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Structure Prediction and Synthesis of Pyridine-Based Macrocyclic Peptide Natural Products

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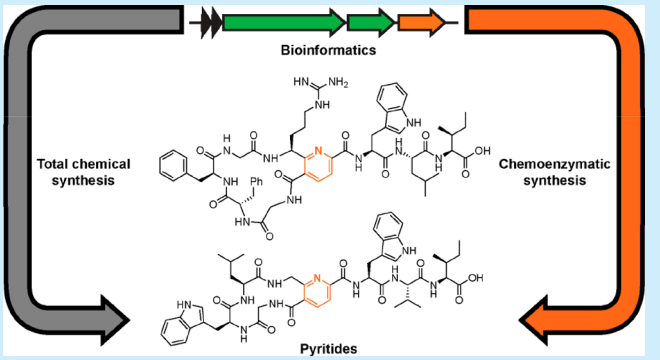
ABSTRACT: The structural and functional characterization of natural products is vastly outpaced by the bioinformatic identification of biosynthetic gene clusters (BGCs) that encode such molecules. Uniting our knowledge of bioinformatics and enzymology to predict and synthetically access natural products is an effective platform for investigating cryptic/silent BGCs. We report the identification, biosynthesis, and total synthesis of a minimalistic class of ribosomally synthesized and post-translationally modified peptides (RiPPs) with the responsible BGCs encoding a subset of enzymes known from thiopeptide biosynthesis. On the basis of the BGC content, these RiPPs were predicted to undergo enzymatic dehydration of serine followed by [4+2]-cycloaddition to produce a trisubstituted, pyridine-based macrocycle. These RiPPs, termed "pyritides", thus contain the same six-membered, nitrogenous heterocycle that defines the thiopeptide RiPP class but lack the ubiquitous thiazole/thiazoline heterocycles, suggesting that thiopeptides should be reclassified as a more elaborate subclass of the pyritides. One pyritide product was obtained using an 11-step synthesis, and the structure verified by an orthogonal chemoenzymatic route using the precursor peptide and cognate pyridine synthase. This work exemplifies complementary bioinformatics, enzymology, and synthesis to characterize a minimalistic yet structurally intriguing scaffold that, unlike most thiopeptides, lacks growth-suppressive activity toward Gram-positive bacteria.



Advances in genome sequencing and analysis have rapidly expanded our knowledge of natural product biosynthetic space.[1,2] This is especially true for the ribosomally synthesized and post-translationally modified peptides (RiPPs), where characterization of new compounds is outpaced by an increasingly inundating number of biosynthetic gene clusters (BGCs).[3−6] Natural product discovery efforts often involve time-consuming and frequently unsuccessful techniques, such as traditional "grind-and-find" or heterologous BGC expression.[7] Given sufficient knowledge of the biosynthetic enzymes, the structure of the final product can be predicted, permitting access to the compound by chemical synthesis.[8−11] RiPP structures are often more readily predicted from genomic information compared to other natural product classes given their direct ribosomal origin.[12,13] Structural predictions can enable directed synthetic efforts, which offer several advantages, including an unparalleled capacity for analogue generation (often with scalability) and obviating the need for tedious isolation from a producing organism.

Recently, we reported a comprehensive genomic landscape survey of the thiopeptides, an extensively modified RiPP class.[14] All thiopeptides feature azole/azoline heterocycles (from Cys, Ser, and Thr), dehydroamino acids (from Ser/Thr), and (dehydro)piperidine/pyridine post-translational modifications (PTMs).[12,14,15] While certain thiopeptides contain a significant number of ancillary modifications, the class-defining PTM is the six-membered N-heterocycle, which arises from an enzyme-catalyzed [4+2]-cycloaddition of two dehydroalanine (Dha) residues and the amide backbone.

We sought to determine if orthologs of the thiomuracin pyridine synthase, TbtD,[15,16] occurred in contexts beyond canonical thiopeptide BGCs. A bioinformatic search yielded several RiPP-like BGCs that lacked an azoline-forming cyclodehydratase while still possessing a split LanB, putatively responsible for Ser glutamylation and elimination, to yield Dha,[17,18] as well as a pyridine synthase (Figure 1A, Figure S1, and Supplementary Data Set 1).[19,20] No other local genes were conserved, suggesting pyridine formation as the sole PTM for these RiPP products. The neighboring precursor peptides also generally lacked Cys, which would be required for thiazole/thiazoline formation, a PTM ubiquitous to all known

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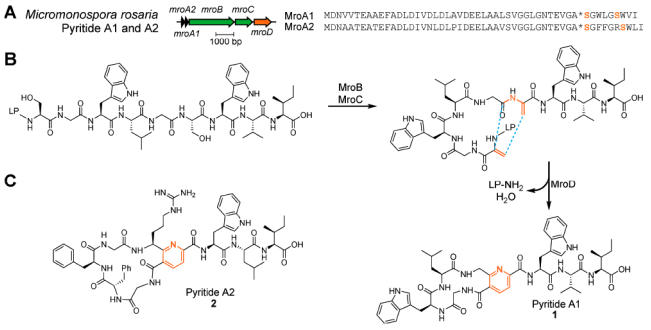


Figure 1. Biosynthesis of pyritides. (A) BGC from *M. rosaria*, encoding two precursor peptides, split LanB dehydratase, and pyridine synthase. (B) Proposed biosynthetic route to pyritide A1 (**1**). (C) Structure of pyritide A2 (**2**). LP, leader peptide.

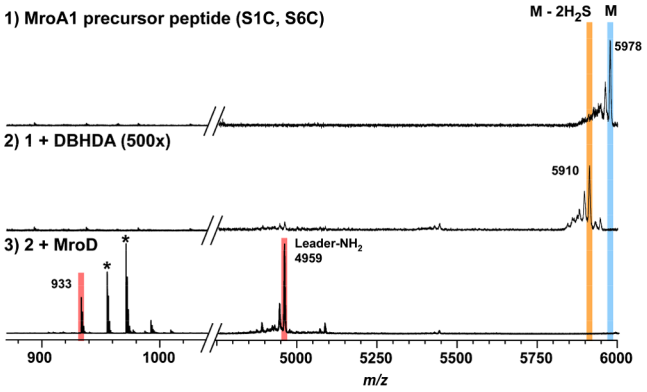


Figure 2. MALDI-TOF mass spectra detailing the chemoenzymatic synthesis of **1**. Compound **4** (m/z 5978) was didehydrothiolated to **5** (m/z 5910) followed by reaction with MroD. The resulting masses correspond to the $[M + H]^+$ ions for **1** (m/z 933) and the eliminated leader peptide carboxyamide (**6**, m/z 4959). Asterisks denote Na^+/K^+ ions.

thiopeptides. Given the lack of sulfur (and thus, thiazole/thiazoline rings), we termed these RiPPs "pyritides".

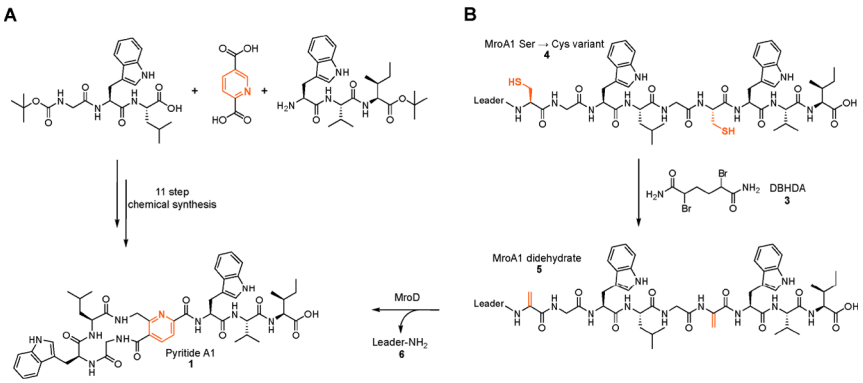
We next tested our genome-guided structural prediction using the pyritide BGC from *Micromonospora rosaria* owing to strain availability. The C-terminal core regions of MroA1 and MroA2 (precursor peptides) contained two Ser residues (Figure 1), both presumably converted to Dha. A subsequent [4+2]-cycloaddition would result in 14- and 17-atom macrocyclic products, hereafter pyritide A1 (**1**) and pyritide A2 (**2**), respectively. To assess the validity of these predictions, we attempted to isolate **1** and **2** from the native producer; however, we were unsuccessful in detecting either pyritide from *M. rosaria*. Instead, an orthogonal, 11-step total synthesis (Figure S2) and chemoenzymatic route were devised to obtain **1** (Scheme 1 and Supplementary Methods).

With respect to the chemoenzymatic approach, we recombinantly expressed and purified the MroA1 precursor peptide and MroD pyridine synthase as fusions to maltose-binding protein (MBP) (Table S1 and Figure S3). To sidestep the tRNA dependency of MroB/C, we carried out a dehydrothiolation reaction using dibromohexanediamide (**3**, DBHDA)[20−23] of an MroA1 variant with Ser1 and Ser6 replaced with Cys (**4**). The resulting didehydrated intermediate (**5**) was detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Figure 2). As the predicted substrate for the pyridine synthase, **5** was then reacted with MroD, yielding masses congruent with the predicted structure of **1** and the leader peptide (LP) bearing a C-terminal carboxamide, **6**.

This chemoenzymatic route was then carried out on a larger scale to produce a quantity of **1** that was sufficient for spectroscopic comparison to the total synthesis product. Approximately 30 L of MBP-MroA1 and 8 L of MBP-MroD culture were used to produce 2.5 mg (post-HPLC yield) of **1**. Several analytical techniques were used to characterize the synthetic and chemoenzymatic samples of **1**, including high-resolution and tandem mass spectrometry (HR-MS/MS), C_{18} reverse-phase HPLC retention time, and ^{1}H NMR (Figures S4−S9 and Tables S2 and S3). Data from all three methods confirmed that the structure of **1** was correctly predicted and the synthetic and chemoenzymatically prepared samples were indistinguishable.

Using the chemoenzymatic route, we obtained an equivalent quantity of pyritide A2 (**2**). The principal difference between **1** and **2** is the presence of an Arg in **2** adjacent to the pyridine. This position is conserved in several other bioinformatically predicted pyritides (Figure S1). Analogous to MroA1, the two Ser residues within the core region of MroA2 were substituted with Cys prior to chemical dehydrothiolation by **3**. This resulted in the production of a mass consistent with the predicted structure of **2**, which was verified by ^{1}H NMR and

Scheme 1. Orthogonal Routes to 1, Including (A) Chemical Synthesis Beginning from Pyridine-2,5-dicarboxylic Acid and Protected Tripeptides (see Figure S2) and (B) Chemoenzymatic Synthesis Using MroA1 Double-Cysteine Variant to Allow DBHDA-Mediated (3) Dehydrothiolation and Subsequent Enzymatic [4+2]-Cycloaddition



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HR-MS/MS (Figures S10−S13 and Tables S2 and S3). The LP byproduct of MroA2 was detected as a C-terminal carboxamide (Figure S14). Purified **1** and **2** were then subjected to a brief panel of microbial growth-suppression assays; however, unlike the well-known activity of thiopeptides toward Gram-positive bacteria,[24] we did not observe any antimicrobial activity for **1** or **2** individually or in combination (Table S4). Further studies are warranted to investigate other potential bioactivities of pyritides.

Our data show that MroD catalyzes the formation of pyridine-based macrocycles that are 14 or 17 atoms in size, mirroring the smallest size reported using enzymes and engineered substrates from the lactazole biosynthetic pathway.[25] MroD is unique among characterized RiPP-related pyridine synthases in that it does not require preinstallation of azole heterocycles prior to yielding a macrocyclic product.[16,26] It should be noted that while a chemoenzymatic route offers convenient access to pyritide analogues, a drawback is that installing Dhb from Thr-containing ribosomal peptides cannot employ a dehydrothiolation route. However, alternate strategies using unnatural amino acids to incorporate Dhb have been developed.[27,28] In addition, total synthesis provides a complementary avenue for producing Dhb-containing pyritide analogues.

Another pitfall of the chemoenzymatic approach is that it assumes one knows the extent of dehydration to a precursor peptide. In the cases of **1** and **2**, the predicted structures were unambiguous as only two Ser residues were present and both were required for pyridine formation. For other pyritide core sequences that contain additional Ser, Thr, or Cys residues, several distinct structures could result, which requires further exploration.

Advances in genomics have led to a renaissance in natural product discovery. Bioinformatic analyses offer a means of prioritizing BGCs predicted to give rise to novel compounds, mitigating the rediscovery of known natural products. This paradigm shift is further enhanced by the unification of biosynthetic enzymology and total synthesis as it provides a dual platform for accessing new molecules. Here, we applied this methodology by identifying an unusual genomic context for RiPP-related pyridine synthases, leading to the structural prediction, chemical synthesis, and enzymatic verification of the pyritides. Given that the only PTM present in pyritide A1 and A2 is a trisubstituted pyridine (formed via an aza-[4+2]-cycloaddition), and the same PTM is class-defining for the thiopeptides, we propose that thiopeptides be reclassified as thiazole/thiazoline-containing pyritides.

We anticipate future investigations into pyritides will yield greater insight into the requisite enzymology and biological activity, which will undoubtedly be augmented by complementary chemical synthesis. Moreover, we predict that the interplay between natural product enzymology and chemical synthesis will become an increasingly useful means of accessing natural products and analogues thereof.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c02699.

Experimental methods and supporting figures (PDF)

Output from the bioinformatics program RODEO detailing the BGC architecture for all identified pyritide BGCs (Supplementary Data Set 1) (ZIP)

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Notes

The authors declare no competing financial interest.

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