

Characterization of the Stereoselective P450 Enzyme BotCYP Enables the *In Vitro* Biosynthesis of the Bottromycin Core Scaffold

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Supporting Information Placeholder

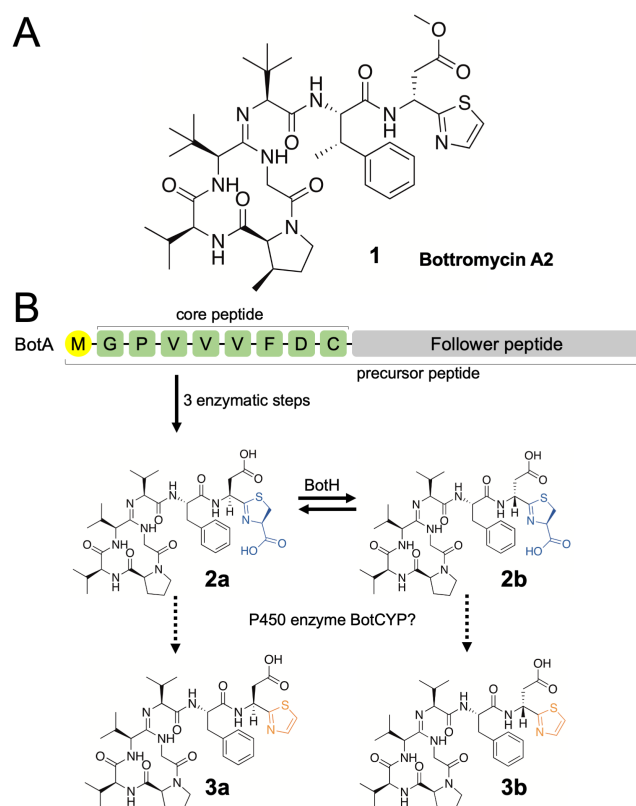
ABSTRACT: Bottromycins are ribosomally synthesized and post-translationally modified peptide natural product antibiotics that are effective against high-priority human pathogens such as methicillin-resistant *Staphylococcus aureus*. The total synthesis of bottromycins involves at least 17 steps with a poor overall yield. Here, we report the characterization of the cytochrome P450 enzyme BotCYP from a bottromycin biosynthetic gene cluster. We determined the structure of a close BotCYP homolog, and used our data to conduct the first large-scale survey of P450 enzymes associated with RiPP biosynthetic gene clusters. We demonstrate that BotCYP converts a C-terminal thiazoline to a thiazole via an oxidative decarboxylation reaction and provides stereochemical resolution for the pathway. Our data enable the 2-pot *in vitro* production of the bottromycin core scaffold and may allow the rapid generation of bottromycin analogues for compound development.

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a rapidly growing natural product superfamily with interesting and diverse bioactivities.^{1,3} Bottromycins (**1**, Scheme 1A) are RiPPs with potent activity against Gram-positive bacteria, including the problematic human pathogens MRSA and VRE.^{4,5} The bioactivity triggered several attempts at total synthesis, which was finally achieved by an inspired route requiring at least 17 steps.⁵ *In vitro* and *in vivo* analyses of bottromycin biosynthesis have largely established the order of steps: Removal of the N-terminal methionine⁷ is followed by thiazoline and macroamide formation^{8,9} and removal of the follower peptide to yield **2a** (Scheme 1B).¹⁰ This intermediate is then converted to **2b** by the epimerase BotH, which results in a mixture of the two epimers (Scheme 1B).¹¹ The D-Asp configuration of **2b**, tentatively assigned by Marfey's analysis of the 2 epimers and protein crystallography,¹¹ is also found in the final natural product **1**.^{5,12}

Five-membered heterocycles that are enzymatically derived from serine, threonine or cysteine residues are frequently found in RiPPs.^{1,3} Their oxidation state, azoline or azole, can have a profound effect on RiPP bioactivity,¹³⁻¹⁴ and all enzymes linked with azoline oxidation in RiPP biosynthesis thus far are flavin-dependent.¹⁵⁻¹⁷ In contrast, it has been proposed that the oxidative decarboxylation reaction of the thiazoline in bottromycin biosynthesis is catalyzed by a P450 enzyme (BotCYP) found in the bottromycin biosynthetic gene cluster (BGC, Figure S1).⁶ To investigate the role of this enzyme *in vitro*, we used heterologously expressed and purified

BotCYP in spectral analyses to confirm an intact heme-containing protein. Incubation of BotCYP with sodium dithionite led to the observation of the typical Soret band at 448 nm in the spectrum with bound carbon monoxide, indicating the chemical reduction of the heme iron (Figure S2).¹⁸

Scheme 1. A Structure of bottromycin A2. B *In vitro* biosynthesis of 2a/b, with a proposed role for the P450 enzyme BotCYP.



Notably, a typical reductase-ferredoxin pair required as a cofactor for cytochrome P450 enzymes is not found in the Bottromycin BGC. Enzymatic reduction of the heme iron was thus attempted via several other P450 reductase-ferredoxin pairs and the highest peak at 448 nm observed using the P450 reductase-ferredoxin pair BmCPR-Fdx2 from *Bacillus megaterium* (Figure S2).¹⁹ We then produced the putative substrate mixture **2a/b** biochemically *in vitro* using three enzymes (Details can be found in the supplementary online material).^{7-8, 10} **2a/b** was incubated with an optimized BotCYP :

BmCPR : Fdx2 ratio and a molar excess of NADPH in the absence and presence of BotCYP at 30 °C over night. The reaction mixture was subsequently analyzed by high-resolution liquid chromatography-mass spectrometry (HR-LCMS, Figures 1A and 1B). In the absence of BotCYP, we observed no effect on **2a/b** ($[M+H]^+$ calc_{mono.}: 799.3807 Da; $[M+H]^+$ obs_{mono.}: r.t. 3.18 min 799.3801 Da, error -0.75 ppm, r.t. 3.02 min 799.3796 Da, error -1.38 ppm). In the presence of BotCYP, however, one major and one minor peak appeared, which had identical masses that corresponded to the decarboxylated, oxidized reaction product **3a/b** ($[M+H]^+$ calc_{mono.}: 753.3752 Da, $[M+H]^+$ obs_{mono.}: r.t. 3.41 min 753.3743 Da, error -1.19 ppm, r.t. 3.22 min 753.3743 Da, error -1.19 ppm) (Figure 1B). Analysis of both peaks by tandem mass spectrometry (MS²) strongly supports the proposed structures (Figures 1C, Tables S1 and S2).

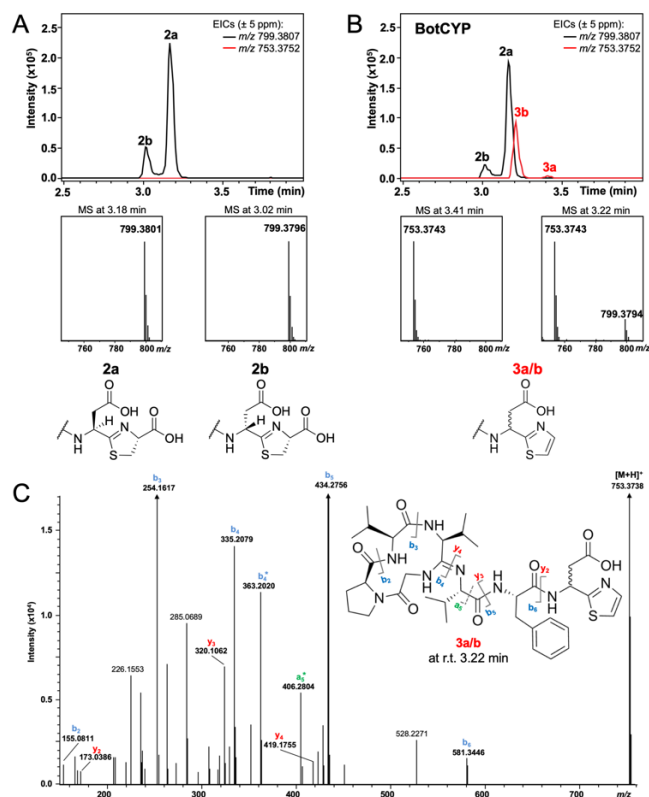


Figure 1: **A** Incubation of putative BotCYP substrates **2a/b** with all cofactors results in no detectable change in the HR-LCMS spectrum. **B** Same as A, but with BotCYP added. The new species **3a/b** showed a loss of 46 Da, which is in agreement with the expected product. **C** MS² spectrum of **3a/b**. a, b and y ions are indicated. The peak list and structures of the fragments b₂ and b₃ can be found in Tables S1 and S2.

These data establish BotCYP as sufficient for the oxidative decarboxylation of the C-terminal thiazoline to a thiazole. We estimate the yield of this reaction to be very poor (< 25%). The presence of a very small second product peak indicated that the enzyme may be stereospecific and selects one of the two available substrate epimers. Using an enzymatically produced, roughly equimolar mixture of **2a** and **2b** in time-course experiments allowed us to demonstrate that the D-Asp containing **2b** peak is preferentially consumed by BotCYP (Figure 2A). To better understand BotCYP, we attempted to determine its crystal structure, but extensive screening did not yield crystals. The close homolog SalCYP from *Salinispora tropica* (Figure S1) was also able to perform the oxidative decarboxylation reaction on similar time-scales and with comparable yields using identical

redox partners (Figures 2B and S3-4). The high-resolution (1.85 Å) structure of SalCYP was determined using single-wavelength anomalous dispersion from iron. All data collection and refinement statistics can be found in Table S3. The overall structure of SalCYP reveals a heme b bound at the reactive centre, which is freely accessible via a solvent channel that leads to a large active site cavity suitable for binding very bulky substrates (Figure S5). The heme b is coordinated by Cys354, forms two salt bridges between the two heme carboxylic acid moieties and Arg104 and Arg298 side chains of SalCYP as well as extensive hydrophobic interactions (Figure S6). Surprisingly, we found the N-terminus (residues 1 – 20) to be ordered and extended away from the protein, with the amino group of the N-terminus coordinating the heme iron of a symmetry mate (Figure S7). To allow an unbiased analysis of the binding pocket, we engineered a new crystal contact. This mutant protein, SalCYP^T, retained enzymatic activity (Figure S8), readily crystallized in space group P4₁ and the structure was determined to 1.50 Å resolution. The active site was now unoccupied by protein, but retained its shape and solvent channel at the entrance to the active site cavity.

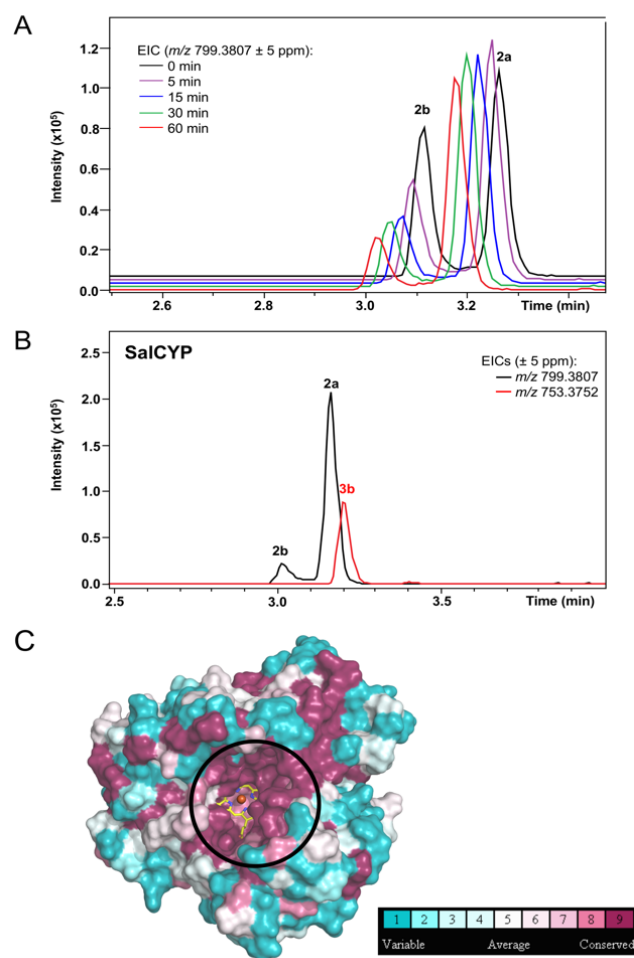


Figure 2: **A** HR-LCMS data show that BotCYP preferentially consumes **2b**. **B** Analysis of a SalCYP reaction with **2a/b**. One product peak is observed when SalCYP is used. **C** ConSurf map showing the conservation of residues around the SalCYP active site (circle) for SalCYP homologues from other bottromycin biosynthetic gene clusters (Figure 3A).

A search for structural homologs using the DALI server²⁰ revealed the camphor hydroxylase CYP101D²¹ as the closest structural homolog (PDB ID 4dxy) with a C_α rmsd of 2.4 Å over 355 residues (Figure S9). The structural homology of SalCYP to the well-

characterized OleTJE (PDB ID 5m0n),²² which catalyzes the oxidative decarboxylation of fatty acids, was calculated as a C_{α} rmsd of 2.9 Å over 310 residues (Figure S9). The most striking difference between SalCYP and virtually all close structural homologs was the wide and deep active-site cleft found in SalCYP (Figure S10). The exception was the structure of TbtJ1 (PDB ID 5vws), which is the only other P450 enzyme structure from a RiPP pathway.²³ TbtJ1 catalyzes the hydroxylation of thiomuracin^{24,25} and possesses a similar active site topology. The residues found at the SalCYP active site are very well conserved (Figure 2C), which may reflect the need for stereocontrol for this step of bottromycin biosynthesis. Taken together, these structures thus indicate that RiPP P450 enzymes have evolved to perform selective reactions within complex linear and cyclic RiPP core peptides (Figure S10).

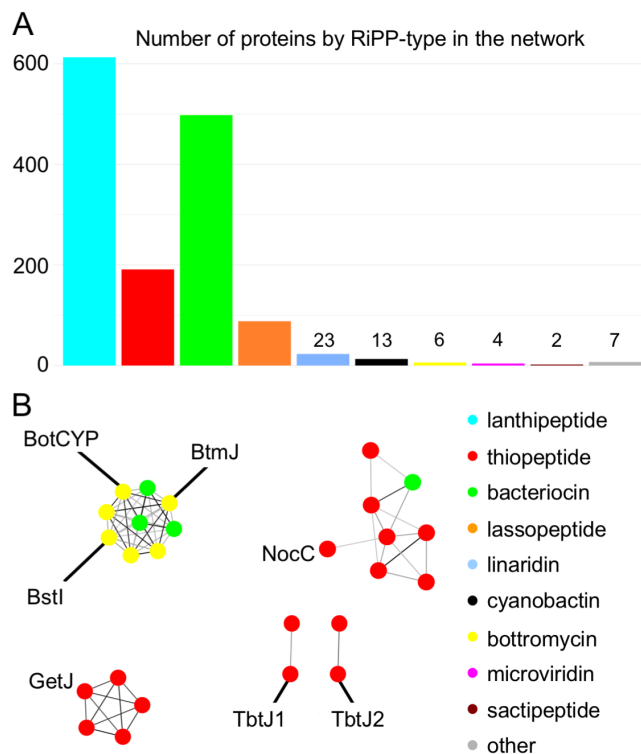


Figure 3: **A** Histogram displaying the number of P450 enzyme containing predicted RiPP BGCs, grouped and colored by RiPP-type (See Figure S11). **B** Clusters with characterized enzymes are annotated. MibO and NocV (see main text) are singlets and not displayed. Manual curation of the BotCYP-containing cluster identifies the three bacteriocin nodes as bottromycin BGCs.

We computed a sequence similarity network for BotCYP to explore the distribution of P450-like enzymes in RiPP BGCs (Figures 3 and S11). Although P450-like enzymes are widespread in bacteria (109,039 members of the Pfam family PF00067), and more than 15,000 of them are located in BGCs, only ~1.5% (1,786) were found in a predicted RiPP cluster. Sequence diversity of these proteins is very large (mean sequence identity 28%), and they are found in diverse Proteobacteria, as well as Actinobacteria, Cyanobacteria, Chloroflexi, and Bacteroidetes. BotCYP is a part of a small cluster that contains all P450 enzymes from putative bottromycin biosynthetic gene clusters, which echoes the findings for the amidohydrolyase PurAH.¹⁰ We would like to note that only a handful of RiPP P450 enzymes within this network have an assigned biosynthetic function to date: MibO (microbisporicin), TbtJ1 and TbtJ2 (thiomuracin), TsrR (thiostrepton) and NocV (nocathiacin) all

catalyze the hydroxylation of amino acids.²⁴⁻²⁷ GetJ from GE37468 biosynthesis catalyzes the conversion of isoleucine to δ -hydroxyproline and TsrP, also involved in thiostrepton biosynthesis, catalyzes an epoxidation that triggers additional changes.^{26, 28} None of these proteins catalyze an oxidative decarboxylation reaction, nor are they involved in heterocycle biochemistry. BotCYP thus expands the catalytic scope of P450 enzymes involved in RiPP biosynthesis, and the map of the RiPP P450 landscape we provide here may serve as a very valuable starting point to explore the functionalities of P450 enzymes in RiPP biosynthesis and discover novel transformations.

The spontaneous exchange between **2a** and **2b** is slow,^{6, 11} and **2b** (D-Asp), the much less abundant epimer, was the preferred substrate for BotCYP. This led us to speculate that the poor yield of the BotCYP and SalCYP reactions may be the result of depleting **2b**. We thus added the recently characterized epimerase BotH,¹¹ which catalyzes the rapid conversion of **2a** to **2b**, to the reaction mixture. Analysis of reactions containing BotH and BotCYP by HR-LCMS demonstrated that the oxidative decarboxylation reaction now nearly goes to completion (Figure 4A). Since **2b** contains D-Asp and was preferentially consumed by BotCYP, it may be intuitive to assume that the reaction product, **3b**, also harbors the D-Asp found in the natural product. To probe the stereochemistry at the Asp C_{α} -position of **3b**, we enzymatically produced substrate selectively labeled with a deuterium at this position (**4a/b**, $[M+H]^+$ calc. mono.: 800.3870 Da; r.t. 3.15 min (**4a**) $[M+H]^+$ obs. mono.: 800.3851 Da, error -2.37 ppm and r.t. 3.00 min (**4b**) $[M+H]^+$ obs. mono.: 800.3852 Da, error -2.24 ppm) (Figure S12).

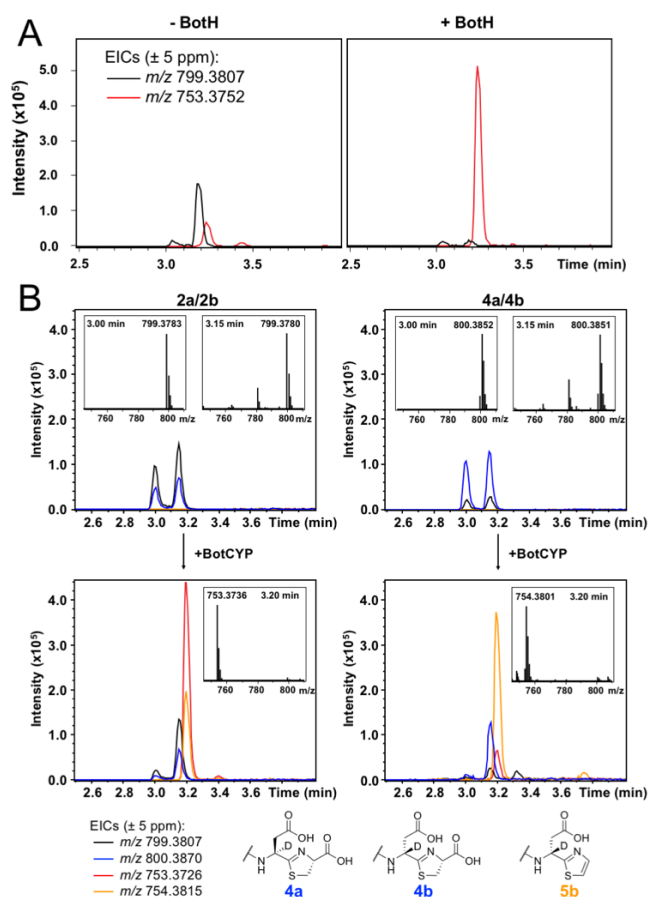


Figure 4: **A** Comparison of the BotCYP reaction with (right) and without (left) BotH. Addition of BotH enables virtually complete turnover. **B** BotCYP reaction using **4a/b** as a substrate. The product **5b** has the same retention time as **3b**, but retains its mass shift of +1 Da.

We then used **4a/b** as a substrate in a BotCYP reaction in H₂O and the resulting peak had the same retention time and fragmentation pattern as **3b** (Figures 4B and S13). Crucially, the product peak (**5b**) retained the mass shift of + 1 Da ([M+H]⁺calc._{mono.}: 754.3815 Da, [M+H]⁺obs._{mono.}: 754.3801 Da, error -1.86 ppm), indicating that the configuration of the Asp C_α-position is retained during the oxidative decarboxylation reaction. Presence of the deuterium at the Asp-position was confirmed by MS² (Figure S13). Our data thus strongly imply that **3b** represents the bottromycin core scaffold (des-methyl **1**) harboring the D-Asp moiety found in bottromycins. Incubation of **5b** with BotH in H₂O does no longer lead to an exchange of the deuterium at the Asp C_α-position, indicating that heterocycle oxidation to a thiazole locks the configuration at the Asp position.¹¹

We have demonstrated that the P450 enzyme found in the bottromycin BGC is sufficient for the oxidative decarboxylation of the C-terminal thiazoline found in the bottromycin biosynthetic intermediate **2a/b**. The stereospecificity of BotCYP (and SalCYP) provides stereochemical resolution for the pathway, since the epimerase BotH produces a product mixture (D- and L-Asp).¹¹ Our data also rationalize the results of a previous study attempting to produce bottromycin derivatives at the Asp-position (Asp to Thr, Ala, or Asn).⁴ Mutations in this position appear incompatible with BotH epimerase activity,¹¹ resulting in very little, if any, D-amino acid substrate for BotCYP and thus no bottromycin derivative production. To function efficiently, BotCYP needs to cooperate with the epimerase BotH,¹¹ but we were unable to detect complex formation between the two proteins (data not shown). This mirrors the results for the bottromycin biosynthetic enzymes PurCD (macrocyclase) and PurAH (amidohydrolase), which are both required for efficient macroamidine formation, but do not form a complex.¹⁰ It is still unclear if the bottromycin biosynthetic machinery is organized within the producing strains, for example by co-localizing enzymes without physical complex formation. The reconstitution of BotCYP also opens a facile, 2-pot *in vitro* biosynthetic route to the bottromycin core scaffold. Consequently, it might be a valuable alternative to synthetic approaches of bottromycin production, which currently still involve steps with only a moderate yield for the final macrocyclization reaction.^{5,29} Our data may thus enable the rapid generation of bottromycin analogues for antibiotic activity testing.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website.

Detailed experimental procedures and supporting information (PDF). Cytoscape session and style files (zip).

Anti-smash annotations for all BGCs including accession codes (txt).

All-to-all sequence identities (gz).

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Notes

No competing financial interests have been declared.

The structures of SalCYP and SalCYP^T have been deposited in the protein databank (PDB IDs 6aba and 6abb, respectively).

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