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Arg-Thz is a minimal substrate for the N^{α},N^{α}-arginyl methyltransferase involved in the biosynthesis of plantazolicin†

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The final biosynthetic step towards plantazolicin (PZN) comprises N^{α},N^{α}-arginyl methyltransferase (PznL) mediated N-terminal bis-methylation. We show that PznL processes truncated desmethyl-plantazolicin analogues, but only those with an N-terminal guanidine side chain derived from arginine. PznL specificity, which is narrow, depends on the side chain of the N-terminal amino acid linked to an azole, and not so much on the number of azoles.

Plantazolicin is a linear azole-containing peptide (LAP) (Fig. 1) with an intriguing structure and interesting biological activities. It was isolated from *Bacillus amyloliquefaciens* FZB42 [1] in 2011.[2,3] It's structure was elucidated in 2011 by ^{15}N-isotope labelling and 2D NMR experiments,[4] and the recent accomplishment of its total synthesis allows for the synthesis of partial structures and derivatives.[5] Plantazolicin is unique amongst the known linear azole-containing peptides (LAPs).[2,6] It features two stretches of five and four azole moieties, respectively, with four conjunct azoles at the C-terminus capped with a rare oxazoline entity. Another distinguishing feature concerns the double methylation of the N-terminal arginyl α-amine. Plantazolicin inhibits the growth of a number of *Bacillus* species, but did not show activity against *Staphylococci* and *Enterococci* species.[2]

Studies on the biosynthesis unravelled the mechanism behind plantazolicin formation to a large extent (Fig. 1A).[2,4,7,8] A ribosomally synthesised 41 residue precursor peptide is post-translationally processed by the concerted (though not understood in detail) action of the gene products PznB, PznC and PznD, designated as a trimeric complex of dehydrogenase, cyclodehydratase and docking protein. Cleavage of the leader peptide by the protease, PznE, yields

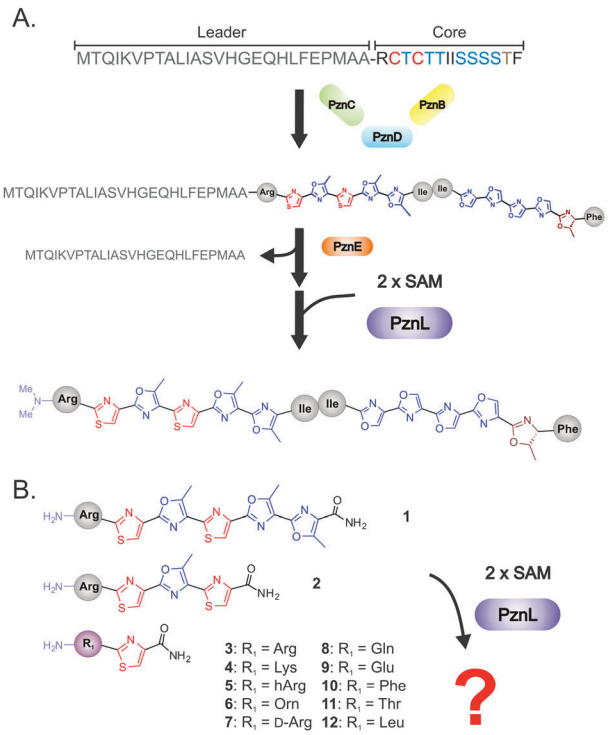

Fig. 1 (A) Suggested biosynthesis of the linear azole-containing peptide (LAP) plantazolicin.[4] (B) Truncated synthetic variants of desmethyl-plantazolicin **1**, **2** and **3** and thiazole side chain variants **4–12** as putative PznL substrates subject of the here-presented study (hArg = homo-arginine).

desmethyl-plantazolicin, which interestingly has not been shown to possess any biological activity. Only after the action of the methyltransferase, PznL, which requires *S*-adenosyl-L-methionine (SAM) as a cosubstrate, biologically active plantazolicin is produced. A recent study by the Mitchell group provided the first insights into the mode of action and specificities of PznL.[8]

Peptide/protein methylation is a widespread modification in nature, yet relatively rarely encountered as a modification of the N-terminal α-amine of ribosomally synthesised and post-translationally modified peptides (RiPPs) or of non-ribosomally synthesised peptides (NRPs).[9–11] Examples of N-terminally

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bismethylated natural products include vancomycin (a NRP) and cypemycin (a RiPP).[12,13] Mitchell and co-workers revealed that PznL acts on desmethyl-plantazolicin and two partially (at the C-terminal oxazoline) hydrolysed forms, but not on some selected oligopeptides lacking a thiazole moiety, but featuring an N-terminal Arg.[8] By making use of synthetic and truncated plantazolicin analogues, we reveal here that PznL in fact requires only a single thiazolyl moiety connected to a guanidine side chain, which emerges by post-translational modification from an N-terminal Arg.

Insight into the molecular mechanisms by which plantazolicin distinguishes between different bacterial strains may be of use in the development of new antibiotics. To this end we set out to express the putative methyltransferase, PznL, and establish its activity profile towards a series of synthetic and truncated substrates differing in both the nature of the amino acid side chains and the number of azoles. Hence, the *pznL* gene was amplified from *Bacillus amyloliquefaciens* RSpMarA2 genomic DNA[2] using primers appropriate for further cloning into Champion™ pET SUMO vector (Invitrogen™) and expressed in *E. coli* Rosetta-gami™ 2(DE3) (Novagen®). The His_6-SUMO-PznL fusion protein was purified by immobilised metal affinity chromatography (IMAC) with a HisTrap column (GE Healthcare) using a ÄKTA 10 purifier system (GE Healthcare) and subsequently re-buffered (50 mM Tris, 300 mM NaCl, 0.5 mM DTT, pH 8.0). We opted to use PznL as an N-terminal His_6-SUMO fusion protein for our studies as we experienced insoluble protein expression using alternative constructs.

The activity of PznL on different truncated plantazolicin substrates was established in an assay mixture consisting of 25 mM Tris–HCl (pH 8.0), 10% glycerol, 60 μM substrate, 500 μM *S*-adenosyl-L-methionine (SAM), 1 mM dithiothreitol and 4.5 μM His_6-SUMO-PznL. For comparison, control samples were incubated with the above buffer but without the enzyme. After incubation for 3 h at 28 °C the reaction was quenched by addition of acetonitrile–water (1 : 1) and the mixture centrifuged. Reaction mixtures were then applied on a reversed phase HPLC column and analysed on an Exactive-Orbitrap mass spectrometer (Thermo Scientific).

First we tested the minimal structure processed by PznL. For this purpose we synthesised peptides **1**, **2** and **3** (Fig. 1 and 2 and Table 1) according to the methodology we developed for the total synthesis of the natural product.[5] As indicated in Fig. 2 all truncated plantazolicin substrates containing one (**3**), three (**2**) and five (**1**) N-terminal thiazoles/5-methyl-oxazoles are fully methylated by PznL. These results downsize the essential recognition motif for plantazolicin methyltransferase PznL to a H-Arg-Thz-NH_2 moiety. Structure elucidation of plantazolicin A revealed the localization of both methyl groups to be at the arginine-derived N-terminus and not at the nitrogens of the guanidine residue.[4,7] To confirm that this is true also for the truncations used here we established that the synthetic N^{α},N^{α}-bismethylated analogue of **1** displays the same elution characteristics on HPLC as *in vitro* bismethylated **1**.

Subsequently, we tested a series of close analogues of H-Arg-Thz-NH_2 (**3**) to assess the substrate specificity of PznL in more detail (see Table 1). Interestingly, none of the substrates containing an alternative basic side chain residue (**4–6**) proved to be viable PznL substrates, and neither the methylene extended homoArg (hArg, **5**) nor the amine analogues ornithine (Orn, **6**) and lysine (Lys, **4**) derivatives were processed by PznL. The same result was observed for compounds **7–11**, where Thz-derivatives of amino acids ranging from non-polar side chains (Leu, **12** and Phe, **10**) to polar neutral (Gln, **8** and Thr, **11**) and acidic (Glu, **9**) side chains were tested. Interestingly, the enantiomer of **3**, H-D-Arg-Thz-NH_2 (**7**), proved to be a good PznL substrate. Finally, H-Arg-Cys-NH_2 (**13**), the unmodified dipeptide corresponding to the minimal substrate, H-Arg-Thz-NH_2 (**3**), did not prove, as expected,[8] to be a viable PznL substrate.

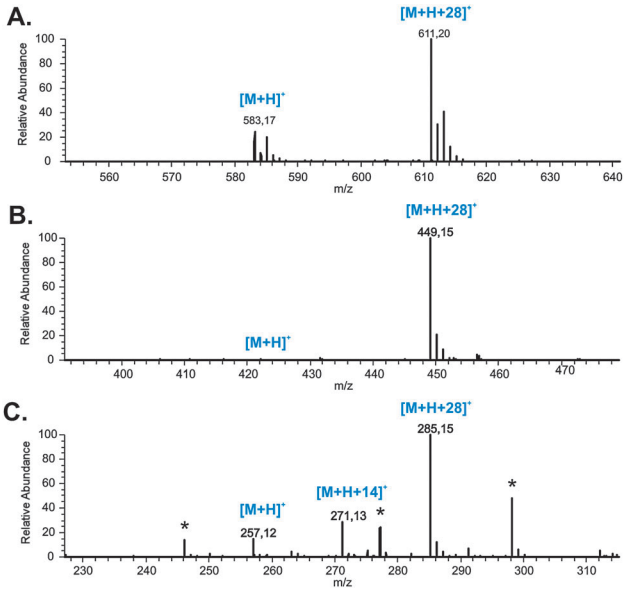

Fig. 2 ESI mass spectra of peptide substrates **1** (A), **2** (B) and **3** (C) incubated with PznL and *S*-adenosyl-L-methionine (SAM).

Table 1 Synthetic substrates for conversions using plantazolicin methyltransferase PznL (Thz = thiazole, Oxz = oxazole, MeOxz = 5-methyl-oxazole)

Compound	Bismethylation
H-Arg-Thz-MeOxz-Thz-MeOxz-MeOxz-NH_2 **1**	+
H-Arg-Thz-MeOxz-Thz-NH_2 **2**	+
H-Arg-Thz-NH_2 **3**	+
H-Lys-Thz-NH_2 **4**	–
H-hArg-Thz-NH_2 **5**	–
H-Orn-Thz-NH_2 **6**	–
H-D-Arg-Thz-NH_2 **7**	+
H-Gln-Thz-NH_2 **8**	–
H-Glu-Thz-NH_2 **9**	–
H-Phe-Thz-NH_2 **10**	–
H-Thr-Thz-NH_2 **11**	–
H-Leu-Thz-NH_2 **12**	–
H-Arg-Cys-NH_2 **13**	–

Having established that PznL does not tolerate modifications in the amino acid side chain grafted onto a thiazole, we looked in more detail at the three truncated desmethyl-plantazolicin analogues **1**, **2** and **3** with the aim to establish which of the three would be the best substrate.

To this end we carried out a number of competition experiments in which we incubated stoichiometric amounts of **1** and **2**, **2** and **3** as well as **1** and **3**, respectively, under the above conditions but with a limiting amount of SAM (substrate : substrate : SAM = 1 : 1 : 2). To qualitatively determine the degree of methylation we compared

the extracted ion chromatograms (XIC) corresponding to singly charged parent ions of the substrate in the assay and negative control (Table S2 and Fig. S5, ESI†). The mixture containing substrates **1** and **2**, featuring three and five azole moieties, gave almost an exclusively bismethylated product in nearly equal amounts, along with starting materials. In contrast, the minimal substrate H-Arg-Thz-NH_2 (**3**) proved to be a poor competitor for both **1** and especially **2** in this experimental set-up, with hardly any bismethylated product stemming from **3** formed along with some monomethylated product (Fig. S4A–C and S5 and Table S2, ESI†).

The above results demonstrate that PznL is capable of producing monomethylated products, and it appears that this happens preferably on relatively poor substrates. To explore this finding further we executed a series of experiments in which **1**, **2** and **3** were incubated with a stoichiometric amount of SAM under otherwise identical conditions and in a time dependent manner. All reactions were started by addition of enzyme and quenched with acetonitrile after 2, 10 or 60 min. For all of the tested compounds signals in the mass spectra corresponding to singly methylated products are observed. Reaction of **1** under these conditions delivers predominantly the bismethylated product, along with considerable amounts of starting material and only marginal amounts of the monomethylated adduct (Fig. S6A, ESI†). In contrast, time-dependent methylation of **2** and **3** under conditions limiting in SAM reveals significantly larger amounts of monomethylated products. These results indicate that the more the structure of the artificial substrate resembles that of the natural substrate (desmethyl-plantazolicin) the higher the ratio of the bismethylated product to the monomethylated product is. Studies of antibacterial activity against the test strain *Bacillus megaterium* were performed in an agar plate diffusion assay of **1**, **2** and **3** and the doubly-methylated analogues of **1** and **3**. However, as suggested earlier[2,7] and in contrast to plantazolicin itself, none of the substrates as well as bismethylated products inhibited bacterial growth (Fig. S2, ESI†).

In summary, we have shown that H-Arg-Thz-NH_2 (**3**), the modified N-terminal fragment of the natural substrate, desmethyl-plantazolicin, is a minimal substrate recognised and processed by the methyltransferase PznL. Although desmethyl-plantazolicin substructures featuring more azole motifs are comparatively better substrates, extended azole stretches are not a requirement for PznL-mediated (bis)methylation. In contrast, the guanidine function of Arg is a decisive structural element, although H-D-Arg-Thz-NH_2 proved to be a viable substrate as well. Extension of the alkylguanidine moiety by one carbon, or substitution with an alkylamine provided non-reactive substrates, and the same holds true for aliphatic, aromatic, polar or acidic analogues. Interestingly, in the recently published crystal structure of PznL, the side chain of an aspartic acid residue (D161) is located in proximity to the SAM binding domain, and the narrow substrate binding tunnel is suggested to accommodate the arginyl side chain of the substrate. The tight fit of the arginyl side chain combined with the charge complementarity of D161 could enforce the observed high substrate selectivity (Fig. S8, ESI†). Furthermore, this observation is in line with a PznL D161A mutant which is deficient in methylation activity.

Our results nicely complement previous ones, which revealed that PznL acts on desmethyl-plantazolicin and two close analogues, but not on peptides featuring a sequence resembling that of the plantazolicin core peptide.[8] Our work further defines the substrate specificity and reveals that depending on the substrate and the available amount of the co-substrate, SAM, PznL is in fact also capable of executing a monomethylation reaction (previously shown to occur only for the PznL Y182F mutant (ref. 8)). Moreover, our work demonstrates that unlike other small-molecule methyltransferases, PznL does not tolerate changes of the N-terminal arginyl-Thz residue,[11] and we therefore suggest to classify PznL as an N^{α},N^{α}-arginyl-thiazole specific methyltransferase.

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