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Insights into Methyltransferase Specificity and Bioactivity of Derivatives of the Antibiotic Plantazolicin

Yue Hao^{#1}, Patricia M. Blair^{#2}, Abhishek Sharma², Douglas A. Mitchell^{2,3,4,†}, and Satish K. Nair^{1,2,4,5,†}

¹Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

²Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

³Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

⁴Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

⁵Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

[#] These authors contributed equally to this work.

Abstract

Peptide antibiotics represent a class of conformationally-constrained natural products of growing pharmaceutical interest. Plantazolicin (PZN) is a linear, polyheterocyclic natural product with highly selective and potent activity against the anthrax-causing bacterium, *Bacillus anthracis*. The bioactivity of PZN is contingent on dimethylation of its *N*-terminal Arg residue by an *S*-adenosylmethionine-dependent methyltransferase. Here, we explore the substrate tolerances of two homologous PZN methyltransferases by carrying out kinetic analyses of the enzymes against a synthetic panel of truncated PZN analogs containing the *N*-terminal Arg residue. X-ray cocrystal structures of the PZN methyltransferases with each of these heterocycle-containing substrates provide a rationale for understanding the strict substrate specificity of these enzymes. Kinetic studies of structure-guided, site-specific variants allowed for the assignment of residues governing catalysis and substrate scope. Microbiological testing further revealed that upon dimethylation of the *N*-terminal Arg, a pentaheterocyclized PZN analog retained potent anti-*B. anthracis* activity, nearly equal to that of full-length PZN. These studies may be useful in the biosynthetic engineering of natural product analogs with different bioactivity profiles, as demonstrated by our identification of a truncated plantazolicin derivative that is active against methicillin-resistant *Staphylococcus aureus* (MRSA).

[†]To whom correspondence should be addressed: Douglas A Mitchell, 600 S. Mathews Ave., Roger Adams Lab Room 345, University of Illinois at Urbana-Champaign, Urbana, IL 61801, 217-333-1345, douglasm@illinois.edu, Satish K Nair, 600 S. Mathews Ave., Roger Adams Lab Room 430, University of Illinois at Urbana-Champaign, Urbana, IL 61801, 217-333-0641, snair@illinois.edu.

Data deposition: The atomic coordinates and structures have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes: to be inserted].

SUPPORTING INFORMATION

Additional experimental procedures, structural characterization details, and supporting figures as mentioned in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no conflict of interest.

Ribosomally synthesized and post-translationally modified peptides (RiPPs) encompass a large and widely distributed class of bioactive secondary metabolites (1), which include the thiazole/oxazole-modified microcins (TOMMs) (2). The natural product plantazolicin (PZN) (1), produced by the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42 (3, 4), is biosynthesized from a 41-residue precursor peptide to yield a product that contains ten azol(in)es, produced through the cyclodehydration and subsequent oxidation of Cys/Ser/Thr characteristic of TOMMs (5, 6). Installation of the heterocyclic moieties is catalyzed by an ATP-dependent cyclodehydratase that converts Cys and Ser/Thr to thiazoline and (methyl)oxazoline, respectively (7), followed by a flavin mononucleotide-dependent dehydrogenation (8) to yield the corresponding thiazole and (methyl)oxazole. Twelve out of the fourteen residues of the core region of the PZN precursor peptide are modified in the fully mature, bioactive natural product. Following the proteolytic excision of the 21-residue leader sequence from the deca-heterocyclized peptide, an *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase modifies the *N*-terminus (9). The resultant product PZN bears an *N*^α,*N*^α-dimethylArg as the *N*-terminal residue, five contiguous azoles, two hydrophobic spacer residues, followed by another stretch of five contiguous heterocycles, and terminates with a *C*-terminal Phe (Figure 1A). Genome mining has identified four other bacterial strains with the capacity to generate PZN-like molecules, with *Bacillus pumilus* being a demonstrated producer of PZN (6).

PZN displays a narrow spectrum of activity. Using the microbroth dilution method, the only organism susceptible to the action of purified PZN to date has been *Bacillus anthracis*, the etiological agent of anthrax (6, 10). Using the agar disk diffusion method, which allows unrealistically high concentrations of antibiotic to be impregnated into the disk, PZN displayed weak growth inhibitory activity towards other members of the genus *Bacillus* (4). Notably, the biological activity of PZN is contingent on the *N*^α,*N*^α-methylation of the terminal Arg, as desmethylPZN activity is attenuated (6). Reconstitution experiments demonstrated that recombinant BamL (the PZN methyltransferase from *B. amyloliquefaciens*) and BpumL (the orthologous methyltransferase from *B. pumilus*) can install both methyl groups onto desmethylPZN in an SAM-dependent manner (9). Unlike other canonical methyltransferases involved in natural product biosynthesis (11-13), both BamL and BpumL have highly restricted substrate tolerances, as the unmodified precursor peptide, as well as several other peptidic substrates designed to mimic the functionality of desmethylPZN, were not substrates for these enzymes (9). BamL can also accept Arg amide (in which the α-carboxylate is replaced with an amide) as a substrate, but only so under near-single turnover conditions (9). Another study concluded that Arg thiazole (also containing a terminal amide) is the minimal substrate for BamL, and found that residues other than Arg are not tolerated by the methyltransferase (14). Lastly, BamL reactions with Arg-thiazole yielded mainly monomethylated product under stoichiometric (i.e. limiting) concentrations of SAM, and the BamL active site mutant Tyr182Phe also yielded monomethylated product using desmethylPZN as substrate (14).

Our prior crystal structures of BamL (PDB Code: 4KVZ) and BpumL (PDB Code: 4KWC) in complex with *S*-adenosyl-L-homocysteine (SAH) revealed a minimalistic methyltransferase fold that lacked any additional structural decorations (9). Due to the

limited aqueous solubility of desmethylPZN, numerous attempts to solve the structures of BamL/BpumL with bound substrate were unsuccessful. Additionally, substrate solubility limitations precluded kinetic characterization of wild-type and mutant enzymes, as well as determination of binding affinities using isothermal titration calorimetry. As the methyltransferase crystal structures revealed a narrow tunnel adjacent to the bound SAH, inferred to be the substrate-binding site, whose dimensions were sufficient to accommodate roughly the first 5 residues of desmethylPZN, we initiated the chemical synthesis of shorter desmethylPZN fragments (Figure 1A) (15). The largest fragment consisted of the *N*-terminal Arg followed by five azoles (hereafter Arg-Az₅, **4**), and was shown to convert to the dimethylated product, Me₂-Arg-Az₅, when reacted with BamL and SAM (15). Smaller desmethylPZN fragments, consisting of the terminal Arg followed by one (Arg-Az₁, **2**) or three (Arg-Az₃, **3**) azoles, were also accepted as BamL substrates and bound to the enzyme with low micromolar affinity (Figure 1B,C).

Here, we present the cocrystal structures, solved to resolutions between 1.5-1.8 Å, of BamL and BpumL in complex with each of the three azole-bearing substrates, as well as cocrystal structures of BpumL with two monomethylated products. The enhanced aqueous solubility of these analogs compared to desmethylPZN facilitated the determination of the Michaelis-Menten kinetic parameters for both the wild-type enzymes with the three truncated derivatives, as well as the kinetic characterization of several active site variants designed based on the structural data. We also show that both Me₂-Arg-Az₃ and Me₂-Arg-Az₅ display antibiotic activity with a broader-spectrum profile relative to PZN. These combined biophysical and microbiological data provide a rare example of a RiPP tailoring enzyme bound to a processed substrate, and provide the chemical framework and proof-of-concept for the engineering of PZN derivatives with improved bioactivity profiles.

RESULTS and DISCUSSION

Kinetic characterization of truncated desmethylPZN substrates

In order to overcome the poor physicochemical behavior of desmethylPZN in aqueous solution, we had previously synthesized three truncated desmethylPZN analogs consisting of an *N*-terminal Arg followed by one, three, or five azoles (Figure 1A). Each of these compounds was converted to the corresponding dimethylated counterpart, with *N*-terminal dimethylation determined mass spectrometric analysis (SI Figure 1). The aqueous solubility of Arg-Az₁ and Arg-Az₃, and moderate solubility of Arg-Az₅, allowed for kinetic characterization of each of these substrates in a coupled assay using SAH nucleosidase to monitor SAM turnover (the pfs SAH nucleosidase has a catalytic efficiency of $11.6 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, (16) which is greater than that of BamL and BpumL). The catalytic efficiency of BamL toward the truncated desmethylPZN substrates increases with the number of heterocycles (Arg-Az₁, $3.46 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$; Arg-Az₃, $5.75 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$; Arg-Az₅, $7.53 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, Table 1). We only tested the kinetic activity of BpumL towards the Arg-Az₃ substrate, yielding a $k_{\text{cat}}/K_{\text{M}}$ value of $6.38 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ comparable to that for BamL on the same substrate (Figure 1C and Table 1). The values for k_{cat} are similar for all three substrates, with decreases in K_{M} values corresponding to increases in the length of the

substrates. These data are consistent with the notion that the BamL active site likely accommodates the first 5 residues of desmethylPZN.

Cocrystal structures of plantazolicin methyltransferases

Given that several other small molecule methyltransferases demonstrate some degrees of substrate promiscuity (11-13), it was perplexing that BamL did not accept non-heterocyclized substrates. In order to delineate the basis for the strict substrate specificity, we determined the cocrystal structures of BamL-SAH and BpumL-SAH with Arg-Az₁, and Arg-Az₃, to resolutions between 1.5 Å - 1.8 Å, and the structure of BpumL-SAH with Arg-Az₅, to 1.75 Å resolution. Crystallographic phases were determined by the molecular replacement method, and relevant data collection and refinement statistics are provided in SI Table 2. In each ternary complex structure, clear and continuous electron density for the *N*-terminal Arg and all of the azoles of Arg-Az₁ and Arg-Az₃ was observed; however, the Arg-Az₅ structures show strong density for only the first four heterocycles, with much weaker electron density for the fifth heterocycle, which resides outside of the binding pocket (SI Figure 3).

The BamL and BpumL cocrystal structures recapitulate the core class I methyltransferase Rossmann fold consisting of six α -helices and seven β -strands (17). The SAH byproduct is bound across the interior in a bent manner, which would dispose the electrophilic methyl group of SAM adjacent to the α -amine of Arg in each of the (poly)azolic substrates (Figure 2A). The (poly)azolic substrates bind in an extended configuration, perpendicular to the plane of the β -strands, in a narrow tunnel formed by the helices that cap the Rossmann fold core (SI Figures 4-6). The *N*-terminal Arg of the substrate is nestled in this pocket and is situated orthogonally to SAH (Figure 2B,C). BamL residues Leu162 and Tyr187 are along one face, and a short ₃₁₀ helix between Phe134 through Phe137 buttresses the opposing side. The guanidinium side chain of the substrate is held in place through hydrogen bonding interactions with the side chains of Asp161, Gln186, and Ser190, and the backbone carbonyl of Gln186 (BamL numbering). This combination of van der Waals contacts and hydrogen bond interactions establishes a planar, coaxial orientation for the substrate Arg.

The azole rings are all cradled within a hydrophobic tunnel situated along the width of BamL and are encapsulated by several residues within a large α -helix that spans the length of the enzyme (SI Figure 3). A number of hydrophobic residues surround the first two azole rings of the substrate, and these include Tyr33, Leu132, Ile141, Val160, Leu162, Tyr182, Leu183, and Tyr187 (SI Figures 4-6). The Thr-derived methyloxazole ring at position 3 of the peptide substrate (R**T**CTT) makes favorable van der Waals contact with Leu132, and Leu258, but we predict that steric clashes would preclude binding of desmethylPZN analogs containing Thr at position 4 (Cys in the native substrate, RCT**C**TT). The third (and presumably) fourth, and fifth rings of Arg-Az₃ and Arg-Az₅ (R**C**T**C**TT) are enclosed by a capping loop that spans BamL residues Thr244 through Ala257, and includes Met255 that makes contacts with the terminal azole in each substrate (vide infra) (Figure 2A).

To determine structures of a product-bound ternary complex, BpumL was incubated with SAM and Arg-Az₃ and Arg-Az₅. However, the structures reveal BpumL-SAH in complex with monomethylated Me-Arg-Az₃ (1.5 Å resolution) and Me-Arg-Az₅ (1.75 Å resolution)

(Figure 3A,B). Subsequently, we carried out liquid chromatographic and mass spectrometric analyses demonstrating that monoalkylated intermediates are indeed observed under pseudo-single turnover conditions, which was consistent with prior experiments on truncated PZN derivatives carried out under limiting concentrations of SAM (14) (SI Figure 7). The cocrystal structures of BpumL-SAH in complex with Me-Arg-Az₃ and Me-Arg-Az₅ reveal that the monomethylated products are situated within the active site with a nearly identical orientation as the corresponding desmethyl substrates. A comparison of the pre- and post-methyl transfer structures suggest that the α -amino group of Arg is positioned in a relatively hydrophobic environment formed by Phe20, Leu130, and Leu134. Two polar residues (Asp33 and Tyr180) are in the vicinity of the α -amino group, and the contours of the pocket are sufficient to accommodate dimethylation on the Arg of the substrate (Figure 3A,B).

A polymorphic capping region undergoes structural changes in response to (poly)azolic substrate

As noted, in BamL, the (poly)azolic substrate-binding tunnel is flanked by a region of the enzyme encompassing Thr244 through Ala257. In the absence of the substrate, this region in BamL and BpumL forms a loop that links the central Rossmann fold core to a flanking helix (Figure 4A). The co-crystal structures of BamL with the various substrates show that this region is polymorphic and can undergo structural changes to accommodate the length of the bound ligand (Figure 4). The most drastic rearrangement is seen in the BamL/SAH/Arg-Az₁ ternary complex in which Gly247 through Met255 reorganize to form an α -helix (Figure 4B). Similarly, in the SAH/Me-Arg-Az₃ ternary structure, Ser245 through Gly248 form a one-turn helix that precedes the loop (Figure 4C). The net result of these conformational movements is the displacement of Met255 to facilitate the packing of the terminal azole in the respective substrate. Conformational displacement of this region results in the formation of a hydrophobic pocket that is optimized for the appropriate truncated substrate. These residues are located at the C-terminus of the methyltransferase, and are among the least conserved amongst BamL and BpumL. Other suspected PZN methyltransferases completely lack the C-terminal extension that contains these residues (Figure 4D).

Kinetic analysis of active site variants with Arg-Az₃ as substrate

The ternary complex structures reported here suggest potential catalytic roles for a number of residues that are in the vicinity of the bound substrate. Prior functional analysis of BamL and BpumL were limited due to issues with substrate solubility and could only be characterized using semi-quantitative mass-spectrometric analysis of product yield (9). Based on the structures of the ternary complex, several individual site-specific variants of BamL were generated and kinetic parameters were determined using Arg-Az₃ as the substrate. Thr38 is located on a helix that runs parallel to the trajectory of the (poly)azolic substrates and is in van der Waals contact with the first two heterocyclized residues (RCTCTT). The T38F mutation results in a 17-fold decrease in k_{cat}/K_M (Table 2). Similarly, mutation of either Leu132 (in van der Waals contact with ring 2), or Leu162 (contact with ring 1) to Phe results in a decrease in k_{cat}/K_M by a factor of 16 and 48, respectively.

Two other variants of BamL were of further interest and thus were kinetically characterized. In the cocrystal structures, Arg42 is involved in an ionic interaction with the SAH

carboxylate, and the R42A mutation resulted in a 12-fold decrease in k_{cat}/K_M (Table 2). Likewise, Tyr182 is in the vicinity of the α -amino group and the Y182F mutation results in a 17-fold decrease in k_{cat}/K_M . The effects of these mutations are consistent with roles for both residues in catalysis, as direct or indirect constituents in acid-base chemistry. Methyltransferases from the biosynthetic clusters for two other PZN-like molecules bear a Phe at this position but contain a neighboring Tyr that may similarly facilitate deprotonation of the α -amine (9).

The molecular basis for substrate specificity

The PZN methyltransferases demonstrate narrow tolerance for substrates (9), which is unexpected as natural product methyltransferases, in general, are broadly substrate-tolerant (11-13), and the methyltransferase that catalyze similar N^{α},N^{α} -dimethylation of α -amino groups (*e.g.* during the biosynthesis of linaridins) are fairly tolerant of substrates (18, 19). An examination of the active site in the cocrystal structures of BamL and BpumL with SAM/SAH and each of the (poly)azolic truncated desmethylPZN derivatives provides a rationale for this specificity. For example, in order to accommodate unmodified peptide substrates, the residue following the Arg must be in the *cis* conformation to avoid steric clashes. In addition, steric constraints would result in energetically unfavorable Ramachandran torsion angles for the second residue, permitting only a Gly at this position. Conceding all of the steric requirements would still be unfavorable due to the placement of the charged C-terminal carboxylate in the hydrophobic binding tunnel. Finally, the active site contours of BamL and BpumL are constricted in a manner that could only accommodate heterocyclic substrates, and kinetic analysis of site-specific mutations at residues that line the binding site suggest that the changes in the contours of the substrate-binding tunnel are not well tolerated and that the pocket around the first azole is the least resistant to alterations in volume.

The prevalence of hydrophobic residues that line the substrate-binding tunnel provide a rationale for understanding why Arg thiazole (bearing a terminal amide) represents the minimal competent substrate for BamL (14). Similarly, Arg amide is a poorer substrate that is turned over by BamL but only under single-turnover conditions (9). In contrast, the amino acid Arg, which bears a charged α -carboxylate, is not accepted as a substrate, probably for the same electronic reasons noted above. Structure-activity studies demonstrate that an Arg at the amino terminus of substrate is a strict requirement, and this constraint can be understood in the context of the structural data. Both BamL and BpumL make extensive hydrogen bonding contacts with the Arg guanidino group, consistent with the observation that substrate containing either ornithine or lysine are not processed. Likewise, homoArg is a poor substrate because the steric limitations of the active site is adapted for Arg and could not accommodate the extra methylene group of this side chain (14).

Antibacterial profile of truncated PZN derivatives

We have previously shown that potent antibacterial activity of PZN is dependent on the dimethylation of the *N*-terminus. As BamL and BpumL could effectively methylate the truncated PZN derivatives, we characterized the activity of these compounds. As expected, all desmethyl compounds were inactive towards the *B. anthracis* Sterne indicator strain at

concentrations of up to 48 μ M, underscoring the importance of *N*-terminal dimethylation for antibiotic activity (Table 3). Following enzymatic dimethylation and purification of each derivative, these resultant compounds were tested for bioactivity against a panel of ten diverse bacterial strains (seven Firmicutes, three Proteobacteria). As with all of the desmethyl compounds, Me₂-Arg-Az₁ did not show any appreciable bioactivity against any strain. However, both Me₂-Arg-Az₃ and Me₂-Arg-Az₅ displayed minimum inhibitory concentrations (MICs) in the low micromolar range against *B. anthracis* Sterne, with Me₂-Arg-Az₅ being nearly as potent as PZN.

Surprisingly, both Me₂-Arg-Az₃ and Me₂-Arg-Az₅ demonstrated broader-spectrum activity relative to PZN (Table 3). While PZN is not active up to 48 μ M against other representative members of the genus *Bacillus*, both Me₂-Arg-Az₃ and Me₂-Arg-Az₅ were active against these PZN-insensitive strains. In all tested species, Me₂-Arg-Az₃ showed reduced bioactivity relative to Me₂-Arg-Az₅. Importantly, the bioactivity profile of Me₂-Arg-Az₅ was extended to include *S. aureus* ^oNRS384 (a USA300 community acquired-MRSA strain), against which this derivative demonstrates an MIC comparable to that against *B. anthracis* (Table 3). Inhibition of *B. anthracis* is similar for PZN and Me₂-Arg-Az₅ in a microbroth dilution assay, but only Me₂-Arg-Az₅ results in inhibition of *S. aureus* NRS384 (Figure 5A). However, unlike PZN, which induces major cell lysis above the MIC, the treatment of mid-exponential *B. anthracis* with Me₂-Arg-Az₅ displayed less extensive lysis (Figure 5). A compound structurally similar to Me₂-Arg-Az₅, but instead bearing a *C*-terminal amide, has been recently shown by the Süssmuth group to be inactive towards *Bacillus megaterium* in an agar diffusion assay when 6 μ g of the pure compound was spotted (14).

Conclusions

Based to their relaxed specificity for *B. anthracis*, PZN and the truncated analogs may not share the same molecular target(s), but confirmation of this will require extensive additional study. Interestingly, Me₂-Arg-Az₅ retains nearly the same potency as full-length PZN against *B. anthracis*. Hence, it is possible that the bioactive portion of PZN is located in the *N*-terminal half of the molecule, while the *C*-terminal half may govern species specificity. An alignment of the five known PZN precursor peptides shows that the *N*-terminal portion is considerably more conserved than the *C*-terminal portion, which could support the previous conjecture (6). Nevertheless, some modifications to the *C*-terminus are detrimental to the anti-*B. anthracis* activity (20). The combined biochemical, and microbiological analyses of PZN methyltransferase specificity sets the framework for future studies aimed to engineering non-natural PZN variants with expanded and/or orthogonal bioactivities.

METHODS

Protein expression and crystallization

The gene for *E. coli* adenine deaminase was amplified using primers based on the published DNA sequence, and inserted into the pET28b vector. Details for the cloning, expression and purification of BamL and BpumL have already been reported (9). Briefly, the coding region for BamL and BpumL were inserted into a modified pET28-MBP vector for production as a

C-terminal fusion. Protein was purified by nickel affinity chromatography, and following removal of the affinity tag, was further purified by ion exchange and size-exclusion chromatography. Mutant proteins were generated using oligonucleotide sequences provided in SI Table 1, and the resultant proteins were purified in a similar manner. Sample purity was assessed by Coomassie-stained SDS-PAGE chromatography (Figure S2). The (poly)azolic substrates Arg-Az₁, Arg-Az₃, and Arg-Az₅ were synthesized as previously described. SAM was either prepared enzymatically using SAM synthase, or purchased from Sigma immediately prior to use. The integrity of SAM was routinely monitored by mass spectrometry and HPLC analysis prior to biochemical experiments. Crystals of the BpumL or BamL/SAH/Arg-Az_x complexes were grown using the hanging drop method. Briefly, a 50 μ L aliquot of protein (in a buffer of 0.5 M NaCl, 20 mM Tris pH=8.0, and 10% glycerol, at a concentration of 10 mg/mL) was incubated with 2 mM SAM/SAH and 2 mM of the (poly)azolic substrate for 1 h on ice prior to crystallization. Commercially available sparse matrix screens were used to identify initial crystallization conditions that were subsequently refined to yield samples suitable for diffraction experiments. Plate-like crystals were grown using 100 mM Bis-Tris propane, pH=5.5, 200 mM Li₂SO₄, and 22-26% polyethyleneglycol 3350. Crystals were transiently submerged in the crystallization medium supplemented with 10% glycerol prior to vitrification by direct immersion in liquid nitrogen.

Data collection, integration and structure determination

All diffraction data were collected at the Life Sciences Collaborative Access Team (LS-CAT; Sector 21) at the Advanced Photon Source (Argonne, IL) using MAR charge coupled device detectors. The diffraction data were indexed and scaled using HKL2000 (21) or XDS (22) to the appropriate limiting resolution. Initial crystallographic phases were determined by the molecular replacement method (23) as implemented in the Phenix software suite (24), using the coordinates of unliganded BamL (PDB Code: 4KVZ) as a search probe. Rounds of manual rebuilding using COOT (25) were interspersed with crystallographic refinement using REFMAC5 (26) until the free R factor dropped below 30%. All ligands and solvents were incorporated into the model at this stage, with further refinement and rebuilding of the model until convergence. Cross-validation, using 5% of the data for the calculation of the free R factor, was utilized throughout the model building process in order to monitor building bias. The validity of all models were routinely determined using PROCHECK (27), MOLPROBITY (28), and by using the free R factor (28) to monitor improvements during building and crystallographic refinement.

Kinetic analysis of BamL and BpumL methyltransferases

Kinetic analysis of BamL and BpumL utilized a coupled spectrophotometric assay. Reaction mixtures typically consisted of 50 mM Tris, pH=7.8, 200 μ M MnSO₄, 5 μ M *E. coli* pfs nucleosidase, 5 μ M *B. subtilis* adenine deaminase, 80 μ M SAM, and varying concentrations of the Arg-Az₁, Arg-Az₃, and Arg-Az₅. The reaction progress was monitored at 310 K and utilized 0.5 μ M of either MBP-tagged wild-type BamL or BpumL. Concentrations for the mutant enzymes are as follows: 1.4 μ M R42A, 1.325 μ M T38F, 1.34 μ M L132F, 1.36 μ M L162F, and 1.8 μ M Y182F. Reaction progress was monitored as changes in absorption at a wavelength of 265 nm. Initial velocity data were calculated and fitted to the Michaelis-Menten equation using the Origin software package. Due to absorption by substrate at 265

nm, reaction rate using high concentrations (> 80 μ M) of peptide substrate failed to yield reliable data.

Antibacterial assays

Microbroth dilution assays were performed as previously described (6). Briefly, 10 mL stationary phase cultures (two for each bacterial species) were adjusted to OD₆₀₀ of 0.01 in the appropriate medium and added to 96-well plates. Two-fold dilutions of each compound (stock solution at 4 mM in DMSO) were added to the cultures (0.375 μ M – 48 μ M). Plates were covered and incubated at 37 °C for 12 h before determination of the MIC based on culture turbidity. The cell densities after 12 h were determined for MRSA and Sterne. Vehicle (DMSO)-treated, 24 μ M PZN-treated, and 24 μ M Me₂-Arg-Az₅-treated wells were analyzed in duplicate on a FilterMax F5 Multi-mode Microplate Reader (Molecular Devices) using sterile LB as the plate blank and measuring cell density at 595 nm. Error bars indicate standard deviation. P-values were calculated using a two-tailed t-test between DMSO-treated and PZN- or Me₂-Arg-Az₅-treated cells of the same bacterial species.

Microscopy

200 μ L aliquots of a stationary phase culture of *B. anthracis* Sterne was used to inoculate 5 mL fresh LB. The culture was grown to OD 0.5 and 1 mL aliquots were harvested by centrifugation (8,000 $\times$ g, 2 min). Bacterial pellets were resuspended in 100 μ L PBS supplemented with vehicle (DMSO), 4 μ M PZN or 12 μ M Me₂-Arg-Az₅. After mixing at 22 °C for 30 min, cells were washed with PBS and resuspended in 200 μ L PBS. 10 μ L aliquots of each cell suspension were mixed with 10 μ L 2% liquefied low gelling temperature agarose (Sigma-Aldrich) on microscope slides. Differential interference contrast microscopy analyses were conducted at the Core Facility at the Institute of Genomic Biology (University of Illinois) using a Zeiss LSM 700 confocal microscope outfitted with a 405 nm laser. Linear contrast was applied when deconvoluting images in Zen 2012 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Chemical structures of PZN and the three (poly)azolic truncated methyltransferase substrates. (B) Enzymatic conversion of Arg-Az₃ into the corresponding dimethylated compound by BamL, as analyzed by HPLC. *, internal standard. (C) Michaelis-Menten curves for the conversion of each of the (poly)azolic substrates catalyzed by BamL using a fixed SAM concentration of 5 mM.

Figure 2.

(A) Structure of the BamL (purple) in complex with SAH (gray), and Arg-Az₃ (cyan) with the capping loop colored in green. (B, C) Difference Fourier electron density maps of (B) BamL-Arg-Az₁ and (C) BamL-Arg-Az₃ contoured at 2.5σ (blue) and 8σ (red), calculated with coefficient ($F_{\text{obs}} - F_{\text{calc}}$), with phases derived from the final structure with the coordinates of the substrate removed for one round of simulated annealing refinement. The SAH is colored in gray, the substrates are colored in cyan, and important active site residues are shown as yellow ball-and-stick.

Figure 3.

(A,B) Active site views of the structures of BpumL in complex with SAH (gray) and either (A) Me-Arg-Az₃ or (B) Me-Arg-Az₅. The substrates are colored in cyan, and important active site residues are shown as yellow ball-and-stick. The superimposed electron density maps are calculated and contoured as in Figure 2B,C.

Figure 4.

(A-C) Views showing that the capping region (colored in green) is polymorphic and changes from (A) a long loop in the BamL structure without substrate to (B) an α -helix in the BamL-SAH-Arg-Az₁ structure, and (C) a short helix-loop in the BamL-SAH-Arg-Az₃ structure. (D) The capping region is located in the poorly conserved C-terminus of BamL and is missing in methyltransferases from related biosynthetic clusters. Residues conserved among all sequences are shaded in black and those that are similar are shaded in light blue. The C-terminal extension is missing in CurL and BlinL and is designated by a series of periods.

Figure 5.

(A) Treatment with 24 μ M PZN or Me₂-Arg-Az₅ is bactericidal against *B. anthracis* Sterne. However, Me₂-Arg-Az₅ but not PZN displays antibiotic activity against MRSA, as evidenced by cell density measurements after 12 h treatment (595 nm). Error bars, standard deviation. *, $P < 0.01$ vs DMSO-treated *B. anthracis* st. Sterne. #, $P < 0.01$ vs DMSO-treated MRSA. (B-D) Me₂-Arg-Az₅ displays antibiotic activity against *B. anthracis* but unlike PZN does not massively induce cell lysis of mid-exponential phase cells after 30 min. Scale bars, 5 μ m. (B) Vehicle (DMSO)-treated *B. anthracis* vegetative cells. (C) *B. anthracis* treated with 4 μ M PZN shows cell lysis. (D) *B. anthracis* remained mostly intact after treatment with 12 μ M Me₂-Arg-Az₅.

Table 1

Steady state kinetic parameters for BamL and BpumL against truncated PZN substrates.

BamL	K_M (μM)	k_{cat} (10⁻² s⁻¹)	k_{cat}/K_M (M⁻¹s⁻¹)
Arg-Az₁	26.8 ± 3.3	9.3 ± 0.4	3.46 × 10 ³
Arg-Az₃	18.3 ± 0.5	10.5 ± 0.1	5.75 × 10 ³
Arg-Az₅	14.6 ± 2.0	11.0 ± 0.4	7.53 × 10 ³
Arg-Az₃ (BpumL)	22.5 ± 2.3	14.4 ± 0.7	6.38 × 10 ³

Table 2Steady state kinetic parameters for BamL variants against Arg-Az₃

BamL	k_{cat}/K_M (M ⁻¹ s ⁻¹)
Wild-type	57.5×10^2
T38F	3.3×10^2
R42A	4.7×10^2
L132F	3.5×10^2
L162F	1.2×10^2
Y182F	1.7×10^2

Table 3

Bioactivity of PZN truncations compared to PZN. Values reported as minimum inhibitory concentrations (μM).

Organism	Me ₂ -Arg-Az ₁	Me ₂ -Arg-Az ₃	Me ₂ -Arg-Az ₅	PZN
<i>Bacillus anthracis</i> Sterne	>48	12	3	0.75-1.5
<i>Bacillus megaterium</i> 7A1	>48	12	3	>48
<i>Bacillus subtilis</i> 168	>48	24	6	>48
<i>Enterococcus faecium</i> U503	>48	>48	>48	>48
<i>Listeria monocytogenes</i> 4b F2365	>48	>48	>48	>48
<i>Staphylococcus aureus</i> NRS384	>48	>48	3	>48
<i>Streptococcus pyogenes</i> M1	>48	>48	>48	>48
<i>Escherichia coli</i> DH5a	>48	>48	>48	>48
<i>Neisseria sicca</i> ATCC 29256	>48	>48	>48	>48
<i>Pseudomonas putida</i> KT244	>48	>48	>48	>48