Dissecting Bottromycin Biosynthesis Using Comparative Untargeted Metabolomics

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Abstract: Bottromycin A₁ is a structurally unique ribosomally synthesized and post-translationally modified peptide (RiPP) that possesses potent antibacterial activity towards multidrug-resistant bacteria. The structural novelty of bottromycin stems that possesses potent antibacterial activity towards multidrug-resistant bacteria. The structural novelty of bottromycin stems from its unprecedented macrocyclic amide and rare \( \beta \)-methylated amino acid residues. The N-terminus of a precursor

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are natural products that are prevalent throughout nature,[1] and their biosynthetic pathways are capable of transforming simple proteinogenic amino acids into structurally complex compounds that have potent bioactivities.[2–4] However, elucidating the biosynthesis of RiPPs can be hindered by the difficulty of isolating intermediates, as the biosynthesis takes place on a larger precursor peptide, and intermediates may be rapidly proteolyzed. Therefore, improved methods for the identification of RiPP intermediates are desirable. Bottromycin A₁ (1, Scheme 1)[5–8] possesses potent antibacterial activity towards multidrug-resistant bacteria,[9] and is structurally unique owing its unprecedented macrocyclic amide, rare \( \beta \)-methylated amino acids residues, and a terminal thiazole. Nature employs a variety of strategies for peptide macrocyclization,[10–12] but amidine formation has only been observed for bottromycin. Initial studies on bottromycin biosynthesis showed that its amino acids were \( \beta \)-methylated by radical SAM methyltransferases[5,7] (RSMTs), but the rest of the bottromycin pathway represented a biosynthetic black box, where little was known about key steps in the pathway, including the unprecedented macrocyclization. To understand the pathway in detail, an untargeted metabolomic approach that harnesses mass spectral networking was used to assess the metabolomes of a series of pathway mutants. This analysis has yielded key information on the function of a variety of previously uncharacterized biosynthetic enzymes, including a YcaO domain protein and a partner protein that together catalyze the macrocyclization.

Bottromycin A₁ (1) is synthesized and post-translationally modified via a YcaO domain protein and a partner protein that together catalyze the macrocyclization.

Scheme 1. Conversion of BtmD into bottromycin A₁ (1).

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A lack of MS2 fragmentation homology, was an abundant molecule that was not revealed by network analysis, owing to information, Figures S6–S23, Table S3. The only significant analysis of the metabolomes of D, metabolic network (Supporting Information, Figure S5). An analysis of the metabolomes of D, btmE and D, btmI, and D, btmI strains revealed an extensive metabolic network (Supporting Information, Figure S5). An analysis of the metabolomes of D, btmE and D, btmG was used to map molecules produced by these mutants onto this network. Nodes representing species that were not present in D, btmD were manually assessed using MS2 to identify molecules related to the btm pathway. This global metabolomic analysis showed that the bottromycin pathway contributes much more to the total metabolite profile of S. scabies than was previously understood, and identified 14 distinct molecules in the wild-type strain, and at least 6 additional molecules across the mutant strains, with masses and fragmentation patterns that are entirely consistent with bottromycin-like molecules (1–20; Figure 1, Figure 2; Supporting Information, Figures S6–S23, Table S3). The only significant molecule that was not revealed by network analysis, owing to a lack of MS2 fragmentation homology, was an abundant species with m/z 406.27 (17; Figure 2; Supporting Information, Figure S17), which was identified by the initial comparative analysis of LC-MS data. The abundance of various bottromycin-like metabolites in WT S. scabies (Figures 1; Supporting Information, Figure S23) indicates that there are significant bottlenecks in the biosynthetic pathway that preclude the efficient processing of BtmD into bottromycin. Instead, partially processed BtmD can be proteolyzed, and the data show that there are multiple points at which the pathway stalls. The diversity of bottromycin-like molecules produced by the WT could explain why it was difficult in prior studies to identify novel metabolites from mutants.

The macrocyclic amidine of bottromycin is unique in nature, and a plausible biosynthetic route involves the nucleophilic attack of Gly1 onto the amide bond between Val4 and Val5, which could require the activation of the amide carbonyl. YcaO domain proteins activate backbone amide bonds by phosphorylation26,27 or adenylation28 of the carbonyl oxygen, and all YcaO domain proteins with a characterized activity have a partner cyclodehydratase that aids catalysis of cyclization to oxazolines or thiazolines. The bottromycin gene cluster encodes two YcaO domain proteins, BtmE and BtmF, but no cyclodehydratases. Therefore, we hypothesized that one participates in macrocyclization and the other is involved in the formation of the terminal thiazole.

Analysis of the comparative metabolomic and MS2 network datasets revealed two new molecules (m/z 873.45 and m/z 887.47) produced by both D, btmF and the amidohydrolase mutant D, btmI, but not by WT S. scabies. Masses of 873.45 Da and 887.47 Da correspond to the addition of H2O to carboxylated O-desmethylated bottromycins A3 (1) and C3, respectively, which indicated that one of the cyclodehydrations does not occur in D, btmF and D, btmI. MSn revealed that these molecules are not macrocyclized but do feature the thiazoline ring (10 and 11; Figure 3a; Supporting Information, Figure S10), thus indicating that BtmF and BtmI cooperate to catalyze amide ring formation, but are not required for thiazoline formation. Both mutant strains also produced a range of other bottromycin derivatives that contain a thiazoline ring but no macrocycle (Figure 1, Figure 2; Supporting Information, Figure S23). D, btmI did produce trace amounts of macrocyclized 7 and 13, which could reflect inefficient spontaneous cyclization following BtmF-catalyzed amide activation. A cyclization mechanism is proposed (Figure 3b) where BtmF activates the amide bond...
using ATP and BtmI catalyzes cyclization. Further experiments with purified proteins will be needed to verify this, especially in relation to timing of ATP activation. Cyclization is contingent on the removal of the N-terminal methionine, which is usually catalyzed by endogenous aminopeptidases, but these do not function efficiently with an MGP sequence.[30] In vitro analysis of the M17 peptidase[31] BtmM with BtmD demonstrated that BtmM catalyzes this reaction when either Zn$^{2+}$ or Co$^{2+}$ are used as co-factors (Supporting Information, Figures S25 and S26).

In contrast to ΔbtmF, the only abundant species that could be confidently assigned as a BtmD-derived metabolite in ΔbtmE was 17 (Supporting Information, Figure S16), which is a trimethylated tripeptide that is also found in the WT, ΔbtmF, ΔbtmI, and ΔbtmJ strains (Supporting Information, Figure S23). This assignment is consistent with the absence of 17 in ΔbtmD and in the RSMT mutants ΔbtmC and ΔbtmG. Unfortunately, this provided no evidence on Cys8 cyclization; the absence of cysteine-containing peptides could reflect rapid peptide degradation when cyclization does not occur. The lack of any thiazole or thiazoline-containing metabolites does imply that BtmE catalyzes thiazoline formation, although further in vitro characterization is required to confirm this. The absence of macrocyclized metabolites suggests that thiazoline formation is an early step in the pathway. BtmH, the only uncharacterized hydrolytic enzyme in the pathway, is proposed to remove the follower peptide, although it is possible that it could also participate in heterocyclization.

The btm cluster lacks a flavin-dependent dehydrogenase that is required for the biosynthesis of all other thiazole/oxazole-containing RiPPs.[29] Instead, a P450 enzyme, BtmJ, was predicted to catalyze the oxidative decarboxylation of the thiazoline into a thiazole.[5–7] This is an uncommon role for a P450, although it has been reported for thiazole formation in the biosynthesis of the plant alkaloid camalexin[32] and could be mechanistically similar to the fatty acid P450 decarboxylase OleT.[33] Analysis of ΔbtmJ revealed two abundant compounds with m/z 841.43 and 855.44 (Figure 4), which were confirmed to be carboxylated O-desmethyl bottromycins B2 and A2, respectively (12 and 13) using MS2 (Supporting Information, Figure S11). Interestingly, two distinct peaks are observed by LC-MS for both m/z 855.44 and 841.43 (Figure 4a), and each pair of
peptide. However, we cannot rule out the possibility that BtmF and BtmI require a substrate that contains a follower metabolites rather than authentic intermediates, and that suggests that the linear compounds are not exported/imported as effectively as failure of the substrate specificities of BtmJ and BtmB. In contrast, the are true intermediates and supports the proposed roles and porting Information, Figure S27), which implies that D

has a non-proteinogenic reflect a mixture of epimers at the aspartate residue, which is disfavored once the aromatic thiazole is formed (Figure 4 b). This is consistent with previous reports of epimerization of amino acids adjacent to carboxylated thiazolines and we could observe spontaneous interconversion of these peaks at pH 7.5 (Supporting Information, Figure S12).

To further assess whether this proton is exchangeable, we carried out a deuterium labeling experiment. Here, all exchangeable protons were replaced with deuterium in D2O, the thiazoline was then hydrolyzed back to Cys in dilute aq. DCl, and the sample was finally treated with H2O. Theoretically, this would trap a deuterium in the Asp α-position as back exchange would be prevented following loss of the thiazoline. This indeed showed specific incorporation of one deuterium into 13 at Asp7 (Supporting Information, Figures S13 and S14), indicating that this position can readily undergo non-enzymic epimerization.

To investigate whether any of the metabolites reported are authentic pathway intermediates, each mutant strain was co-cultivated with ΔbtmD, which is unable to produce the precursor peptide. Any diffusible molecules produced by mutants that are genuine intermediates should be converted into 1 by the functional enzymes in ΔbtmD. Only the ΔbtmJ + ΔbtmD co-cultivation in the production of 1 (Supporting Information, Figure S27), which implies that 12 and 13 are true intermediates and supports the proposed roles and substrate specificities of BtmJ and BtmB. In contrast, the failure of the ΔbtmF and ΔbtmI co-cultivation experiments suggests that the linear compounds 10 and 11 are shunt metabolites rather than authentic intermediates, and that BtmF and BtmI require a substrate that contains a follower peptide. However, we cannot rule out the possibility that 10/11 are not exported/imported as effectively as 12/13. The lack of an O-methyl group on the α-aspartyl residue in any of the metabolites identified from mutant strains indicates that O-methylation is the last step in the pathway, thereby generating an active antibiotic. This was supported by the in vitro O-methylation of 4 using recombinant BtmB (Supporting Information, Figure S28).

Three RSMTs catalyze four C-methylations in the btm pathway. In S. scabies, bottromycin production is either severely reduced or entirely abolished when either RSMT gene, btmC or btmG, is deleted. BtmG methylates Val4 and Val5, and BtmC methylates Phe6. but it is unclear why the pathway stalls when either step is missed. The metabolic datasets showed that both ΔbtmC and ΔbtmG have highly similar metabolite profiles, and the production of macrocyclized shunt metabolites 6 and 7 indicates that C-methylation is not a prerequisite for cyclization. However, the fully C-methylated metabolites produced by ΔbtmF and ΔbtmI demonstrate that macrocyclization is not a prerequisite for C-methylation either. Also, the production of methylated tripeptides by ΔbtmC and ΔbtmG indicates that the pathway can stall before cyclization when C-methylation is disrupted. The data are consistent with incomplete C-methylation reducing the efficiency of various downstream modification steps.

There has been widespread recent interest in both the biosynthesis and biological activity of bottromycin owing to its unusual structure and potent antimicrobial activity. In this study, we have harnessed untargeted metabolomics to elucidate the biosynthetic pathway to bottromycin.

BtmD MGPDVFDCMTADFLNDDPNAELSAEMEELSWGAWGDEATS

BtmM Methionine hydrolysis

BtmE Thiazoline formation

BtmC BtmG BtmK \(\text{β-methylation}\)

BtmG

BtmK BtmG BtmC

1. \text{BtmF} \text{BtmI} \text{Macrocyclization}

2. \text{BtmH} \text{Follower peptide hydrolysis}

Bottromycin A_2 (1)

Scheme 2. Revised bottromycin pathway.
A1 (Scheme 2; Supporting Information, Table S4). Our analysis identified a wide array of metabolites related to bottromycin, and the untargeted metabolomic data matrix (Supporting Information, Table S5) indicates that there may be further, currently uncharacterized, metabolites produced by this pathway. This study also reveals the first example of YcA domain-catalyzed macrocyclization, which provides the foundation for detailed mechanistic investigations into this step.

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Keywords: biosynthesis · bottromycin · mass spectrometry · natural products · peptides

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Dissecting Bottromycin Biosynthesis Using Comparative Untargeted Metabolomics

A detailed understanding of bottromycin biosynthesis was obtained by harnessing a mass-spectral network (represented by colored nodes in the picture) to analyze a series of pathway mutants. This method provides fresh insights into how this potent antibiotic is assembled, and shows that a YcaO domain protein works with a hydrolase-like protein to catalyze the formation of the macrocyclic amidine.