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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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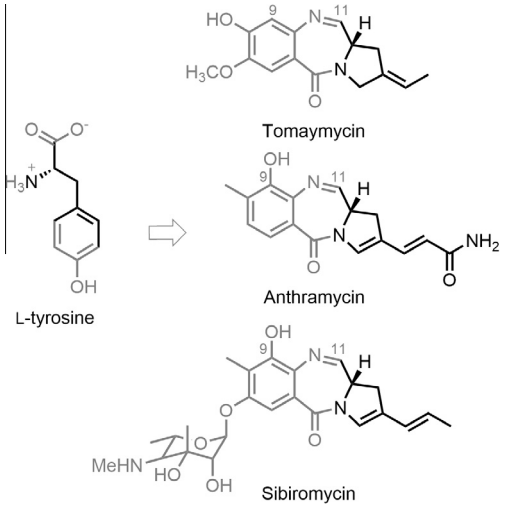
ABSTRACT

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-*seco*dopa (λ_{max} = 368 nm). ^{1}H NMR spectroscopy indicates that L-2,3-*seco*dopa cyclizes into the α-keto acid tautomer of L-4-(2-oxo-3-butenoic-acid)-4,5-dihydropyrrole-2-carboxylic acid (λ_{max} = 414 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 and pyrrolidine biosynthesis within both PBD and non-PBD pathways.

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 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 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



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

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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 desired[22] 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.

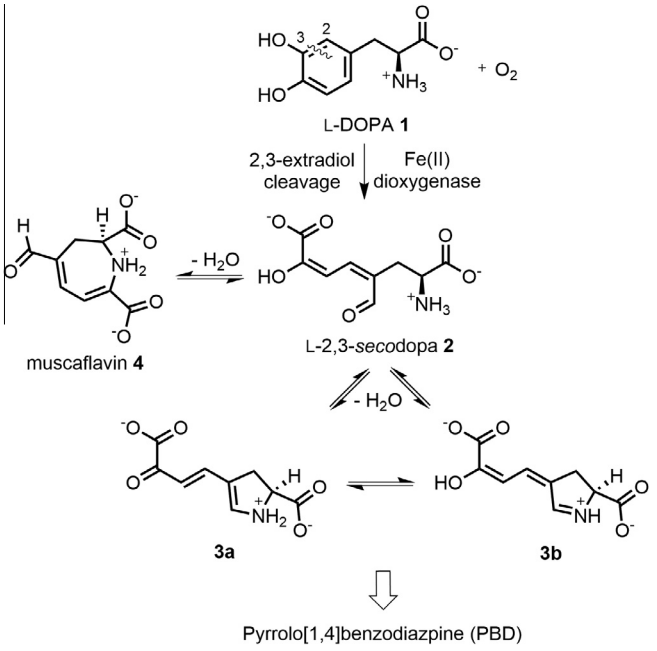
Previous feeding studies first suggested that the dihydropyrrole moiety originates from L-tyrosine,[11] but few biochemical details of the intervening transformations are available. A comparison of the gene clusters responsible for producing the PBDs anthramycin,[20] sibiromycin,[27] tomaymycin,[28] and porothramycin[29] allowed a tentative assignment of gene function and revealed a 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 hormaomycin[34] 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 HrmF[34] 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 tomaymycin[28] biosyntheses 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-*seco*dopa **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 His_6 and His_6-SUMO tag, respectively. Each was then expressed in BL21 (DE3) *Escherichia coli* and purified via Ni-NTA chromatography. The tag of SibV was removed by a SUMO-specific protease (Ulp1)



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

leaving behind a single non-native N-terminal serine. The dioxygenases were finally purified by size exclusion chromatography to homogeneity and reconstituted with Fe^{2+} to yield light blue *holo*-dioxygenases. The final purification step provided ca. 150 mg His_6-Orf12 (Orf12) and 10 mg SibV per liter of growth media (Fig. S1). Since His_6 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) (Fig. S2). Gel filtration was used to determine that LmbB1 is also a dimer in solution[36] 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) (Fig. 1). SibV transformed **1** to the same transient and final products as evident from equivalent changes in UV–visible absorbance (Fig. 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 A[37] and hormaomycin[34] indicating that this transformation is not limited to PBD biosynthesis but general to pyrrolidine biosynthesis of many natural products.

2.3. ^{1}H 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 ^{1}H 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