P450-Mediated Non-natural Cyclopropanation of Dehydroalanine-Containing Thiopeptides

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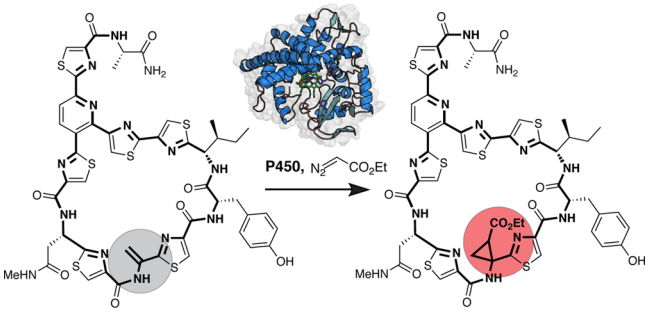
ABSTRACT: Thiopeptides are a growing class of ribosomally synthesized and post-translationally modified peptide (RiPP) natural products. Many biosynthetic enzymes for RiPPs, especially thiopeptides, are promiscuous and can accept a wide range of peptide substrates with different amino acid sequences; thus, these enzymes have been used as tools to generate new natural product derivatives. Here, we explore an alternative route to molecular complexity by engineering thiopeptide tailoring enzymes to do new or non-native chemistry. We explore cytochrome P450 enzymes as biocatalysts for cyclopropanation of dehydroalanines, chemical motifs found widely in thiopeptides and other RiPP-based natural products. We find that $P450_{TbtJ1}$ and $P450_{TbtJ2}$ selectively cyclopropanate dehydroalanines in a number of complex thiopeptide-based substrates and convert them into 1-amino-2-cyclopropane carboxylic acids (ACCAs), which are important pharmacophores. This chemistry takes advantage of the innate affinity of these biosynthetic enzymes for their substrates and enables incorporation of new pharmacophores into thiopeptide architectures. This work also presents a strategy for diversification of natural products through rationally repurposing biosynthetic enzymes as non-natural biocatalysts.



Thiopeptides belong to the broad family of natural products referred to as ribosomally synthesized and post-translationally modified peptides (RiPPs).[1,2] RiPPs are generated from a typically 30−50 residue precursor peptide that is transformed into the bioactive natural product by a series of enzymatic modifications.[3,4] Thiopeptides are known to possess a remarkable range of bioactivities, including antiviral, anticancer, and antiplasmodial activities, as well as antibacterial activity against antibiotic-resistant strains.[2] The potent biological activities of thiopeptides make them attractive drug leads on the one hand, but the complex chemical structures make them challenging synthetic targets on the other. The core enzymes from thiopeptide and other RiPP biosynthetic pathways have proven powerful tools for synthesizing these complex molecules. By changing the sequence of the peptide substrates, new modified variants of these bioactive compounds can be readily prepared. For example, the native chemistries of LctM and PatD have been exploited to create new analogs of lacticin 481 and the patellamides, respectively.[5−7] More recently, we have developed a chemoenzymatic route to thiopeptides, thiocillin and thiomuracin, based on the activity of the pyridine synthases TclM and TbtD; this chemistry allowed the synthesis of several new thiopeptide variants.[8,9]

An alternative route to expand the structural diversity of thiopeptides might be to adapt their biosynthetic enzymes for non-native chemical transformations on their cognate peptides. In particular, heme-dependent cytochrome P450 enzymes have recently been exploited as biocatalysts for non-native chemistries. Arnold, Fasan, and others have shown that P450s, and other heme proteins, catalyze non-native metallocarbenoid and metallonitrenoid insertion reactions, providing novel biosynthetic routes to the formation of C−C, C−N, and other bonds.[10−18] P450-mediated cyclopropanation of aryl olefins using diazoacetate reagents has garnered attention due to the high turnover and diastereoselectivities displayed by native and engineered P450s.[11,19] This reaction has been adapted for the enzymatic synthesis of precursors to pharmaceuticals such as levomilnacipran and ticagrelor.[20,21] In addition, tuning P450 reactivity through mutations of the proximal Cys heme-ligand to Ser or His has led to efficient whole cell cyclopropanation catalysts.[19,20]

We hypothesized that P450s from RiPP biosynthetic pathways may be capable of intermolecular carbenoid-mediated cyclopropanations of olefins displayed on their cognate substrates. In particular, dehydroalanines (Dhas) and related olefins are common features in a number of RiPPs natural

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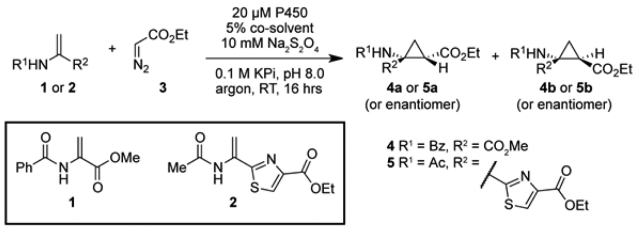
products including lantipeptide and thiopeptide antibiotics. Despite the fact that hemoprotein-catalyzed cyclopropanation reactions have typically been limited to aryl olefins (e.g., styrenes), we speculated that the geminal push−pull olefin present in Dhas would be sufficiently electron-rich to permit modification by P450-metallocarbenoids. On small molecule Dhas, this chemistry would allow access to 1-amino-2-cyclopropane carboxylic acids (ACCAs), important motifs in natural products and pharmaceuticals.[22−24] Alternatively, Dhas might provide reactive handles for ACCA incorporation into complex RiPP natural products.

We began our investigations by targeting cyclopropanation of two model compounds representative of Dhas observed in lantipeptides (**1**) and thiopeptides (**2**). For our first survey of this chemistry, we employed a panel of diverse P450 variants, which were previously identified as highly active and/or stereoselective for cyclopropanation of styrene with ethyl diazoacetate (EDA, **3**).[25] To enrich activity, we also added an active site Thr → Ala mutation that has been found to be generally activating with respect to carbenoid transfer. As summarized in Table 1 (and Figure S1, Figure S2, and Table S1), each variant screened was active for cyclopropanation against **1** and **2**, to yield cyclopropane products **4** and **5**, respectively. Conversions and diastereoselectivities were determined based on UV absorbance compared to authentic standards that were isolated and fully characterized by NMR. Diastereomers of **4** have been previously described in the literature;[26] key NOEs were used to discern diastereomers of compound **5** (see Supporting Methods).

Table 1. Dha Cyclopropanation with P450 Variants[a]



catalyst	conversion (**4**)[a]	TTN(**4**)	*dr* (**4**)	conversion (**5**)[b]	TTN(**5**)	*dr* (**5**)
hemin	10	50	66:34	67	337	82:18
$P450_{BM3}$-T268A	2	8	63:37	1	3	94:06
$P450_{BM3}$-T268A/C400S	6	29	68:32	13	67	82:18
$P450_{BM3\text{-}CIS}$-C400S	6	32	69:31	11	56	66:34
$P450_{Biol}$-T238A	4	21	49:51	13	65	46:54
$P450_{Biol}$-T238A/C344S	18	90	55:45	61	304	72:28
$P450_{EryF}$	12	60	76:24	18	91	72:28
CYP142-T234A	2	12	74:26	3	16	84:16
$P450_{PikC}$-T247A	29	145	83:17	64	321	91:09
CYP119-T213A	19	94	85:15	11	57	86:14
CYP119-T213A/C317S	6	28	57:43	44	221	10:90

[a]TTN = total turnover number. TTNs and diastereomeric ratios determined by HPLC analysis. [b]Conditions: 30 mM **1**, 10 mM **3**. [c]Conditions: 10 mM **2**, 10 mM **3**.

A number of trends can be observed in these results. Most notably, thiazole Dha, **2**, was cyclopropanated more readily when compared to the more electron-deficient Dha ester (**1**); N−H insertion, a preferred target for P450-generated carbenoids with olefin-substituted anilines,[15] was not observed for either substrate. While conversions in excess of 60% could be obtained with **2** (Table 1, Figures S1 and S2), cyclopropanation of **1** gave low conversions (2−29%). In reactions with compound **1**, reduced cyclopropanation conversions are accompanied by competing EDA dimerization, which proceeds in 14−71% conversion (Figure S3 and Table S1). This result suggests that slow insertion into more electron-deficient Dha olefins may enhance off-path dimerization products. As anticipated, the Thr → Ala mutation proved generally helpful. However, the addition of an axial Cys → Ser mutation, which is activating in aryl olefin cyclopropanation,[19,25] led to variable improvements across both substrates. Interestingly, the Cys → Ser mutation had a profound effect on stereoselectivity in CYP119. In general, the C−C *cis* diastereomer comprised the preferred product for either substrate (see Supporting Methods), with moderate *dr* observed for **1** and very good *dr* for **2** (Table 1). With CYP119-T213A-C317S, a loss of stereoselectivity was observed for compound **1**, whereas against compound **2**, the catalyst showed a significant inversion of diastereoselectivity (*dr*: 84:16 → 10:90).

We next expanded our P450 library in order to examine enzymes that evolved to oxidize natural products containing motifs analogous to compounds **1** and **2**. Many thiopeptides undergo post-translational monooxygenation by P450s. For example, thiomuracin and related thiopeptides have hydroxyls and/or epoxides installed on side chains (Figure 1).[1,27−29]

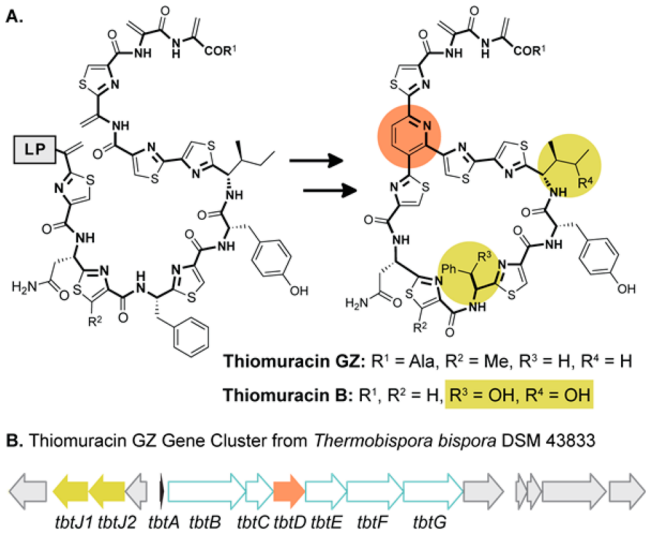


Figure 1. (A) Cyclization of the TbtA precursor by TbtD leads to the pyridine core (orange) of Thiomuracin GZ. LP = leader peptide. (B) Two monooxygenations, highlighted in yellow, are predicted to be carried out by P450 gene products of *tbtJ1* and *tbtJ2*.

Mutagenesis experiments have tied these modifications to P450s, but there have been no reports of *in vitro* reconstitution to date.[29,30] We chose to pursue two such P450s, $P450_{TbtJ1}$ and $P450_{TbtJ2}$, identified from the recently reported thiomuracin GZ pathway from *T. bispora* DSM 43833 (Figure 1).[31] $P450_{TbtJ1}$ and $P450_{TbtJ2}$ variants bearing conserved activating mutations were screened for cyclopropanation of **1** and **2** with EDA. $P450_{TbtJ1}$ and $P450_{TbtJ2}$ variants gave similar efficiencies to library constituents on **1** (Table 2 and Figures S1 and S2) and showed enhanced activity against Dha−thiazole, **2**. Notably, $P450_{TbtJ2}$−T247A provided the best conversion (79%), turnovers, and *dr* of any of the biocatalysts surveyed against **2**. These results suggested that $P450_{TbtJ1}$ and $P450_{TbtJ2}$ variants might be suitable candidates to carry out similar chemistry on Dha-containing analogs of native substrates.

Table 2. Dha Cyclopropanation with $P450_{TbtJ1}$ and $P450_{TbtJ2}$ Variants[a]

catalyst	conversion (**4**)[a]	TTN(**4**)	*dr* (**4**)	conversion (**5**)[b]	TTN(**5**)	*dr* (**5**)
$P450_{TbtJ1}$	2	12	80:20	*nt*	--	--
$P450_{TbtJ1}$-T234A	10	50	74:26	54	267	78:22
$P450_{TbtJ1}$-C340S	12	59	65:35	*nt*	--	--
$P450_{TbtJ1}$-T234A/C340S	16	81	54:46	55	277	81:19
$P450_{TbtJ2}$	4	20	71:29	*nt*	--	--
$P450_{TbtJ2}$-T247A	20	101	90:10	79	393	95:05
$P450_{TbtJ2}$-C353S	6	29	49:51	*nt*	--	--
$P450_{TbtJ2}$-T247A/C353S	9	44	59:41	38	192	69:31

[a]TTN = total turnover number. TTNs and diastereomeric ratios determined by HPLC analysis, nt = not tested. [b]Conditions: 30 mM **1**, 10 mM **3**. [c]Conditions: 10 mM **2**, 10 mM **3**.

In an effort to better understand the differences in activities observed among P450 catalysts for **2**, we determined the crystal structure of substrate-free $P450_{TbtJ1}$ (Table S2, Figure S4, 2.4 Å resolution, PDB: 5VWS). Initial efforts to obtain structures for $P450_{TbtJ2}$ did not yield diffraction-quality crystals. Nevertheless, the $P450_{TbtJ1}$ structure allows a visual comparison of X-ray crystal structures for P450 biocatalysts tested herein. $P450_{TbtJ1}$ exhibits the typical trigonal prism-shaped P450 fold with the heme prosthetic group embedded adjacent to the central I helix (Figure S5).[32] The surface of the protein shows a deep but narrow channel that may accommodate its preferred linear peptide substrate. This large substrate-binding cavity is flanked by the distinctive B−C and F−G loops shared by many P450s.[1] Common to substrate-free crystal structures of other P450s, the B−C loop appears partially disordered (Figures S6 and S7). Dynamic interactions with the B−C loop play an important role in P450 substrate recognition, and substrate binding typically results in organization of the B−C loop in other P450 crystal structures.[33]

Qualitatively, P450s that show high activity against substrate **2** (i.e. $P450_{PikC}$ (PDB: 2C7Z), $P450_{TbtJ1}$ (PDB: 5VWS), and CYP119 (PDB: 1IO7)) are marked by large, solvent-accessible heme binding pockets in their ligand-free form (Figure S8). Alternatively, the hemin of poorly active P450s, including $P450_{BM3}$ and CYP142 (PDB: 1YQO and 3EJB, respectively), is buried within the protein, with only narrow solvent channels leading to the cofactor. This observation is supported by calculations of accessible channel volumes (see Figure S8 for details), which suggest that highly active P450s possess large channels that facilitate substrate access to the metallocarbenoid intermediate.[34] A notable structural difference for $P450_{TbtJ1}$ is the presence of several phenylalanines (F70, F277, and F300, Figure S5) near the active site. These aromatic residues may be involved in $\pi-\pi$ interactions that bind and/or orient native polythiazole substrates and may contribute to the enriched activity observed for $P450_{TbtJ1}$ variants on thiazole Dha, **2**. A similar motif has been proposed for thiazole recognition by EpoK, a P450 involved in epothilone biosynthesis, and similar residues can be found in alignments with $P450_{TbtJ2}$ (Figures S9 and S10).[35] Further structural and biochemical studies to confirm the importance of these features for substrate recognition, as well as $P450_{TbtJ2}$ structural investigations, are ongoing; however, we anticipate that the substrate-free structure will facilitate further engineering and applications of this chemistry on this and other thiopeptides.

With thiomuracin P450 variants in hand, we turned to the synthesis of putative substrates. On the basis of prior mutagenesis work, it seemed likely that these enzymes would function on linear thiopeptide cores prior to installation of the central pyridine core (Figure 1).[30] We exploited a recently disclosed solid-phase route to thiopeptide cores to prepare a substrate analog in which Ile8, which is monooxygenated in a number of thiomuracin variants, was replaced with a Cys residue that could be eliminated to yield a Dha at this position; all other Dhas normally present in this core were replaced with alanine to reduce potential complexity (Figure S11).[8,9] Compound **7** (I8−1Dha, Figure 2A and Table 3) was separately incubated with three of the best biocatalysts for cyclopropanation of **1** and **2** ($P450_{PikC}$−T247A, $P450_{TbtJ1}$−T234A/C340S, and $P450_{TbtJ2}$−T247A), as well as free hemin. Due to the limited solubility of **7**, the maximum peptide concentration investigated was 100 μM. Catalyst concentrations were maintained at 20 μM, consistent with small molecule reactions. $P450_{PikC}$−T247A, $P450_{TbtJ1}$−T234A/C340S, and hemin all gave very low levels of modified products exhibiting different retention times (Figure 2B−D, Table 3). These peaks likely correspond to multiple diastereomers of the expected cyclopropanation product, which are generated in low conversions by these catalysts. In contrast, $P450_{TbtJ2}$−T247A provides robust conversion to a single major peak in the LC trace (**8**, Figure 2A and E, Figures S12 and S13); several minor peaks can also be observed near the baseline. To confirm that this product was the expected cyclopropane and to investigate diastereoselectivity, we repeated the reaction on scale using ^{13}C-labeled EDA in order to facilitate subsequent characterization by NMR. ^{1}H and ^{13}C NMRs of the purified major product confirm that it is a single diastereomer of the cyclopropanation product (see Supporting Methods). A trace amount (<5% based on integration) of an unidentified compound with resonance at ~40 ppm can also be observed in the ^{13}C NMR. Together, these results show that $P450_{TbtJ2}$−T247A catalyzes robust and diastereoselective cyclopropanation on an engineered variant of its natural substrate. As $P450_{TbtJ2}$−T247A exhibits proclivity toward modification of an I8−1Dha core (**7**), this may indicate $P450_{TbtJ2}$'s role in native modifications at the I8 position.

We further investigated the potential for site-specific cyclopropanation on thiopeptide substrates exhibiting multiple Dhas. Substrates F5−3Dha (Figure S14 and S15) and I8−3Dha (**9**, Figure 2F and S16) display three Dhas: one Dha at one of the two different positions that normally undergo P450-mediated monooxygenation (Phe5 or Ile8) and two Dhas at positions 1 and 11, which are natively used as handles for pyridine formation en route to the macrocyclic thiopeptide. Variants of $P450_{TbtJ1}$ and $P450_{TbtJ2}$ provided cyclopropane products (i.e., **10** in Figure 2F) with improved conversion relative to $P450_{PikC}$−T247A or hemin alone (Table 3, Figure 2G and H; Figures S17−S22). Somewhat surprisingly, the major products exhibit modifications at all three olefins (Figures 2, S21 and S22); mono- and dicyclopropanation products are only detectable in the baseline of the mass spectra. Again, $P450_{TbtJ2}$−T247A shows particularly strong conversion (~68%, Figure 2H and Table 3) from substrate **9** and yields primarily a single peak in the UV trace. In order to investigate site-selectivity for enzymatic cyclopropanations by $P450_{TbtJ2}$−T247A, product formation in the presence of **9** was monitored over time. Notably, the reaction nears completion in under an hour, and the tricyclopropanated species was the major product observed even at early time points (Figures S23 and S24). Accumulation of mono- and dimodified products was not observed. These results suggest that the native binding affinity

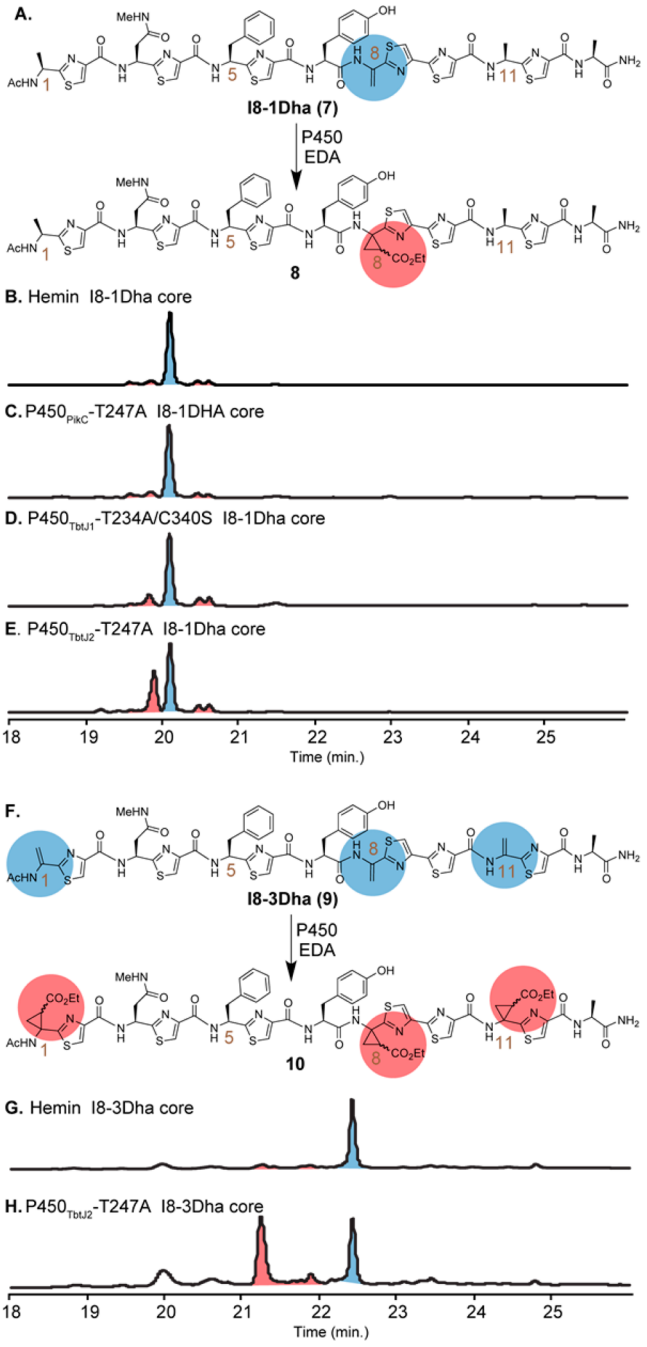


Figure 2. P450-mediated cyclopropanation of linear I8Dha thiopeptide core substrates bearing either one (**7**, panel A) or three (**9**, panel F) Dha olefins. (B–E) UV traces of cyclopropanation reactions on **7** show starting material (blue) and monosubstituted cyclopropane products (**8**, red). (G,H) UV traces of reactions on **9** show starting material (blue) and trisubstituted cyclopropane products (**10**, red). Reaction conditions: 20 μM catalyst, 100 μM thiomuracin derivative, 10 mM EDA, 10 mM $Na_2S_2O_4$, 0.1 M KPi, pH 8, 7.5% cosolvent, argon, 16 h.

of $P450_{TbtJ1}$ and $P450_{TbtJ2}$ may permit processive scanning of the substrate, transiently orienting each olefin within the active site for modification. Several RiPP enzymes are known to act processively on their substrates, although this would be a unique example of a late stage tailoring enzyme that is able to work processively as well.[18] A broader analysis of substrate promiscuity with additional analogs will be necessary to confirm this mechanism.

Table 3. Dha Cyclopropanation on Linear Thiopeptide Cores

catalyst	core	% conversion[a]
hemin	I8–1 Dha (**7**)	14
$P450_{pikC}$–T247A	I8–1 Dha (**7**)	9
$P450_{TbtJ1}$–T234A/C340S	I8–1 Dha (**7**)	29
$P450_{TbtJ2}$–T247A	I8–1 Dha (**7**)	46
hemin	F5–3Dha	11
$P450_{pikC}$–T247A	F5–3Dha	12
$P450_{TbtJ1}$–T234A/C340S	F5–3Dha	44
$P450_{TbtJ2}$–T247A	F5–3Dha	30
Hemin	I8–3Dha (**9**)	14
$P450_{PikC}$–T247A	I8–3Dha (**9**)	25
$P450_{TbtJ1}$–T234A/C340S	I8–3Dha (**9**)	42
$P450_{TbtJ2}$–T247A	I8–3Dha (**9**)	68

[a]% Conversion based on UV_{A254} integration versus that of starting material.

For comparison, we investigated modification of Dha-modified peptides using standard metal catalysts. Rhodium catalysts have been used by Romo and colleagues to catalyze microscale intermolecular C–H amination and olefin cyclopropanation of natural products.[36,37] Attempts to cyclopropanate I8–3Dha (**9**) using a panel of Rh catalysts provided only trace amounts of product (Figure S25). These data may suggest an inhibitory interaction between the peptide and catalyst. In addition, poor peptide solubility prevents high substrate concentrations typically used for Rh-mediated cyclopropanations. Alternatively, catalysis by Fe^{III}–tetraphenylporphyrin provided a complex mixture of cyclopropanation products enriched for one or two cyclopropane modifications (Figure S25).

Finally, in order to access cyclopropanated thiopeptides, we sought to determine whether $P450_{TbtJ1}$ or $P450_{TbtJ2}$ variants can modify a macrocyclic thiopeptide. This would provide a unique enzymatic opportunity for late-stage introduction of new C–C bonds, functional handles, and structural diversity into a natural product or natural product-like molecule. Accordingly, both the F5–3Dha and I8–3Dha core substrates were elaborated with a 15-residue fragment of the thiomuracin leader peptide. In previous work, we reported that a pyridine synthase recognition sequence is located in this region of the thiomuracin leader peptide (Figures S26 and S27).[9,38] Treatment of the F5–3Dha core with the thiomuracin pyridine synthase, TbtD (Figure 1), yielded the pyridine macrocycle (**11**, Figures 3 and S28). In contrast, no cyclization was observed with the I8–3Dha core, suggesting that TbtD is intolerant to this modification. Incubation of the cyclic F5-Dha thiomuracin analog (**11**, Figure 3A) and EDA with $P450_{TbtJ1}$–T234A/C340S or $P450_{TbtJ2}$–T247A afforded the cyclopropanated thiopeptide product (**12**, Figure 3B and C; confirmation by LC-MS/MS in Figures S29 and S30). We additionally screened $P450_{TbtJ1}$ and $P450_{TbtJ2}$ variants against two olefin-containing thiopeptides, nosiheptide and thiostrepton, derived from alternative biosynthetic pathways. Only baseline detection of cyclopropanated nosiheptide was observed, whereas thiostrepton showed no modification (data not shown). These results suggest that $P450_{TbtJ1}$ and $P450_{TbtJ2}$ variants show preference for linear and cyclic thiopeptide analogs of their natural substrate for cyclopropanation.

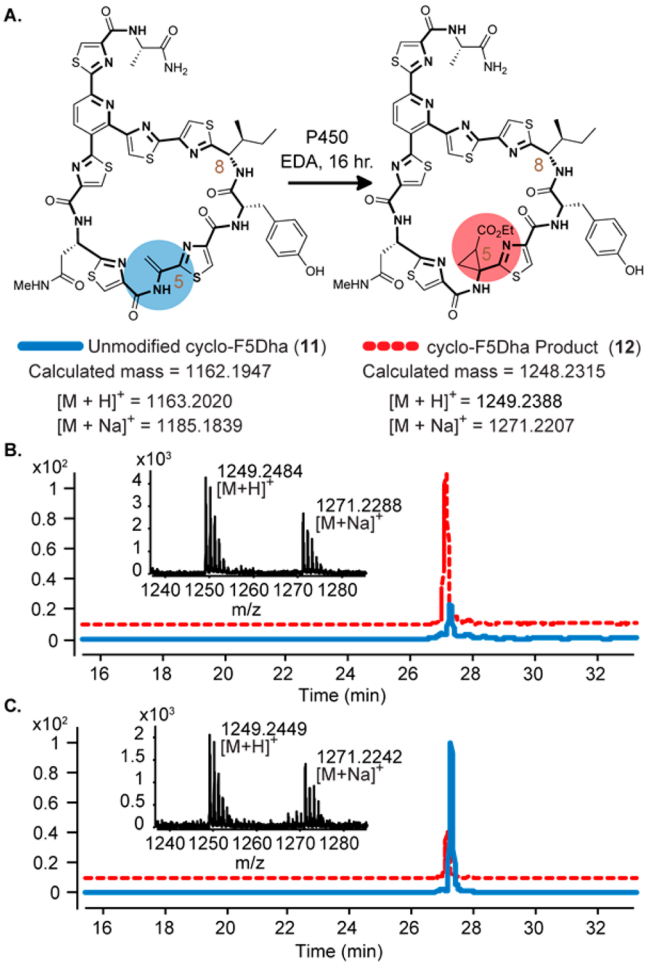


Figure 3. Non-natural enzymatic modification of a cyclic thiopeptide. (A) Cyclic F5Dha peptide precursor (**11**) and cyclopropane product (**12**). (B) Extracted ion chromatograms from $P450_{TbtJ1}$−T234A/C340S cyclopropanation reaction with cyclo-F5Dha. (C) Extracted ion chromatograms from $P450_{TbtJ2}$−T247A cyclopropanation reaction with cyclo-F5Dha. Cyclo-F5Dha is shown in blue, and the cyclopropane product is shown in red. Reaction conditions: 20 μM P450, 100 μM thiomuracin derivative, 10 mM EDA, 10 mM $Na_2S_2O_4$, 0.1 M KPi pH 8, 7.5% cosolvent, argon, 16 h.

To summarize, we have shown that enzymes from thiopeptide biosynthesis can be recruited to introduce non-natural functional groups on complex molecular scaffolds related to their native products. We initially demonstrated that P450 mediated cyclopropanation could be extended to modification of Dha-containing model substrates resembling those found in RiPP natural products. Both electron-rich and electron-deficient Dhas can be transformed in this way, resulting in the enzymatic synthesis of ACCAs. Engineered variants of $P450_{TbtJ1}$ and $P450_{TbtJ2}$, putative tailoring enzymes from the biosynthesis of thiomuracin, displayed weak activity on model Dha **1** but were more efficient catalysts with thiazolyl model substrate **2** and thiopeptides resembling their native substrates. In comparison, hemin and other P450s proved to be inferior biocatalysts, while Rh catalysts typically used in cyclopropanation of synthetic compounds displayed very poor yield and selectivity. These results suggest that the innate substrate recognition facilitates and enhances the non-natural enzymatic modification of these natural products and their analogs. This work sets the stage for investigations of the substrate scope and engineering improved variants of $P450_{TbtJ1}$ and $P450_{TbtJ2}$, as well as assessing the biological activity of ACCA-containing analogs. Similar efforts could expand the scope of chemical functionality that can be introduced by these catalysts. For example, a diverse array of metallocarbenoid and metallonitrenoid chemistries have been demonstrated in P450s[10−12,14−18] and Ir-substituted heme proteins,[39,40] suggesting that P450-mediated modification of natural products can be extended to other non-natural C−C, C−N, and C−X modifications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00358.

Experimental details, synthetic schemes, figures (PDF)

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Notes

The authors declare no competing financial interest.

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