed by methylation, in a *S*-adenosylmethionine (SAM)- and methylcobalamin (MeCbl)-dependent step yields (*S*)-2-hydroxypropylphosphonate ((*S*)-2-HPP).^{10,15–18} Epoxidation of (*S*)-2-HPP, carried out by the mononuclear, non-heme Fe(II)-dependent enzyme HppE, completes the biotransformation to produce fosfomycin.^{19–21}

Investigations into the novel mechanism utilized for the SAM/MeCbl-dependent methylation were hampered by the poor aqueous solubility of heterologously expressed Fom3, the enzyme that is proposed to catalyze this step.¹⁵ Attempts to identify homologous enzymes with more suitable solution properties focused on pseudomonads, as *P. viridiflava* PK-5, *P. fluorescens* PK-52,²² and *P. syringae* PB-5123²³ had been reported as producers of fosfomycin and various analogs. Genomic sequencing of *P. syringae* PB-5123 identified a putative phosphonate biosynthetic cluster that encodes a characteristic PEP mutase (called Psf1) involved in C-P bond formation (Figure 1A).²⁴ While this cluster also contains a putative HppE (termed Psf4), genes homologous to enzymes that catalyze any of the other biosynthetic steps in *Streptomyces* are absent. Rather, some of the genes in the *P. syringae* PB-5123 cluster show similarity to genes present in the biosynthetic pathways of other phosphonates, such as FR-900098,²⁵ and phosphinothricin.^{26,27} For example, the *P. syringae* PB-5123 pathway lacks the ubiquitous PnPy decarboxylase that is coupled with PEP mutase to thermodynamically drive the reaction forward. Instead, the unfavorable equilibrium for PnPy formation is driven forward by the addition of an acetyl group from acetyl-CoA by a citrate synthase-like enzyme Psf2 (Figure 1A).²⁴

While *in vitro* reconstitution of the remaining enzymes in the pathway was not demonstrated, a chemically plausible pathway for fosfomycin biosynthesis, starting from phosphonomethylmalate (Pmm, the product of the Psf2-catalyzed reaction) could be envisaged (Figure 1A).²⁴ As in the *Streptomyces* pathway, the terminal reaction in the *P. syringae* PB-5123 cluster is the epoxidation of (*S*)-2-HPP to yield fosfomycin.^{21,28} While the *P. syringae* Psf4 is only distantly related to the *S. wedmorensis* enzyme HppE, prior studies have confirmed identical epoxidase activity of Psf4.^{28,29} However, detailed analyses of the similarities and differences between Psf4 and the structurally well-characterized HppE²⁰ have yet to be carried out. The pseudomonad pathway harbors two genes, *psf3* and *psf6*, whose products show similarities to dehydrogenases, suggesting that a likely substrate for the penultimate step may be 2-oxopropylphosphonate (2-OPP). However, enzymatic activity for neither Psf3 nor Psf6 has been demonstrated. In addition, reduction of 2-OPP to 2-HPP must occur to provide the correct *S* stereochemistry, as only this enantiomer can form the product fosfomycin. Both Psf4 and HppE can also utilize the other stereoisomer (*R*)-2-HPP as a substrate by performing an oxidation to form the methylketone 2-OPP (Figure 1B,C).^{30–32}

Here, we present *in vitro* characterization of Psf3 as a reductase that catalyzes the NADPH-dependent conversion of 2-OPP to (*S*)-2-HPP, and we report Michaelis-Menten parameters for the wild-type enzyme. In order to discern the molecular basis for this stereospecific transformation, we determined the 2.05 Å resolution crystal structure of Psf3 with bound cofactor and 2-OPP substrate. The roles of several active site residues in catalysis were established through kinetic analysis of site-directed variants. We also present several cocrystal structures of the Psf4 epoxidase with bound substrates, which allow for comparisons with the previously determined structure of HppE.^{20,31} These biochemical studies provide mechanistic insights into the final steps in the biosynthesis of fosfomycin in pseudomonads and reveal new information regarding the middle reactions of the biosynthetic pathway that are unaccounted for in this convergent method of fosfomycin production.

RESULTS AND DISCUSSION

Reconstitution and stereospecificity of Psf3 reductase activity

Based on the *in vitro* characterization of the acetyltransferase Psf2, we had previously proposed a chemical route for the formation of fosfomycin starting with Pmm. Although we could not establish activity for any of the enzymes in these steps, the known substrate for Psf4, (*S*)-2-HPP, was presumed to have derived from the reduction of 2-OPP. In order to ascertain the enzyme involved in this step, we expressed and purified the candidate gene product Psf3, which showed sequence similarity to oxidoreductases. The substrate 2-OPP was produced through chemical deprotection of the commercially available dimethyl-2-OPP, and the integrity of the compound was established by ¹H and ³¹P NMR spectroscopy. Psf3 carried out the reduction of 2-OPP to (*S*)-2-HPP (Figure 2A and S1A). In order to determine the preferred hydride donor for the reduction, initial rates were determined using either NADH or NADPH, showing a 50-fold faster rate with NADPH as the cofactor (Table S1). Kinetic parameters, with respect to 2-OPP and determined by monitoring the consumption

of NADPH, showed that Psf3 catalyzed reduction with a catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) of $1.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (Table S2), which is a value comparable to that of other reductases.³³

In order to establish the stereospecificity of the transformation, we examined the reverse reaction, i.e. the oxidation of the two enantiomers of 2-HPP by Psf3 in the presence of excess NADP⁺ (Figure 2B). Spectroscopic analysis of each reaction using ³¹P NMR spectroscopy showed that the reaction with (*S*)-2-HPP resulted in a new peak that is consistent with 2-OPP (as determined by spiking with authentic standards), while no reaction could be observed with (*R*)-2-HPP. These data establish that Psf3 catalyzes the NADPH-dependent reduction of 2-OPP, with (*S*)-2-HPP as the product.

Crystal structure of Psf3 and basis for the stereospecificity of hydride transfer

Although bioinformatics analysis identifies Psf3 as an NAD(P)H-dependent dehydrogenase, attempts at structure-based modeling are precluded by the low (around 23%) sequence identity with similar proteins of known structure. Thus, we determined the 2.05 Å resolution cocrystal structure of Psf3 with bound ligands NADP⁺ and 2-OPP. The use of the oxidized form of the cofactor prohibited catalytic turnover of 2-OPP and facilitated structure determination of the ternary complex. The overall structure is similar to that of other enzymes within the 3-hydroxyacid dehydrogenase fold family, and consists of an N-terminal Rossmann fold, composed of residues Ala3 through Glu165, appended to a helical C-terminal dimerization domain formed by Gly167 through His288 (Figure 3A). As in other enzymes within this fold family, Psf3 forms homodimers, both in solution and in the crystal. Dimer formation occurs entirely via the C-terminal helical domain wherein two large α helices from each monomer engage each other to form a central coiled-coil around which four smaller helices from the respective monomers are stacked. The resultant dimer interface is extensive and results in the burial of roughly 1900 Å² of surface area, as determined using Chimera.³⁴

A DALI³⁵ search against the Protein Data Bank³⁶ identifies members of the 3-hydroxyacid dehydrogenase family as the closest structural homologs, despite only modest conservation in primary sequence (Figure 4). Examples include 6-phosphogluconate dehydrogenase (PDB code 3PEF; 26% sequence identity; Z-score of 36.3; RMSD of 1.9 Å over 285 aligned Cα atoms),³⁷ tartronate semialdehyde reductase (PDB code 1VPD; 21% sequence identity; Z score of 35.5; RMSD of 1.9 Å over 285 aligned Cα atoms),³⁸ and L-serine dehydrogenase (PDB code 3OBB; 26% sequence identity; Z score of 33.2; RMSD of 2.5 Å over 283 aligned Cα atoms).³⁹ The conservation of the overall fold persists in spite of a low sequence identity. However, unlike canonical 3-hydroxyacid dehydrogenase enzymes, the substrate for Psf3 is not an acid but rather a phosphonate, illustrating how a prevalent protein fold has been adapted to function on a divergent type of substrate.⁴⁰

The NADPH cofactor is housed within the N-terminal Rossmann-fold domain, where the adenine ring is stabilized through stacking between Arg33 and helix α4 formed by Ser69-Ala78. The 2'-phosphate of NADPH is engaged by interactions with Arg33 and Lys37 (Figure 3B). In the structure of the NADH-dependent tartronate semialdehyde reductase (PDB code 1VPD), an equivalent Arg33 protrudes into the active site and would occlude binding of the 2'-phosphate from an NADPH cofactor,³⁸ illustrating that this residue is not

predictive of NADP(H) use. The nicotinamide is bound on one side by the side chain of Met13, which precludes substrate binding to this face. The substrate 2-OPP is bound directly above the cofactor and is located in a cleft between the Rossmann-fold and dimerization domains.

Two main active-site features specific to Psf3 likely contribute to the utilization of a phosphonate, rather than a carboxylate, substrate. First, the Psf3 active site is composite, and both monomers of the homodimer contribute residues that can engage the phosphonate substrate. For example, Glu180 and Tyr217 from one monomer are both poised to interact with the phosphonate oxygens (Figure 3C), whereas Arg212 (located in a loop following helix α 10) from the other monomer caps the base of the binding site to provide additional interactions with a phosphonate oxygen. The R212K mutation in Psf3 results in a 10^3 -fold decrease in the catalytic efficiency (Table S2, Figure S2). Secondly, helix α 11, located in the dimerization domain, is shifted into the substrate-binding site relative to its position in canonical 3-hydroxyacid dehydrogenases, and this movement positions Tyr217 at a suitable location to interact with one of the oxygen atoms of the phosphonate substrate (Figure 3C). In other enzymes of this family, the equivalent residue is a small aliphatic Ala/Val, or is otherwise positioned away from the active site. Similar composite active sites have been observed in the structures of other phosphonate biosynthetic enzymes such as 2-hydroxyethylphosphonate dioxygenase⁴¹ and HppE.²⁰

In the Psf3 ternary complex structure, the methylketone of 2-OPP is positioned directly above the nicotinamide ring of the cofactor, where it would be suitably poised for hydride transfer (Figure 3B). The C3 methyl group of 2-OPP is positioned into a small hydrophobic pocket formed by Val234, Val238, and the methylene carbons of Arg239. Accommodation of the C3 methyl into this pocket is achieved by the presence of Val234 in place of the larger Phe residue that is located at this equivalent position in most other 3-hydroxyacid dehydrogenases (Figure 4).³³ A number of residues surrounding the carbonyl oxygen may participate in a hydrogen-bonding network with the substrate and a water molecule. Alanine mutations of these residues were made to investigate their involvement in Psf3 activity. The keto oxygen is positioned 2.4 Å away from Lys173, which may help to polarize the carbonyl for favorable conversion into the alcohol, and/or could serve as a proton donor to the alkoxide. This Lys is conserved in other 3-hydroxyacid dehydrogenases (Figure 4).^{33,42} Accordingly, the K173A variant does not demonstrate any discernable activity (Table S2, Figure S1F). Other residues that are in near vicinity to the oxygen include Thr98 and Asn177, and the Ala variants at each of these residues demonstrate a 10^3 -fold loss in catalytic efficiency; neither one of these variants could be saturated in substrate, precluding the determination of k_{cat} or K_{M} (Table S2 and Figures S1, S3, and S4). Lastly, although Asp242 is nearly 4.6 Å away from the carbonyl oxygen, a static bridging water molecule can be observed in all four independent copies of Psf3 in the crystal structure (Figure 3C). This water molecule may be another candidate for donating a proton to the alkoxide during catalysis. The D242A mutation results in a 100-fold decrease in k_{cat} , further affirming a role of this residue in catalysis (Figure and S1E and S5 and Table S2). These various interactions observed in the structure provide a rationale for the stereospecificity of the hydride transfer. Binding of the cofactor and substrate results in the orientation of the *Re* face of 2-OPP above the C4 of the nicotinamide at a distance between 2.6 – 2.7 Å. The numerous residues that are

within interaction distance to the carbonyl oxygen, and the hydrophobic pocket that houses the C3 carbon of the substrate fix the orientation of 2-OPP, resulting in the formation of only the *S* stereoisomer following reduction. In the Psf3 oxidative reaction, the X-ray structure shows that the hydride is added to the *Si* face of the nicotinamide from C2 of (*S*)-2-HPP. For the corresponding reductive reaction the pro-*S* hydride of NADPH is delivered to C2 of 2-OPP.

Reconstitution studies and crystal structures of Psf4 epoxidase

The epoxidase involved in the final step in fosfomycin biosynthesis has been shown to be operative in both the *Streptomyces* and *Pseudomonas* pathways.^{21,28} While prior biochemical studies of both Psf4²⁸ and HppE²¹ utilized molecular oxygen and NADH/FMN as an exogenous source for the electrons required for epoxide formation, recent studies with HppE demonstrate hydrogen peroxide can also be used as oxygen source, thus obviating the need for reducing equivalents.⁴³ We carried out *in vitro* reconstitution of Psf4 with Fe(II), under anaerobic conditions, using (*S*)-2-HPP as a substrate. The reaction was initiated by the slow infusion of H₂O₂, as previously described for HppE,⁴³ and analysis of the products by ³¹P NMR spectroscopy demonstrated conversion of the substrate to fosfomycin (Figure S6A). Addition of catalase to the reaction, which decomposes hydrogen peroxide into water and oxygen, resulted in a near loss of product formation (Figure S6C). Thus, like HppE, the *Pseudomonas* homolog Psf4 can function as a peroxidase. In order to understand the biochemical basis for the biosynthesis of fosfomycin by Psf4, and to derive a structure-based comparison to the well characterized HppE, we obtained crystal structures of *P. syringae* PB-5123 Psf4 in complex with a variety of substrates.

Cocrystal structures of the physiologically relevant Fe(II)-bound Psf4 were determined under anaerobic conditions, while structures of the inert Mn(II)-substituted enzyme were determined from crystals grown aerobically. As there were no significant changes between structures with the different metals (Figure S7), and as crystals of the inert Mn(II) enzyme diffracted to higher resolutions, detailed descriptions will focus on these structures. Psf4 revealed an overall fold consisting of an N-terminal domain composed of residues Arg4 through Phe60 and a C-terminal cupin fold that encompasses residues Gly80 through Tyr189 with an intervening 18-residue linker that connects the two domains (Figure 5A). While the cupin fold resembles the similar domain observed in the structure of HppE (Figure 5B), the N-terminal domain is distinct. Specifically, the Psf4 N-terminal domain contains an α/β -fold formed by two β -strands that are flanked by two α -helices. Analysis of this domain using the DALI³⁵ server to query the Protein Data Bank fails to identify any structurally similar domains. In the structures of HppE, the equivalent N-terminal domain contains a structurally unrelated helical bundle (Figure 5B).²⁰

Psf4 forms a tetramer in the crystallographic asymmetric unit. Under the buffer conditions used for our studies, size exclusion chromatography is consistent with, but not proof of, a dimeric assembly (Figure S8A). However, dynamic light scattering (DLS) data could not accurately assign an oligomerization state for the polypeptide (Figure S8B). In the crystal structure, oligomerization is facilitated by interactions between the interdomain linker with the adjacent cupin fold, and through the N-terminal domain, resulting in a tetrameric

organization similar to that in HppE, albeit using different structural scaffolds (Figure 5A–C and Figure S9). In both Psf4 and HppE, the N-terminal domains interact with each other through mirrored hydrophobic environments. For example, in Psf4, residues Leu6, Leu42, and Phe60 from one monomer interact with the same constellation of residues within a second monomer (Figure S10). Despite the fact that the HppE N-terminal domain contains an entirely different topology, a similar manner of mirrored hydrophobic interactions facilitates the formation of the tetramer. The oligomerization domains of both Psf4 and HppE also contact the respective cupin domains of the adjacent monomer, positioning active site residues in a crossover fashion (Arg20/Lys21 in Psf4 and Arg18/Lys23 in HppE) (Figure 5C).

The jellyroll β -barrel cupin domain of Psf4 shows strong structural conservation with that of HppE, and the two domains can be aligned with an RMSD of 1.6 Å over 105 C α atoms (Figure 5A and 5B). The catalytically essential Fe(II) is housed in this domain and is coordinated by a facial triad composed of His128, Glu132, and His171 (Figure 5D and 5E). In the 2.44 Å resolution structure of Psf4 in complex with (*S*)-2-HPP, the phosphonate of the substrate is hydrogen-bonded to enzyme residues Tyr95, Arg87, and Asn125 from one monomer, and via a cross-over interaction with Lys21 from an adjacent monomer (Figure 5D). The γ -carbon of the (*S*)-2-HPP substrate is accommodated into a hydrophobic pocket composed of residues Leu112, Val134, Ile184, and Ala186. The ligand coordinates the metal in a bidentate fashion with both the β -hydroxyl and phosphonate oxygens. This interaction properly positions the substrate α -carbon for hydrogen atom abstraction by a reactive iron species, whether an Fe(IV)-oxo or Fe(III) superoxo intermediate as has been previously proposed for HppE.^{31,43}

The 2.49 Å resolution cocrystal structure of Psf4 in complex with (*R*)-2-HPP illustrates that a near identical set of enzyme residues are poised to interact with this stereoisomer (Figure 5E). However, with (*R*)-2-HPP, Psf4 catalyzes the oxidation of the β -hydroxyl to a carbonyl rather than cyclization to form the epoxide.²⁸ A comparison of the binding orientations of the two stereoisomers at the metal center provides a rationale for the formation of the different products. Both ligands bind to the metal in a bidentate manner via a phosphonate oxygen and the substrate β -hydroxyl. Bidentate binding of (*S*)-2-HPP optimally orients the substrate α -carbon for hydrogen abstraction. However, binding of (*R*)-2-HPP now optimally positions the β -carbon, rather than the α -carbon, for hydrogen abstraction, forming the β -carbonyl containing product 2-OPP. Thus the different reactivities of the two stereoisomeric substrates can be explained by differences in their respective binding orientations, as has been previously observed in the case of HppE.³¹

Based on the findings in this and previous reports, we hypothesized that we could leverage the combined activities of Psf3 and Psf4 to produce fosfomycin from a racemic mixture of 2-HPP. In this hypothesis, Psf4 would convert (*S*)-2-HPP directly to fosfomycin, whereas (*R*)-2-HPP would be converted to 2-OPP, which in turn would be reduced to (*S*)-2-HPP by Psf3 (Figure S11). The net result would be comparable to a dynamic kinetic resolution, converting both enantiomers of 2-HPP into fosfomycin in an enantioconvergent process. Indeed, we observed complete conversion of racemic 2-HPP to fosfomycin in a one-pot assay performed aerobically with Psf3 and NADPH, and the NADH/FMN reduction system

described previously for Psf4²⁸. The latter conditions were chosen instead of using hydrogen peroxide because of incompatibility with the NADPH required for Psf3 activity.

CONCLUSIONS

We describe here the *in vitro* activity reconstitution and crystallographic analysis of the last two enzymes involved in fosfomycin biosynthesis in pseudomonads. The fosfomycin biosynthetic pathways in *Streptomyces* and *Pseudomonas* are likely the products of convergence²⁴. Although biochemical reconstitution of the entire pathway has not yet been established, a plausible route for the production of 2-OPP has been proposed starting from phosphonomethylmalate,²⁴ the last biochemically detected intermediate in this pathway. The reduction of 2-OPP to (*S*)-2-HPP by Psf3, followed by epoxidation by Psf4 to yield fosfomycin completes the latter half of this pathway. The structure of Psf4 shares strong structural similarities with *S. wedmorensis* HppE in the cupin domain, but the oligomerization domains contain entirely different folds. This organization is functionally consequential as oligomerization of both proteins is necessary to generate the composite active site that orients an Arg/Lys pair from one monomer into the active site of another. Despite the use of topologically distinct N-terminal domains to facilitate oligomerization, the proper position of residues in the composite active sites occurs in both Psf4 and HppE.

An intriguing aspect of fosfomycin production that derives from this study relates to the observed tolerance of HppE and Psf4 for both stereoisomers of 2-HPP. Using an oxygen-dependent activation system (FMN), Liu and coworkers have shown that HppE is a competent catalyst utilizing either the non-physiological substrate (*R*)-2-HPP (k_{obs} of 0.58 $\pm$ 0.02 min⁻¹) or the physiological *S* isomer (k_{obs} of 0.31 $\pm$ 0.06 min⁻¹).³² The molecular basis for the altered regioselectivity is explained through the structural data for HppE.³¹ A similar trend is observed in Psf4 where the k_{obs} for (*R*)-2-HPP (0.33 $\pm$ 0.04 min⁻¹) is roughly twice as fast as that for (*S*)-2-HPP (0.17 $\pm$ 0.02 min⁻¹).²⁸ We were able to use this “off-pathway” reaction of Psf4 to our advantage by successfully driving the formation of 2-OPP through the Psf3 and Psf4 “on-pathway” transformations to make fosfomycin from racemic 2-HPP. Ongoing studies are aimed at elucidating the full biosynthetic pathway to fosfomycin in pseudomonads. Understanding the convergent biosynthetic routes to fosfomycin may also facilitate access to analogs of this potent antibacterial agent.

METHODS

Organisms, Media, and Reagents

All oligonucleotides used in this study were purchased from Integrated DNA Technologies (Table S3). Reagents used for molecular biology experiments and Luria-Bertani (LB) medium were purchased from New England BioLabs, Thermo Fisher Scientific, or Gold Biotechnology. Plasmid sequencing was performed by ACGT Inc. unless otherwise noted. *Escherichia coli* strain DH5 α was used for plasmid maintenance and *E. coli* Rosetta 2 DE3 pLysS was used for protein overexpression. *Pseudomonas syringae* PB-5123 was used for the cloning and amplification of both *psf3* and *psf4*. Detailed notes of organisms and plasmids used in this study are provided in Table S4, S5.

Determination of Psf3 Activity by ^{31}P NMR Spectroscopy

The assay (500 μL) contained 50 mM HEPES pH 7.5, 30 μM Psf3 WT or variant, 2 mM NADPH, and 2 mM 2-OPP. The reaction was incubated at room temperature for 13 h and was quenched by removal of the enzyme using a Millipore centrifuge filter (10 kDa molecular weight cut off, 5 min, $16,000 \times g$). D_2O (100 μL , 20% final v/v) was added to the flow-through before sample measurement. Reactions were performed in duplicate. Spectra were analyzed using MestReNova software version 8.0.0. In order to perform the reverse reaction, the assay components were the same except that 2 mM NADP^+ and 1 mM (*S*)-2-HPP or (*R*)-2-HPP were substituted for NADPH and 2-OPP and 15 μM Psf3 WT was used instead of 30 μM .

Protein Expression and Purification

Detailed experimental conditions and procedures are described in the Supplemental Information associated with this article.

Determination of Kinetic Parameters for Wild-type and Variant Psf3

Michaelis-Menten parameters for Psf3 with respect to 2-OPP were determined using reactions (200 μL) that contained 50 mM HEPES pH 7.5, 0.25 μM Psf3 WT or 5 μM Psf3 variant, 250 μM NADPH, and varying amounts of 2-OPP (0.05–8 mM). Hellma® Suprasil® quartz cuvettes (1 cm path length) were used and the change in absorbance at 340 nm was recorded. Reactions were performed in triplicate. Rates were measured using the Cary WinUV kinetics software version 6.0.0 1547 and data were fit to the Michaelis-Menten equation using Igor Pro version 6.32A. The same reaction was also performed with varying NADPH concentrations (200 and 350 μM) and saturating 2-OPP (4 mM). The initial rates recorded were the same as with 250 μM NADPH. Hence, the assay contained saturating levels of NADPH. Activity assays with WT Psf3 were performed with either NADPH or NADH and rates were analyzed in the same way as above, with the reaction containing 50 mM HEPES pH 7.5, 0.25 μM Psf3, 250 μM NAD(P)H, and 4 mM 2-OPP.

Determination of Psf4 Activity by ^{31}P NMR Spectroscopy

Psf4 reconstitution with Fe(II) and the activity assay were performed under anaerobic conditions. Psf4 (200 μM) was reconstituted with 1 molar equivalent of $\text{Fe(II)(NH}_4)_2(\text{SO}_4)_2$ in 50 mM HEPES pH 7.5 and incubated for 10 min prior to addition to the assay components. The assay mixture contained 50 mM HEPES pH 7.5, 50 μM Psf4, 1 mM (*S*)-2-HPP, 10 mM L-ascorbic acid, and 1 mM H_2O_2 for a final volume of 500 μL . When indicated, catalase (5 μM final) was added. The reaction was initiated by the slow infusion of H_2O_2 by turning the pipet dial while mixing. The reaction was left for 2 h at room temperature before treatment with Chelex (~100 μL) for 20 min while shaking. Protein was removed by centrifugal filtration using a Millipore 10 kDa molecular weight cut off filter. D_2O (100 μL , 20% final v/v) was added before NMR analysis. Experiments were performed in duplicate. Spectra were analyzed using MestReNova software version 8.0.0.

Determination of Fosfomycin Production from Racemic 2-HPP

The assay was performed aerobically. Psf4 (200 μ M) was reconstituted with 1 molar equivalent of $\text{Fe(II)(NH}_4)_2(\text{SO}_4)_2$ in 50 mM HEPES pH 7.5 prior to addition to the assay components. The assay mixture contained 50 mM HEPES pH 7.5, 100 μ M Psf4, 250 μ M FMN, 1.6 mM NADH, 15 μ M Psf3, 2 mM NADPH, 250 μ M (*S*)-2-HPP, and 250 μ M (*R*)-2-HPP for a final volume of 500 μ L. The reaction was incubated for 2.5 h at room temperature before quenching and preparation for NMR analysis as described for the Psf4 activity assay. The reaction was repeated with (*R*)-2-HPP alone (500 μ M) and full conversion to fosfomycin was again observed.

Crystallization, data collection, structure determination and refinement

Detailed protocols for crystallization and structural elucidation procedures are described in the Supporting Information associated with this article (Supplementary Methods and Supplementary Tables 4, 5).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Convergent biosynthetic pathways for the production of fosfomycin as found in different genera. For the pathway in pseudomonads (top), the reactions that have been confirmed by experimental data are shown as single transformations. For the pathway from streptomycetes (bottom), only the methyl transfer step has not been definitively confirmed and is shown with dashed lines. For the pathway in *Pseudomonas*, the epoxide oxygen in fosfomycin is hypothesized to originate from the β -carbonyl of PnPy (shown in red). This has been confirmed for the pathway in *Streptomyces*.¹⁶ (B-C) Reactions catalyzed by the orthologous enzymes Psf4 and HppE on different enantiomers of 2-HPP. The reactions are drawn using oxygen and an electron source as performed initially,²¹ but recent studies show that the reactions can also proceed using hydrogen peroxide as a co-substrate.⁴³

Figure 2.

(A) Michaelis-Menten curve for the wild-type Psf3 reaction using 2-OPP as a substrate. Error bars represent the standard deviations calculated from measurements made in triplicate. (B) ³¹P NMR spectroscopic analysis of the reverse (oxidation) activity of Psf3 using the two enantiomers of 2-HPP. Reactions using (S)-2-HPP resulted in a new peak that is consistent with 2-OPP (as determined by spiking with authentic standards), while no new product could be observed with (R)-2-HPP.

Figure 3.

(A) Overall structure of the Psf3 homodimer showing the orientation of the Rossmann fold (in cyan and black) and dimerization domains (in green). The second monomer is colored in gray. The location of bound ligands NADP⁺ (yellow) and 2-OPP (purple) are as shown, and Arg212 that is involved in crossover interactions between the two monomers is also indicated in brown sticks. (B) Simulated annealing difference Fourier maps (Fo-Fc) of Psf3 complexes contoured to 2.5 σ (blue) showing the bound NADP⁺, and (C) 2-OPP. The coordinates for the ligand were omitted prior to map calculations. The final refined coordinates of the complexes are superimposed with important active site residues and ligands shown in stick representation. Hydrogen bond interactions are represented with dashed lines.

Figure 4.

Structure-based multiple sequence alignment of Psf3 with other members of the 3-hydroxyacid dehydrogenase family. Secondary structural elements are shown above the alignment. Residues in Psf3 that interact with NADP⁺ are indicated with a blue triangle, and residues that contact 2-OPP are indicated with a yellow circle.

Figure 5.

(A) Overall structure of Psf4 showing the cupin (purple) and dimerization (green) domains. A linker region (brown) completes the β -sheet cupin fold of an adjacent monomer (in gray) (B) Structure of HppE that contains a cupin domain (pink) that is structurally conserved with that in Psf4 and a dimerization domain (olive) that contains a separate protein fold. A linker region (tan) completes the β -sheet cupin fold of an adjacent monomer (in gray) (C) Structure of the Psf4 tetramer showing the crossover interactions that occur as residues Arg20 and Lys21 from one monomer are diverted into the active site of a different monomer. The

catalytically requisite metal ion is shown as a gray sphere. (D) Close-up view of the active site of Psf4 in complex with (*S*)-2-HPP (brown) and (E) (*R*)-2-HPP (cyan). Important active site residues are shown as purple sticks. A simulated annealing difference Fourier maps (Fo-Fc), calculated with the coordinates of the bound substrate omitted, is shown at a contour level of 2.5σ (green) and 5σ (red).