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Identification of the dioxygenase-generated intermediate formed during biosynthesis of the dihydropyrrole moiety common to anthramycin and sibiromycin

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Abstract

A description of pyrrolo[1,4]benzodiazepine (PBD) biosynthesis is a prerequisite for engineering production of analogs with enhanced antitumor activity. Predicted dioxygenases Orf12 and SibV associated with dihydropyrrole biosynthesis in PBDs anthramycin and sibiromycin, respectively, were expressed and purified for activity studies. UV-visible spectroscopy revealed that these enzymes catalyze the regiospecific 2,3-extradiol dioxygenation of L-3,4-dihydroxyphenylalanine (L-DOPA) to form L-2,3-*secodopa* ($\lambda_{\text{max}} = 368 \text{ nm}$). ¹H NMR spectroscopy indicates that L-2,3-*secodopa* cyclizes into the α -keto acid tautomer of L-4-(2-oxo-3-butenic-acid)-4,5-dihydropyrrole-2-carboxylic acid ($\lambda_{\text{max}} = 414 \text{ nm}$). Thus, the dioxygenases are key for establishing the scaffold of the dihydropyrrole moiety. Kinetic studies suggest the dioxygenase product is relatively labile and is likely consumed rapidly by subsequent biosynthetic steps. The enzymatic product and dimeric state of these dioxygenases are conserved in dioxygenases involved in dihydropyrrole or pyrrolidine biosynthesis within both PBD and non-PBD pathways.

Keywords

Extradiol dioxygenase; L-DOPA; *secodopa*; Dihydropyrrole; Biosynthesis; Anthramycin; Sibiromycin; Pyrrolo[1,4]benzodiazepine

1. Introduction

Pyrrolo[1,4]benzodiazepines (PBDs) are tricyclic secondary metabolites of actinomycetes that have emerged as anticancer drug candidates due to their ability to alkylate double

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Supplementary data

Supplementary data associated with this article can be found in the online version.

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stranded DNA.^{1–3} PBDs contain an anthranilate and dihydropyrrole or pyrrolidine moiety fused to each side of a diazepine ring that bears an electrophilic imine carbon at C11, the site of alkylation (Scheme 1).^{4–7} Their efficacy is due in part from their ability to bind DNA in a sequence selective manner within the minor groove^{8,9} and the resistance of subsequent PBD-DNA adducts to proofreading machinery that repairs DNA.¹⁰

Among PBDs, sibiromycin displays the highest affinity for DNA and greatest cytotoxicity due to the unique presence of an appended amino sugar. Molecular dynamics and docking studies indicate that this sugar protrudes from the minor groove and likely blocks transcription factors from binding their targets.¹² Sibiromycin displays activity against ovarian, plasmacytoma and leukemia cancer cell lines.¹³ However, the pharmacological utility of this natural product is limited by its cardiotoxicity that has been traced to the hydroxyl group at C9 of the anthranilate moiety.^{14,15} To circumvent this harmful side effect, monomeric and dimeric PBD analogs were synthesized chemically but their lengthy protocols were plagued by the lability of intermediates, low yields and limited stereochemical control.^{16–19} Biosynthesis offers a complementary and appealing strategy to generate new PBD candidates. For instance, delineating the biosynthesis of the anthranilate moiety in PBDs^{20,21} allowed for its reprogramming to create an analog of sibiromycin lacking its C9 hydroxyl group. The resulting 9-deoxysibiromycin displays reduced cardiotoxicity as desired²² confirming the potential for creating a range of new PBDs that suppress unwanted side effects.

Creating a dihydropyrrole moiety within a PBD to abolish the bending caused by DNA alkylation is an attractive goal since the resulting adduct has the potential to evade DNA repair enzymes that detect alterations in DNA curvature.¹⁰ Some of the distortion in DNA is already ameliorated by the right-handed twist of PBDs that complements right-handed DNA.^{6,7,23–26} Structure perturbation still persists and includes bending of the helix around the newly formed covalent bond. The degree of DNA bending induced by the adduct (5.0–8.9° with anthramycin and 8.2–14.5° with tomaymycin) inversely correlates with the degree of PBD twist (35.4° in anthramycin and 9.1° in tomaymycin).¹ Before natural processes can be harnessed to develop new dihydropyrroles within PBD to escape DNA repair, the biosynthesis of these groups must be identified.

Previous feeding studies first suggested that the dihydropyrrole moiety originates from L-tyrosine,¹¹ but few biochemical details of the intervening transformations were available. A comparison of the gene clusters responsible for producing the PBDs anthramycin,²⁰ sibiromycin,²⁷ tomaymycin,²⁸ and porothramycin²⁹ allowed a tentative assignment of gene function and revealed common strategy for generating these PBDs. Biochemical studies have confirmed that all of these pathways begin with an enzyme-catalyzed *ortho*-hydroxylation of L-tyrosine to form L-3,4-dihydroxyphenylalanine (L-DOPA) **1**.^{30,31} Non-PBD natural products lincomycin A^{32,33} and hormaomycin³⁴ contain a pyrrolidine ring that also originates from the equivalent hydroxylation step. *In vitro* assays showed that LmbB1^{35–37} from lincomycin A biosynthesis and HrmF³⁴ from hormaomycin biosynthesis next catalyze an extradiol dioxygenation of **1** to form a yellow product. Homologous enzymes identified by the consensus sequence signature of extradiol dioxygenases in the

anthramycin,²⁰ sibiromycin,²⁷ and tomaymycin²⁸ biosynthesis were consequently expected to promote a dioxygenation reaction as the second step of the pathways.

The dioxygenases involved in all PBD biosyntheses are expected to transform **1** into L-2,3-secodopa **2** that has the potential to cyclize into the five-membered ring found in the PBD dihydropyrrole moiety (Scheme 2). However, multiple sites of cleavage and alternative cyclizations observed with other 2,3-extradiol dioxygenases not involved in PBD biosynthesis illustrate the plethora of possible products. The goal of the project was to identify and characterize the product(s) formed by the dioxygenase along the path to the dihydropyrrole moiety.

2. Results and Discussion

2.1 Expression and purification of PBD dioxygenases

The putative dioxygenases associated with formation of the dihydropyrrole moiety in *Streptomyces refuineus*'s anthramycin (Orf12) and *Streptosporangium sibiricum*'s sibiromycin (SibV) were selected as representative of the general transformation. Orf12 and SibV were alternately fused with an *N*-terminal His₆ and His₆-SUMO tag, respectively. Each was then expressed in BL21(DE3) *E. coli* and purified via Ni-NTA chromatography. The tag of SibV was removed by a SUMO-specific protease (Ulp1) leaving behind a single non-native *N*-terminal serine. The dioxygenases were finally purified by size exclusion chromatography to homogeneity and reconstituted with Fe²⁺ to yield light blue *holo*-dioxygenases. The final purification step provided *ca.* 150 mg His₆-Orf12 (Orf12) and 10 mg SibV per liter of growth media (Figure S1). Since His₆ tags sometimes interfere with protein oligomerization,^{38,39} the oligomeric state of SibV was examined by gel filtration and confirmed to form its expected dimer in solution (observed molecular mass of 34.6 kDa, theoretical monomeric mass of 17.1 kDa) (Figure S2). Gel filtration was used to determine that LmbB1 is also a dimer in solution³⁶ indicating that the oligomerization state of dioxygenases is conserved among the PBD and lincomycin A biosynthetic pathways.

2.2 UV-visible spectroscopic characterization of the transient and final products formed by dioxygenase treatment of **1**

Transformation of **1** by Orf12 generated a transient compound (A_{max} at 378 nm) that subsequently diminished concurrent with formation of a yellow compound (A_{max} at 414 nm) (Figure 1). SibV transformed **1** to the same transient and final products as evident from equivalent changes in UV-visible absorbance (Figure S3). Thus, these two dioxygenases appear to promote identical reactions. The same A_{max} at 414 nm was observed previously after turnover of dioxygenases LmbB1 and HrmF of lincomycin A³⁷ and hormaomycin³⁴ indicating that this transformation is not limited to PBD biosynthesis but general to pyrrolidine biosynthesis of many natural products.

2.3 ¹H NMR spectroscopic analysis of the product formed by dioxygenation of **1** catalyzed by Orf12

The yellow compound generated in the dioxygenase catalyzed oxidative cleavage of **1** was isolated by extraction and characterized by ¹H NMR spectroscopy. This required a

concentrated sample but oxygen-dependent inactivation of the enzyme made it necessary to add Orf12 in multiple aliquots to compensate for its loss of activity in the presence of reductant. The enzyme was subsequently removed by wash with CHCl_3 . The remaining reaction mixture was spiked with 10% D_2O and analyzed by NMR spectroscopy. No signals associated with **1** were observed indicating its complete consumption (Figure 2). Assignment of the ^1H signals was based on a model compound, 3-propylidene- -pyrroline-5-carboxylic acid, previously proposed as a downstream intermediate in the biosynthesis of the dihydropyrrole moiety.⁴⁰ The chemical shifts, integration values and coupling constants were all consistent with L-4-(2-oxo-3-butenic-acid)-4,5-dihydropyrrole-2-carboxylic-acid, **3a**. The signals located upfield of the HDO resonance were diagnostic of the protons attached to sp^3 hybridized carbons and were assigned to H5 α (3.11 ppm), H5 β (2.72 ppm) and H4 (4.59 ppm) (Figure 2). These three signals are each a doublet of doublets arising from an AMX spin system that forms from coupling between the diastereotopic methylene protons H5 α and H5 β ($^2J_{\text{HH}} = 16$ Hz) and are further split by H4 at the adjacent chiral center. Vicinal coupling constants between H5 α and H4 ($^3J_{\text{HH}} = 12$ Hz) and between H5 β and H4 ($^3J_{\text{HH}} = 6.2$ Hz) satisfied the Karplus relationship⁴¹ that correlates vicinal coupling constants to dihedral angles (predicted to be 2.9° and 117.2° by Chem3D Pro, respectively). The resonance furthest downfield (7.59 ppm) was a singlet and consistent with proton H3 located on the unsaturated carbon in the dihydropyrrole. The remaining pair of doublets H1 (5.52) and H2 (7.58 ppm) were assigned to vicinal protons. The large HDO signal distorted the baseline beneath the H1 and H4 resonances and suppressed their integration from the expected value of one. ^1H - ^1H COSY analysis confirmed the expected connectivities between the protons with the exception of H4. Its resonance is located near that of the solvent and was not observed due to solvent suppression (Figure S4). No aldehydic protons were observed that would have indicated the formation of alternative products as discussed below (Scheme 2).

The multiplicity of signals for the pair of vicinal protons H1 and H2 were used to distinguish between the tautomers **3a** and **3b**. These protons produced a large coupling constant ($^3J_{\text{HH}} = 16$ Hz) that is consistent with vicinal coupling constants of *trans*-olefinic protons ($^3J_{\text{HH expected}} = 12\text{--}18$ Hz) such as those in the α -keto acid tautomer **3a** but not in the α -enol acid tautomer **3b** since this alternative would have exhibited a lower vicinal coupling constant ($^3J_{\text{HH expected}} < 11$ Hz).⁴² A pair of smaller signals (7.12 and 5.80 ppm) exhibits a coupling constant (8.5 Hz) consistent with the equivalent protons on **3b**. Integration of these signals indicate the presence of approximately 5% **3b**. The measured extinction coefficient for **3a** at pH 8.0 ($\epsilon_{414\text{nm}} = 50 \pm 3 \text{ mM}^{-1}\text{cm}^{-1}$) closely matches that reported for the LmbB1 product ($\epsilon_{413\text{nm}} = 48 \pm 2$ and $\epsilon_{414\text{nm}} = 45 \pm 2 \text{ mM}^{-1}\text{cm}^{-1}$)^{36,43} and provides confirmation that Orf12 generates the same product as LmbB1. However, LmbB1 was reported earlier to form **3b**.³⁷ This was based on a ^1H NMR spectrum of an equal mixture of product and starting material for which the coupling constant (8 Hz) of a substrate signal (6.94 ppm) was erroneously assigned to the product and used to rule out the formation of **3a**.³⁷ A ^1H NMR spectrum of the starting material confirms our reassignment (Figure S5). Conditions used to isolate the enzyme products did not appear to alter their structure. NMR analysis of the crude product confirmed the predominance of **3a** rather than **3b** despite the greater conjugation of **3b**. Differentiating between these tautomers will become important again

when identifying substrates of the enzymes downstream in the biosynthetic pathways. We speculate that **3a** is similarly generated during lincomycin A and hormaomycin biosynthesis as well. Equivalent ESI-MS data were recorded for the products formed by both HrmF³⁴ and Orf12.

Previously, a 2,3-extradiol dioxygenase involved in betalain biosynthesis in the fungus *Amanita muscaria* was reported to form the seven-membered ring, muscaflavin **4** after formation of **2** (Scheme 2).⁴⁵ This raises the possibility that a non-productive pathway may act competitively during dihydropyrrole biosynthesis. However, only **3a** was detected from cyclization of **2**. A characteristic aldehydic signal expected in the ¹H NMR spectrum of **4** was not observed. The ¹H NMR signals for the product assigned as **3a** did not agree with literature values reported for **4**.⁴⁵ This indicates that the intramolecular cyclization of **2** to **4** observed in betalain biosynthesis⁴⁵ does not occur in PBD dihydropyrrole biosynthesis. It is unclear if Orf12 prevents this process that had been described as spontaneous.⁴⁵

The *A. muscaria* 2,3-extradiol dioxygenase is also known to cleave at the alternative C4–C5 bond of **1** to form betalamic acid **6** via L-4,5-*secodopa* **5**.⁴⁵ This suggests that the dioxygenases involved in PBD dihydropyrrole biosynthesis might have the potential to cleave the aromatic ring of **1** at multiple sites. However, no products resulting from the 4,5-cleavage of **1** by Orf12 were observed (Scheme 3) as indicated by the absence of ¹H NMR signals for the aldehydic protons of **5** or **6**. This indicates that Orf12 acts regiospecifically to cleave only the expected C2–C3 bond of **1**. Alternative cleavage to form **5** and **6** would have prevented formation of the five-membered dihydropyrrole.

2.4 Is the transient acyclic compound **2** a biosynthetic intermediate of PBD biosynthesis?

The transient product observed by UV-Vis spectroscopy during dioxygenation is consistent with **2** since its absorbance maximum was similar to that exhibited by 2,3-*secocaffeic* acid ($A_{\text{max}} = 384 \text{ nm}$).³⁷ The extra conjugation of this analog explains the red-shift (16 nm) of its absorbance maximum relative to that of **2**. UV-visible spectroscopy revealed that the equilibrium between **3a** and the colorless compound **2** (A_{max} at 368 nm) can be shifted by increasingly acidic conditions (pH 3–5) (Figures S6). The absorbance maximum of **2** was slightly lower than that implied during its formation by dioxygenation ($A_{\text{max}} = 378 \text{ nm}$) of **1** due to the additive effect of the absorbance from **3a**. The transient compound was verified to be **2** ($\epsilon_{368 \text{ nm}} = 27 \pm 3 \text{ mM}^{-1}\text{cm}^{-1}$ at pH 4.0) by its ability to reconvert to **3a** upon exposure to base (Figure S6) and by the presence of a downfield resonance (9.08 ppm) in its ¹H NMR spectrum (Figure S7) that corresponded to its aldehydic proton. The assignment was further confirmed by the coupling constant ($^3J_{\text{HH}} = 7.4 \text{ Hz}$) between vicinal protons H1 (6.01 ppm) and H2 (7.11 ppm) that is diagnostic of an anti-Karplus type arrangement of protons ($^3J_{\text{HH expected}} < 11 \text{ Hz}$) found in **2** and missing in **3a**. Partial degradation of **2** occurred within the time (1 h) required for NMR characterization, as indicated by a decreasing signal at its maximal absorbance of 368 nm. The degradation products likely contributed the small signals in the ¹H NMR spectrum that remain unannotated.

The formation of **2** catalyzed independently by Orf12 and SibV was consistent with the turnover of other 2,3-extradiol dioxygenases that cleave catechols to form acyclic 2,3-

secocatechols.⁴⁶ For **2**, reaction continues as its nucleophilic amine condenses with the proximal aldehyde and subsequently undergoes dehydration to form **3a**, to yield the dihydropyrrole moiety (Scheme 4). This cyclization occurs spontaneously since its rate is independent of enzyme concentration in the LmbB1-dependent production of **2**.⁴³ Whether downstream biosynthetic enzyme(s) prefer the acyclic compound **2** or cyclic compound **3a** as substrates is not yet known.

The lifetime of **2** at pH 8 (250 mM sodium phosphate) is unusually short for further processing by subsequent biosynthetic enzymes. Exposure to a basic environment slows the cyclization of **2**⁴³ suggesting that dioxygenation reactions performed at pH > 8 may shift the equilibrium from **3a** to **2**. To assess the effects of increasing basicity on the equilibrium between **2** and **3a**, the dioxygenation reaction was performed over a range of pH conditions (Figures 1, S3 and S8). Not surprisingly, **2** was too transient to be observed under neutral (pH 7) or slightly acidic conditions (pH 6). However, **2** persisted longer when it formed at pH 9 (A_{max} at 378 nm disappeared within 80 sec) compared to when it formed at pH 8 (A_{max} at 378 nm disappeared within 40 sec). However, the transiency of **2** even at pH 9 suggests that **3a** is the only candidate that would persist long enough for further processing to assemble the PBD dihydropyrrole moiety.

2.5 Lifetime of 3a

The lability and non-enzymatic degradation of **3a** was apparent from the decrease of its absorbance at 414 nm over hours (Figure S9). At pH 8.0, its half-life was approximately 36 h at 25 °C and 25 h at 37 °C. A ¹H NMR spectrum of the resulting degradation showed a new downfield singlet (8.91 ppm) corresponding to an aldehydic proton but there were also at least another 16 new signals that could not be definitively assigned to any mixture of aldehyde containing isomers of **3a** (**4**, **5**, or **6**). The instability of **3a** may also arise from polymerization via nucleophilic addition at the α , β -unsaturated γ -keto acid. The observed lability of **3a** suggests that its consumption by the next enzyme in the biosynthetic path must be rapid *in vivo*. This property is important to consider when manipulating this pathway in the future for engineering new PBD derivatives.

3. Conclusion

The dioxygenases associated with biosynthesis of PBDs catalyze a cleavage of the aromatic ring in **1** to form **2**. This in turn spontaneously cyclizes to **3a**. Together, these processes are crucial for generating the scaffold of the dihydropyrrole moiety common to all PBDs. The oligomeric state and enzymatic product among the dioxygenases Orf12, SibV, LmbB1 and HrmF are conserved in functionally distinct natural products (PBDs as well as lincomycin A and hormaomycin). These observations suggest a likely commonality in all dihydropyrrole and pyrrolidine biosynthetic pathways originating from L-tyrosine. Further studies will examine how **3a** is incorporated into the PBD.

4. Experimental procedures

4.1 Materials

The pET28b plasmid and BL21(DE3) strain were obtained from Novagen (Darmstadt, Germany). The pSMT3 plasmid and Ulp1 expression vector⁴⁷ were generously donated by Dr. C. Lima at the Sloan Kettering Institute (New York, NY). Electrocompetent GeneHogs strain of *Escherichia coli* (*E. coli*) was obtained from Invitrogen (Carlsbad, CA). Ni-NTA agarose was obtained from Qiagen (Valencia, CA). Sephacryl S-200 HR and a gel filtration calibration kit containing low molecular weight standards were obtained from GE Healthcare (Piscataway, NJ). An Econo-Pac 10DG column prepacked with Bio-Gel P-6DG gel was obtained from Bio-Rad (Hercules, CA). **1** was obtained from Sigma (St. Louis, MO). H₂O was purified to a resistivity of 18.2 MΩ-cm. All other reagents were purchased at the highest grade commercially available and used without further purification.

4.2 General methods

Protein purification was performed using an ÄKTA Prime Liquid Chromatography from GE Healthcare. Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) made with 12% resolving and 4% stacking acrylamide layers. Protein concentrations were calculated using extinction coefficients (ϵ) at 280 nm predicted by ExPASy ProtParam.⁴⁸ All UV-visible absorption spectra were obtained using a Varian UV-vis Cary 100 Spectrophotometer (Walnut Creek, CA). ¹H NMR experiments were performed on a Bruker 500 MHz with H₂O suppression using a presaturation pulse sequence and COSY experiments were performed on a Bruker 400 MHz with H₂O suppression.⁴⁹ Mass spectra were obtained in the ESI⁺ mode using a Waters Acquity UPLC-Xevo-G2-aTof-MS (Milford, MA) equipped with a 2.1 x 50 mm BEH-C18 column (1.7 μ m and 300 Å pore size) using a 0–80% aq. acetonitrile gradient over 12 min (0.3 mL/min). Quaternary structure was characterized with a gel filtration column (Superdex 200 HR 10/30) provided by the Kelman Lab at University of Maryland Biotechnology Institute (UMBI) (Rockville, MD). Dihedral angles were predicted by Chem3D Pro 13.0.2 from PerkinElmer (Waltham, MA). All assays were performed at 25 °C unless stated otherwise.

4.3 Cloning of dioxygenase genes

The consensus sequence signature (accession number PDOC00078) was used to identify putative extradiol dioxygenases in PBD biosynthesis. The genes encoding Orf12 and SibV were amplified from the pANT-1³⁰ and pSuperSib1²⁷ cosmids, respectively, using the appropriate primers (Table S1). Each PCR product was gel purified and digested with restriction enzymes (Table S1). These were ligated with the appropriate linearized plasmid to generate pET28b/*orf12* containing a His₆ encoding tag on the 5' side of the gene and pETDuet-1/*sibV*. The *sibV* gene was subcloned from pETDuet-1/*sibV* into the pSMT3 vector using BamHI and HindIII restriction sites. This generated pSMT3/*sibV* containing a His₆-SUMO encoding tag on the 5' side of the gene. All constructs were confirmed by sequencing at UMBI.

4.4 Expression of apo-dioxygenases

BL21(DE3) starter cultures, transformed with the appropriate plasmid, were diluted 200-fold in Miller LB broth supplemented with kanamycin (50 µg/mL) and grown at 37 °C with agitation until the OD_{600 nm} reached 0.9. The cells were then induced with D-lactose (0.2%) at 30 °C with agitation (12 h), harvested by centrifugation at 5,000 rpm at 4 °C (15 min) and flash frozen in N₂ (l) for storage at –80 °C.

4.5 Purification of apo-dioxygenases

All purification steps were performed at 4 °C. Cells alternatively expressing His₆-Orf12 or His₆-SUMO-SibV were resuspended in buffer A (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 1 mM fresh dithiothreitol (DTT) and 20 mM imidazole). Protease inhibitors benzamidine (1 mM) and phenylmethylsulfonyl (PMSF) (1 mM) were added and then cells were lysed by four passages through a French Press at 1000 psi. Cell debris was removed by centrifugation at 15,000 rpm (30 min) and the supernatant was loaded onto a Ni-NTA agarose (11 mL) column pre-equilibrated with buffer A. The resin was washed with buffer A and the dioxygenase was eluted with buffer B (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 1 mM fresh DTT and 250 mM imidazole) at 1 mL/min. The SibV fusion underwent additional steps to remove the SUMO tag by proteolysis using Ulp1 (1:100 w/w). The digestion was dialyzed (2 x 1 L) (12–14 kDa MWCO) against buffer A, reapplied to the Ni-NTA agarose column pre-equilibrated with buffer A and eluted with buffer A. His₆-Orf12 and SibV were then separately dialyzed (2 x 1 L) against buffer C (50 mM sodium phosphate pH 8.0, 10% glycerol and 1 mM fresh DTT), concentrated in a pressurized stirred cell (EMD Millipore) under N₂ (g) (40 bars) with a membrane (10,000 NMWL) to a volume of 600 µL, and applied onto a Sephacryl S-200 HR column (240 mL) pre-equilibrated with buffer C. The dioxygenases were then eluted with buffer C at 0.4 mL/min. The resulting *apo*-dioxygenases were 95% pure as evident by ImageQuant analysis after their separation by denaturing gel electrophoresis and staining with Coomassie Brilliant Blue. The enzymes were flash frozen in liquid droplets with N₂ (l) and stored at –80 °C until needed.

4.6 Reconstitution of apo-dioxygenases with Fe²⁺

Both *apo*-dioxygenases (3.2 mM) were each reconstituted in buffer C with FeSO₄·7H₂O (2.5 mM) in the presence of DTT (15.6 mM) and L-ascorbic acid (sodium salt, 15.6 mM) for 25 min at 4 °C producing red solutions. The reconstituting agents were removed via buffer exchange into buffer D (100 mM sodium phosphate pH 8.0) with a P-6DG desalting column (10 mL) pre-equilibrated with buffer D. The resulting light blue *holo*-dioxygenases were flash frozen in liquid droplets with N₂ (l) and stored at –80 °C. *Holo*-dioxygenases used for assays were subjected to a maximum of one freeze and thaw cycle.

4.7 Determination of the oligomeric state of SibV

Ribonuclease A (13.7 kDa), ovalbumin (44.0 kDa), conalbumin (75.0 kDa) and aldolase (158.0 kDa) were used as molecular weight standards and blue dextran (2,000.0 kDa) was used to determine the void volume (V_o). *Holo*-SibV and the molecular weight standards (20 µg) were individually dissolved in buffer E (50 mM sodium phosphate pH 8.0, 150 mM NaCl, and 1 mM fresh DTT) and loaded onto the Superdex-200 resin column that had been

pre-equilibrated with buffer E. Each sample was eluted with buffer E at 0.4 mL/min at 4 °C. The partition coefficient (K_{av}) was calculated by Equation 1 using elution volume (V_e), V_o , and packed resin volume (V_c). A calibration curve was generated by plotting K_{av} against the log of the molecular weight (M_R).

$$K_{av} = (V_e - V_o) / (V_c - V_o) \quad (\text{Eq. 1})$$

4.8 Generation and purification of the product **3a** formed by Orf12

Holo-Orf12 (0.4 mM) was added in aliquots (44) every 30 sec to a solution of **1** (17.7 mM) in sodium phosphate (30 mM, pH 8.0). The enzyme was precipitated by washing the reaction mixture with chloroform (3 x 3 mL). Residual chloroform was removed from the aqueous layer under reduced pressure for 20 min to yield a yellow solution. **3a**: ^1H NMR (10% D_2O , 500 MHz) δ 7.59 ppm (s, 1H), 7.58 ppm (d, J = 16 Hz, 1 H), 5.52 ppm (d, J = 16 Hz, 0.5 H), 4.59 ppm (dd, J = 6.2, 12 Hz, 0.8 H), 3.11 ppm (dd, J = 12, 16 Hz, 1 H), 2.72 ppm (dd, J = 6.2, 16 Hz, 1 H). ESI⁺-MS: m/z 212.06 (M)⁺, 166.05, 148.04.

4.9 Acid catalyzed hydrolysis of **3a** to form **2**

A solution of **3a** (13 mM) in sodium phosphate (20 mM, pH 8.0) was spiked with deuterium chloride (DCl) (1.6% v/v) to form **2**. ^1H NMR (10% D_2O , 400 MHz) δ 9.08 ppm (s, 1H), 7.11 ppm (d, J = 7.4 Hz, 1 H), 6.01 ppm (d, J = 7.4 Hz, 1 H), 3.44 ppm (m, 2 H), 2.34 ppm (d, J = 15 Hz, 1 H).

4.10 Determination of extinction coefficients for **3a** and **2**

A fixed concentration of **1** (7.6 mM) determined spectrophotometrically ($\epsilon_{280\text{ nm}} = 2.63\text{ mM}^{-1}\text{cm}^{-1}$)⁵⁰ was transformed into **3a** by batchwise addition of *holo*-Orf12 (0.2 mM) described above. ^1H NMR analysis confirmed full conversion to **3a** based on the lack of signals associated with **1**. The absorbance of **3a** at 414 nm was measured at pH 8.0 and Beer's law was used to calculate the $\epsilon_{414\text{ nm}}$ of **3a**. A fixed concentration of **3a** (15 μM) determined spectrophotometrically was then transformed into **2** with acid (a 1000-fold scaled down version of the procedure described above). Disappearance of an absorbance maximum at 414 nm and appearance of an absorbance maximum at 368 nm confirmed full conversion to **2**. The $\epsilon_{368\text{ nm}}$ of **2** was back-calculated at pH 4.0 using the $\epsilon_{414\text{ nm}}$ of **3a** and the absorbance values at those respective wavelengths for both compounds (15 μM). These studies were done in triplicate and the error represented one standard deviation of uncertainty.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

UV-visible spectroscopy of transient ($A_{\text{max}} = 378 \text{ nm}$) and yellow ($A_{\text{max}} = 414 \text{ nm}$) species during dioxygenation of **1** catalyzed by Orf12. The reaction between **1** (1.0 mM) and Orf12 (4.9 μM) was performed in sodium phosphate (250 mM) at pH 8.0. Scans were recorded every 20 sec for a total of 120 sec.

Figure 2. Annotated ¹H NMR (500 MHz, 10% D₂O, H₂O suppression with presaturation) spectrum of **3a** formed by dioxygenation of **1** catalyzed by Orf12. The solvent signal was truncated for clarity.

Scheme 1.

Pyrrolo[1,4]benzodiazepines (PBDs) tomaymycin, anthramycin and sibiromycin include a dihydropyrrole or pyrrolidine moiety shown in black.¹¹

Scheme 2.

Two tautomers, **3a** and **3b** and a side product muscaflavin **4** may form via L-2,3-secodopa **2** that is generated from oxidative cleavage of L-dopa **1**.

Scheme 3.

PBD biosynthesis does not involve a competitive oxidation of **1** to generate a non-productive intermediate, betalamic acid **6**.

Scheme 4.

Orf12 and SibV catalyzed the dioxygenation of **1** to form **2**, a transient species observed only within the first minute of dioxygenation at pH 8 before it cyclized spontaneously to **3a**.