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Structural Characterization of the Highly Cyclized Lantibiotic Paenicidin A via a Partial Desulfurization/Reduction Strategy

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Supporting Information

ABSTRACT: Lantibiotics are ribosomally synthesized antimicrobial peptides produced by bacteria that are increasingly of interest for food preservation and possible therapeutic uses. These peptides are extensively post-translationally modified, and are characterized by lanthionine and methyllanthionine thioether cross-links. *Paenibacillus polymyxa* NRRL B-30509 was found to produce polymyxins and tridecaptins, in addition to a novel lantibiotic termed paenicidin A. A bacteriocin termed SRCAM 602 previously reported to be produced by this organism and claimed to be responsible for inhibition of *Campylobacter jejuni* could not be detected either directly or by genomic analysis. The connectivities of the thioether cross-links of paenicidin A were solved using a novel partial desulfurization/reduction strategy in combination with tandem mass spectrometry. This approach overcame the limitations of NMR-based structural characterization that proved mostly unsuccessful for this peptide. Paenicidin A is a highly cyclized lantibiotic, containing six lanthionine and methyllanthionine rings, three of which are interlocking.

Bacteriocins, ribosomally synthesized antimicrobial peptides produced by bacteria, have great potential for use in food preservation, livestock protection, and medical applications. Bacteriocins from Gram-positive bacteria are diverse with respect to their size, structure, and modifications, and have been classified according to these properties.[1] Class I bacteriocins, more commonly referred to as lantibiotics, are grouped on the basis of their extensive post-translational modifications.[2,3] Lantibiotics are characterized by the presence of lanthionine (Lan) and methyllanthionine (MeLan) residues, which are biosynthesized via a two-step process. First, serine and threonine residues are enzymatically dehydrated, forming dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively.[3] Then, a cysteine thiol nucleophilically attacks the β-position of a Dha or Dhb residue, forming a Lan or MeLan residue, respectively.[3] Nisin, the most well-studied lantibiotic, is currently being used for food preservation in many countries.[2] Several lantibiotics have been chemically synthesized,[4] and the bioengineering of lantibiotics[2] is an active area of research.

Paenibacillus polymyxa NRRL B-30509 was previously reported to produce a class II (or non-lanthionine-containing)[1a] bacteriocin termed SRCAM 602 that inhibited the growth of *Campylobacter jejuni* (Figure 1A).[5] Due to our interest in this unusual activity, we hoped to further characterize this bacteriocin. Using deferred inhibition assays, we found that this organism produced antimicrobial activity against both Gram-positive and Gram-negative bacteria. However, we found no evidence for the production of SRCAM 602, using the reported purification protocol. Following genome sequencing of *P. polymyxa* NRRL B-30509, we found no genetic determinants for the production of SRCAM 602 (unpublished data). Furthermore, a SRCAM 602 structural gene was not found via a PCR-based approach using degenerate oligonucleotide primers [see Figure S1 in the Supporting Information (SI)]. While the production of polymyxins E1 and E2 was detected (Figures 1B and S2), they were not found to be active against *C. jejuni*. This activity was instead attributed to the production of variants of tridecaptin A (Figure 1C). The activity against Gram-positive bacteria was attributed to paenicidin A, a novel lantibiotic.

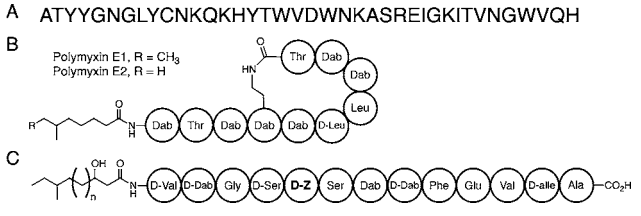

Figure 1. Structures of (A) SRCAM 602,[5] (B) polymyxins E1 and E2, and (C) tridecaptin A_1 (n = 1, Z = Trp), A_3 (n = 3, Z = Trp), and A_4 (n = 3, Z = Phe). Dab = 2,4-diaminobutyric acid; aIle = *allo*-isoleucine.

The amino acid sequences of the tridecaptin A variants were determined using LC-MS/MS (Figure S3). Two variants predominated, which we have termed tridecaptin A_3 and A_4. Tridecaptin A_3 has an amino acid sequence identical to that of tridecaptin Aα (which we suggest be renamed A_1, in addition to renaming tridecaptin Aβ to A_2),[6,7] whereas tridecaptin A_4 only differs based on the substitution of tryptophan-5 with phenylalanine. The MS/MS data further indicate that the lipid chains of the tridecaptin variants reported herein have a mass 28 Da greater than the lipid chain of tridecaptin Aα (or A_1), suggesting the presence of an additional two methylene units. Analysis of the derivatized tridecaptin lipid chains via

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GC-MS showed the presence of two structurally different lipid chains. Based on a comparison of the EI-MS data with previous reports[8] and with a synthetic standard of methyl 3-hydroxy-6-methyloctanoate (unpublished data), these lipid chains were determined to be 3-hydroxy-8-methyldecanoyl and 3-hydroxy-9-methyldecanoyl groups (Figures 1C, S4, and S5). The relative abundance of the two lipid peaks shows a strong preference for the 3-hydroxy-8-methyldecanoyl lipid chain, whereas only a small amount of the other form is incorporated into the mature peptide (Figure S4).

The lantibiotic paenicidin A ($[M+H]^+$ = 3376.5) was analyzed by MS/MS sequencing, but only limited fragmentation was observed. Still, a short amino acid sequence was obtained from these data, judged to be toward the C-terminus of the peptide. The genome sequence was then searched for an open reading frame encoding this amino acid sequence. Taking into consideration the size of the peptide, in addition to the estimated position of the known amino acid sequence within the peptide, a putative structural gene was identified. This gene was clustered with other genes predicted to play a role in lantibiotic maturation and export (Figure 2A).[2,3]

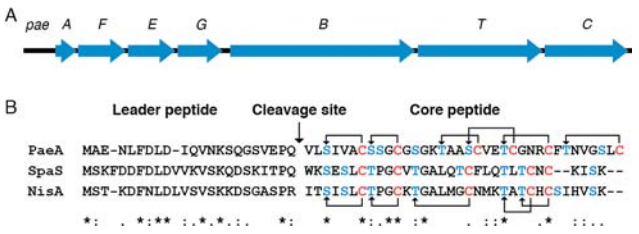

Figure 2. (A) Paenicidin A biosynthetic gene cluster. (B) Comparison of the paenicidin A prepropeptide with that of subtilin (SpaS) and nisin (NisA). Cysteines are indicated in red, and serines and threonines are in blue.

In addition to the lantibiotic structural gene (*paeA*), the gene cluster encodes separate dehydratase (PaeB) and cyclase (PaeC) enzymes.[2,3] Lantibiotic dehydratases are responsible for dehydrating serine and threonine residues, whereas the cyclase enzymes catalyze the nucleophilic attack of a cysteine onto the dehydrated residues in a Michael fashion.[3] A dedicated lantibiotic ABC transporter (PaeT) was also encoded, likely required for the export of paenicidin A. Further, the genes *paeFEG* encode a putative ABC transporter that might provide immunity to the bactericidal effects of paenicidin A.[9] PaeE and PaeG are predicted to be the membrane-spanning domains of the ABC transporter, whereas PaeF is likely a cytosolic ATP binding protein. Notably absent from the gene cluster was a protease (i.e., *lanP*) responsible for cleaving the leader peptide of the paenicidin A precursor peptide. However, this cleavage could be accomplished by a protease encoded elsewhere in the genome, as has been reported for other lantibiotics, such as subtilin.[10] Also lacking in this gene cluster was a dedicated immunity protein (i.e., *lanI*-type)[9] to protect the producer organism from the bactericidal effects of paenicidin A. The nucleotide sequence has been deposited into GenBank (accession number JX679672).

The amino acid sequence of the paenicidin A precursor peptide was compared with that of other lantibiotics, including subtilin and nisin (Figure 2B). This alignment revealed relatively high homology between the leader peptides, and suggested a cleavage site following the amino acid sequence PQ, as has been observed for other lantibiotics.[3b] Comparison of the predicted monoisotopic mass of the paenicidin A core peptide (3483.5 Da) with the monoisotopic mass of mature paenicidin A (3375.5 Da) suggested that the peptide is dehydrated six times during maturation. The formation of dehydroalanine and dehydrobutyrine residues (and subsequently Lan and MeLan residues) requires the loss of water. As paenicidin A has six cysteine residues, it is possible that they are all involved in Lan and MeLan formation. To exclude the possibility of disulfide bridges, paenicidin A was first incubated with dithiothreitol, and then iodoacetamide. No alkylation of cysteine residues was observed via MALDI-TOF MS (data not shown). This, taken with the predicted loss of six water molecules, indicates that paenicidin A contains six Lan and MeLan residues. The absolute configurations of the Lan and MeLan residues were determined using GC-MS, which showed the presence of DL-Lan and DL-MeLan exclusively (data not shown), as has been observed for other lantibiotics.[3a,4]

In addition to six cysteines, the paenicidin A precursor peptide contains nine serines and threonines (Figure 3A). This

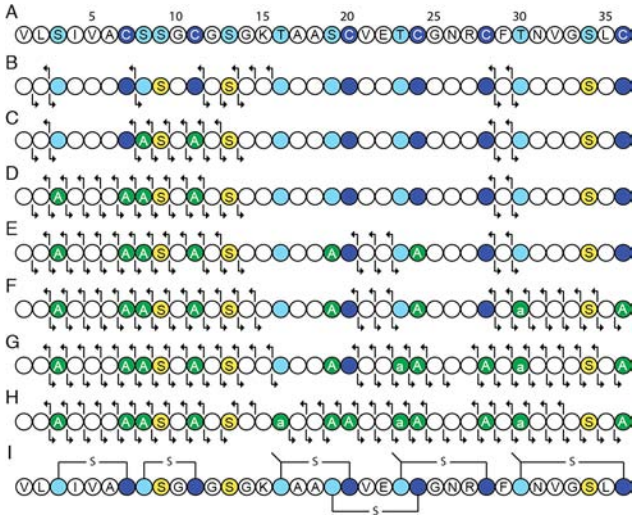

Figure 3. Partial desulfurization/reduction MS/MS results. (A) Primary sequence of paenicidin A. MS/MS data summarized for (B) natural paenicidin A, (C) monodesulfurized, (D) didesulfurized, (E) tridesulfurized, (F) tetradesulfurized, (G) pentadesulfurized, and (H) fully desulfurized paenicidin A (b-ions indicated above, y-ions below). Serines and threonines are indicated in light blue, cysteines in dark blue, unmodified serines in yellow, and desulfurized residues in green. 2-Aminobutyric acid is represented by the letter a. (I) Connectivity of paenicidin A.

gives rise to a large number of possible connectivities for the Lan and MeLan residues. Although the MS/MS sequencing results provided only limited fragmentation data, the lack of fragmentation in certain regions was found to be informative (Figure 3B). Fragmentation between amino acids situated within Lan and MeLan-containing rings is typically not observed by MS/MS, and so it was possible to map out some of the connectivity based on which regions did not show any fragmentation. Following this approach, the tentative assignment of three Lan and MeLan rings was obtained. No fragmentation was observed between residues 3 through 7, whereas fragmentation was observed between residues 7 and 8, with a b-ion mass indicating the dehydration of Ser-3. This suggests that residues 3 and 7 are modified into a Lan residue, wherein Ser-3 is first dehydrated and then nucleophilically

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attacked by Cys-7. Based on similar evidence, connections between Ser-8 and Cys-11, and between Thr-30 and Cys-36 are suggested. With a tentative assignment of the connectivity of the termini, we then turned to the central portion of paenicidin A. However, no fragmentation was observed in this region, preventing any structural determination. This is as expected, as the sequence of the cysteine, serine, and threonine residues is such that the three remaining Lan and MeLan rings are necessarily interlocking.

We initially attempted to determine the connectivity of paenicidin A using NMR spectroscopy, but the solubility was poor under the conditions tested. Instead, we decided to try reductive desulfurization, a chemical modification approach that has been previously used to linearize lantibiotics, allowing for the study of cyclic regions.[11−13] Treatment with nickel boride and hydrogen gas cleaves the thioether bridges found in Lan and MeLan residues, rendering the linearized peptide amenable to analysis by Edman sequencing[11] or MS/MS analysis.[12,13] We fully desulfurized paenicidin A and analyzed the resultant peptide with MS/MS (Figure 3H). However, no information was obtained regarding the connectivity of the central interlocking region of paenicidin A, as all of the Lan and MeLan residues were desulfurized into Ala and 2-aminobutyric acid (Abu) residues. These data did, however, confirm that Ser-9, Ser-13, and Ser-34 are not dehydrated and are not involved in Lan or MeLan formation.

Following the desulfurization reaction, it was observed that paenicidin A was not fully desulfurized. Several different states of desulfurization were observed, ranging from the tridesulfurized peptide to the fully desulfurized peptide. Treatment of paenicidin A with the desulfurization conditions for a shorter period of time yielded a different population of desulfurized peptides, ranging from the monodesulfurized peptide to the tetradesulfurized peptide. This prompted us to analyze the MS/MS data for these partially desulfurized peptides. It was our hope that desulfurization of just one of the three central interlocking rings would allow for fragmentation to be observed in the central region, and reveal the connectivity. Indeed, LC-MS/MS analysis of one of the tridesulfurized states allowed for the determination of the full connectivity of paenicidin A (Figure 3E). Fragmentations were observed between residues 20 through 23, which were absent from the mono- and didesulfurized states (Figure 3C,D). This indicated that a Lan residue was formed from Ser-19 and Cys-24. Furthermore, this required that the two remaining MeLan rings be formed between Thr-16 and Cys-20, and between Thr-23 and Cys-28 (Figure 3I). This was supported by the MS/MS data for the other desulfurized states of paenicidin A (Figure 3). On the basis of these data, the hexacyclic structure of paenicidin A was determined (Figure 4). To our knowledge, this is the first time that a partial desulfurization/reduction approach has been used to determine the connectivity of a lantibiotic.

The order in which Lan and MeLan residues were desulfurized appeared to depend on two factors. First, Lan residues were desulfurized more readily than MeLan residues, potentially due to steric effects. Second, Lan and MeLan residues near the termini were preferentially desulfurized, which was likely a matter of accessibility. Still, these trends were not absolute. For instance, different monodesulfurized peptides were observed which had been desulfurized in different positions.

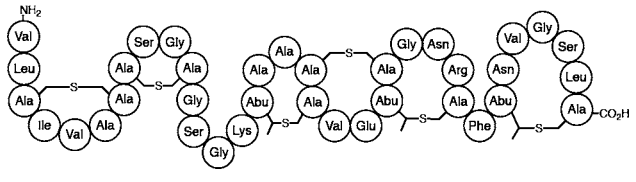

Figure 4. Primary structure of paenicidin A.

In an attempt to confirm the Lan and MeLan connectivities determined through MS/MS, we acquired TOCSY and NOESY data sets for paenicidin A. Despite the poor solubility of the peptide, it was possible to assign the majority of the proton chemical shifts (see SI). The NOESY data set was examined for NOEs between β- and/or γ-protons belonging to different spin systems across the thioether bridges.[11,14] These results confirm the presence of a Lan residue between positions 8 and 11, a MeLan residue between positions 16 and 20, and a MeLan residue between positions 23 and 28 (Figure S6). However, confirmation of the three other Lan and MeLan residues was not possible based on these NMR experiments due to poor NOE intensities and spectral overlap.

The primary structure of paenicidin A (Figure 4) features six Lan- and MeLan-containing rings, three of which are interlocking. This extent of cyclization in a lantibiotic comes second only to geobacillin I, which is heptacyclic.[14] The positions and sizes of the two N-terminal Lan-containing rings resemble the well-studied N-terminal region of nisin (Figure 2B).[2] This may suggest that it plays a similar role in lipid II-binding and inhibition of peptidoglycan biosynthesis.[15] To our knowledge, the tricyclic interlocking central region of paenicidin A represents a novel structural motif. This region may be involved in pore formation, as has been found for other lantibiotics.[2]

The difficulties encountered during our attempts to structurally characterize paenicidin A via NMR spectroscopy demonstrate the usefulness of our partial desulfurization/reduction MS/MS approach. Importantly, this approach is not dependent on obtaining concentrated solutions of the lantibiotic, which was not possible for paenicidin A. Furthermore, microgram quantities of peptide are sufficient for this approach, instead of the low milligram quantities often required for NMR experiments and which may not be easily attainable for some lantibiotics. Sample preparation is also simplified, as the coupling of MS/MS with LC greatly decreases the need for sample purity relative to NMR. Proton chemical shift assignment can be labor-intensive, whereas the analysis of MS/MS data is far simpler. Finally, MS/MS experiments can be conducted in a matter of hours, whereas acquiring NMR spectra may require several days. Based on these qualities, the partial desulfurization/reduction and MS/MS analysis of lantibiotics should prove to be a generally applicable method for the structural characterization of lantibiotics, especially those containing interlocking rings. As more genomic data are amassed, many more lantibiotics will be identified in the near future, and this methodology will likely prove useful for their characterization. Furthermore, this methodology may also be used for the structural characterization of the growing number of non-lantibiotic thioether bridge-containing peptides, including peptides such as those similar to thurincin H.[16]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, genetic probing results, polymyxin and tridecaptin MS/MS and GC-MS data, paenicidin A

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spectrum of activity, paenicidin A gene cluster analysis, and paenicidin A proton chemical shifts. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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