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A Latent Oxazoline Electrophile for N−O−C Bond Formation in Pseudomonine Biosynthesis

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Nitrogen−heteroatom bonds figure prominently in the structural, chemical, and functional diversity of natural products. For example, homolytic cleavage of the weak N−O bond of mitomycin relative FR900482 triggers formation of the active mitosene antitumor agent,[1] and protein tyrosine phosphatases generate a transient N−S linkage by capture of the sulphenic acid present in the inactive form of the enzyme.[2] A different class of N−X bonds, the N−O-containing hydroxamates, is featured in small molecule ligands excreted by bacteria to scavenge ferric iron.[3] In the case of *Pseudomonas* metabolite pseudomonine,[4] however, the hydroxamate moiety is found uncommonly configured in an isoxazolidinone ring.[5] While less prevalent than uncyclized hydroxamates, these cyclic N−O variants have surfaced in several naturally occurring metabolites including well-known tuberculosis antibiotic D-cycloserine[6] and β-lactamase inhibitor lactivicin[7] (Figure 1).

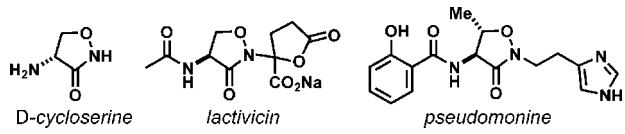


Figure 1. Naturally occurring isoxazolidinones include antibiotics D-cycloserine and lactivicin and *Pseudomonas* siderophore pseudomonine.

Flavin monooxygenases are often implicated in the formation of the ubiquitous N−O bond,[8,9] yet the pathway of hydroxylamine incorporation into the isoxazolidinone has not been delineated. Through in vitro reconstitution of the pseudomonine synthetase, we have uncovered a previously uncharacterized mode of heterocyclization that involves an oxazoline electrophile for N−O−C bond formation. Our analysis suggests an S_N2 mechanism and establishes the oxygen of a hydroxamate intermediate as the nucleophile in this instance of isoxazolidinone biosynthesis.

Pseudomonine is a blue-fluorescent siderophore first isolated from the surface of the Lake Victoria Nile perch.[4] The biosynthetic genes associated with production have been identified in the genome of *Pseudomonas fluorescens*[10] and have been putatively assigned by analogy in *Pseudomonas entomophila*.[11] As illustrated in Figure 2a, five core proteins are responsible for the functionalization of L-His and subsequent ligation with Thr and salicylic acid (SA) to generate the pseudomonine scaffold. Comparison of the pseudomonine non-ribosomal peptide synthetase (NRPS) with those of related siderophores vibriobactin[12] and acinetobactin[13] reveals similar domain organization (Figure 2b) yet does not account for the observed structural differences in the heterocyclic products. In particular, acinetobactin and pseudomonine are nearly constitutional isomers with variation consisting only of the second hydroxyl substituent of the aromatic acid. In the well-studied vibriobactin system, the cyclization domains, as condensation subtypes,[14] have been shown to act in tandem: Cy2 of VibF promotes formation of

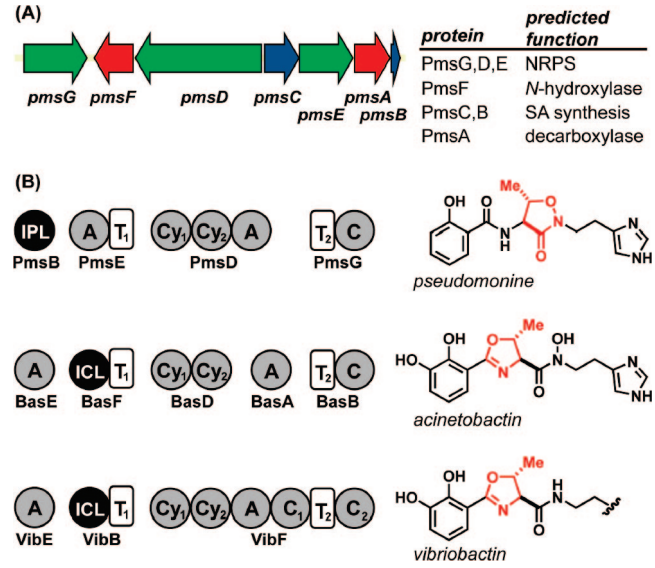
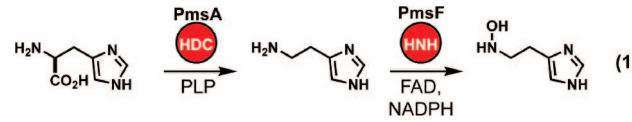


Figure 2. (a) The 21-kb pseudomonine biosynthetic gene cluster and predicted function of encoded proteins. (b) Variations in NRPS domain organization among related heterocyclic siderophores. Pseudomonine is a close structural relative of oxazoline acinetobactin. IPL = isochorismate pyruvate lyase; ICL = isochorismate lyase; A = adenylation, T = thiolation, Cy = cyclization, and C = condensation domain.



a salicyl amide, which then becomes the site of Cy1-catalyzed cyclization of the Thr-hydroxyl side chain and dehydration to deliver the oxazoline.[12] The pseudomonine synthetase appears to be a relative of VibF, albeit severed into two proteins. PmsD harbors an analogous set of cyclization domains, while alignment with VibF indicates remnants of a condensation (C1) domain at the C-terminus of PmsD and N-terminus of PmsG.[15] Despite this resemblance, we anticipated that in vitro analysis of the pseudomonine assembly line would elucidate a pathway for isoxazolidinone biosynthesis distinct from that for the formation of oxazoline-containing siderophores.

The five requisite genes for pseudomonine biosynthesis *pmsADEGF* were amplified from genomic DNA isolated from *P. entomophila* and separately expressed in *E. coli* to provide the derived C- or N-terminal His_6-fusion constructs. Annotation of PmsA and PmsF suggested these proteins are involved in provision of *N*-hydroxyhistamine, which could serve as a nucleophile in the release of a PmsG-tethered acyl product (eq 1). Although PmsA was isolated as the apoprotein, PLP-bound enzyme was generated upon titration with the cofactor as indicated by the characteristic

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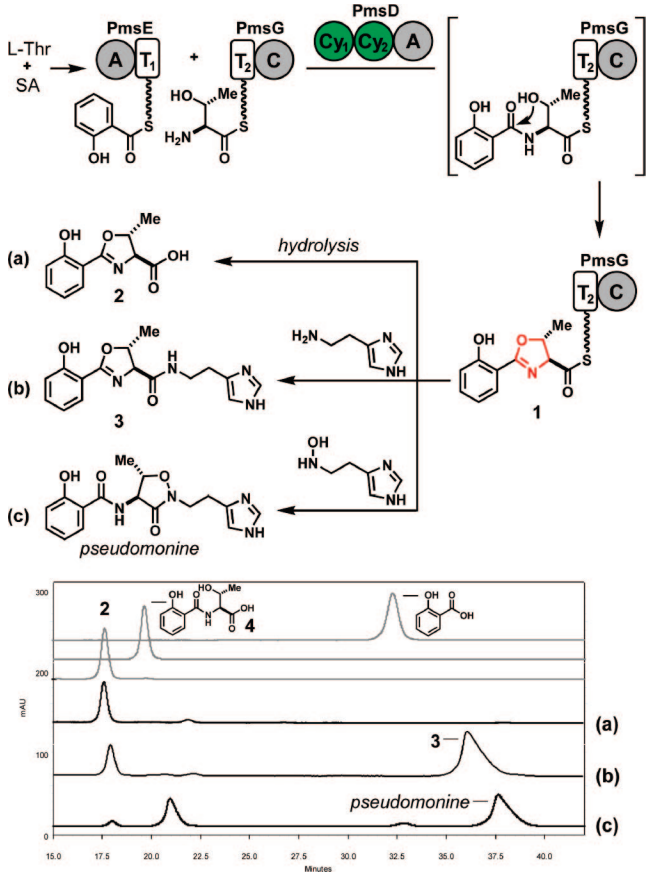
Scheme 1. Early Condensation Events in Pseudomonine Biosynthesis and Evidence for Oxazoline Intermediate **1**[a]



[a] (a) Hydrolysis of thioester **1** and identification of oxazoline **2** by HPLC. (b) Capture of **1** with histamine yields **3** (m/z = 314.70). (c) In the presence of synthetic *N*-hydroxyhistamine, PmsE, G, and D convert SA and L-Thr to pseudomonine (*m*/*z* = 331.14).

UV absorbance of the Schiff base at 416 nm.[16] Decarboxylase activity of PmsA in the presence of L-His was subsequently confirmed by HPLC analysis. PmsF was characterized as a two-component flavoprotein as indicated by NADPH oxidation in the presence of FAD and histamine; catalytic turnover could also be observed by the FAD and NADPH-dependent consumption of histamine by HPLC. These studies corroborate the proposal of *N*-hydroxyhistamine as a somewhat unstable yet soluble intermediate in pseudomonine biosynthesis. Elucidation of the pathway for incorporation of this amine into the pseudomonine isoxazolidinone necessitated the functional examination of NRPS components PmsE, G, and D (Scheme 1).

On the basis of genetic analysis, we expected that N-acylation of a Thr residue with an aromatic acid derivative is a common starting point in the biosynthesis of the pseudomonine, acinetobactin, and vibriobactin. In vitro confirmation of this assumption required the posttranslational modification of the carrier T domains of PmsE and G with phosphopantetheinyl substrate attachment sites, accomplished by incubation of each with CoA and Sfp, a promiscuous phosphopantetheinyl transferase from *B. subtillus*.[17] An ATP−^{32}PPi exchange assay established that the A domains of PmsE and PmsD are each active for substrate adenylation. We propose that, unlike the A domain of PmsE, that of PmsD functions in trans to generate substrate-bound PmsG. Although the embedded Thr moiety of pseudomonine is thought to be of the *allo*-configuration,[4] we discovered that that PmsD activates proteinogenic L-Thr preferentially over L-*allo*-Thr or D-Thr.

Our assessment of amide-bond formation catalyzed by PmsD was carried out as indicated in Scheme 1; reaction analysis was simplified by the fact that the thioester of the PmsG-tethered condensation product undergoes adventitious hydrolysis, thus allowing for catalytic turnover of the synthetase. Unexpectedly, comparison of the released carboxylic acid with authentic synthetic standards[18] by HPLC cleanly identified oxazoline **2** as the hydrolysis product and implicated thioester **1** in PmsD catalysis (Scheme 1, path a). The distinct UV profiles for the salicylate-derived oxazoline **2** and the uncyclized salicyl amide **4** helped to confirm product assignment; this difference was a useful property for the analysis of subsequent reconstitution experiments. The formation of oxazoline **2** reveals that, in analogy to VibF, PmsD is capable of promoting both amide coupling, and subsequent cyclodehydration. It is possible that this mode of heterocyclization is off-pathway; however, our findings strongly suggest that the isoxazolidinone of pseudomonine arises via an unusual mechanism, one which most likely does not directly involve the action of PmsD.

In order to ascertain the intermediacy of oxazoline **1** in pseudomonine biosynthesis, we next tested the condensation activity of PmsG. The C domain of PmsG is proposed to catalyze acylation of a soluble amine monomer with the protein-bound thioester; this event constitutes the final step in chain extension and product release. Condensation assays involved the in situ generation of **1** through the combination of PmsE, G, and D in the presence of L-Thr and SA; the addition of either histamine or synthetic *N*-hydroxyhistamine revealed that oxazoline **1** could be efficiently captured by an amine nucleophile. The C domain of PmsG readily accepts histamine as a substrate (Scheme 1, path b); the resulting product is assigned as the intact oxazoline **3** based on LCMS data and the characteristic UV absorption spectrum.[18]

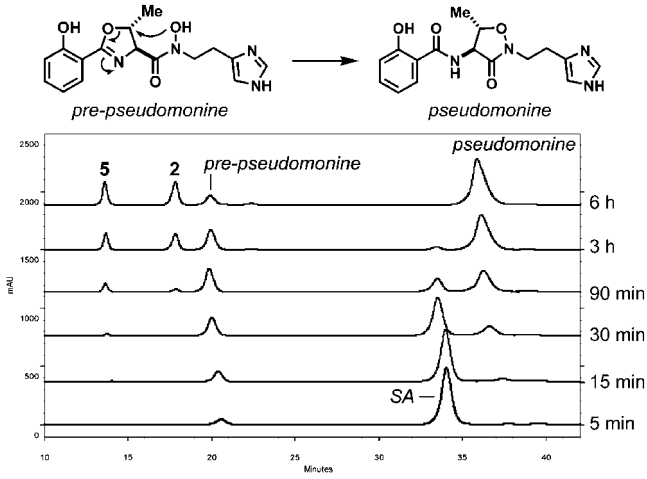
In contrast, the presence of *N*-hydroxyhistamine in the condensation assay results in complete reconstitution of pseudomonine biosynthesis[19] (Scheme 1, path c). The observed assembly line product is identical to pseudomonine isolated from cultures of *P. fluorescens* as determined by HRMS, ^{1}H NMR, and UV absorption data. Pseudomonine is also produced in reconstitution experiments that include the combination of histamine, the *N*-hydroxylase PmsF, and requisite cofactors FAD and NADPH (data not shown). This outcome further supports the assigned function of PmsF and establishes *N*-hydroxyhistamine as the native substrate in pseudomonine biosynthesis.

These data led us to propose that the isoxazolidinone of pseudomonine is accessed through the rearrangement of an initial oxazoline hydroxamate condensation product, named "pre-pseudomonine". As illustrated in Scheme 2, S_N2 attack at the β-carbon of a Thr-derived oxazoline results in the N−O−C bond connectivity of the isoxazolidinone moiety. Close monitoring of the pseudomonine reconstitution assay described in Scheme 1, path c reveals the formation of a transient compound suspected to be pre-pseudomonine (Scheme 2). Although this intermediate has the same mass as pseudomonine (by LCMS analysis), the associated UV absorption spectrum suggests it bears a salicyl-oxazoline.[20]

The proposed intramolecular S_N2 reaction involves an inversion of configuration at the β-carbon of the Thr side chain. This stereochemical outcome accounts for the observation that, whereas L-Thr is activated by the A domain of PmsD, the pseudomonine isoxazolidinone bears a modified L-*allo*-Thr residue with substituents positioned with trans relative stereochemistry about the heterocycle.[21]

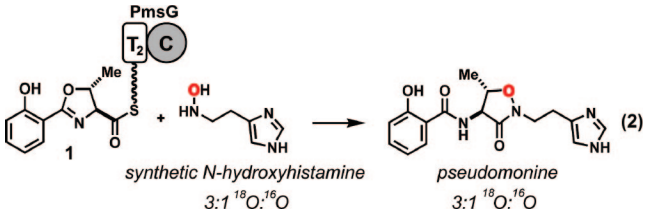
Additional evidence supporting the rearrangement mechanism was garnered from the ^{18}O-labeling study shown in eq 2. The synthetic route developed for access to *N*-hydroxyhistamine could

Scheme 2. Proposed Rearrangement Mechanism for the Formation of the Pseudomonine Isoxazolidinone Involving an Oxazoline Electrophile[a]



[a] A time course of pseudomonine reconstitution reveals the intermediacy of pre-pseudomonine. Compound **5**, eluting at 13 min, is assigned as the salicyl-Thr-*N*-hydroxyhistamine adduct, the relative stereochemistry of which is unknown. Absorbance monitored at 247 nm.

be modified for the incorporation of ^{18}O at the hydroxylamine oxygen. Use of the resulting labeled substrate in reconstitution assays gave the expected pseudomonine product with a comparable level of enrichment as measured by MS analysis. Although an orthogonal set of experiments with SA labeled with ^{18}O at the carboxylate group generates unlabeled pseudomonine, this result was complicated by the partial loss of the SA label under the reaction conditions. Taken together, however, these data indicate that the isoxazolidinone oxygen originates from the *N*-hydroxyhistamine precursor.



The proposed initial product of the pseudomonine synthetase, pre-pseudomonine, is nearly identical to acinetobactin (Figure 2), yet pre-pseudomonine appears to undergo facile conversion to the isoxazolidinone. In this regard, it remains to be seen if both pre-pseudomonine and pseudomonine are physiologic siderophores. Our results suggest that rearrangement is spontaneous; the rate of conversion of isolated pre-pseudomonine, prepared enzymatically, to pseudomonine at pH 7 is not altered by the presence of PmsG. It is not clear how the additional hydroxyl substituent of acinetobactin precludes an analogous transformation, and how pH and temperature influence the reaction. The pre-pseudomonine rearrangement is reminiscent of the postulated mechanism of retaining hexosaminidases, which involves the hydrolysis of a substrate-derived oxazolinium intermediate.[22] However, to the best of our knowledge, an oxazoline electrophile in natural products enzymology has not been described.[23] Our results characterize the hydroxamate oxygen as a nucleophile in isoxazolidinone biosynthesis and may be indicative of a general strategy for the production of this class of N−O−C bonds in nature.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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