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Structure and Biosynthesis of the Antibiotic Bottromycin D

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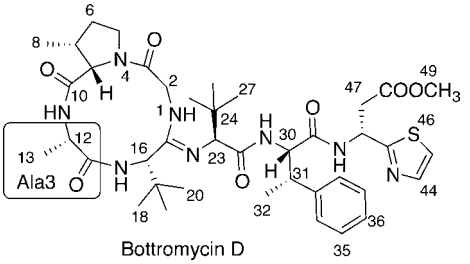
ABSTRACT



Drug resistant infectious diseases are quickly becoming a global health crisis. While *Streptomyces* spp. have been a major source of antibiotics over the past 50 years, efficient methods are needed to identify new antibiotics and greatly improve the rate of discovery. LCMS-based metabolomics were applied to analyze extracts of 50 *Streptomyes* spp. Using this methodology, we discovered bottromycin D and used whole genome sequencing to determine its biosynthesis by a ribosomal pathway.

Drug resistant infectious diseases are leading a global health crisis. For example, one resistant organism, methicillin-resistant *Staphylococcus aureus* (MRSA), causes more deaths in the United States each year than HIV/AIDS, Parkinson's disease, and emphysema combined.[1] Drug resistant infections are now the second leading cause of death worldwide.[1] Historically, a major source of antibiotics has been soil-derived bacteria, primarily from the genus *Streptomyces*, but this genus has been extensively studied for more than 80 years, meaning that the frequency of rediscovery of known compounds is high.

Efficient methods to address this problem could greatly assist with the discovery of new antibiotics. Therefore, we have employed UHPLC/HRMS-based metabolomics for strain selection/dereplication and to efficiently discover novel small molecules from *Streptomyces* and other actinomycetes.[2] Similar methodology has been applied to strains of *Myxococcus* spp. with success.[3,4]

In particular, we have used principal component analysis (PCA) as a metabolomics tool to efficiently, effectively, and statistically analyze LCMS profiles from bacteria to identify those producing novel small molecules. We recently used the methodology to aid in the discovery of the microtermolides isolated from an insect-associated *Streptomyces* sp.[5] Herein, we report the discovery of a new bottromycin A_2 analog, bottromycin D (**1**), using LCMS-PCA to analyze 50 marine ascidian-derived *Streptomyces* spp. as outlined in the Supporting Information (SI). The producing strain WMMB272 was quickly identified as having unique metabolites using LCMS-PCA, leading to the quick discovery of this new antibiotic. Given the

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interest in this class of antibiotics, we subsequently sequenced the genome of WMMB272, discovering a highly novel ribosomal biosynthetic pathway, reported herein.

The structure of bottromycin A_2 was recently confirmed through total synthesis and showed potential as an effective antibiotic against MRSA and VRE.[6] More recently, it was shown that bottromycin A_2 is rapidly hydrolyzed to the carboxylic acid, likely by esterases, rendering it inactive.[7] Semisynthesis to replace the ester with a ketone led to a potent and stable analog with improved pharmacological properties.[7] The new analog showed *in vivo* efficacy in a mouse model of MRSA and was orally active, albeit at a high dose.[7] Therefore, new analogs of this class represent important potential therapeutics to help combat drug resistant infectious disease. Upon the discovery of bottromycin D (**1**) in the current report, we sequenced the genome of the producing organism and identified the biosynthetic pathway, creating an opportunity to improve pharmacokinetics via biosynthetic engineering. Herein, we present the discovery of a new anti-MRSA natural product as well as its biosynthesis.

Strain WMMB272 was isolated from the ascidian, *Didemnum psammathode*, collected in the Florida Keys. Analysis of the 16S rRNA gene sequence revealed that the strain was most closely related to (99% 16S identity) *Streptomyces sulphureus* NRRL B-1627. Using LCMS-PCA on 50 *Streptomyces* spp., two major classes of putative novel natural products were quickly identified (see Figure S1). The first was a rare class of pyridinopyrones discovered independently in our lab just preceding the publication of the structures by Fenical and co-workers.[2,8] Two additional compounds were identified from analysis by PCA: one at t_R 5.7 min and m/z 781.4072 and another at t_R 6.5 min and m/z 795.4228, which we termed bottromycin D (**1**). A query of Antibase indicated that these were new natural products, and subsequent purification of bottromycin D (**1**) confirmed that it was predominantly responsible for the activity against MRSA seen from the WMMB272 extract. On the basis of the accurate mass, the compound with m/z 781.4072 contained one less CH_2 than bottromycin D (**1**), but was only a minor component. The initial isolation of **1** was from a solid agar medium, but our yield was only approximately 10 μg, estimated using NMR. Therefore, we optimized fermentation conditions and greatly improved the yield (to nearly a 10 mg/L isolation yield from liquid media), which facilitated structure determination.

The structure determination was achieved using standard NMR methods but was complicated due to the presence of a 1:1 ratio of conformers in $CDCl_3$. Changing the solvent to 2:1 CD_3CN/DMSO resulted in three major conformers. To rule out the possibility of a diastereomer, a combination of using low temperature NMR and changing the ratio of CD_3CN/DMSO were used to investigate conformer populations. The ratio of the major conformers changed at low temperature in a manner inconsistent with the presence of a diastereomer (see SI).

The planar structure was determined using a combination of COSY and TOCSY to assign the spin systems for each amino acid. Subsequently, ROESY and HMBC spectra were used to confirm the amino acid sequence and the presence of t-Leu. Once the structure was assembled, the difference between all other bottromycin analogs and bottromycin D (**1**) was determined to be the presence of an Ala in **1** instead of a Val in the known analogs. The absolute configurations of most amino acids were determined using the advanced Marfey's method (see SI).[9] Both the Thia-β-Ala and the t-Leu racemized during hydrolysis as would be expected. One of the structures observed in $CDCl_3$ had nearly identical chemical shifts to those previously reported for bottromycin A_2, which indicated that the configuration of the t-Leu and Thia-β-Ala was identical to bottromycin A_2.

In order to assess its antibiotic activity, we determined the MICs for bottromycin D (**1**) while using bottromycin A_2 and vancomycin as standards. To obtain bottromycin A_2, we fermented *Streptomyces bottropensis* NRRL ISP2-5262 from the USDA culture collection. Bottromycin A_2 was isolated after fermentation and purified by HPLC. MIC values for bottromycin A_2 were in agreement with published values of 1 μg/mL while MIC values for bottromycin D (**1**) were 2 μg/mL against methicillin-resistant *Staphylococcus aureus* (MSSA) and MRSA. Perhaps this lower activity is due to the conformational flexibility of bottromycin D (**1**).

As noted above, a known problem in the development of bottromycins as antibiotics is the lability of the side chain ester to hydrolysis *in vivo*, leading to inactive metabolites.

Synthetic approaches have attempted to circumvent this problem, but rational biosynthetic engineering provides a complementary approach. The biosynthesis of these compounds was unknown, but two biosynthetic routes seemed likely. Based upon structural considerations, bottromycin D (**1**) could be synthesized via the nonribosomal thiotemplate mechanism, or it could be part of the growing family of ribosomally synthesized and post-translationally modified peptides (RiPPs).[10] In RiPPs, a precursor peptide is first ribosomally synthesized and then highly tailored to yield the final natural product. In particular, the RiPP route is interesting because simple modification of the codons could potentially lead to libraries of derivatives.

In order to determine the biosynthetic origin of bottromycins, we sequenced the genome of WMMB272 using Illumina HiSeq and performed bioinformatic analysis. BLAST analysis did not yield any candidate nonribosomal pathways. Instead, the precise amino acid sequence encoding bottromycin D (GPAVVFDC) was found within the context of a short precursor peptide, BstA (Figure 1). *bstA*

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Table 1. Genes in Putative Bottromycin D (**1**) Gene Cluster in WMMB272 and *S. scabies*

genes		% identity			
WMMB272	*S. scabies*	amino acid	nucleotide	proposed function	closest homologue (% identity/accession)
bstK	scab_566711	71.8	72.2	MFS-family/H^+-antiporter	MFS transporter [*Microlunatus phosphovorus*] (43/YP_00457499)
bstB	scab_566701	66.7	71.1	*O*-methyltransferase	*O*-methyltransferase domain-containing protein [*Frankia* symbiont of *Datisca glomerta*] (50/YP_004583867)
bstC	scab_56691	76.0	73.0	radical SAM dependent enzyme	Radical SAM domain protein [*Bacillus* sp. 1NLA3E] (33/ZP_09600822)
bstA	scab_56681	81.8	82.6	precursor peptide	
bstD	scab_56671	63.5	70.0	YcaO protein	hypothetical protein yber0001_29470 [*Yersinia bercovieri* ATCC 43970] (21/ZP_04627890)
bstE	scab_56661	73.1	74.4	YcaO protein	hypothetical protein [*Streptosporangium roseum* DSM 43021] (29/YP_003341786)
bstF	scab_56651	77.8	78.2	radical SAM dependent enzyme	radical SAM domain-containing protein [*Salinispora tropica* CNB-440] (38/YP_001159120)
bstG	scab_56641	69.2	74.1	α–β hydrolase	hydrolase [*Rhodococcus equi* ATCC 33707] (39/ZP_08156955)
bstH	scab_56631	65.2	69.5	amidohydrolase	Amidohydrolase [*Alkaliphilus oremlandii* OhILAs] (26/YP_001514298)
bstI	scab_56621	74.8	78.0	cytochrome P450	cytochrome P450 *Thermomonospora curvata* DSM 43183 (37/YP_003300966)
bstJ	scab_56611	74.3	72.4	radical SAM dependent enzyme	Radical SAM domain protein [*Bacillus* sp. 1NLA3E] (35/ZP_09600822)

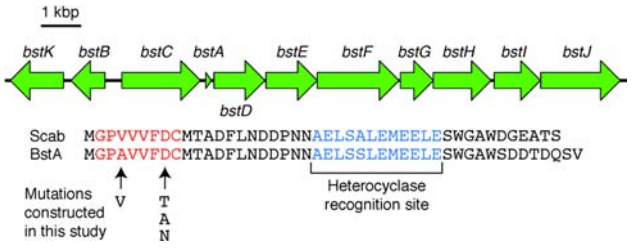

Figure 1. Gene map of the bst cluster in WMMB272 and alignment of the precursor peptide sequence (BstA) with scab_56681. Mutations evaluated in this study are highlighted.

was flanked by genes encoding putative post-translational modifying enzymes. This region was used to search GenBank, leading to the identification of a homologous cluster in the genome of *Streptomyces scabies*.[11] Indeed, the *S. scabies* putative bottromycin cluster contained a precursor peptide that was nearly identical to bottromycin D (**1**), with one major exception: the precursor peptide encoded the sequence GPVVVFDC, indicating an Ala3→Val substitution in comparison to BstA. This sequence is identical to that of the previously described compound, bottromycin A_2.[6] It thus seemed likely that we had correctly identified the bottromycin gene cluster, which we named *bst*.

To further demonstrate that these genes were responsible for bottromycin production, we engineered derivatives of the precursor peptide. *bstA* was cloned into the *E. coli-Streptomyces* shuttle vector, pSET152,[12] to generate pSET-bstA. This sequence was mutated to generate a series of derivatives. In particular, we constructed derivative pSET-bstA1, containing an Ala3→Val substitution. This vector mimicked the natural bottromycin A_2 peptide sequence found in *S. scabies*, but was not found in the wild-type genome of strain WMMB272. Vectors were transformed into strain WMMB272 by conjugation. Fermentation of the pSET-bstA1 mutant led to production of authentic bottromycin A_2, as demonstrated by high-resolution mass spectrometry (Figure S3). By contrast, bottromycin A_2 was not found in fermentation of the wild-type strain, nor was it found in the pSET-bstA mutant strain. These results verify that *bst* encodes the pathway to bottromycin D (**1**). The pSET vector was inserted into a different (ectopic) site on the chromosome, and not into the *bst* pathway. Therefore, all strains produced

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bottromycin D (**1**), even in the case where bottromycin A_2 was also produced.

The genome of WMMB272 as well as PCR and sequence alignments showed that *bst* is highly similar to and syntenic with its complement in *S. scabies* (Table 1). The predicted enzymes in the cluster were ~66−78% identical to those in *S. scabies*, and the predicted precursor peptides were 82% identical in the amino acid sequence. Based upon the identified genes, a rational biosynthetic pathway can be proposed. However, further work is required to confirm that the identified cluster contains all of the necessary genes for biosynthesis. For example, a YcaO-like protein could form the heterocyclic thiazole residue.[13] Several radical SAM proteins are likely involved in β-C-methylation. Previous work has shown that the methyl group in Me-Pro in bottromycin A_2 is derived from SAM.[14] An *O*-methyltransferase likely modifies Asp, while other hydrolases are probably involved in ring formation and cleavage. Interestingly, as found in the telomestatin-like natural product YM-216391, the leader sequence in *bstA* is not a leader at all, but instead follows the core peptide sequence that encodes the natural product.[15] The leader sequence encodes a short helical segment that we previously identified as being important in the interaction with heterocyclase enzymes from the PatD group.[16]

We were interested in ascertaining whether *bst* could be genetically modified to eliminate the ester hydrolysis problem involving Asp7. Therefore, mutants were synthesized in which Asp7 was replaced with Thr, Ala, and Asn. Preliminary experiments using several dozen transformants under various conditions failed to lead to a successful variant at this position. The relatively low yield of the Ala3→Val mutant indicates that this genetic platform likely requires additional optimization for producing bottromycin derivatives in a significant yield, and this work is ongoing. For example, in constructing the vectors to express bottromycin derivatives, we placed the *bstA* derivatives under control of the *ermE* promoter.[17] In one series of these vectors, the Met start site was placed immediately in frame with the normal promoter context. In another, an additional 54 nt from the *bstA* regulatory region was included between *ermE* and *bstA*. Only constructs containing this additional regulatory unit led to products (bstA*), indicating that regulation of precursor expression is limiting in these experiments.

In summary, a new analog of bottromycin, herein named bottromycin D (**1**), was discovered, along with its biosynthetic pathway. To the best of our knowledge, this is the first analog that has an amino acid substitution in the heterocyclic ring. The Val3→Ala substitution appears to make bottromycin D (**1**) more conformationally flexible, perhaps due to decreased steric interactions in the three-dimensional structure, which has recently been determined.[18] Importantly, the activity of bottromycin D (**1**) was on par with other analogs, indicating that some substitutions to the heterocyclic ring may be tolerated. Overall, the rapid discovery of bottromycin D (**1**) demonstrates the power of LCMS-based metabolomics coupled with PCA to identify novel antibiotics and antibiotic analogs, while its production by a RiPP biosynthetic cluster indicates the importance of this biosynthetic route for natural products.

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Supporting Information Available. Experimental details, details surrounding PCR primers used, nucleotide sequences, tables of NMR assignments, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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