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Synthesis of Plantazolicin Analogues Enables Dissection of Ligand Binding Interactions of a Highly Selective Methyltransferase

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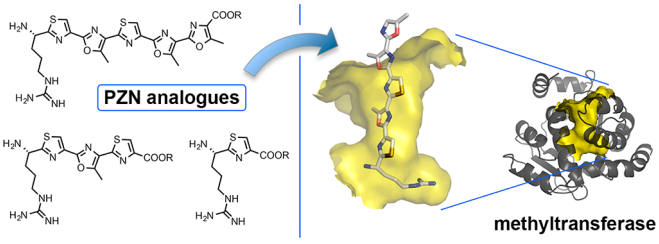
ABSTRACT



A convergent strategy for the synthesis of truncated analogues of plantazolicin (PZN), a member of the thiazole/oxazole-modified microcin (TOMM) class of natural products, has been developed. These *N*-terminal mono-, tri-, and pentazole substructures of PZN were utilized to probe the substrate requirements and thermodynamic ligand binding parameters of an unusually selective PZN methyltransferase (BamL) by isothermal titration calorimetry. Our results demonstrate that the presence of a single *N*-terminal azole permits efficient processing by BamL; however, the substrate binding becomes stronger with increased polyazole chain length.

Plantazolicin (PZN, **1**, Figure 1)[1] is a member of the thiazole/oxazole-modified microcin[2] (TOMM) class of natural products and is produced by select strains of *Bacillus amyloliquefaciens* and *Bacillus pumilus*. PZN possesses highly discriminating antibacterial action against *Bacillus anthracis*, the causative agent of anthrax.[1b] The biosynthesis of PZN involves the post-translational modification of a 41-residue precursor peptide into a polyazole framework (desmethylPZN) via heterocyclization of Cys, Ser, and Thr residues. Subsequent leader peptide cleavage, followed by *N*-terminal dimethylation at Arg, affords the product.[1a] Our previous studies[1a,3] on the biosynthesis of PZN have led to the identification and structural elucidation of a highly selective, *S*-adenosyl-L-methionine (SAM) dependent methyltransferase (BamL) which only methylates the *N*-terminus of compounds highly similar to desmethylPZN. Intriguingly, Arg-containing tetrapeptides, meant to serve as substrate mimics (RGGG, RAAA), were also unprocessed by BamL,[3] which is unusual behavior for a tailoring methyltransferase.[4]

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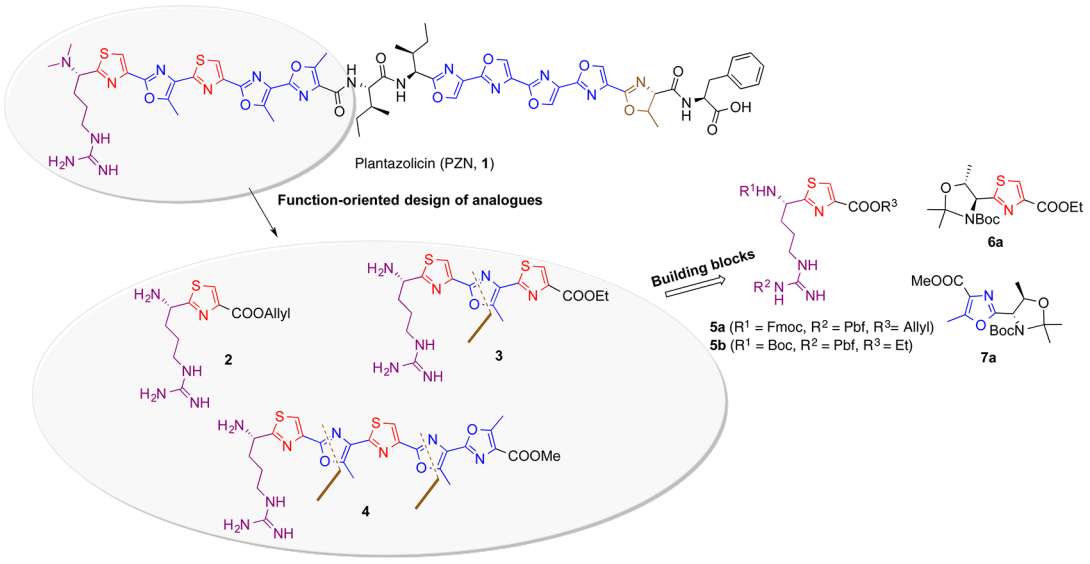

Figure 1. Structure of plantazolicin (**1**), its envisaged analogues (**2**–**4**), and their retrosynthesis into building blocks (**5**–**7**).

Motivated by the remarkable preference of BamL for the Arg-polyazole framework of desmethylPZN, we initiated studies toward a modular synthesis of truncated analogues of PZN. X-ray crystallographic analysis of BamL in complex with S-adenosyl-L-homocysteine (SAH) revealed a deep, narrow tunnel running from the surface of the protein to the active site.[3] As the length of this tunnel is sufficient to accommodate the first 4–5 residues of desmethylPZN, while its narrowness would exclude uncyclized peptides, we hypothesized that it served as the desmethylPZN-binding pocket. Therefore, we envisioned three different Arg-(poly)-azole fragments (Figure 1, **2**–**4**) of increasing length and complexity as putative substrates for probing BamL activity and selectivity. Our synthetic strategy was designed to enable access to sufficient quantities of a panel of biosynthetically inaccessible PZN variants.[5] Such a function-oriented approach[6] would allow us to characterize the relative effect of adding heterocycles to the *N*-terminal framework on BamL-dependent binding and methylation. The envisaged variants were also expected to offer practical utility, as their increased aqueous solubility would facilitate biochemical assays such as isothermal titration calorimetery (ITC) studies with BamL, which was otherwise not possible with the poorly soluble natural substrate, desmethylPZN.[3] In the course of our above work, the Süssmuth group reported the total synthesis of PZN.[7]

Herein, we disclose a convergent synthesis of various PZN analogues and demonstrate their utility to probe the substrate specificity and thermodynamic parameters of BamL activity using ESI-MS end point and ITC binding assays.

Retrosynthetic analysis of the targeted PZN analogues (**2**–**4**, Figure 1), with a goal of a convergent synthetic design, led to the identification of three azole building blocks (**5**–**7**, Figure 1), wherein the latent functional groups were strategically masked by mutually orthogonal protecting groups.[8] Moreover, one of the building blocks (Arg-Thz, **5**, Figure 1) in its deprotected form also served as our simplest synthetic target (**2**, Figure 1).

The synthesis of **2** began with the HCTU/HOBt-promoted coupling of Fmoc-Arg(Pbf)-OH (**9**, Pbf = 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl) with Fmoc-Cys(Trt)-OAllyl (**8**, Scheme 1). The resulting dipeptide **10** was subjected to a one-pot Trt-deprotection and cyclodehydration using Tf_2O/PPh_3O[9] to yield the respective thiazoline. Unfortunately, the chromatographic separation of this highly polar thiazoline from PPh_3O was difficult due to similar retention factors. Gratifyingly, a one-pot procedure proved useful, wherein, after cyclodehydration, the thiazoline was immediately oxidized with MnO_2 to afford the thiazole (**5a**). Unlike the thiazoline, thiazole **5a** was readily separable from PPh_3O (25% yield). Subsequent Fmoc deprotection of **5a**, followed by cleavage of the Pbf group in the presence of TFA/TIPS/H_2O (94:3:3), furnished **2**, our first truncated target. Although we were able to obtain **5a** by a one-pot cyclodehydration–oxidation, the

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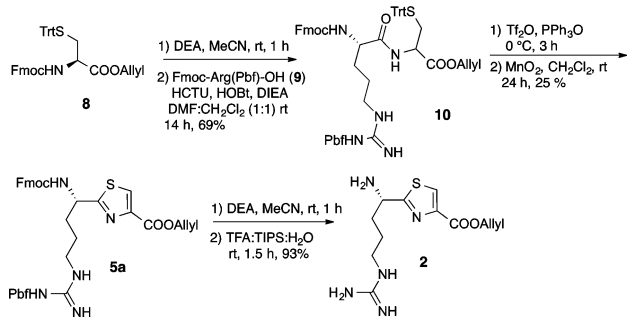
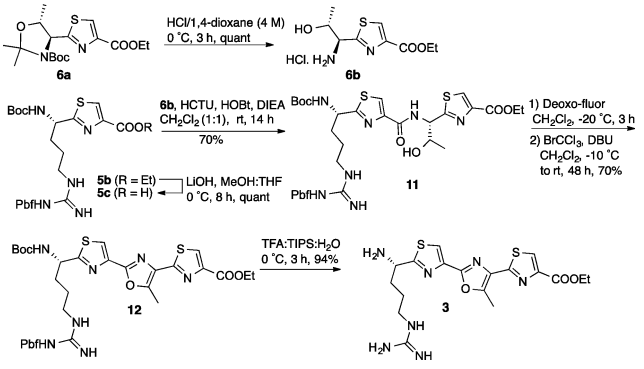
Scheme 1. Synthesis of Monoazole *N*-terminal PZN analogue (**2**)



relatively low yield, coupled with a necessity for sufficient quantities for further chain-elongation steps, led us to adopt the Hantzsch route[10] for the synthesis of related Boc-protected analogue **5b** (Supporting Information (SI)).

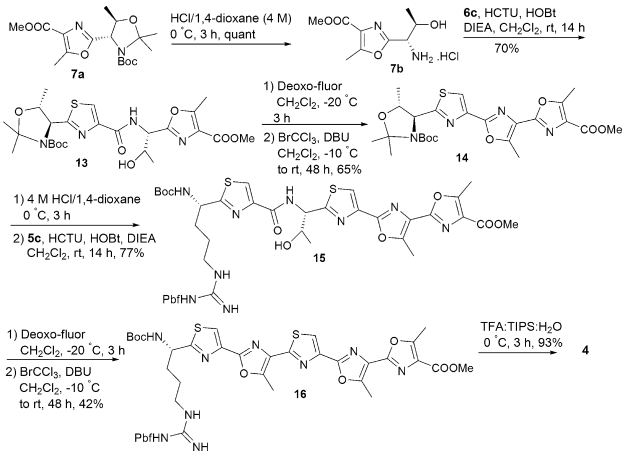
Progressing into the next phase of synthesis (Scheme 2), deprotection of the acetonide-Boc motif of thiazole building block **6a** (SI) revealed the HCl salt of amino alcohol **6b**. In parallel, the ethyl ester of **5b** was hydrolyzed to carboxylic acid **5c**, which was condensed with **6b** using HCTU/HOBt and DIEA. The resulting bisthiazole intermediate **11** was subjected to cyclodehydration by employing Deoxo-fluor,[11] followed by oxidation with $BrCCl_3$/DBU, to afford the triazole (**12**). Finally, simultaneous deprotection of the *N*-Boc and Pbf groups by TFA/TIPS/H_2O furnished the desired triazole analogue (**3**).

Scheme 2. Synthesis of Triazole *N*-terminal PZN analogue (**3**)



Next, we turned our attention to the construction of the complete left-hand fragment of desmethylPZN. The synthesis commenced with the oxazole building block **7a** (SI), whose treatment with HCl revealed the free amino alcohol **7b** (Scheme 3). In parallel, the thiazole ester (**6a**) was saponified to its carboxylic acid (**6c**). The two fragments

Scheme 3. Synthesis of left hand fragment of desmethylPZN (**4**)



(**7b** and **6c**) were then condensed by the action of HCTU/HOBt and DIEA into the bisazole hydroxy amide (**13**). Cyclodehydration of **13** by Deoxo-fluor at −20 °C installed the oxazoline residue, which was oxidized with $BrCCl_3$/DBU (4.2 equiv, 48 h) to give **14**. Treatment of **14** with HCl (4 M, 1,4-dioxane) unmasked the amino alcohol, which was subsequently coupled to **5c**. The resulting tetrazole hydroxy amide **15** was subjected to sequential cyclodehydration−oxidation to furnish the contiguous pentazole framework **16**. In the final step, acid induced dismantling of the *N*-Boc and Pbf groups gave the desired left-hand fragment of desmethylPZN (**4**).

With the mono-, tri-, and pentazole *N*-terminal PZN analogues in hand, we first evaluated whether these compounds were substrates for BamL. Each analogue (**2**−**4**) was screened for conversion into the respective methylated product by treatment with BamL in the presence of SAM. Like desmethylPZN, all three were found to be dimethylated by BamL (as determined by ESI-MS/HRMS and LC-MS, SI) after a 16 h end point assay (condition A, SI). Under these conditions, **3** gave full conversion, while **2** and **4** left detectable levels of unconverted starting material. A more stringent 1 h assay with lower concentrations of enzyme and substrate (condition B, SI) was also conducted to obtain greater resolution into the relative substrate efficiencies. As expected with condition B, we observed decreases in peak intensity by ESI−MS for all of the dimethylated products relative to condition A. However, this decrease was substantially greater for reactions with **2** and **4**, as reaction with **3** was nearly complete under condition B (SI). This suggests that while the presence of one azole is sufficient for BamL processing, the reaction efficiency increases substantially when the substrate has three azole rings (**3**).

To gain deeper insight into the binding interaction of **2**−**4** with BamL, we conducted a series of ITC binding assays (Figure 2). While we obtained binding curves for the monoazole (**2**, $K_d = 2.2\,\mu M$) and triazole fragments (**3**, $K_d = 1.7\,\mu M$), the limited aqueous solubility of pentazole **4** resulted in unreliable data. The addition of an organic

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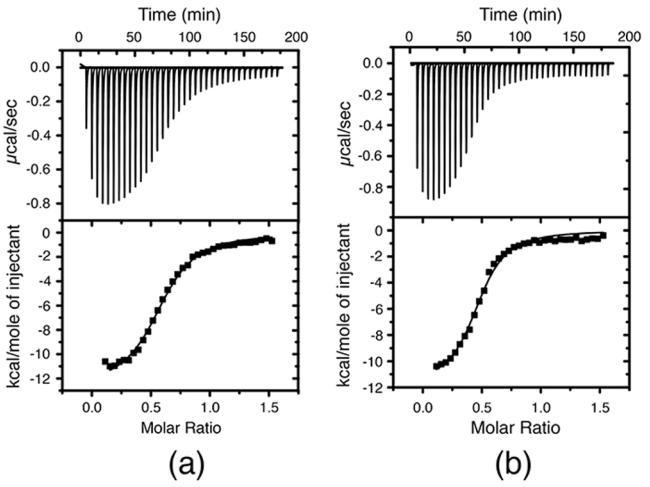
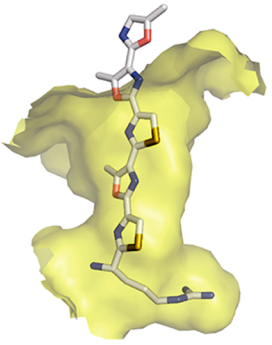


	Ligand 17	2	3
K_d (µM)	225	2.2	1.7
ΔG (kcal/mol)	-4.9	-7.7	-7.8
ΔH (kcal/mol)	-10.7	-12.6	-11.8
TΔS (kcal/mol)	-5.8	-4.9	-4.0

(c)



(d)

Figure 2. ITC data and fitting curves for the binding of **2** (a) and **3** (b), the thermodynamic parameters of binding for the ITC titration of PZN analogues (**2**–**3**) and Arg-NH_2 (**17**) with BamL (c), and a model of the tunnel depicting the binding site for PZN analogues (d).

cosolvent (i.e., 2% v/v DMSO) solubilized **4**, yet the ITC binding curve was uninterpretable.[12] This poor solution behavior of **4** paralleled the highly hydrophobic desmethylPZN, which also yielded uninterpretable data by ITC.[3] Our results underscore the benefit of using small molecule synthesis to probe complex protein–substrate interactions.

Previously, we reported that an even more minimal structure, Arg-NH_2 (**17**), was inefficiently converted to $(CH_3)_2$-Arg-NH_2 by BamL.[3] We again employed ITC to evaluate binding to BamL and measured a K_d of 225 μM (Figure 2c and SI). It is noteworthy that addition of a single thiazole to Arg-NH_2 (**2** vs **17**) increased the affinity for BamL by 2 orders of magnitude. Analysis of the Gibbs free energy terms indicated that the stronger interaction obtained with **3** as compared to **2** was primarily entropically driven. The ITC data also show that differences in BamL affinity were not solely responsible for the enhanced processing of **3** compared to **2**.

In conclusion, we have disclosed a convergent synthesis of biosynthetically inaccessible analogues of PZN comprising the Arg-mono-, tri-, and -pentazole frameworks. The developed building block approach utilizes a strategic combination of Deoxo-fluor-promoted cyclodehydration and Hantzsch thiazole synthesis to install azoles on a peptide backbone. The synthetic PZN analogues enabled the interrogation of ligand binding interactions with BamL, an unusually selective small molecule methyltransferase. All three synthetic fragments were dimethylated in a SAM- and BamL-dependent fashion. ITC studies indicated that the triazole fragment (**3**) possessed marginally higher affinity for BamL than the monoazole (**2**). Due to their superior aqueous solubility compared to desmethylPZN, the synthesized PZN analogues may be more suitable ligands for X-ray crystallography studies with BamL.

(12) The use of 2–2.5% *v*/*v* DMSO as a cosolvent did not lead to inactivation of BamL, as evidenced by extensive dimethylation of **4** under reaction condition A (SI).

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Supporting Information Available. Experimental procedures, characterization data for compounds, and details of enzyme assays. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.