Insights into the evolution of lanthipeptide biosynthesis

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Abstract: Lanthipeptides are a group of posttranslationally modified peptide natural products that contain multiple thioether crosslinks. These crosslinks are formed by dehydration of Ser/Thr residues followed by addition of the thiols of Cys residues to the resulting dehydroamino acids. At least four different pathways to these polycyclic natural products have evolved, reflecting the high efficiency and evolvability of a posttranslational modification route to generate conformationally constrained peptides. The wealth of genomic information that has been made available in recent years has started to provide insights into how these remarkable pathways and their posttranslational modification machineries may have evolved. In this review, we discuss a model for the evolution of the lanthipeptide biosynthetic enzymes that has recently been developed based on the currently available data.

Keywords: lantibiotics; dehydration; cyclic peptide; posttranslational modification; glutamylation; nisin; phosphothreonine lyase; kinase; lanthionine; labionin

Introduction

The genome sequencing efforts of the past decade have illustrated that ribosomally synthesized and

posttranslationally modified peptides (RiPPs) are much more prevalent than previously anticipated.¹ RiPPs are encoded in the genomes of organisms in

Abbreviations: Dha, dehydroalanine; Dhb, dehydrobutyryne; Lan, lanthionine; LanA, lanthipeptide precursors; LanB, lanthipeptide dehydratase for class I lanthipeptides; LanC, lanthipeptide cyclase for class I lanthipeptides; LanCL, LanC-like proteins; LanKC, lanthionine/labionin synthetase for class III lanthipeptides; LanL, lanthionine synthetase for class IV lanthipeptides; LanM, lanthionine synthetase for class II lanthipeptides; MeLan, methylanthionine; RiPPs, ribosomally synthesized and posttranslationally modified peptides.

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BROADER AUDIENCE STATEMENT: Antibiotic resistance is a growing health concern. With the rapid increase of the number of

bacteria with fully sequenced genomes, researchers can now scan these genomes for genes that may be involved in the production of antibiotics. Ribosomally synthesized and posttranslationally modified peptides (RiPPs) are particularly suited for such genome mining efforts. These studies have not only resulted in the discovery of new antibiotics, but have also provided a wealth of information regarding their potential evolutionary origins.

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all domains of life, their structural diversity is vast, and their biological activities are equally diverse. Most RiPPs are biosynthesized from a larger precursor peptide that consists of a core peptide that is converted to the final product and an N- or C-terminal extension called the leader or follower peptide, respectively. These extensions guide many, but not always all, of the posttranslational modifications that take place in the core peptide.² RiPPs are typically macrocyclic, similar to the high incidence of cyclization in non-ribosomal peptides. Cyclization can offer several advantages such as increased metabolic stability, improved cellular uptake, and preorganization to recognize cellular targets.

Lanthionine-containing peptides, or lanthipeptides, are the largest group of RiPPs based on the frequency of their biosynthetic gene clusters in the currently available genomes. The lanthionine structure, abbreviated as Lan, consists of two alanine residues that are linked through a thioether that connects their β -carbons (Fig. 1). Many lanthipeptides also contain methylanthionines, abbreviated as MeLan, which carry an additional methyl group on one of the β -carbons (Fig. 1). Biosynthetically, lanthionines and methylanthionines originate from Ser and Thr residues that are first dehydrated to generate dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues respectively [Fig. 1(A)].³ Subsequently, the thiol of a Cys is added across the carbon–carbon double bond of these dehydroamino acids in a Michael-type addition to produce the Lan and MeLan structures, respectively [Fig. 1(B)]. Since typical lanthipeptide substrate peptides have multiple Ser, Thr, and Cys residues, the final products are polycyclic and display remarkable diversity in structure as illustrated by a select set of lanthipeptides depicted in Figure 2. Currently known lanthipeptides have a range of different activities including antimicrobial (called lantibiotics),⁴ morphogenetic,⁵ antiviral,⁶ and antiallodynic.⁷ The longest known family member, nisin (Fig. 2), has been used for more than 50 years as a food preservative to combat food-borne pathogens.⁸ In addition, several other lanthipeptides are currently under development for various therapeutic applications including a semisynthetic analog of actagardine,⁹ the chlorinated lantibiotic microbisporicin,^{10,11} duramycin,¹² and labyrinthopeptin A2 (Fig. 2).⁷

Four Routes to Lanthipeptides: Possible Evolutionary Origins

Although all lanthipeptides are made by dehydration of Ser and Thr residues followed by the addition of Cys residues to the resulting dehydroamino acids, the catalytic machinery that carries out these reactions is remarkably diverse. At present, four different enzymatic processes to lanthionines have been identified,³ some of which are evolutionarily related (Fig. 3). For nisin and other class I lanthipeptides,

the dehydration reaction is carried out by LanB dehydratases and the cyclization by LanC cyclases. The LanB enzymes are large, about 120 kDa, and do not have homology with any other characterized proteins in the databases. NisB, the LanB dehydratase involved in nisin biosynthesis, requires ATP, glutamate, and an as yet unidentified macromolecule to carry out the dehydration of eight Ser/Thr residues in the precursor peptide.¹³ In this process, the hydroxyl groups of Ser and Thr are first converted to glutamyl esters, which are then eliminated to form the carbon–carbon double bonds [Fig. 1(A)]. The transient glutamylation constitutes a novel posttranslational modification of a peptide or protein; the only other known examples of glutamylation of Thr occur not on a peptide or protein substrate but rather on the enzymes glutaminase-asparaginase and γ -glutamyltranspeptidase as a catalytic acyl enzyme intermediate during the hydrolysis of glutamine and glutathione, respectively.^{14,15} At present, it is not known whether the glutamylation of lanthipeptide precursors involves the α or γ carboxylate of Glu.

The addition of the thiol of Cys to Dha and Dhb catalyzed by NisC, the cyclase involved in nisin biosynthesis, is believed to involve an active site Zn^{2+} ion that was identified by both spectroscopic and crystallographic studies.^{16,17} Analogous to other enzymes that activate thiol nucleophiles such as farnesyl transferase,¹⁸ the Zn^{2+} ion is believed to activate the Cys thiols in the substrate peptide [Fig. 1(B)]. Not only is the mechanism of catalysis similar, NisC also has structural homology with farnesyl transferase, sharing the same α, α -barrel toroidal fold, with a Zn^{2+} at the top of the barrel.¹⁷ Interestingly, whereas LanB enzymes have no sequence homologs other than other putative dehydratases, LanC-like (LanCL) proteins of unknown function are also found in a wide variety of higher organisms including plants,¹⁹ insects, and mammals.^{20,21}

The three other classes of lanthipeptides are produced by multifunctional lanthionine synthetases (Fig. 3). The class II enzymes, generically called LanM, first phosphorylate Ser/Thr residues in the substrate peptides, followed by elimination of the phosphate to generate the dehydroamino acids [Fig. 1(A)].²² This net dehydration activity is located in the N-terminal domain of LanM proteins, which are about 110–120 kDa in size. Like the LanB proteins, the dehydratase domains of LanM proteins have no clear sequence homologs in the protein databases other than other class II lanthionine synthetases, but the C-terminal cyclase domains of LanM proteins have clear sequence homology with the class I LanC cyclase enzymes, including the conserved Zn^{2+} ligands (Fig. 3).

Class III and IV lanthionine synthetases, generically called LanKC and LanL, respectively,

A. Dehydration

B. Cyclization

Figure 1. A. The dehydration step in lanthionine formation involves activation of the side chain hydroxyl group of Ser and Thr to generate dehydroamino acids (blue). For the class I enzyme NisB involved in nisin biosynthesis, activation involves glutamylation (magenta). As discussed in the text, it is currently not known if the ester bond is made with the α or γ carboxylate of glutamate. For class II–IV antibiotics, activation of the hydroxyl group involves phosphorylation (orange) followed by phosphate elimination. B. The cyclization step in lanthipeptide biosynthesis also comes in different flavors. For class I, II, and IV lanthipeptides it is believed that cyclization involves activation of the Cys nucleophile (red) by an active site Zn^{2+} . For class III lanthipeptides, the Zn^{2+} is not present in the cyclase and thiol activation is achieved by an alternative, currently unknown mechanism. Class III cyclization is also unusual in that it results either in the formation of (methyl)lanthionines or the formation of labionins, which contain a carbon–carbon crosslink formed from one Cys (red) and two dehydroalanines (blue and green).⁷ An example is labyrinthopeptin A2 shown in Figure 2. Abu, 2-aminobutyric acid; Lab, labionin.

display an interesting domain architecture. These enzymes contain a central domain with clear sequence homology with Ser/Thr protein kinases (Fig. 3).²³ Indeed both classes of enzymes have been shown to phosphorylate the Ser and Thr residues in

the substrate peptide that are destined to be dehydrated,^{24,25} similarly to the catalytic strategy employed by the class II LanM enzymes. However, the elimination of the phosphate group to form the alkenes appears to have evolved differently because

Figure 4. A. Cytoscape diagram showing the various cyclase-containing enzymes. In green are the class I LanC clade, in red the class II LanM clade, in magenta the class III LanKC enzymes, in blue the class IV LanL proteins, and in yellow and turquoise, the mammalian LanCL1 and LanCL2 proteins, respectively. B. Bayesian MCMC phylogeny of the class I, II, and IV cyclization enzymes as well as the LanCL proteins found in *Eukarya*. The ProcM-like enzymes are found in a separate subclade in the LanM clade.³⁶ Color scheme is the same as in panel A.

For class III and IV this is most obvious, as fusion of protein kinase and pThr lyase domains created a dehydratase. Whereas most protein kinases and pThr lyases have evolved to be highly specific with respect to their substrates, the respective domains of class III and IV lanthipeptide synthetases appear to have maintained the low substrate specificity of primitive progenitors, but they have become dependent on the leader peptides for activation. For class II LanM enzymes, it remains to be established whether phosphorylation and elimination occur in the same or different active sites. The origin of the class I LanB dehydratases is less clear. Presumably, their ancestor was involved in activation of glutamate or a glutamate derivative, but for what purpose is currently not clear, especially because an unknown macromolecule is required for LanB activity.¹³ Identification of this component may provide insights into the evolutionary origin of LanB proteins.

It is interesting that whereas the dehydration enzymes apparently have independently evolved at least three times, the cyclization domains all have clear sequence homology. The closer relation of the four cyclization domains/enzymes is somewhat surprising since Michael-type addition of Cys residues to dehydroamino acids is a relatively facile process that even takes place readily in the absence of any enzyme.^{31–35} Hence, a priori one might have expected greater divergence in the enzymes that catalyze the cyclization reaction. For the Zn-dependent enzymes, presumably their ancestor had a different activity involving a Zn^{2+} and they were recruited because they accelerated the Michael addition process and/or they fortuitously favored the formation of products with a ring topology that provided an evolutionary beneficial activity. Interestingly, the phylogenetic analysis shows that the eukaryotic LanCL proteins are closer related to the cyclization

domain of class II LanM proteins than the standalone LanC enzymes [Fig. 4(A)].

Lanthipeptide Precursor Peptides and Their Possible Evolutionary History

The most challenging task for the lanthipeptide biosynthetic enzymes is control over the ring topology of their products. As can be seen from the structures of a small subset of currently known lanthipeptides in Figure 2, each cyclase enzyme generates a series of rings that all have different sequences and sizes. To provide an idea of this challenge, for nisin the dehydrated substrate peptide contains five Cys and eight dehydroamino acids (Fig. 5). Thus, the number of isomers differing in ring topology that can be generated by a cyclization process lacking any selectivity would be 6720.³⁷ When also considering all of the possible stereoisomers that can be formed, this number would be at least 8.6×10^5 . However, NisC generates a single product out of all these potential structures. How the cyclization enzymes achieve these feats, which would be very challenging for a chemist, is still an open question, but some insights have recently started to emerge. One possibility is that the fully dehydrated intermediate drawn in Figure 5 actually is never formed. For instance, it is possible that the dehydratase and cyclase may be alternating in modifying the substrate peptide such that cyclization commences well before all dehydrations have taken place.^{38,39} This scenario would decrease the number of electrophiles that each Cys has to distinguish. Indeed, directionality has been observed for several lanthipeptide synthetases.^{28,38–40}

One enzyme, one substrate, one product

For many years, the lanthipeptide biosynthetic gene clusters that were sequenced all contained a single

Figure 5. Biosynthesis of nisin A.

gene encoding the precursor peptide and a single set of genes encoding the biosynthetic enzymes acting on that peptide. These enzymes were believed to produce just a single product, or in some cases a few structurally highly related products (e.g., nisin is made as a mixture of compounds in which Ser33 sometimes escapes dehydration). Importantly, the ring topology of the products was always identical. Lanthipeptide engineering studies meanwhile showed that both *in vivo* and *in vitro*, lanthionine synthetases were highly tolerant with respect to their substrates; that is they were able to catalyze formation of products very different from the ones for which they evolved. The key to this demonstrated substrate tolerance is that the non-native peptide sequences need to be attached to a native leader peptide.^{41–44} The leader peptide has been proposed to activate the biosynthetic enzymes via an allosteric type mechanism^{37,45}; once activated, the biosynthetic enzymes display remarkable substrate tolerance with respect to the sequence of the core peptide. The dehydration reaction is most forgiving, whereas the substrate tolerance of the cyclization step is typically more restricted. The evolutionary

origin of the leader peptide is not clear, but some interesting observations have been made. For instance, the operon structure and leader peptide sequence of several class II lanthipeptides have strong similarity to those involved in the biosynthesis of non-lanthionine-containing bacteriocins.^{46–48} This observation suggests that the recruitment of the *lanM* gene may have resulted in the transformation of a non-posttranslationally modified bacteriocin to a class II lanthipeptide.

One enzyme, many substrates, many products

Although the substrate tolerance demonstrated by various lanthipeptide biosynthetic enzymes has been used for the preparation of various non-native cyclic peptides,^{41–44} this characteristic was thought to be non-physiological until some naturally occurring examples emerged in which more than one substrate was acted upon by a single set of biosynthetic enzymes to make structurally diverse products. The first such reported example was the production of two peptides with different ring topology, ericin A and S, that were made by one set of class I biosynthetic enzymes in *Bacillus subtilis* A1/3 [Fig.

Figure 6. A. Structures of ericin A and S that have different ring topologies in their C-termini but are made by one set of biosynthetic enzymes. Color coding is as in Figure 2. B. Sequences of 29 substrate peptides encoded on the genome of *Prochlorococcus* MIT 9313. Sixteen of these that have been investigated thus far are substrates for ProcM.⁵⁰ The leader peptides are highly conserved and display homology with Nif11 nitrogen fixing proteins,⁵¹ but the core peptides are highly diverse. Fully conserved residues in the leader peptide are shown in light blue and highly conserved residues are shown in green. Ser/Thr residues in the core peptide are shown in dark blue and Cys residues in red. C. Representative group of prochlorosin (Pcn) products that are generated from the corresponding substrates in panel B.

6(A)].⁴⁹ This study received relatively little notice but heralded the discovery that the genomes of various genera of marine cyanobacteria such as *Prochlorococcus* and *Synechococcus* contain a single gene encoding a class II lanthipeptide synthetase but tens of genes encoding substrate peptides.⁵⁰ These substrates have highly conserved leader peptides, but hypervariable core peptides [e.g., Fig. 6(B)]. For the LanM synthetase ProcM from *Pro-*

chlorococcus MIT9313, it has been shown that the enzyme converts a highly diverse set of substrates termed ProcAs into a library of cyclic peptides [Fig. 6(B,C)].^{50,52} This system provides a novel example of natural combinatorial chemistry, the process in which a pool of different compounds are converted to a panel of different products by a common set of chemical reactions.⁵³ The ProcM products that have been characterized thus far by NMR spectroscopy

and/or mass spectrometry are remarkably diverse with respect to ring topology, including rings of different sizes and sequence [Fig. 6(C)].⁵² How one enzyme might generate this highly diverse group of structures is still unresolved in the absence of structural information on the ProcM enzyme, but it is difficult to envision how one active site could actively enforce the formation of all of these rings. We have suggested that perhaps the substrate peptides themselves might have a propensity to form certain ring topologies, possibly promoted by interactions with the surface of the modification enzymes (e.g., burying of hydrophobic side chains).³⁶

Interestingly, the class II ProcM-like synthetases from *Prochlorococcus* and *Synechococcus* that based on genomic data appear to have a large number of physiological substrates are all in the same clade of the phylogenetic tree of Zn-dependent cyclization enzymes [Fig. 4(B)]. This observation suggests that there is something special about these enzymes that allows them to act on so many different substrates. One aspect in which they differ from other cyclization enzymes is that they have three Cys ligands to the Zn²⁺ instead of two Cys and one His.⁵² Increasing the number of thiolates on Zn²⁺ ions is known to increase the reactivity of the thiolates,^{54–57} which may be related to the substrate tolerance. Not only are the enzymes that appear to engage in combinatorial biosynthesis all found in one clade, the substrate peptides for many members of this group of enzymes are also very different from those of other lanthipeptides. The leader peptides of these substrates are much longer, typically about 70–80 amino acids [Fig. 6(B)], and they appear to have been repurposed from Nif11 nitrogen-fixing proteins,⁵¹ the first clue as to the precursors from which the leader peptides may have evolved. Another interesting question is whether this clade of enzymes is primitive and has not yet evolved to make a single product or alternatively whether the enzymes are highly evolved for broad substrate tolerance. For the latter case, it would raise the question of how such substrate tolerance would be maintained during evolution. One possibility is that at least two of the many products provide an evolutionary advantage. If those two products have very different structures, it may prevent the enzyme from evolving such that it efficiently makes one beneficial product.

Some support for the model that the sequence of the core peptide may determine the ring topology of the product formed by ProcM has been obtained. For instance, the enzyme was able to correctly process a chimeric substrate consisting of a ProcA leader peptide and the core peptide of a completely unrelated lanthipeptide called lactacin 481.³⁶ Also, the nisin biosynthetic enzymes NisB and NisC were able to produce a bioactive epilancin 15X analog from a chi-

meric substrate containing the NisA leader and ElxA core peptides. In addition, similar ring topologies found in diverse lanthipeptides from different organisms are produced by enzymes that have low phylogenetic similarity, consistent with a potential preference for the ring topology being inherent to the core peptide. Of course, it is also possible that similar ring topologies are the outcome of convergent evolution driven by the biological activity of certain ring structures.

A recent study based on stereochemistry supports a model for the outcome of the cyclization process in which the core peptide sequence is more important than previously anticipated. Cytolysin is a lytic substance produced by *Enterococcus faecalis* that is believed to be a virulence factor.⁵⁸ Its structure was recently determined and it consists of the two lanthipeptides shown in Figure 7.⁵⁹ Determination of the stereochemistry of the Lan and MeLan residues provided a surprise. The C-ring of the large subunit and the B-ring of the small subunit displayed the expected *meso* stereochemistry (2*S*,6*R*, Fig. 7, R = H) that had been found for lanthionines in all lanthipeptides up to that point. However, the A and B-rings of the large subunit and the A-ring of the small subunit have different stereochemistry that results from addition to the opposite face of the alkene.⁵⁹ It is difficult to envision how the lanthionine synthetase active site might force addition to one face of the double bond for some of these rings and the opposite face for the other rings, especially since all rings in cytolysin are made up of five amino acids. On the other hand, the rings that form the unusual stereochemistry all have in common that they are made by cyclization of a DhxDhx XxxXxxCys motif (Dhx = Dha or Dhb; Xxx is any amino acid except Ser, Thr, or Cys).⁵⁹ Hence, it appears that the conformation imparted by two consecutive dehydroamino acids presents the opposite face of the alkene to the zinc-bound Cys thiolate to result in the observed alternative stereochemistry. Indeed, a search for this sequence motif in other lanthipeptides, and subsequent determination of the stereochemistry of the (Me)Lan residues,⁵⁹ identified other examples of the alternative stereochemistry suggesting that this is a general determinant of stereochemistry that is governed by the sequence of the core peptide and not the enzyme.

One enzyme, one substrate, possibly several products

Another interesting observation related to the cyclization process was reported for class III lanthipeptides. Genome database mining identified a number of organisms containing genes encoding class III synthetases.⁶⁰ Screening of *Streptomyces avermitilis* DSM 46492, *Saccharopolyspora erythraea* NRRL 2338, and *Streptomyces griseus* DSM 4023 by mass

Figure 7. A. Structures of the large and small subunits of cytolysin from *E. faecalis*. B. The observed stereochemistry of the different rings. The stereochemistry of the α -carbon originating from Cys is *R* in both cases, but the stereochemistry at the α and β carbons originating from Thr ($R = \text{Me}$) is inverted showing that the two types of rings are formed by *anti* addition to opposite faces of the dehydrobutyrine (or dehydroalanine for $R = \text{H}$).

spectrometry demonstrated that they indeed produced lanthipeptides. The sequence of the precursor peptides contained the characteristic SerXxxXxx SerXxxXxxXxxCys motifs that lead to labionin (Lab) formation in labyrinthopeptins⁷ and catenulipectin²⁸ [Figs. 1(B) and 2], but lanthionine formation in SapB²³ (Fig. 2) and curvopeptin.²⁹ Because there seems to be no phylogenetic correlation for enzymes making Lan or Lab rings,³⁶ it is currently not understood how the respective class III synthetases govern the formation from Lab or Lan. Interestingly, GC-MS analysis of the products from *S. avermitilis*, *S. erythraea*, and *S. griseus* showed that they contained both Lab and Lan.⁶⁰ Because of the small amounts of products obtained, the authors could not determine whether these peptides were homogeneous containing one Lab and one Lan, or whether the peptides were produced as a mixture of compounds, some containing Lan and some containing Lab. Regardless of which of these possibilities turns out to be correct, it shows that the same class III lanthipeptide cyclase domains can form either Lan or Lab structures from the same sequence motif, which is remarkable given the large difference between these structures.

Summary

The availability of the rapidly growing number of bacterial genomes is starting to provide the first insights into the possible mechanisms by which lanthipeptide biosynthesis may have evolved. Most of the “modern” lanthipeptide synthetases are highly evolved and make single polycyclic products that are the result of a remarkably well-orchestrated set of reactions. The discovery of class III and IV lanthi-

peptide synthetases suggest that the biosynthetic machinery may have evolved from stand-alone kinases, phosphoThr/phosphoSer lyases, and Zn^{2+} -dependent enzymes with a very common α -toroidal core structure. The low sequence specificity of primitive progenitor enzymes appears to have been maintained with respect to the phosphorylation and elimination reactions, but an allosteric regulation mechanism that depends on a leader peptide has evolved that assures that the dehydratases do not non-specifically act on any nascent polypeptide produced by the ribosome. The evolutionary origin of class I and II dehydratase machinery is less clear, but both involve activation of the Ser and Thr side chains, and presumably they have evolved from other enzymes that utilized nucleotides and Glu. Structural information will be needed to provide additional insights.

The evolution of the precursor peptides and hence the structure of the final products is more complex. For some systems, the progenitors of the precursor peptides can be inferred from the sequences of their leader peptides, with a group of class II peptides in cyanobacteria having descended from nitrogen-fixing proteins.⁵⁷ But for the majority of lanthipeptide precursor peptides, their origins are unclear. Intriguing potential insights into the evolution of the posttranslational modification process that turns a linear peptide into a highly structured product that recognizes its target with exquisite selectivity^{61,62} is provided by a subgroup of class II lanthipeptides in cyanobacteria.⁵⁰ The observation of a large number of precursor peptides with highly conserved leader peptides but highly diverse core peptides that are converted into a library of products

by one synthetase suggests that this may be a snapshot of a system under evolution featuring a non-specific synthetase that can act on a large number of core peptide sequences. Some support has been obtained that the topology and stereochemistry generated by such a synthetase may be determined at least in part by inherent conformational preferences of the core peptide sequence, perhaps reinforced by interaction of the core peptide with the molecular surface of the synthetase. During evolution, such ancestral synthetases would have evolved to more specific enzymes when they made products that were beneficial to the organism, ultimately resulting in proteins that make one product very efficiently. As discussed in this review many questions still remain for this model including how a non-specific synthetase is maintained, whether such a synthetase is primitive or has evolved to have low substrate specificity, and how the biosynthetic enzymes interact with their leader and core peptides. Future studies will hopefully provide answers to these questions.

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