Enzyme Catalysis

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Elucidation of Pseurotin Biosynthetic Pathway Points to Trans-Acting *C*-Methyltransferase: Generation of Chemical Diversity**

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Abstract: *Pseurotins comprise a family of structurally related Aspergillal natural products having interesting bioactivity. However, little is known about the biosynthetic steps involved in the formation of their complex chemical features. Systematic deletion of the pseurotin biosynthetic genes in A. fumigatus and in vivo and in vitro characterization of the tailoring enzymes to determine the biosynthetic intermediates, and the gene products responsible for the formation of each intermediate, are described. Thus, the main biosynthetic steps leading to the formation of pseurotin A from the predominant precursor, azaspirene, were elucidated. The study revealed the combinatorial nature of the biosynthesis of the pseurotin family of compounds and the intermediates. Most interestingly, we report the first identification of an epoxidase C-methyltransferase bifunctional fusion protein PsoF which appears to methylate the nascent polyketide backbone carbon atom in trans.*

Pseurotins[1] make up a family of fungal secondary metabolites which exhibit wide-ranging biological activities of medicinal importance. For example, pseurotin A (**8**; see Scheme 1) was reported to inhibit monoamine oxidase[2] and induce cell differentiation in PC12 cells.[3] Previously, the gene cluster responsible for biosynthesizing **8** was predicted and identified by deletion and overexpression of the polyketide synthase nonribosomal peptide synthetase (PKS-NRPS) hybrid enzyme gene, *psoA*, in *Aspergillus fumigatus* Af293.[4] PsoA was shown to be responsible for the biosynthesis of the core structure of **8**, that is the 1-oxa-7-azaspiro-[4,4]non-2-ene-4,6-dione skeleton having five chiral centers. Recently, a detailed analysis of the pseurotin biosynthetic gene cluster describing its genetic organization was reported.[5] However, the mechanism of pseurotin biosynthesis, such as the formation of the spiro-ring core structure, still remains undefined (Scheme 1 and Figure 1 A). Another interesting aspect of pseurotin biosynthesis is the formation of a large number of closely related bioactive compounds, such as azaspirene (**2**)[6] and synerazol (**7**).[7] This diversity of pseurotin-type natural products is thought to be generated during the post-PKS-NRPS modification steps. However, it is often difficult to resolve the biosynthetic steps involving multiple enzymes and complex intermediates. Here, we carried out knockout experiments for the pseurotin biosynthetic genes in the Δ*pyrG*/Δ*ku70* strain of *A. fumigatus* A1159, named AfKW1 (see the Supporting Information), and conducted in vivo and in vitro characterization of four modification enzymes, PsoC, PsoD, PsoE, and PsoF, to reveal the unique mechanism involved in the biosynthesis of the pseurotin family of natural products.

Sequence analysis (see the Supporting Information) indicated that PsoF is a single polypeptide comprised of an unusual combination of two domains, one homologous to a methyltransferase (MT) and another to an FAD-containing monooxygenase (FMO). Deletion of *psoF* abolished production of **8**, **9**, **13**, and **14**, thus indicating the essential role of PsoF in pseurotin biosynthesis (Figure 2 i versus ii). Surprisingly, the deletion also resulted in the formation of **22** (see Table S15 and Figures S45–S48 in the Supporting Information), a C3-desmethylated analogue of **2** (Figure 2 i versus ii). A mixture of the geometric isomer **23** and the reduced product **24**, both lacking the C3 methyl group (see Table S16 and Figures S49–S52), were also isolated from Δ*psoF*/AfKW1. Close analysis revealed that the MT domain of PsoA actually exhibited residual activity as judged by marginal formation of **4** in the Δ*psoF*/AfKW1 strain (see Figures S5 ii and viii). These results allowed us to propose a mechanism of how the C3 methyl group is introduced into the pseurotin scaffold (Figure 1 A). For the FMO domain of PsoF, lack of formation of the pseurotin-type compounds **8**, **9**, **13**, and **14** suggested its role in the formation of the 10,11-epoxide.

To characterize in detail the biochemical functions of the *C*-MT and FMO domains of PsoF, we cloned *psoF* from

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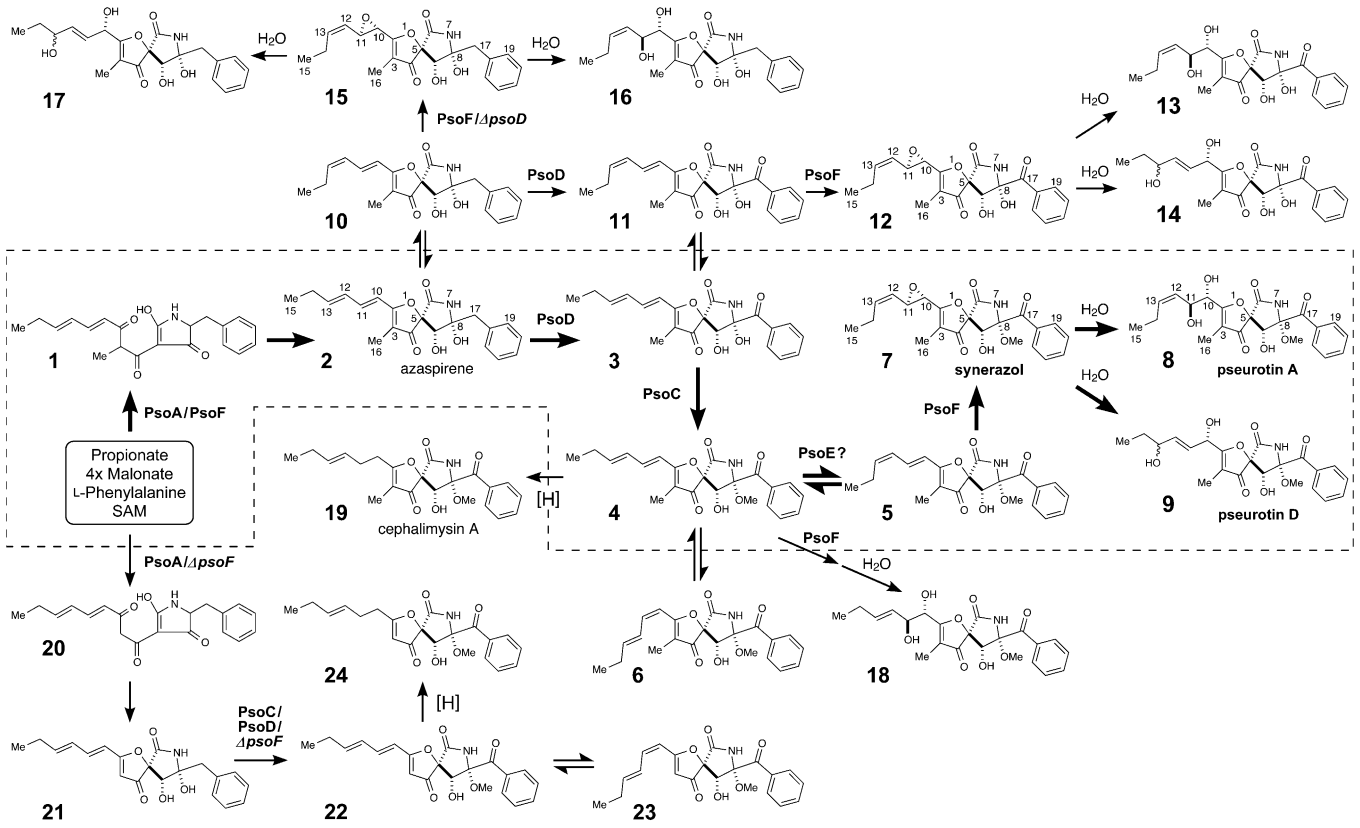


Scheme 1. Biosynthetic pathway for the transformation of **1** into the pseurotins **8** and **9** involving methylation and multiple oxidation steps. The pathway enclosed within the broken-line box with thick arrows represents the proposed main pathway toward formation of **8** and **9**.

mRNA and expressed it heterologously in *Escherichia coli*. The *C*-methylation step was examined in vitro using an *N*-acetylcysteamine (NAC) thioester[8] which mimics the natural substrate, phosphopanteteine-linked **25** (see the Supporting Information). The purified PsoF was able to catalyze methylation of **25** to form **26** (Figure 1B and Figure 3). Interestingly, we did not observe the formation of the epoxidated products of **25** or **26**, thus suggesting those early intermediates are not recognized as substrates by the FMO domain of PsoF. For functional analysis of the FMO domain, initially **2** was subjected to an in vitro assay with the recombinant PsoF. The assay revealed that PsoF was completely unreactive to **2**. Since the presence of an epoxide moiety in synerazol (**7**; see Table S7 and Figure S22) hinted that **7** could be formed by the activity of an FMO, we subjected the mixture of geometric isomers **4** and **6** to PsoF as substrates in an in vitro reaction. In this reaction, PsoF converted the substrates into four products: the epoxide-containing **7** and the spontaneously hydrolyzed epoxide products, **8**, **9**, and **18** (Figure 4). The chemical structures of all compounds, **4** (Table S5 and Figures S14–S17), **6** (Table S6 and Figure S18–S21), **7**, **8** (Table S8 and Figure S23), **9** (Table S9 and Figure S24–S27), and the new compound **18** (Table S13 and Figure S40–S43) were elucidated by HRESIMS, and ^{1}H NMR and ^{13}C NMR spectroscopy (see the Supporting Information). In vitro transformation of **4** into **18** by PsoF indicated that the FMO domain is capable of accepting the 13*E*-containing compound as its substrate. However, a complete lack of formation of any 13*E*-containing products in the wild-type strain (Figure 2i) suggests that 13*Z*-configured compounds are likely to be the true substrate of PsoF. Furthermore, formation of epoxidized or hydroxylated products from the 11*Z*-containing compound **6** was not observed in the reaction. Thus, the preferred substrate of PsoF is considered to be **5**, an 11*E*,13*Z*-configured isomer. Based on the chemical structures of the products **8** and **9**, we propose that those products are formed from **5** through epoxidation catalyzed by the FMO domain of PsoF. After epoxidation of **5** by PsoF to form **7**, **7** can be attacked by a water molecule nonenzymatically at two different positions to yield two diol products, **8** and **9**, through a S_N2 and S_N2' reaction, respectively (Scheme 2). For the formation of **8**, the water molecule attacks **7** at C11 from the opposite face of the epoxide in an antiperiplanar manner to yield the 10*S*,11*S*-diol of **8**. In contrast, **9** is formed by the water molecule attacking **7** at C13. Subsequent allyl migration and epoxide opening yields the diol product. Taken together, these results show PsoF to be a unique bifunctional enzyme catalyzing two different types of reactions at completely separate steps of the pseurotin biosynthetic pathway.

Next, we examined *psoC* and *psoD*, which are predicted to code for a MT and a cytochrome P450, respectively. To understand the function of PsoC, we analyzed the mutant strain, Δ*psoC*/AfKW1. The UV traces from HPLC analysis of the Δ*psoC*/AfKW1 culture extract showed accumulation of the O8-unmethylated products **13** (Table S10 and Figure S28–S31) and **14**, with loss of **8** and **9** (Figure 2i versus iii). This

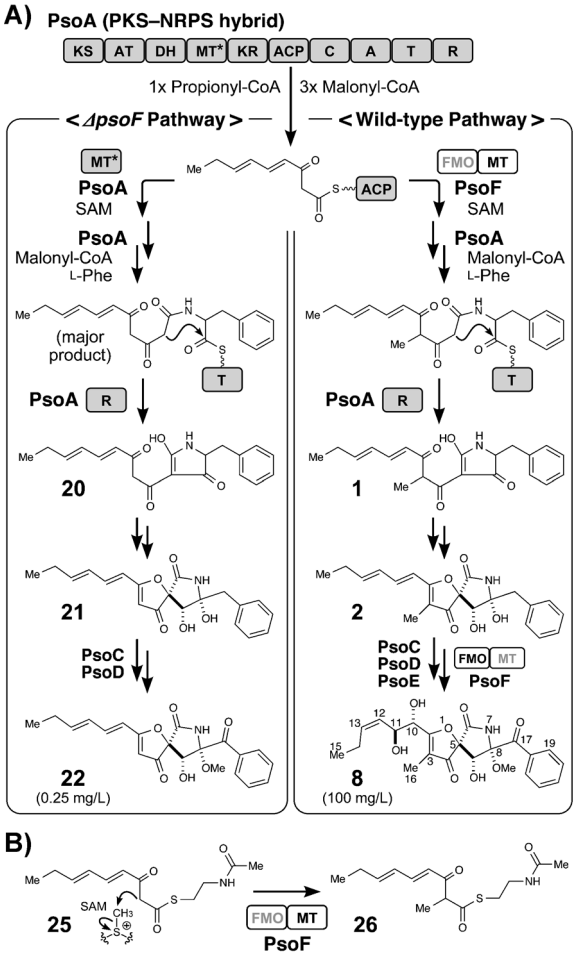


Figure 1. Modular organization of the uncharacterized iterative PKS-NRPS encoded by *psoA* (12.3 kb) and *C*-methylation activity of PsoF. A) Proposed biosynthetic pathway for the formation of **8** and **22**. The iterative PKS-NRPS is shown with its predicted core domains. Pathways toward formation of **8** versus **22**, depending on the presence of PsoF, are shown. B) C methylation of the polyketide backbone core by the MT domain of PsoF. Functional analysis was performed with a chemically synthesized NAC thioester **25**, which was converted into a methylated product **26** by PsoF. A = adenylation, ACP = acyl carrier protein, AT = acyltransferase, C = condensation, DH = dehydratase, KR = ketoreductase, KS = ketosynthase, MT = methyltransferase, MT* = partially active methyltransferase, R = reductase, SAM = *S*-adenosyl-L-methionine, T = thiolation.

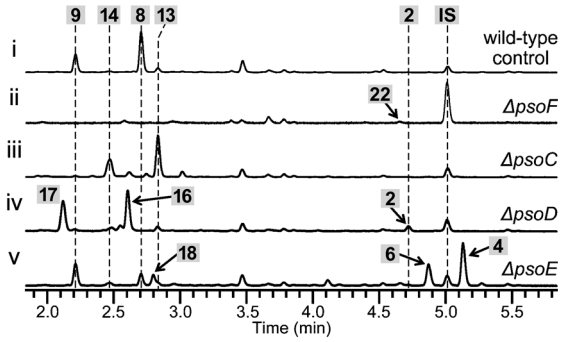


Figure 2. HPLC traces of metabolic extracts from the cultures of various *A. fumigatus* A1159-based deletion strains to identify the genes responsible for the biosynthetic steps involved in the formation of **8** during the pseurotin biosynthesis. All deletion strains were prepared in AfKW1 by replacing the target gene with *pyrG* by homologous recombination. All cultures were grown in MYG medium. All HPLC traces were monitored at $\lambda = 280$ nm. Extract of the culture of i) AfKW1 harboring pKW20088 for the expression of *pyrG* as a wild-type control, ii) Δ*psoF* strain, iii) Δ*psoC* strain, iv) Δ*psoD* strain, and v) Δ*psoE* strain. IS = internal standard, which is the *N*-Boc-L-tryptophan methyl ester (Boc-Trp-OMe).

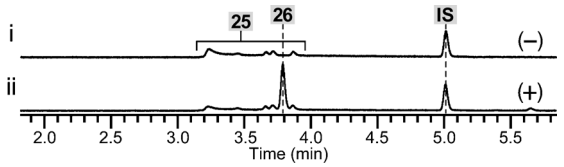


Figure 3. HPLC analysis of **26** biosynthesis using purified PsoF. HPLC traces of the i) control reaction without PsoF and ii) in vitro reaction with PsoF, thus affording the methylated product **26** after 1 h at 30 °C with 0.2 mM of substrate, which was a keto–enol equilibrated mixture of **25** and its geometric isomers; 1 mM SAM and 0.1 M NaCl in 0.1 M Tris-HCl (pH 7.4). All HPLC traces were monitored at $\lambda = 280$ nm.

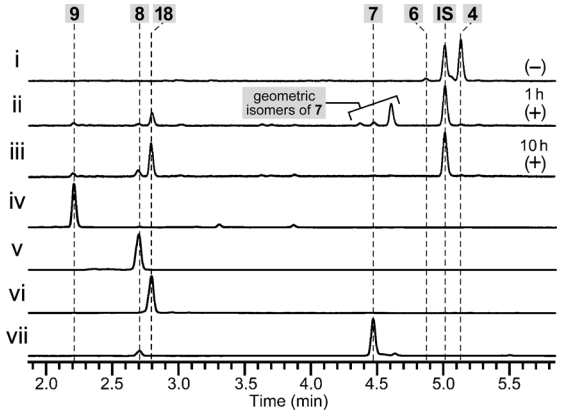
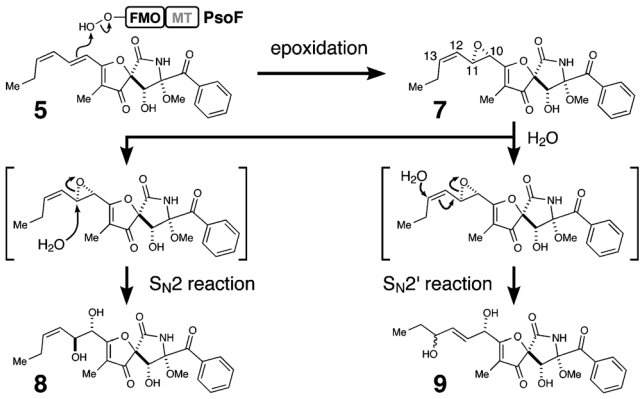


Figure 4. In vitro characterization of PsoF for the formation of **7**, **8**, **9**, and **18**. All HPLC traces were monitored at $\lambda = 280$ nm. Analysis of in vitro reaction of PsoF with a mixture of the geometric isomers **4** and **6** as substrate. i) Negative control using heat-inactivated PsoF. ii) Epoxide intermediates were formed from **4** and **6** by PsoF after 1 h at 30 °C. iii) the three products **8**, **9**, and **18** were formed from the substrates by PsoF at 30 °C after 10 h. Authentic references of iv) **9**, v) **8**, vi) **18**, and vii) **7** are also shown.

result indicated that PsoC likely performs O methylation of the hydroxy group of C8 in **3** to form **4** (Scheme 1). To characterize the *O*-methylation activity of PsoC in detail, we examined recombinant PsoC in vitro using **13** as a substrate. Interestingly, PsoC did not convert **13** into **8**. This result indicates that PsoC does not tolerate modifications to the diene side chain of the azaspirene skeleton as its substrate. To investigate the role of PsoD, we prepared another mutant strain, Δ*psoD*/AfKW1. HPLC analysis of the extract showed an accumulation of **16** (Table S11 and Figures S32–S35) and **17** (Table S12 and Figures S36–S39) and a loss of **8** and **9** (Figure 2i versus iv), thus indicating that this cytochrome P450 is responsible for installing the ketone group at C17 (Scheme 1). To confirm the activity of PsoD, in vivo



Scheme 2. Proposed reaction mechanism of diol formation (**8** and **9**) by either S_N2 or S_N2' reaction from the substrate **7**, which is formed using PsoF.

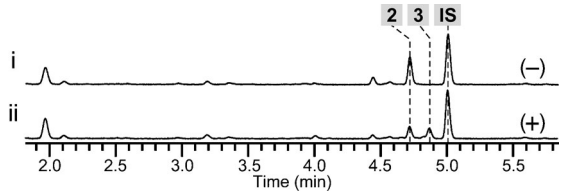


Figure 5. In vivo characterization of the cytochrome P450 PsoD for the formation of **3**. UV trace at $\lambda = 280$ nm from HPLC analysis of the conversion of **2** into **3** by PsoD. i) *Saccharomyces cerevisiae* BY4705 without *psoD* as a negative control. ii) *S. cerevisiae* BY4705 harboring the expression vector carrying the *psoD* gene. Cultures were incubated at 30 °C for 3 h after feeding **2** to the strains.

conversion of **2**, fed exogenously to the culture of *Saccharomyces cerevisiae* expressing the *psoD* gene (Figure 5), as well as the in vitro assay for the same conversion (Figure S12), were conducted. The observed conversion of **2** into **3** confirmed the idea that *psoD* codes for an oxidoreductase which is responsible for the oxidation of C17 in **2** and, by extension, the oxidation of **10**. Lack of formation of O8-methylated products in the Δ*psoD*/AfKW1 mutant indicates that PsoC requires the presence of the C17 ketone group in its substrate for activity. This finding places the PsoD-catalyzed step upstream of the PsoC-catalyzed step in the overall pseurotin biosynthetic pathway. In addition, inactivity of PsoD against **16** and **17** (Figure S57) indicates that, like PsoC, PsoD is also sensitive to modifications to the diene side chain. Lastly, based on the proposed activities of PsoC and PsoD, we predict that **22**, isolated from the Δ*psoF* strain, is derived from **21**, the analogue of **2** which is lacking the C3 methyl group because of the absence of PsoF.

Characterization of the disruption mutants and heterologously produced enzymes also identified multiple analogues arising from *cis*/*trans* isomerization of the diene portion of the azaspirene core. Disruption of *psoE*, a predicted glutathione *S*-transferase (GST) gene, resulted in a dramatic loss of **8**, new formation of **18**, and a significant accumulation of **4** and **6** as compared to that of the wild-type control (Figure 2 v versus i). The compounds **4** and **6** are both 13*E* isomers, and they are predicted to be formed immediately before the PsoF-catalyzed epoxidation step. As discussed earlier, the preferred substrate for PsoF is predicted to be 13*Z*-configured compound. Thus, accumulation of **4** and **6** in the Δ*psoE* strain, and previous reports of isomerase activity of certain GSTs,[9] suggest that PsoE may be involved in the *trans*-to-*cis* isomerization of the C13 olefin. As shown in the in vitro assay of PsoF (Figure 4 ii and iii), **18** is formed by C10,C11 epoxidation by PsoF at low efficiency as **4** accumulates in the Δ*psoE* cell. In contrast, formation of **8** in this mutant strain can be explained by the low level of isomerization of the C13 olefin converting **4** into **5**. This isomerization was also observed in the in vitro assay of PsoF, where a small amount of **8** was formed in the absence of the 13*Z*-containing substrate (Figure 4 iii). As to the stereoisomerism at C11, concomitant accumulation of the 11*E*- and 11*Z*-configured geometric isomers, **22** and **23**, respectively, in the Δ*psoF*/AfKW1 strain and similar accumulation of **4** and **6** upon disruption of another gene, *psoE*, suggest that *cis*/*trans* isomerization of the C11 olefin is non-enzymatic. We also observed the formation of **24** in the Δ*psoF* strain and cephalimysin A (**19**)[10] in the Δ*psoB* strain (Figure S6, Table S14, and Figure S44). Those compounds appear to be formed by reduction by a nonspecific oxidoreductase, since no other oxidoreductase gene is found in or near the gene cluster.

In this study, we combined targeted gene deletion, heterologous in vivo bioconversion, and an in vitro assay to identify the main pathway leading to the formation of pseurotins through the formation of azaspirene and synerazol (Scheme 1). Through the study, we identified eight of the intermediates formed and the enzymes responsible for the oxidation and methylation steps taken during the transformation of **2** into **8**. PsoF is unique in that its gene is encoded separately from the pseurotin biosynthetic gene cluster and embedded within the unrelated fumagillin biosynthetic gene cluster.[5] However, even more unexpected was that PsoF predominantly performed an apparent *trans C*-methylation of the backbone of the growing polyketide chain, with the MT domain of the PKS-NRPS enzyme PsoA maintaining residual methyltransferase activity. Furthermore, PsoF turned out to be a bifunctional enzyme, where its FMO domain catalyzes the stereospecific epoxidation of the C11,12 olefin. Presumed spontaneous conversion of the epoxide into a diol by either an S_N2 or S_N2' reaction leads to the formation of pseurotin A (**8**) or D (**9**), respectively, as well as **13**, **14**, **16**, and **17**. The in vitro assay of PsoF with the NAC thioester of **25** showed that PsoF catalyzed the *C*-methylation to form **26** (Figure 3). However, **26** was converted into neither the epoxide nor diol product. This result suggests that PsoF performs the C3 methylation prior to epoxidation, and the FMO domain requires extension of the polyketide chain with a phenylalanine residue and possibly spiro-ring formation for activity. While CTB3 from *Cercospora nicotianae*, involved in the cercosporin biosynthesis, was also reported to be a potential bifunctional enzyme predicted to have an N-terminal, *O*-MT, and C-terminal FMO-like domain,[11] PsoF is the first enzyme of the kind whose activity has been characterized in detail. While bifunctional enzymes often catalyze two consecutive steps of a metabolic pathway,[12] PsoF represents an

example of a highly unusual enzyme which is experimentally shown to catalyze two completely unrelated steps within the same biosynthetic pathway. Our current study also identified that the enzymes responsible for the post-NRPS-PKS assembly modifications, such as PsoC, PsoD, and PsoF, are capable of accepting more than one compound as their substrate. Moreover, possible combination of enzymatic and non-enzymatic isomerization of the conjugated side chain further multiplies the number of compounds generated. These findings reiterate the notion[13] that redox enzymes and MTs possess considerable substrate tolerance and play a vital role in forming various intermediates while introducing complexity into the chemical structures of those compounds.

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