Polyketides

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Oxidative *trans* to *cis* Isomerization of Olefins in Polyketide Biosynthesis

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Abstract: *Geometric isomerization can expand the scope of biological activities of natural products. The observed chemical diversity among the pseurotin-type fungal secondary metabolites is in part generated by a trans to cis isomerization of an olefin. In vitro characterizations of pseurotin biosynthetic enzymes revealed that the glutathione S-transferase PsoE requires participation of the bifunctional C-methyltransferase/epoxidase PsoF to complete the trans to cis isomerization of the pathway intermediate presynerazol. The crystal structure of the PsoE/glutathione/presynerazol complex indicated stereospecific glutathione–presynerazol conjugate formation is the principal function of PsoE. Moreover, PsoF was identified to have an additional, unexpected oxidative isomerase activity, thus making it a trifunctional enzyme which is key to the complexity generation in pseurotin biosynthesis. Through the study, we identified a novel mechanism of accomplishing a seemingly simple trans to cis isomerization reaction.*

Geometric isomerization can significantly alter the physical and biological properties of natural products.[1] Pseurotin-type fungal natural products are a prominent example of geometric isomerization yielding a collection of related but distinct compounds. Identifying and understanding the mechanism of geometric isomerization is of great interest not only for understanding natural product biosynthesis but also for developing effective methods for preparing geometric isomers. The structurally interesting and biologically active secondary metabolites synerazol (**1**)[2] and azaspirene (**2**)[3] are representative members of the pseurotin family of compounds which exhibit various biological activities of medicinal importance[4] (Scheme 1). The chemical diversity of the pseurotin-type compounds is thought to be generated during the post-polyketide synthase/nonribosomal peptide synthetase modification steps.[5] Recently, we discovered a bifunctional enzyme, PsoF, which catalyzes a C-methylation and an epoxidation of various intermediates to generate multiple products, including pseurotin A (**3**) and D (**4**).[6] However, involvement of multiple enzymes and complex intermediates in the pseurotin biosynthetic pathway prevented determination of the exact activity of PsoE, a predicted glutathione S-transferase (GST). Herein, we present the successful biochemical characterization and crystallographic analysis of the biosynthetic enzymes and reveal the unique mechanism involved in the *trans* to *cis* isomerization of an olefin in the azaspirene-type intermediates to form the 12,13*Z*-configured products.

Our previous gene knockout study in the *A. fumigatus ΔpyrG/Δku70* strain AfKW1[6] allowed us to determine the involvement of PsoE in the formation of the C12–C13 *cis* olefin. Thus, we decided to undertake in vitro analyses of this GST-type enzyme to establish its function clearly. When recombinantly produced PsoE was mixed with glutathione (GSH) and its substrate, presynerazol (**5**), no reaction took place (Figure 1 i, ii). However, when PsoF was added to the reaction mixture, the 12,13*E*-configured **5** was completely converted into the 12,13*Z*-configured **1** and **3** (Figure 1 iii, iv). The chemical structures of the compounds discussed above were characterized with electrospray ionization LC-HRMS, as well as ^{1}H NMR and ^{13}C NMR spectroscopy as shown in our previous report.[6] We chose to include PsoF in the reaction because it was proposed to be responsible for the epoxidation step immediately following the isomerization of the 12,13*E*-configured olefin, which was previously speculated to be catalyzed by PsoE in the pseurotin biosynthetic pathway.[6] The results from the in vitro assays carried out with PsoE and PsoF clearly established that PsoE indeed played a role in the *trans* to *cis* isomerization of the C12–C13 olefin for the ultimate formation of the 12,13*Z*-configured product **1** from the 12,13*E*-configured substrate **5**. The lack of isomerization of the C12–C13 olefin in the absence of PsoF also revealed that PsoF, a previously established bifunctional methyltransferase epoxidase, is also indispensable to the *trans* to *cis* isomerization step.

Next, we examined the reaction conditions in detail to better understand how the task of isomerization is allocated

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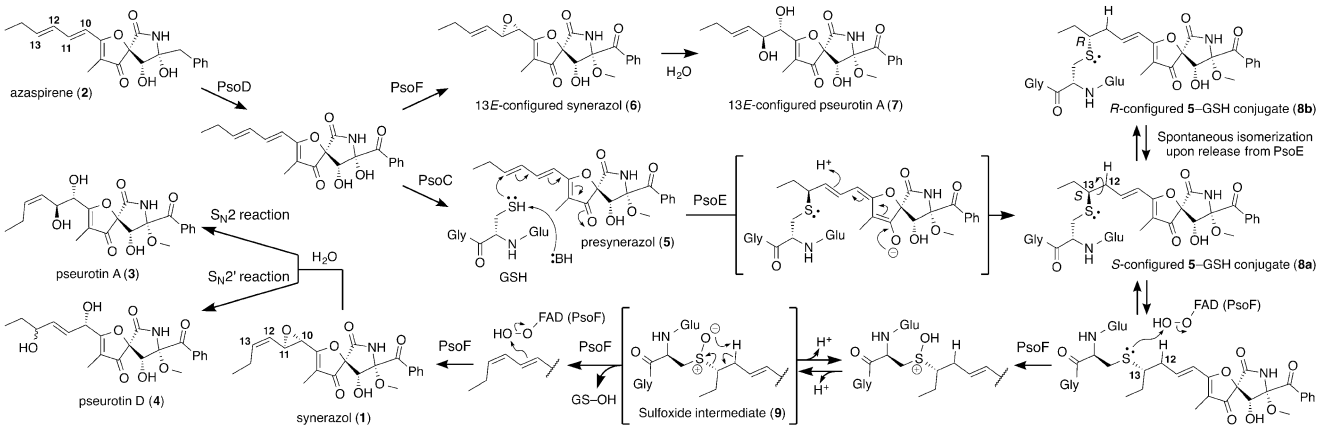

Scheme 1. Proposed mechanism of the transformation of azaspirene (**2**) into synerazol (**1**) catalyzed by PsoE and PsoF from the pseurotin biosynthetic pathway.

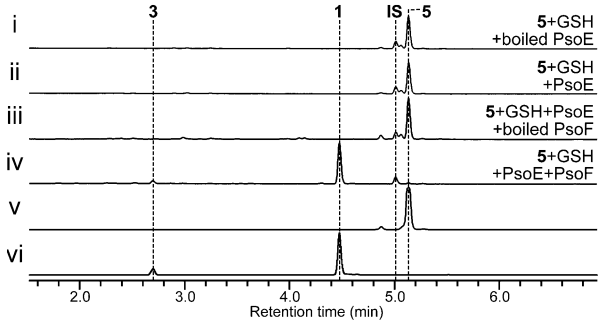

Figure 1. In vitro analyses of the activity of PsoE, alone or with PsoF, against **5** in the presence of glutathione (GSH). See the Supporting Information for details. HPLC profiles of a reaction mixture containing i) the heat-inactivated (boiled) PsoE with **5** and GSH, ii) PsoE with **5** and GSH, iii) PsoE and heat-inactivated PsoF with **5** and GSH, and iv) PsoE and PsoF with **5** and GSH. Traces are standardized to the height of the internal standard (IS) peak. All traces were monitored at $\lambda = 260$ nm. The authentic reference of **5** (v) and **1** (vi) are also given.

between PsoE and PsoF. When the concentration of GSH was lowered in the reaction mixture containing PsoE and PsoF, we observed a substantially reduced formation of **1** and an increased production of 13*E*-configured synerazol (**6**; see Figure S2 i–iii in the Supporting Information). Similar product yield, which is higher than the enzyme concentration (20 μM), in the presence of 1 mM or 10 μM GSH suggests that the isomerization is enzyme-dependent and GSH is required. In addition, omission of NADPH from the reaction mixture prevented the formation of both **1** and **6**, as well as non-epoxidized products (see Figure S2 iv), thus indicating the requirement for a reducing agent in this transformation. Also, concomitant analysis of the in vitro reactions for the presence of GSH-conjugated intermediates identified peaks having a mass/charge ratio corresponding to the **5**/GSH conjugate **8** and its monoxide, presumably the sulfoxide intermediate **9**, as determined by LC-HRMS (see Figure S3). These results suggest that PsoE forms **8**, which is isomerized and then epoxidized by PsoF to form **1**. Low yield of **8** at low concentration of GSH leads to the formation of the shunt product **6**.

To gain a better understanding of the role of PsoE, we determined the crystal structure of PsoE complexed with its native substrates **5** and GSH at 2.5 Å resolution using the single-wavelength anomalous dispersion method (see Tables S2 and S3). Diffraction-grade crystals were obtained only for the PsoE/**5**/GSH ternary complex. The overall fold of PsoE (Figure 2 A) is characteristic of the GST family of proteins.[7] Like other fungal GSTs, PsoE also shows low sequence similarity to other GSTs,[8] but the Theta-class human GST T2–2[9] is found to be most similar to PsoE (see Figure S4). The main structural difference between PsoE and T2-2 is the absence of a C-terminal α-helix α9 in PsoE. In GST T2–2, α8-α9 loop and α9 helix cover up the active site to bury the bound ligand deep inside the active-site pocket (see Figure S5 iii). However, the C-terminal residues of PsoE assume a random coil which is positioned away from the active site, thus leaving the ligand-binding site (H-site) without a lid (see Figure S5 i and ii vs. iii). While lack of an α9 helix also occurs in other classes of GSTs, including Zeta GSTs such as the *Ralstonia* sp. strain U2 maleyl pyruvate isomerase[10] (see Figure S5 iv) and Sigma GSTs such as human prostaglandin synthases[11] (see Figure S5 v and vi), a shortened β2-α2 loop and a shift in the positioning of α4-α4a loop and α4a helix reduce the depth of the H-site pocket in PsoE. Electron density for the bound ligand permitted modeling of **8**, a true reaction intermediate with the GSH and **5** covalently linked in an *S* configuration (Figure 2 A). We designated the *S*-configured **8** as **8a** and the *R*-configured counterpart as **8b** (Scheme 1). PsoE forms hydrogen bonds with the GSH moiety using residues Arg 37, Lys 49, Val 50 (backbone amide and carbonyl groups), Glu 63, and Cys 64 (Figure 2 B) from the N-terminal domain which form the G-site that is conserved among cytosolic or canonical GSTs (cGSTs)[8] (see Figure S4, in bold red and purple letters). Only one residue from the C-terminal domain, Asn 99, forms a hydrogen bond with the GSH moiety. The formation of **8a** likely involves Gln13, Cys 14, and Arg15 in PsoE, which structurally corresponds to Ser 13, Ser 14, and Cys15 in human GST Z1-1, and was shown to be important in catalysis and substrate binding.[12] Those residues in PsoE are thought to participate in catalysis by promoting the formation of the

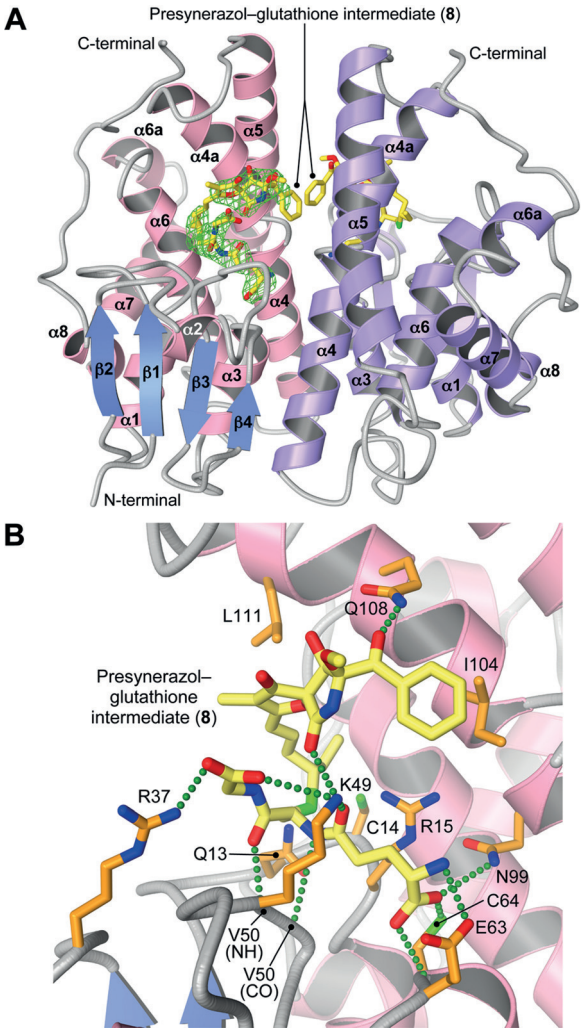

Figure 2. Crystal structure of PsoE complexed with the GSH conjugate of presynerazol **8a**. A) Overall structure of PsoE (PDB ID: 5F8B) as a dimer. In the first PsoE molecule (left), α-helices and β-strands are colored in pink and blue, respectively, whereas the second molecule (right) is colored only in purple. Carbon atoms in the stick model of the bound ligand **8a** are in yellow, whereas oxygen, nitrogen, and sulfur atoms are in red, blue and green, respectively. Electron density for **8a** in the first PsoE molecule ($2F_o-F_c$ map contoured at 1.2 σ, green mesh) is shown. B) The active site of PsoE, showing the interactions between **8a** and PsoE. The protein side chain carbon atoms are in orange. Green dashed lines represent hydrogen bonds.

thiolate form of the bound GST. While the GSH sulfur atom and the side-chain sulfur atom of Cys 14 are separated by 4.97 Å in our crystal structure, a conformational change in PsoE and an altered binding conformation of **5** can allow Cys 14 to interact with GSH prior to the formation of **8a**. A similar shift in the GSH–catalytic residue interaction under different reaction conditions was observed in the Pi-class GST P1-1.[13]

As to substrate recognition, the H-site pocket is normally enclosed by residues from α1, α4, α4a, and α6 helices and β1-α1 and β2-α2 loops in cGSTs[8] (see Figure S4, bold green and purple letters). However, PsoE employs only the exterior surface of the α4a helix at the dimer interface to interact with **5** (Figures 2B; see Figure S5i), thus making the substrate-binding mode of PsoE highly unique among other GSTs. PsoE–**5** interactions are primarily hydrophobic, but hydrogen bonds between **5** and Lys 49 and Gln 108 side chains may help align the C12–C13 olefin of **5** and the GSH thiol for the ensuing C–S bond formation. However, because of the exposed nature of the substrate binding site, PsoE does not seem to bind **5** rigidly. Loose binding of **5** was also observed experimentally in the PsoE/GSH/**5** complex crystalized in the absence of a cobalt ion (see Table S3), as indicated by sparse electron density for the bound ligand, particularly for **5** (see Figure S6A). Inclusion of cobalt in the crystallization conditions altered the packing of PsoE dimers such that the α2-containing loop from the symmetry-related molecule covers the open active site, thus limiting the wiggle space for the bound **5** (see Figure S6B). Nevertheless, very limited specific interactions with **5** in the loosely packed substrate binding site of PsoE (Figure 2; see Figure S5 i,ii) suggests that PsoE may be predisposed to releasing **8a** relatively easily. These observations suggest a possible reaction mechanism of the *trans* to *cis* isomerization reaction involved in the biosynthesis of synerazol.

Cis–trans isomerization reactions are known to be catalyzed by several different GSTs, including the Zeta-class GSTs which perform isomerization of maleyl (*cis*) substrates to fumaryl (*trans*) products.[14] However, our earlier study has established that PsoE alone is unable to perform the isomerization. Examination of the PsoE/**8a** complex structure also identifies no residue in the active site which is suitably positioned for acid-base catalysis of the isomerization reaction. Thus, it appears that PsoE is designed only to perform the stereospecific conjugation of GSH with **5** to form **8a** and pass the product to PsoF for isomerization and epoxidation to form **1**. The transfer of the substrate from PsoE to PsoF may be facilitated by the formation of a transient complex between those two enzymes. Such an interaction may be promoted by the C-terminal segment of PsoE, which is about 30 residues long and does not assume an ordered conformation in our crystal structure.

Based on these results, we theorize that PsoE catalyzes the stereospecific conjugation of GSH and **5** to form **8a**, which converts the C12–C13 olefin into a C12–C13 σ bond. The *cis* olefin can be formed by rotating around the C12–C13 σ bond and then eliminating the GSH group to regenerate the C12–C13 olefin with a *Z* configuration. The crystal structure of PsoE has revealed that PsoE is not equipped to carry out the isomerization step. However, the uniquely open active site of PsoE would allow **8a** to be released from PsoE and passed to PsoF. The flavin-containing monooxygenase domain of PsoF can oxidize the sulfur atom of **8a** with its flavin hydroperoxide to generate **9**, thus setting the molecule up for a subsequent pericyclic *syn*-elimination which leads to the release of the oxidized GSH and the formation of the 12,13*Z*-configured stereoisomer of **5**. Lastly, the isomerized product remaining in the PsoF active site undergoes a subsequent epoxidation[15] of the C10–C11 olefin to form **1**. For PsoF to be able to perform the *trans* to *cis* isomerization of **8a**, its active site needs to have sufficient room to allow binding of the relatively bulky GSH moiety in the vicinity of the C12–C13 olefin. The fact

that PsoF can accept substrates having either a *E* or *Z* configured C12–C13 olefin for the epoxidation of the C10–C11 olefin suggests that the section of the active site pocket which accommodates the terminal portion of the synerazol diene side chain is somewhat spacious. The bulky GSH group of **8a** can force it to be bound in the least hindered, extended conformation, which is in fact the pro-*cis* conformation, inside the PsoF active site, to promote the formation of the *cis* olefin at C12–C13 upon release of the GSH molecule. There was a report of another GST which was shown to catalyze a *cis*/*trans* isomerization reaction involved in the biosynthesis of hypothemycin. This GST, Hpm2, was shown to isomerize 7′,8′-*trans*-containing aigialomycin A and 7′,8′-*cis*-containing hypothemycin to an equilibrated mixture comprising 85% *trans* and 15% *cis* products.[16] While Hpm2 catalyzes the isomerization reaction alone, it is unable to shift the equilibrium toward the formation of the higher-energy *cis* product. In the pseurotin biosynthesis, employment of an additional enzyme, namely PsoF, to perform the isomerization reaction at the expense of FAD and NADPH seems to be driving the conversion of *trans*-containing **5** into *cis*-containing **1**.

In conclusion, our study has shown that PsoE is a unique GST which works with another enzyme PsoF to accomplish the *trans* to *cis* isomerization of an olefin in the azaspirene-type compounds, thus giving rise to a family of geometric isomers. This unexpected finding adds yet another function to PsoF, a previously established bifunctional enzyme,[6] thus making it a trifunctional enzyme capable of performing C-methylation, isomerization, and epoxidation reactions on a range of substrates. Through the study, we have successfully uncovered the activities of key enzymes that play a critical role in the biosynthetic strategy employed for generating chemical diversity of pseurotin family of natural products.

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