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Three Siderophores from One Bacterial Enzymatic Assembly Line

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Iron is required for the survival of bacteria and is growth-limiting at the low concentrations present during vertebrate infections.[1] In response to a low [$Fe^{3+/2+}$] microenvironment, bacteria up-regulate the production of enzymes that synthesize and transport small iron-scavenging molecules called siderophores.[2] These compounds often serve as virulence factors enabling the maintenance of infection for pathogenic bacteria.[3] The key functional attribute of siderophores is high affinity for ferric iron (the predominant form in oxidizing environments); nature utilizes several types of coordinating functionality assembled in small-molecule scaffolds to provide multidentate Fe^{3+} ligation.[2]

A major biosynthetic strategy in pathogenic bacteria is the use of nonribosomal peptide synthetase (NRPS) assembly logic and protein machinery to assemble three types of iron-chelating moieties: phenols and catechols, oxazolines and thiazolines, and *N*-OH amides (hydroxamates). Some siderophores, such as enterobactin,[4] have only catechol functional groups, while desferrioxamine[5] displays an arrangement of hydroxamates as ferric iron ligands. The *Pseudomonas* siderophore pyochelin[6] predominantly reflects the dihydrocyclization strategy (thiazoline). Combinatorial biosynthetic evolution has led to siderophores with mixed chelating functional groups, including catechols and oxazolines in vibriobactin[7] and phenols and thiazolines in yersiniabactin[8] from the bacteria responsible for cholera and plague, respectively. All three types of functional groups (catechol, oxazoline/thiazoline, and hydroxamate) are present in the reported structure of acinetobactin (**1**) from the Gram-negative pathogen *Acinetobacter baumannii*[9] and in anguibactin (**2**) from the fish pathogen *Vibrio anguillarum* (Figure 1).[10] Despite efforts to engineer NRPS pathways, selectivity conferred by adenylation and condensation domains often limits the range of monomers accepted at each stage of assembly.[11]

The biosynthetic gene clusters for acinetobactin and anguibactin have been identified and resemble that of vibriobactin and a structural relative, pseudomonine (**4**).[12a] We recently purified the five-protein pseudomonine biosynthetic assembly line from *Pseudomonas entomophila,* which uses salicylate, threonine, and histamine as building blocks, and we reconstituted in vitro the biosynthesis of isoxazolidinone siderophore **4**.[12b] Notably, a flavoprotein hydroxylase, PmsF, converts histamine to *N*-OH-histamine, which is then used as the chain-terminating nucleophile to capture a hydroxyphenylmethyloxazolinyl acyl group tethered covalently on the pantetheinyl arm of a carrier protein domain of the protein PmsG. This yields a close homologue of **1**, which we termed prepseudomonine (**3**). Under physiologic buffered conditions, **3** rearranges nonenzymatically to **4**, establishing the oxazoline to be a latent electrophile trapped intramolecularly by the hydroxamate oxygen. Detection of the oxazoline as the initial product was facilitated by the use of histamine rather than *N*-OH-histamine as the chain-termination substrate, yielding a vibriobactin-like oxazolinyl amide **8** (rather than oxazolinyl-*N*-OH amide), which cannot be processed by intramolecular rearrangement (Figure 2A).

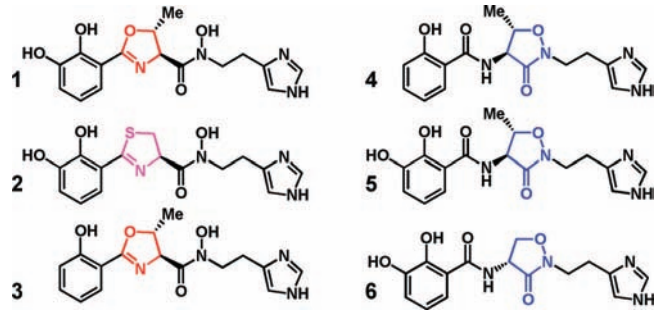

Figure 1. Siderophore natural products contain a variety of iron-chelating functionalities.

Because prepseudomonine **3** and structure **1** for acinetobactin are so similar, we investigated whether the purified pseudomonine assembly line proteins (PmsDEG) would utilize 2,3-dihydroxybenzoate (DHB) in place of 2-hydroxybenzoate (salicylate). Purified PmsE generates 2,3-DHB-AMP and then transfers it covalently to the pantetheinyl arm of its thiolation domain. In turn, PmsD transfers the DHB acyl group to threonyl-*S*-pantetheinyl-PmsG to yield the dihydroxyphenylmethyloxazolinyl-*S*-PmsG covalent acylenzyme intermediate. The C domain of PmsG can then use synthetic *N*-OH-histamine as a nucleophilic substrate and release a siderophore with the correct molecular weight for **1**. However, that molecule rapidly rearranges nonenzymatically to the stable compound **5** over a 60 min time period (Figure 2B).

This phenomenon is similar to what we previously observed for conversion of prepseudomonine to pseudomonine.[12] Indeed, it is likely the same *N*-OH-oxazoline to isoxazolidinone rearrangement occurs in the acinetobactin scaffold. This was validated by ^{1}H NMR analysis of **5**. Comparison of the spectrum of enzymatic **5** with the spectroscopic data reported in the original acinetobactin isolation and structure assignment paper suggests that the structure for acinetobactin is isoxazolidinone **5**.[9] Accordingly, we redefine structure **1** as preacinetobactin. To further evaluate the substrate scope of the *N*-OH-oxazoline to isoxazolidinone siderophore scaffold rearrangement under physiologic conditions, we next tested benzoic acid as a surrogate for salicylate and DHB. Surprisingly, the pseudomonine assembly line produced an initial oxazoline that rearranges to isoxazolidinone **10**. Of particular interest was the relative rate of rearrangement, which we found to increase in direct proportion to the electron density of the aryl moiety (relative rates of rearrangement: **1** > **3** > pre-**10**).

Given the promiscuity of the pseudomonine synthetase assembly line for analogues of 2-hydroxybenzoate and *N*-OH-histamine, we next evaluated whether threonine could be substituted by the amino acids serine and cysteine. Indeed, serine is accepted as a substrate when either salicylate or DHB is the acyl donor and likewise is converted to the stable isoxazolidinone. The serine-derived product **6** was then targeted for synthesis to positively confirm the structural

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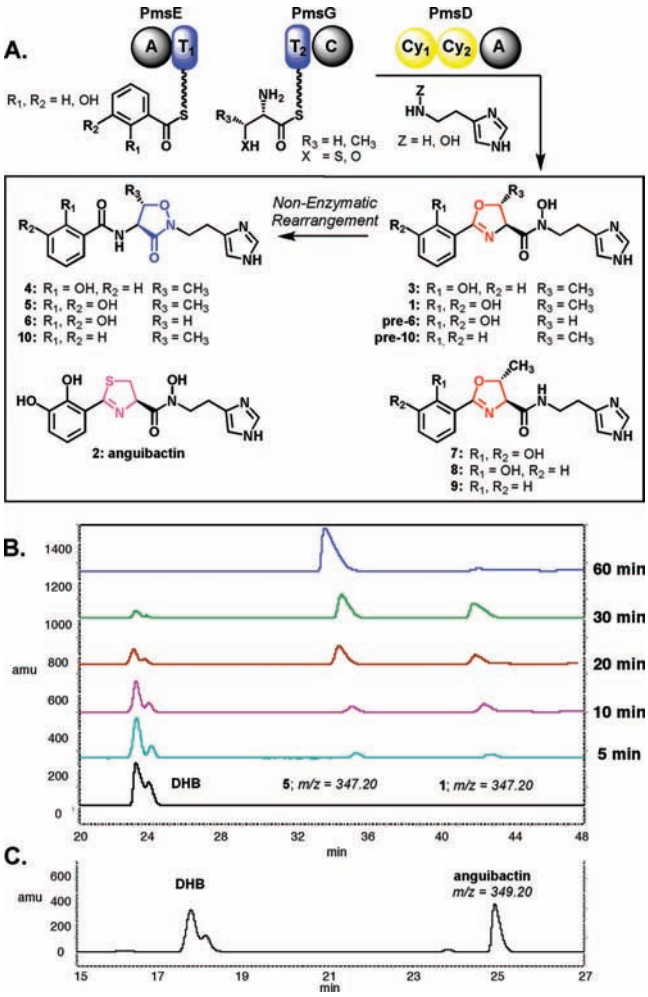

Figure 2. (A) PmsDEG assembly line production of a family of siderophore and "siderophore-like" compounds. (B) Time-course reconstitution assay with PmsDEG to produce acinetobactin (**5**) and preacinetobactin (**1**). (C) Reconstitution assay with PmsDEG to produce anguibactin (*m/z* 349.20).

assignment of the isoxazolidinone scaffold in this desmethyl acinetobactin congener (as cycloserine is commercially available). The synthesis of **6** was completed in four steps and provided material identical to that prepared enzymatically, as indicated by ^{1}H NMR and HPLC analysis (see the Supporting Information).

The use of cysteine as a substrate revealed that the PmsDEG assembly line can make a third known siderophore, anguibactin (**2**) (Figure 2C). In this case, the initial amide bond between DHB and Cys-*S*-PmsG is formed, after which PmsD-mediated cyclization provides the thiazoline intermediate. Acyl transfer to the nitrogen of *N*-OH-histamine mediated by the C domain of PmsG then yields anguibactin. Unlike the nascent oxazolinyl *N*-OH-histamine siderophores that undergo facile intramolecular rearrangement, the thiazolinyl ring of **2** persists in the presence of the hydroxamate group. This may be due to the decreased electrophilicity of C_β of the thiazoline ring relative to that of the oxazoline ring. Propensity toward rearrangement does not appear to depend on the CH_3 substituent at C_β, as both oxazolinyl (from Ser) and methyloxazolinyl (from Thr) rearrange to isoxazolidinones **6** and **5**, respectively.

Nature commonly generates structural diversity by the differential tailoring of a core scaffold through late-stage functional group modification.[13] However, a more unusual source of diversification involves the use of different starting monomers to generate new structures. Through in vitro study of this highly dissociated three-component NRPS, we have unmasked a cryptic promiscuity that enables the synthesis of a family of siderophores and siderophore-like products. The products formed include anguibactin and acinetobactin, which are produced by two pathogens unrelated to *Pseudomonas*: *V. anguillarum* and *A. baumannii*, respectively. The inherent capability of these biosynthetic enzymes to produce different molecules depending on substrate availability points to the evolvability of the pseudomonine synthetase.

This work also reassigns the structure of acinetobactin and further underscores the biological relevance of the *N*-OH-oxazoline to isoxazolidinone internal rearrangement in siderophore diversification. In addition, our study sheds light on functional group compatibility within these densely functionalized natural product small-molecule frameworks. The proximal location of hydroxamate and thiazoline for Fe^{3+} multidentate chelation is stable, presumably because of the electronic difference between S and O in lowering the C_β electrophilicity in thiazoline frameworks relative to oxazoline ones.

The rearranged isoxazolidinone scaffold in acinetobactin and pseudomonine is predicted to retain sufficient affinity for ferric iron to provide its producers a selective advantage in iron-limited environments. Hydroxamate and oxazoline functional groups are compatible in siderophore scaffolds if they are distal and not prone to intramolecular reaction in a five-membered transition state, as shown by the structure of the *Mycobacterium tuberculosis* siderophore mycobactin.[14] Because acquisition of iron is such a central survival function, the combinatorial evolution and shuffling of pathogenic bacterial siderophore assembly lines reveals how highly functionalized small-molecule chemical manifolds are explored and functionally diversified.

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Supporting Information Available: SI Figures 1−17, experimental procedures, and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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