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***In vitro* biosynthesis and substrate tolerance of the plantazolicin family of natural products**

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Abstract

Plantazolicin (PZN) is a ribosomally synthesized and post-translationally modified peptide (RiPP) natural product that exhibits extraordinarily narrow-spectrum antibacterial activity towards the causative agent of anthrax, *Bacillus anthracis*. During PZN biosynthesis, a cyclodehydratase catalyzes cyclization of cysteine, serine, and threonine residues in the PZN precursor peptide (BamA) to azolines. Subsequently, a dehydrogenase then oxidizes most of these azolines to thiazoles and (methyl)oxazoles. The final biosynthetic steps consist of leader peptide removal and dimethylation of the nascent *N*-terminus. Using heterologously expressed and purified heterocycle synthetase, the BamA peptide was processed *in vitro* concordant with the pattern of post-translational modification found in the naturally occurring compound. Using a suite of BamA-derived peptides, including amino acid substitutions as well as contracted and expanded substrate variants, the substrate tolerance of the heterocycle synthetase was elucidated *in vitro*, and the residues crucial for leader peptide binding were identified. Despite increased promiscuity compared to what was previously observed during heterologous production in *E. coli*, the synthetase retained exquisite selectivity in cyclization of unnatural peptides only at positions which correspond to those cyclized in the natural product. A cleavage site was subsequently introduced to facilitate leader peptide removal, yielding mature PZN variants after enzymatic or chemical dimethylation. In addition, we report the isolation and characterization of two novel PZN-like natural products was predicted from genome sequences but whose production had not yet been observed.

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SUPPORTING INFORMATION

Supporting methods, supplementary figures, and supplementary tables can be found in the Supporting Information. This material is available free of charge at <http://pubs.acs.org>.

INTRODUCTION

Natural products are a rich source of chemically and mechanistically diverse antibiotics (1). Within this broad space, the ribosomally synthesized and post-translationally modified peptide (RiPP) natural products comprise a class garnering increased attention (2). Plantazolicin (PZN) is an anti-*B. anthracis* RiPP produced by *Bacillus velezensis* (formerly *Bacillus amyloliquefaciens*) FZB42 (3–6) and a member of a subclass of RiPPs termed the linear azol(in)e-containing peptides (LAPs) (7, 8). LAPs, thiopeptides, and azol(in)e-containing cyanobactins can also collectively be referred to as thiazole/oxazole-modified microcins (TOMMs) (2). During TOMM biosynthesis, a trimeric heterocycle synthetase (BCD) converts select Cys, Ser, and Thr residues in the C-terminal (core) region of the precursor peptide to thiazole, oxazole, and methyloxazol(in)e moieties (Supplementary Figure 1a) (7). The D-protein (a member of the YcaO superfamily), in partnership with the C-protein, first installs azolines through an ATP-mediated cyclodehydration which proceeds through a phosphorylated hemiorthoamide (9, 10), and the B-protein [flavin mononucleotide (FMN)-dependent dehydrogenase] then oxidizes select azolines to azoles (11). The C-protein serves to bind the N-terminal (leader) region of the precursor peptide, enhancing the rate of processing (12–14). Following heterocyclization, the biosynthesis of PZN is completed by removal of the leader peptide, likely by the locally encoded type II CaaX protease (15, 16), and dimethylation of the new N-terminus by an S-adenosylmethionine (SAM)-dependent methyltransferase (17, 18).

Though originating from a peptide, the structure of mature PZN after post-translational modification is remarkably non-peptide-like (Figure 1a), with two sets of five contiguous heterocycles conferring rigidity and hydrophobicity (4, 5). In addition to the PZN biosynthetic gene cluster (BGC) found in *B. velezensis*, homologous gene clusters have been identified in a number of other bacterial strains (Figure 1b, Supplementary Figure 1) (8), although only *Bacillus pumilus* has been demonstrated to produce PZN (4). The putative precursor peptides associated with these BGCs exhibit a highly conserved core region, which would result in similar groupings of contiguous heterocycles if indeed post-translationally modified like PZN (Figure 1c, Supplementary Figure 1). The unique structure of PZN endows it with an extremely narrow spectrum of activity and a membrane-targeting mode of action unlike other known antibiotics (19). PZN exhibits bactericidal activity against *B. anthracis*, the causative agent of anthrax, with a minimum inhibitory concentration (MIC) of 1–2 µg/mL, while not killing other species, even closely related ones (4, 19). It remains to be seen whether the other potential natural products in the PZN family, if produced, would display similar activities.

The characterization of structural analogs of bioactive compounds can benefit the study of mode of action and aid in the establishment of structure-activity relationships. RiPPs are particularly attractive natural product engineering targets, as their genetically encoded precursor peptides provide an avenue for the facile introduction of diversity via mutagenesis of the coding gene. The RiPP biosynthetic enzymes can then be used to generate variant compounds from the unnatural precursor peptides, provided that the enzymes accept those peptides as substrates. Precursor peptide replacement has been employed to investigate and capitalize on the biosynthetic pathways of lanthipeptides (20–26), lasso peptides (27–29),

and thiopeptides (30–35). The patellamide biosynthetic enzymes are notably permissive toward unnatural substrates, allowing for the generation of many new natural product analogs without the need for intensive total synthesis (36–40).

Previously, we used an *Escherichia coli* expression system to examine the substrate tolerance of the PZN biosynthetic pathway and to generate analogs to explore structure-activity relationships (41). Heterologous production of PZN analogs in *E. coli* prevented the interception of biosynthetic intermediates and also, determination of the order of post-translational events. Nonetheless, the enzymes involved in PZN biosynthesis proved to be extremely selective for their substrates, with the cyclodehydratase in particular appearing incapable of processing any substrates with non-cyclizable residues in positions that were normally cyclized (41). In the present study, we reconstitute the PZN synthetase *in vitro* and use the expanded substrate scope of this cell-free system to more thoroughly probe the biosynthesis of PZN, including the identification of residues crucial for leader peptide binding. Lastly, we demonstrate the production of a new PZN analog from its native producer as well as an additional, predicted PZN via *in vitro* enzymatic synthesis.

RESULTS AND DISCUSSION

In vitro reconstitution of the PZN heterocycle synthetase

In order to determine the activity of the PZN heterocycle synthetase *in vitro*, the precursor peptide (BamA), dehydrogenase (BamB), and two-component cyclodehydratase (BamC, BamD) were each heterologously expressed in *E. coli* as fusions to maltose-binding protein (MBP) and affinity purified (Supplementary Figure 2). Unfortunately, all attempts to express MBP-BamC yielded only degraded protein. A codon-optimized version of the gene for *E. coli* expression (BamC_{opt}, Supporting Methods) was synthesized and heterologously expressed to yield a modest amount of enzyme (~2 mg/L, Supplementary Figure 2), but size-exclusion chromatography indicated that BamC_{opt} did not form an observable complex with BamB and BamD, which could impair the activity of the synthetase (Supplementary Figure 3a). In order to obtain more significant quantities of properly functioning protein, we elected to substitute BamC with a closely related homolog, which is an approach that has been successful for other heterocycle synthetases (42, 43). The C-protein from the PZN BGC in *Bacillus pumilus* (BpumC, accession number WP_041117410; Figure 1b) shares 91% amino acid similarity (63% identity) with BamC (4), and when expressed as an MBP fusion protein in *E. coli*, yielded over 90 mg/L following affinity purification (Supplementary Figure 2). In contrast to BamC_{opt}, BpumC formed a complex with BamB and BamD as assessed by size-exclusion chromatography (Supplementary Figure 3b). Gratifyingly, when these MBP-tagged proteins (BamB, BpumC, and BamD) were combined with MBP-BamA, TEV protease (to remove the MBP tags), and ATP, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) indicated a mass loss consistent with the formation of nine azoles and one azoline (-198 Da) onto the precursor peptide (Figure 2a). Subsequent Fourier transform ion cyclotron resonance tandem mass spectrometry (FT-ICR-MS/MS) was used to localize these modifications, which was identical to the *bona fide* natural product (Supplementary Figure 4). While BamC_{opt} did not appear to form a stable complex with other components of the synthetase (Supplementary Figure 3a), overnight

reactions with BamC_{opt} did form the expected -198 Da product (Supplementary Figure 5). Ultimately, the more robust protein yield and activity of BpumC warranted its usage in all subsequent assays.

Bolstered by our success with *in vitro* reconstitution of the *Bam* heterocycle synthetase, we also set about reconstituting the homologous synthetase from *Corynebacterium urealyticum* (*Cur*, Figure 1b) (4, 8). Size-exclusion chromatography indicated that CurB, CurC, and CurD formed a complex (Supplementary Figure 6), and combination of the MBP-tagged synthetase proteins with ATP, TEV, and MBP-CurA resulted in a mass loss of 200 Da for CurA, consistent with the installation of ten azoles (Figure 2b).

Although some TOMM cyclodehydratase enzymes are capable of activity in the absence of their cognate dehydrogenase, or even in the absence of their cognate C-protein (9, 43), this was not the case for either the *Bam* or *Cur* cyclodehydratases, as the exclusion of any one component of the synthetase from either BGC abolished activity (Supplementary Figure 7). For these synthetases, all three components of the heterocycle synthetase were required for activity, as has been demonstrated for most other studied TOMM clusters, including microcin B17 and streptolysin S (7, 42, 44). Thus, in order to reveal the order of biosynthetic events, the enzymatic activities of the cyclodehydratase and dehydrogenase needed to be disentangled. Towards this goal, we prepared two inactive forms of the dehydrogenase, BamB-Y206A and BamB-R93A. BamB-Y206A contains an alanine substitution at a key active site residue (11, 45), while BamB-R93A no longer co-purified with FMN upon heterologous expression in *E. coli* but still associated with BpumC/BamD by size-exclusion chromatography (Supplementary Figure 3c). When MBP-BamA was treated with BpumC, BamD, and either BamB-R93A or BamB-Y206A, the cyclodehydratase installed at most four azoline heterocycles, which were localized to the four most *N*-terminal positions in the core peptide (Figure 3, Supplementary Figure 8). Subsequent treatment of this species with synthetase containing an active dehydrogenase yielded a fully-modified substrate (nine azoles and one azoline). While the four-azoline species proved to be a competent *in vitro* intermediate, it is not necessarily a natural, on-pathway biosynthetic intermediate. Nevertheless, one of these four azolines is most likely the first to be installed during the normal course of biosynthesis. All attempts to isolate additional intermediates from the *Bam* or *Cur* synthetase reactions were unsuccessful due to the apparent high processivity of the enzymes (Supplementary Figure 9). However, the observation that the cyclodehydratase cannot install all ten azolines in the absence of an active dehydrogenase is strongly suggestive that during the normal reaction course, azole formation begins at the *N*-terminal residues prior to completion of azoline formation at the *C*-terminal residues.

Substrate tolerance of the PZN synthetase *in vitro*

With the PZN heterocycle synthetase reconstituted *in vitro*, we next turned our attention to determining the parameters of substrate tolerance for these enzymes with variant precursor peptides. MBP-tagged precursors with core region substitutions were generated using site-directed mutagenesis and *E. coli* heterologous expression. After treating the panel of purified variant peptides with the heterocycle synthetase *in vitro*, the extent of processing was determined by MALDI-TOF-MS, with subsequent FT-ICR-MS/MS to localize the sites of

post-translational modification (Figure 4, Supplementary Figures 10–36). Overall, the ability of the synthetase to process variant substrates was increased over what was previously observed in *E. coli* (41), presumably due to a greater level of control over the reaction conditions (*e.g.* higher concentrations of reaction components are possible *in vitro*). Nonetheless, many of the variant peptides remained incompletely processed, reaffirming that the PZN cyclodehydratase is remarkably substrate selective.

Initially, we examined the limits of substrate scope for the *Bam* synthetase by creating a series of variant peptides wherein a Cys, Ser, or Thr residue was replaced with Pro, which has been used as a crude steric mimic for an azoline in previous studies (43, 46). While the *Bam* synthetase was able to install heterocycles in the expected positions *N*-terminal to the interrogated Pro residue, neither the residue proximal to Pro, nor any residues following Pro were cyclized (Figure 4a, Supplementary Figures 10–15). Presumably, the conformational restriction imparted on the peptide backbone by the presence of Pro in the +1 position prevented the *Bam* cyclodehydratase from installing a heterocycle at that position, perhaps due to an inability to properly fit the substrate in the active site or some other catalytic deficiency. While such an intolerance for Pro in the +1 position is consistent with previous studies using the cyclodehydratase from *Bacillus* sp. Al Hakam (43), the *Cur* cyclodehydratase, among other TOMM natural products (47, 48), is capable of accepting Pro in the –1 position (Figure 1c, Figure 2b).

Similar *N*-terminal but not *C*-terminal processing was also observed for a number of Ala substitutions, though the cyclodehydratase was able to cyclize residues with Ala in the +1 position (Figure 4b, Supplementary Figures 16–25). In a few cases involving Ala near the middle of the core peptide, the cyclodehydratase was capable of installing an azoline in the next heterocyclizable position after the Ala, but this was always a minor species, perhaps due in part to hydrolysis of the azoline. Dehydrogenation of this azoline to the azole was never observed in the position following Ala, nor was cyclodehydration observed in more than one position following Ala. This abortive trend was also observed in cases where an Ala residue was inserted between two wild-type residues, including between the two central Ile residues in the core peptide (^8A, ^12A; ^ indicates insertion). Together, these results further support the hypothesis that heterocyclization during PZN biosynthesis proceeds in an *N*- to *C*-terminal direction.

The single notable exception to this trend of Ala preventing significant heterocyclization of *C*-terminal residues was in the case of the BamA-T3A substrate, for which the synthetase fully cyclized both Cys2 and Cys4 to thiazoles (Figure 4b, Supplementary Figure 16). Further heterocyclization *C*-terminal to Cys4 was not observed, consistent with the other Ala-substituted precursor peptides. It is possible that the increased nucleophilicity of the Cys side chain relative to that of Ser/Thr, plus the more electrochemically favorable oxidation to thiazole relative to oxazole, were responsible for the ability of the synthetase to overcome the introduction of Ala in the preceding (–1) position. To test this hypothesis, three double substitutions were constructed and assayed for modification in which the Ala residue was followed by Cys in the next position normally heterocyclized: T5A/T6C, T6A/S9C, and S12A/T13C. In each of these cases, the synthetase was able to convert the unnatural Cys to a thiazole, though some Cys remained unprocessed in the S12A/T13C peptide (Figure 4c,

Supplementary Figures 26–28). Together, these results demonstrate that the increased reactivity of Cys, in essence, chemically rescues processing when the activity of the synthetase would normally be halted by Ala. However, when Cys was placed at positions not heterocyclized in the wild-type PZN (*i.e.* I7C and I8C), susceptibility to iodoacetamide labeling demonstrated that these Cys residues remained unmodified, whereas other positions of BamA were processed as in the wild-type peptide (Figure 4c, Supplementary Figure 29). Such exquisite site-selectivity is consistent with that previously observed in *E. coli* (41).

To further examine the effects of disrupting the natural arrangement of heterocyclizable residues, several precursors with contracted core peptides were constructed and assayed for modification by the *Bam* synthetase. Shortening the precursor peptide by one residue in the center of either block of five heterocyclizable residues (C4_Δ, S11_Δ; _Δ indicates deletion) did not significantly disrupt processing, as assessed by the MALDI-TOF-MS endpoint assay, resulting in the maximum of nine heterocycles installed (Figure 4d, Supplementary Figures 30–31). Likewise, truncation of the precursor peptide to remove the *C*-terminal half of the core peptide (I8*; * indicates a stop codon) also led to the installation of five heterocycles (Figure 4d, Supplementary Figure 32). Finally, removal of the *C*-terminal Phe residue (F14*) prevented heterocyclization only at the preceding residue, Thr13 (Figure 4d, Supplementary Figure 33), indicating that the *Bam* synthetase is unable to install a *C*-terminal heterocycle.

In addition to the precursors with one or two substituted residues, three potential substrates with more extensive alterations were also assayed. These chimeric peptides consisted of the leader peptide from BamA and the core peptides from three other BGCs in the PZN family. These peptides were previously assayed in *E. coli*, where no post-translational modifications were observed (41). However, the reaction conditions using purified enzymes now enabled modification of all three chimeric peptides (Figure 4e, Supplementary Figures 34–36). The ability of the synthetase to modify the BamA-CurA and BamA-BlinA chimeras is especially notable, as each contains an internal Pro residue. As previously demonstrated in this work, the presence of a Pro residue in the BamA core peptide prevented heterocyclization in positions *C*-terminal to the Pro (Figure 4a). Yet in the case of BamA-CurA and BamA-BlinA, Pro8 is followed immediately by several Cys residues. We hypothesize that the increased nucleophilicity of the Cys sidechain again may be chemically rescuing an otherwise unprocessed substrate, as was the case for the Ala/Cys double mutants (Figure 4c). The processing of BamA-CurA also demonstrates site-selectivity on the part of the PZN dehydrogenase, as it did not oxidize the penultimate position to the thiazole, whereas CurB oxidized all azolines in CurA to the corresponding azoles (Figure 2b, Supplementary Figure 37).

In addition to the heterocyclizable residues, the other key position in BamA at which substitution was not tolerated by the heterocycle synthetase in *E. coli* was Arg1 (41). Previously, it was unclear whether the intolerance toward even the most conservative substitution (Arg to Lys) at this position was due to selectivity by the cyclodehydratase or prevention of leader peptide cleavage. With the *Bam* synthetase reconstituted *in vitro*, its tolerance for alternative residues at the *N*-terminus of the core peptide could now be assessed in the absence of the leader peptidase. *In vitro*, the R1K construct was accepted by the *Bam* synthetase, though not robustly, as indicated by the presence of a number of

incompletely cyclized species (Supplementary Figure 38a). In addition to the “conservative” R1K substitution, a variety of other alterations to this position were also assayed. The *Bam* synthetase was revealed to be more permissive to small residues (R1G, R1S) than to bulky (R1I, R1F) or negatively-charged (R1D) residues, though only in the case of R1K could the synthetase install the full complement of ten heterocycles (Supplementary Figure 38a). The initial enzymatic rate was also determined for the synthetase in the presence of each of these Arg1 mutants, and those substrates which permitted the installation of more heterocycles also generally had greater initial velocities (Supplementary Figure 38b). The *Bam* synthetase thus appeared to strongly favor Arg1.

In addition to determining the relative rates of processing for the Arg1 variants, several other precursor peptides were kinetically assayed using the *Bam* synthetase (Table 1). The observed V_o values for these substrate variants were relatively unperturbed regardless of the number of heterocycles installed or the substituted position in the core. Such similarity suggested that the first cyclodehydration event (Cys2) is the rate-determining step, and this result offers further support that modification proceeds in an *N*- to *C*-terminal direction. Indeed, many data reported herein would be difficult to reconcile if an alternative processing order was operating. Meanwhile, in contrast to the similar V_o values, the apparent K_m values varied greatly. Although it can be difficult to interpret kinetic parameters for a multistep reaction where substrate binding is primarily dictated at a location distal to the active site (*i.e.* the C-protein engages the leader peptide, *vide infra*) (12), lower apparent K_m values correlated with an increased total number of modifications, such as with the native substrate and T13P (Figure 4a). Such a trend suggests that substrates receiving more modifications interact more productively with the active site, which is expected for any substrate with a lower K_m . The higher apparent K_m for the T3A and T6A variants also suggests that these Thr residues enhance productive interactions with the enzyme, perhaps through the β -methyl substituent on their side chains.

Leader peptide recognition and binding by the PZN synthetase

In order to more fully understand the substrate scope of the heterocycle synthetases, we next investigated the contribution of the leader peptide towards substrate binding and processing. Previous work has shown that CurC, and not CurD, interacts with a CurA leader peptide, exemplifying the previously described role for the C-protein component of the heterocycle synthetase in leader peptide binding via the RiPP recognition element (RRE) domain (12). Building on these previous results, we examined the interaction between an *N*-terminally FITC-labeled BamA leader peptide (FITC-BamA-LP, Supplementary Table 1) and the synthetase proteins by fluorescence polarization (FP). Unexpectedly, BpumC on its own was incapable of avid FITC-BamA-LP binding, with an extrapolated K_d of approximately 100 μ M (Figure 5). In the presence of BamD, the K_d decreased to 20 ± 2 μ M, though BamD alone did not substantially bind the leader peptide (Figure 5), indicating that BpumC was indeed the primary engaging protein, in line with previous results. The surprisingly weak binding by BpumC alone was not a result of the non-cognate nature of the BamA/BpumC interaction, since the binding of FITC-BpumA-LP (the cognate sequence) to BpumC gave comparable results ($K_d \sim 100$ μ M, extrapolated; Figure 5). In light of the observation that the full, trimeric PZN heterocycle synthetase (BamB/BpumC/BamD) was required to modify

BamA (Supplementary Figure 7), we hypothesized that BpumC requires its associated partners for efficient leader peptide binding. To test this, BamB-R93A, which lacks the FMN cofactor, was employed so that the intrinsic fluorescence of the flavin would not interfere in the FP assay. When BamB-R93A, BpumC, and BamD were combined to form the complete heterocycle synthetase, leader peptide binding was considerably enhanced ($K_d = 1 \pm 0.1 \mu\text{M}$, Figure 5). This result is in stark contrast to several previously studied TOMM synthetases, where the C-protein, which contains the ~90 residue RRE, was sufficient for effective peptide binding (12, 46). We hypothesize that some C-proteins must leverage their association with other synthetase components to induce structural rearrangements that potentiate leader peptide binding (49).

To investigate the importance of various precursor peptide regions for substrate binding, we constructed a series of C-terminal truncations (Table 2). The results indicated that not only did the core region not detectably contribute to the overall binding of the peptide, but also that the first 24 residues of the precursor peptide were minimally necessary to maintain wild-type binding affinity. Next, Ala scanning of BamA was performed to identify individual residues of the leader peptide (designated by negative numbers) responsible for interactions with the synthetase. As measured by a competition FP assay (Table 2), BamA and BpumA had nearly equal IC_{50} values for the synthetase, which were also similar to the K_d of their fluorescent derivatives. Overall, most of the single Ala substitutions had only modest effects on the binding of the peptide, although P(-21)A and L(-18)A had a much more substantial effect, which was borne out by kinetic assays (Table 2).

To ensure that these observations were not *in vitro* artifacts, a previously described *E. coli* heterologous production system was used to assess processing of the BamA variants *in vivo* (41). In this assay, the production of the mature PZN-I7V variant (a known, well-tolerated core peptide substitution) from a plasmid-borne copy of the *bamA* gene containing a leader peptide substitution was compared to the production of the wild-type PZN from a fosmid-borne copy, as measured by MALDI-TOF-MS (Table 2; Supplementary Figure 39). Overall, these *in vivo* results corroborated those obtained *in vitro*, indicating that two residues, Pro(-21) and Leu(-18), are primarily responsible for the peptide's interaction with the synthetase in what appears to be a $_{-21}\text{PXXL}_{-18}$ recognition motif. Interestingly, in other precursor peptides within the PZN family, this Pro is highly conserved, whereas the Leu is less so (Supplementary Figure 1c). Furthermore, these results indicate that the conserved residues at the C-terminus of the leader region (FEPXAA), which are likely part of a conserved protease recognition motif, contribute only modestly to the overall peptide affinity.

Select CurA peptides with substitutions at the equivalent positions in the leader peptide were also assayed for binding and processing by the *Cur* synthetase *in vitro* (Table 2). In line with the conserved nature of the position (Supplementary Figure 1c), Pro(-23) in CurA was crucial for binding, but not the neighboring non-conserved Pro(-22). Unsurprisingly, the binding of CurA-N(-26)S, whose leader peptide is identical to that of BlinA (12), was virtually no different from that of wild-type CurA. Taken together with the leader peptide binding experiments using the *Bam* synthetase, these results identify the critical residues that mediate recognition by the PZN heterocycle synthetases.

Beyond being necessary for leader peptide binding, we next sought to determine if these key residues were sufficient for binding by assaying a variety of precursor peptides from the PZN family with non-cognate synthetases. In the FP assay, the *Bam* synthetase exhibited weakened binding to CmsA compared to BamA or BpumA and none to BlinA, while CurC (sufficient for precursor peptide binding) exhibited binding only to the highly similar BlinA and CmsC only to CmsA (Supplementary Table 2). These observations were borne out by a MALDI-TOF-MS processing assay, which demonstrated that the *Bam* synthetase was capable of installing ten heterocycles on either BamA or BpumA, but only six on the less-similar CmsA, and none on either BlinA or CurA (Supplementary Figure 40). Likewise, the *Cur* synthetase installed heterocycles only on CurA or BlinA, and not on BamA, BpumA, or CmsA (Supplementary Figure 40). As the C-protein provides the main contact for binding to the leader peptide, it was feasible that substituting the C-protein in these synthetase complexes could enable binding, and thus processing, of non-cognate precursor peptides. However, these “hybrid synthetases” were not active, likely due in part to the inability to form productive complexes between a C-protein and its non-cognate partners (Supplementary Figure 41). Additional attempts to confer binding of non-cognate substrates through simply swapping the RRE domains of these C-proteins were unsuccessful (data not shown).

These non-cognate experiments demonstrated that relatively few conserved leader peptide residues [*i.e.* Pro(-21) in BamA] are necessary but not sufficient for modification. These results also suggest that synthetase recognition is encoded across the length of the peptide, even though affinity is primarily localized to a few “hot spots” (50). Even though most single substitutions had a minor effect, multiple residue differences in the leader peptide (*e.g.* between BamA and CurA) clearly prevented non-cognate processing, as the *Bam* synthetase was able to fully process non-cognate core peptides when its cognate leader was present (Figure 4e). Interestingly, CmsA does not contain the important Leu(-18) relative to BamA but can still be partially processed by the *Bam* synthetase, likely due to the overall greater similarity between leader peptides for CmsA and BamA, compared to BamA and CurA (Supplementary Figure 40). Overall, the ability of these various synthetases to process non-cognate substrates appears to correlate with evolutionary distance.

Production of novel PZN analogs

Having investigated the substrate tolerance of the *Bam* synthetase, it was of interest whether these variant precursor peptides could be used to generate mature PZN analogs. Following heterocyclization, the next biosynthetic step is removal of the leader peptide, which is putatively performed by the protease encoded in the BGC (*bamE*, Figure 1b) (15, 16). As a predicted type II CaaX protease, BamE is thought to be an integral membrane protein (51); thus, we treated BamA that had been heterocyclized *in vitro* with a crude preparation of *B. velezensis* membranes. Unfortunately, no proteolytic product was observed (data not shown). To facilitate the *in vitro* production of PZN analogs, we introduced a cleavage site for a soluble, commercial reagents, as has been previously successful for the production of other RiPPs (52–55). Using site-directed mutagenesis, Ala(-1) was changed to one of several other residues: Lys (trypsin), Phe (chymotrypsin), Glu (endoprotease GluC), and Met (cyanogen bromide, CNBr). A(-1)M was the best-tolerated substitution in combination with

enzymatic heterocyclization, as determined by MALDI-TOF-MS (Supplementary Figure 42a). Subsequent treatment of the heterocycle-containing peptide with CNBr in the presence of formic acid (56, 57) yielded a species whose mass was consistent with that of hydrolyzed desmethylPZN, indicating cleavage of the leader peptide (Supplementary Figure 42b). Unfortunately, the acidic conditions necessary for the CNBr reaction also hydrolyzed the methyloxazoline normally present in PZN (Figure 1a).

The A(-1)M substitution was introduced into select BamA variants, in addition to CurA, to enable chemical removal of the leader peptide. Following CNBr treatment, these peptides were then subjected to *N*-terminal dimethylation by the SAM-dependent methyltransferase involved in PZN biosynthesis (BamL), whose activity had previously been reconstituted *in vitro* (16–18). BamL dimethylated each of these PZN variants, including the CurA variant, as determined by MALDI-TOF-MS (Supplementary Figure 43). Though the methyltransferase has significant substrate selectivity owing to its narrow substrate-binding channel (17), all of the PZN variants used in these reactions contained both the *N*-terminal Arg and at least one azole, which is minimally necessary for robust methyltransferase activity. Although enzymatic dimethylation was successful, more robust modification was achieved by reductive alkylation with formaldehyde and borane-pyridine (Supplementary Figure 44) (58). Regardless of the method of dimethylation, this sequence of reactions enabled the production of a new collection of PZN variants beyond what was previously achievable *in vivo* (41).

Through the system of treating CurA-A(-1)M with the *Cur* synthetase, CNBr-based removal of the leader peptide, and reductive alkylation of the *N*-terminus, we obtained sufficient quantities of the PZN-like natural product from *C. urealyticum* to enable FT-ICR-MS/MS analysis of the purified compound, which corroborated the proposed structure containing ten azoles and *N*-terminal dimethylation (Figure 6a, Supplementary Figure 45). ¹H NMR also established the presence of sharp singlets consistent with azole formation (Supplementary Figure 46). Similar to the name “plantazolicin,” we have named this anticipated natural product “coryneazolicin” (CZN). Our production of CZN demonstrates the use of *in vitro* biosynthesis to generate a predicted natural compound whose production has not been observed from the native producer. To establish if CZN is naturally produced will require further investigation.

After obtaining CZN, we next turned our attention to recently identified PZN-like BGCs from other bacteria (Supplementary Figure 1). Several of these BGCs encode precursor peptides with substitutions in the core region compared to BamA, the products of which could aid in further determination of the structure-activity relationships of the PZN class of natural products. Although the unannotated precursor peptide from *Bacillus badius*, BadA, lies in a different location relative to other genes in the cluster (Figure 1b), it only differs from BamA by two residues in the core region (Figure 1c). Methanolic extracts of *B. badius* contained an *m/z* 1300 species consistent with the expected mass of the precursor peptide with PZN-like modifications. Furthermore, a less intense species was found with *m/z* 1318, consistent with a PZN-like compound where the methyloxazoline moiety was hydrolyzed (Supplementary Figure 47) (3). Purification of “badiazolicin” (BZN) enabled FT-ICR-MS/MS analysis, which supported a PZN-like structure containing nine azoles, one azoline,

and *N*-terminal dimethylation (Figure 6b, Supplementary Figure 48). After quantification by ^1H NMR, BZN exhibited a minimum inhibitory concentration (MIC) of 2 $\mu\text{g/mL}$ against *B. anthracis* by microbroth dilution assay, but no growth inhibitory activity towards *B. subtilis* (MIC > 16 $\mu\text{g/mL}$) or methicillin-resistant *S. aureus* (MIC > 32 $\mu\text{g/mL}$).

In conclusion, we have demonstrated the *in vitro* reconstitution of two heterocycle synthetases involved in the biosynthesis of PZN by *B. velezensis* and CZN by *C. urealyticum*. Overall, the synthetases *in vitro* afforded a greater degree of biosynthetic insight than previous work with PZN biosynthesis in cells, enabling characterization of synthetase complex assembly and substrate binding properties. Using the *Bam* heterocycle synthetase, we have explored substrate tolerance in the PZN precursor peptide and proposed that cyclodehydration occurs in an *N*-terminal to *C*-terminal direction. These synthetases were then used to achieve the total *in vitro* biosynthesis of a number of PZN variants, culminating in the isolation of two novel natural products, CZN and BZN.

METHODS

In vitro synthetase assays—Heterocycle synthetase reactions to assess substrate tolerance or necessity of individual components contained 100 μM of MBP-tagged precursor peptide, 10 μM of each of the pertinent MBP-tagged synthetase enzymes, and 40 $\mu\text{g/mL}$ TEV protease in synthetase buffer [50 mM Tris (pH 7.5), 125 mM NaCl, 20 mM MgCl_2 , 10 mM dithiothreitol, and 3 mM ATP] (9). Reactions were carried out in 100 μL volume for 18 h at 22 $^\circ\text{C}$, at which point they were quenched by addition of 100 μL MeCN to precipitate the large proteins. After centrifugation for 5 min at 16,000 $\times g$, the supernatant was either dried by speedvac or diluted to 12.5% MeCN by addition of water to facilitate desalting before analysis by mass spectrometry.

For synthetase reactions where isolation of intermediates was attempted, synthetase enzymes were pre-treated with TEV to remove MBP and present at 2 μM in the reaction. Other components were present at the concentrations listed above, and the reaction was quenched by addition of 100 μM MeCN at 10 and 30 minutes.

Mass spectrometry—For MALDI-MS, peptide samples were desalted by C18 ZipTip according to manufacturer instructions and analyzed using a Bruker Daltonics UltrafleXtreme MALDI-TOF/TOF instrument operating in reflector/positive mode with α -cyano-4-hydroxycinnamic acid as the matrix.

For high-resolution mass spectrometry and MS/MS, desalted peptides were resuspended in 80% (v/v) MeCN, 1% AcOH and centrifuged at 11000 $\times g$ for 5 min prior to analysis by direct infusion Fourier transform mass spectrometry (FT-MS). An Advion NanoMate 100 was used to directly infuse samples to an LTQ-FTMS/MS (ThermoFisher) operating at 11 T. The MS was calibrated weekly, following the manufacturer's instructions, and tuned daily using Pierce LTQ Velos ESI Positive Ion Calibration Solution (ThermoFisher). Spectra were collected with a resolution of 100,000. Ions were selected for ion trap fragmentation or FT-MS/MS fragmentation based on signal intensity, and spectra were collected using the following parameters: isolation width of 5 m/z , normalized collision energy of 35, activation

q value 0.4, activation time 30 ms. Data analysis was performed using the Qualbrowser application within ThermoFisher Xcalibur v 2.2.

In vitro kinetics assay—Peptide processing kinetics were measured using a previously described purine nucleoside phosphorylase (PNP)-coupled assay to detect ADP production from ATP hydrolysis (9, 59). Because ATP consumption is 1:1 with heterocycle formation, this assay provides a measure of the rate of heterocycle formation. In general MBP-BamB, MBP-BpumC, and MBP-BamD were pretreated with TEV protease at room temperature to cleave the tag, but for the Cur synthetase proteins, TEV treatment was performed at 4 °C because extended incubation at room temperature caused protein precipitation. After adding the enzyme cleavage mixture to a cuvette for a final concentration of 5 μ M BCD, enzymatic reactions were initiated by the addition of a peptide substrate mixed with synthetase buffer, 200 μ M 7-methyl-6-mercapto-7-methylpurineriboside (Berry and Associates), and 0.2 U of PNP at 22 °C. Maximal initial rates were determined by averaging the slope over the first two minutes of the reaction. The rate of processing for leader peptide mutants was determined with 30 μ M BamA/BpumA or 25 μ M for CurA and their respective synthetase complexes whereas the BamA-R1X mutants were tested at 100 μ M. Kinetic parameters for the Bam/Bpum complex were determined using variable substrate concentrations and were calculated using the nonlinear Michaelis-Menten fit with OriginPro9.1 (OriginLab). All experiments were performed in triplicate.

Fluorescence polarization binding assay—The interaction between FITC-conjugated precursor peptides and different synthetase proteins was measured with a previously employed fluorescence polarization (FP) assay (12). In brief, the assay was performed in nonbinding-surface, 384-black-well polystyrene microplates (Corning) with serial dilutions of the indicated protein(s) and 25 nM of the indicated fluorescent peptide in storage buffer. After 30 minutes of incubation at room temperature, the dilutions were measured using a FilterMax F5 multimode microplate reader (Molecular Devices) with $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 538$ nm. All assays were performed in triplicate and K_d values were determined from a non-linear dose-response curve in OriginPro9.1 (OriginLab). Error bars represent standard error of the mean.

To assess the interaction of non-fluorescent peptides with the PZN synthetase, a competition FP binding assay was used as previously described (49). MBP-tagged precursor peptides were serially diluted and mixed with 25 nM of a fluorescent peptide and synthetase component(s). The BamA/BpumA peptides were competed with FITC-BamA-LP to bind to 3 μ M BamB-R93A, BpumC, and BamD whereas the CurA mutants were competed with FITC-CurA-LP to bind 25 μ M CurC. IC_{50} values were determined from the 50% inhibition point calculated using a dose response curve with Origin Pro 9.1 (OriginLab).

Leader peptide cleavage—Dried precursor peptide (after synthetase reaction and MeCN precipitation) was resuspended in 60% formic acid and 1–2 small crystals of cyanogen bromide (CNBr) were added. The reaction was allowed to proceed for 4 h at 22 °C, at which point it was quenched by addition of 5 volumes of water and desalted by ZipTip, then dried by speedvac.

Methylation—Enzymatic dimethylation using BamL was performed using <50 μM peptide (exact concentration not known), 10 μM MBP-BamL, 10 μM Pfs, and 3 mM *S*-adenosylmethionine in 50 mM Tris (pH 8), for 18 h at 37 °C, as described previously (17). Chemical dimethylation was performed using <1 mM peptide (exact concentration not known), 200 mM formaldehyde, and 300 mM borane-pyridine in 10 mM NH_4CO_3 with 50% MeOH, for 18 h at 22 °C (58).

Production of CZN—Synthetase reactions using MBP-tagged CurA, CurB, CurC, and CurD were performed as described above, with the exceptions that 250 μM MBP-CurA was used, the buffer contained 6 mM ATP, and reactions were carried out in replicates of 200 μL each. After precipitation with MeCN and centrifugation, the supernatants were dried by speedvac, then resuspended and pooled in 60% formic acid (v/v) for CNBr digestion as described above. After the CNBr reaction was quenched with water, the solvent was removed by blowing air. The dried reaction mixture was then resuspended in water to remove water-soluble components and centrifuged for 10 min at 16,000 $\times g$, after which the supernatant was removed and the remaining solids resuspended in MeOH to extract CZN. After centrifugation for 10 min at 16,000 $\times g$, the MeOH supernatant (containing crude CZN) was removed and used in the chemical methylation reaction as described above.

This crude CZN solution in 50% MeOH (v/v) was reverse phase purified using a Thermo BETASIL C18 column (250 mm $\times$ 10 mm; pore size: 100 Å; particle size: 5 μm) at a flow rate of 4 mL min^{-1} . A gradient of 40–95% MeOH with 10 mM NH_4HCO_3 in the aqueous phase over 41 min was used. The fractions containing CZN (as monitored by A_{254} and later verified by MALDI-MS) were collected into 20 mL borosilicate vials and the solvent was removed *in vacuo*.

Production of BZN—*Bacillus badius* ATCC14574 was grown in Luria-Bertani (LB) broth (10 mL/18mm glass culture tube) at 37 °C overnight. Sterilized aluminum trays (16-7/16" $\times$ 11-5/8" cake pans) containing nutrient agar (1 L/tray) were inoculated with 3 mL of overnight culture and incubated for 48 h at 37 °C. Cells were harvested with a razor blade and Tris-buffered saline (TBS) (160 mL/tray) and pelleted by centrifugation (4,000 $\times g$, 20 min, 10 °C). The supernatant was decanted and the pellets were stored at –20 °C until extraction.

Crude BZN was extracted by resuspending the pellets in MeOH (160 mL/tray) through vortex agitation, and the resuspended cells were equilibrated for 4 hours at 22 °C on a shaking platform. The supernatant was retained after centrifugation (4,000 $\times g$, 20 min, 10 °C), vacuum filtered with Whatman filter paper, and concentrated by rotary evaporation. Lyophilization of the extract yielded a yellow solid (200 mg/tray). The crude material was dissolved in 50% MeCN, where the sample separated into two layers. Both layers were added to Celite, which was dried by rotary evaporation. The dried Celite was packed into a cartridge for solid loading of BZN onto a RediSep Rf 130g C18 column (Teledyne Isco) for purification by MPLC using a Combiflash system (30–95% MeCN/10 mM aqueous NH_4HCO_3 over 16 column volumes). The fractions containing BZN were pooled, concentrated by rotary evaporation, and lyophilized to dryness. BZN was then reverse phase purified using the same method as for CZN, except that a gradient of 85–95% MeOH with

10mM aqueous NH_4HCO_3 over 20 min was used. The fractions containing BZN were collected in 20 mL borosilicate vials and the solvent removed *in vacuo*. The isolated yield of HPLC-purified BZN was 0.7–0.8 $\mu\text{g/L}$ of nutrient agar.

NMR—Samples were dissolved in 600 μL of CD_3OD (99.95 atom % D, Cambridge Isotope Labs). NMR spectra were obtained with an Agilent VNMRs 750 MHz narrow bore magnet spectrometer equipped with a 5 mm triple resonance (^1H - ^{13}C - ^{15}N) triaxial gradient probe and pulse-shaping capabilities. Samples were held at 298 K during acquisition. Standard Varian pulse sequences were used; a relaxation delay (d1) of 28 s was used during acquisition for quantitative NMR (qNMR), and 90° pulse widths were calibrated and used (8.50 μs for CZN acquisition; 8.75 μs for BZN acquisition). 320 transients were recorded for BZN; 2048 transients were recorded for CZN. Apodization (0.4 Hz line-broadening), phase correction, integration, integral normalization, and automated baseline correction were applied prior to qNMR processing. Spectra were recorded with VNMRJ 4.2 software, and data was processed using MestReNova 8.1.1. Chemical shifts (δ , ppm) were referenced internally to the solvent peak (methanol). For quantitative NMR, the probe was calibrated using the qEstimate tool VNMRJ on the standard ^{31}P sample (48.5 mM triphenylphosphate in CDCl_3 , Varian part #: 00-968120-97).

Antibacterial activity assays—The concentration of BZN was calculated by qNMR, which has been used for accurate quantification of microgram quantities of natural products (60). The native qNMR functionality within VNMRJ 4.2 was used to quantify BZN based on the average integration of itsazole peaks, which occur as distinct singlets in the aromatic region. Determination of MIC values for BZN was performed as described previously (19).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Structure of plantazolicin (PZN). (b) Biosynthetic gene clusters responsible for the production of PZN and an abridged list of homologous clusters. *Bam*, *Bacillus velezensis* FZB42; *Bpum*, *B. pumilus* ATCC 7061; *Bbad*, *B. badius*; *Cur*, *Corynebacterium urealyticum* DSM 7109. (c) Precursor peptide sequences for PZN and select related family members. Colors indicate post-translational modifications in the mature product: green, N^{α},N^{α} -dimethylarginine; red, thiazole; blue, (methyl)oxazole; brown, azoline; *, leader peptide cleavage site. A full listing of gene clusters and precursor peptides for the PZN family are shown in Supplementary Figure 1.

Figure 2.

(a) Reaction of the PZN precursor peptide BamA with the *Bam* heterocycle synthetase *in vitro* results in the installation of nine azoles and one azoline, consistent with PZN produced from the native host. (b) Reaction of the precursor peptide CurA with the *Cur* heterocycle synthetase *in vitro* results in the installation of ten azoles.

Figure 3.

Reaction of BamA (1) with a heterocycle synthetase containing the inactive BamB-R93A results in the installation of up to four azolines, without their oxidation to azoles (2). Subsequent treatment of this species mixture with acid hydrolyzes the azolines to their original amino acids (3), whereas treatment with a fully wild-type synthetase yields the full complement of nine azoles and one azoline (4), comparable to treatment of unmodified BamA with wild-type synthetase (5). Substitution of the catalytically inactive BamB-Y206A for BamB-R93A yields equivalent results.

Figure 4.

Processing of mutant BamA peptides by the *Bam* synthetase *in vitro*. Mutated and uncyclized residues are shown in solid black circles; half-filled circles indicate a mixture of species. **(a)** Mutation of most residues to Pro prevents cyclization both *C*-terminal and in the -1 position to the Pro. Wild-type BamA (WT) is shown for comparison. **(b)** Mutation of a normally-cyclized position to Ala, or insertion of Ala between two wild-type residues, generally prevents cyclization *C*-terminal to the Ala. **(c)** A Cys residue in the next heterocyclizable position relative to Ala enables cyclodehydration of that residue, though Cys in a position not normally cyclized remains unmodified. **(d)** Deletion of a residue does not affect processing by the synthetase, though one residue must remain uncyclized at the *C*-terminus. **(e)** The *Bam* synthetase *in vitro* is capable of processing substrates with the core peptides of other members of the PZN family.

Figure 5.

Fluorescence polarization (FP) binding assay with BamB-R93A/BpumC/BamD and FITC-labeled BamA leader peptide (LP) (**a**) or FITC-labeled-BpumA-LP (**b**). K_d values not shown are $>150 \mu\text{M}$. Error bars represent s.d. of at least three independent measurements. Error on K_d values are given as the s.e.m. from regression analysis. For binding curves that do not reach saturation, K_d values were extrapolated.

Figure 6. Proposed structures for the novel natural products coryneazolicin (CZN) (**a**) and badiazolicin (BZN) (**b**), as supported by FT-ICR-MS/MS (see also Supporting Figures 45 and 48).

Table 1Kinetics of select BamA substrates with the *Bam* synthetase.

Mutant	V_o ($\mu\text{M Pi min}^{-1}$)	Apparent K_m (μM)	Heterocycles installed
WT	1.76 ± 0.09	3.22 ± 1.08	10
T3A	1.71 ± 0.13	26.8 ± 5.9	2
T6A	2.74 ± 0.25	55.5 ± 11.3	4
I8P	2.35 ± 0.12	7.61 ± 2.28	5
S12A	2.35 ± 0.19	8.36 ± 2.86	8
T13P	2.48 ± 0.09	5.73 ± 1.26	8
C4	1.88 ± 0.22	9.37 ± 4.3	9
BamA-CurA	1.78 ± 0.12	12.3 ± 2.8	10

Table 2

Binding and processing of leader peptide mutants.

Peptide	IC ₅₀ (μM)	<i>in vitro</i> (%) ^b	<i>in vivo</i> ^c
BamA-WT	2.8 ± 0.3	100 ± 9.8	+++
BamA-T(-26)A	5.4 ± 0.6	108 ± 6.7	+++
BamA-Q(-25)A	4.4 ± 0.6	94 ± 9.8	++
BamA-I(-24)A	5.5 ± 0.7	117 ± 5.4	+
BamA-K(-23)A	5.9 ± 0.6	89 ± 5.1	++
BamA-V(-22)A	4.8 ± 0.3	102 ± 1.0	+
BamA-P(-21)A	>100	54 ± 8.6	— ^d
BamA-T(-20)A	5.5 ± 0.7	95 ± 2.7	++
BamA-L(-18)A	>100	63 ± 2.9	—
BamA-I(-17)A	2.7 ± 0.3	99 ± 7.1	++
BamA-S(-15)A	9 ± 2	100 ± 1.3	+
BamA-V(-14)A	8.2 ± 0.9	110 ± 11.7	+
BamA-H(-13)A	13 ± 3	106 ± 1.4	+
BamA-G(-12)*	58 ± 8	n.d.	n.d.
BamA-Q(-9)*	13.5 ± 2.7	n.d.	n.d.
BamA-F(-6)*	6.8 ± 1	n.d.	n.d.
BamA-M(-3)*	2.4 ± 0.3	n.d.	n.d.
BpumA-WT	1.9 ± 0.1	105 ± 5.3	+
CurA-WT	22 ± 4	108 ± 4	n.d. ^e
CurA-N(-26)S ^a	24 ± 4	100 ± 9	n.d.
CurA-P(-23)A	>150	7.1 ± 0.7	n.d.
CurA-P(-22)T	53 ± 9	105 ± 1	n.d.
CurA-V(-20)A	>150	44.4 ± 0.3	n.d.

^aCurA-N(-26)S represents leader sequence of BlinA^bNormalized to wild-type BamA or CurA, respectively^cDefined relative to that of the plasmid-borne wild-type BamA: 100–75% (+++), 75–25% (++), 25–5% (+)^dLimit of detection estimated to be 5% based on requiring that the signal be 3 times greater than baseline noise^en.d., not determined