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REVIEW ARTICLE

Structural characterization of thioether-bridged bacteriocins

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Bacteriocins are a group of ribosomally synthesized antimicrobial peptides produced by bacteria, some of which are extensively post-translationally modified. Some bacteriocins, namely the lantibiotics and sactibiotics, contain one or more thioether bridges. However, these modifications complicate the structural elucidation of these bacteriocins using conventional techniques. This review will discuss the techniques and strategies that have been applied to determine the primary structures of lantibiotics and sactibiotics. A major challenge is to identify the topology of thioether bridges in these peptides (i.e., which amino-acid residues are involved in which bridges). Edman degradation, NMR spectroscopy and tandem MS have all been commonly applied to characterize these bacteriocins, but can be incompatible with the post-translational modifications present. Chemical modifications to the modified residues, such as desulfurization and reduction, make the treated bacteriocins more compatible to analysis by these standard peptide analytical techniques. Despite their differences in structure, similar strategies have proved useful to study the structures of both lantibiotics and sactibiotics.
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INTRODUCTION

Bacteriocins are a diverse group of antimicrobial peptides and proteins that are ribosomally synthesized by bacteria. A multitude of bacteriocins have been reported, exhibiting a range of sizes and post-translational modifications.[1] Many of the more extensively post-translationally modified bacteriocins feature cyclic structures. Some of these cyclized structures arise from amide bond formation, such as the circular bacteriocins that feature an amide bond linking the N and C termini.[2] Others are cyclized through the formation of a covalent bond between a cysteine thiol and a carbon atom of another residue, thereby forming a thioether bridge. These thioether linkages are primarily found in two groups of bacteriocins: the lantibiotics and the sactibiotics, which are the focus of this review.[1] Although some bacteriocins contain other sulfur-containing bridges, such as disulfides, they will not be further discussed.

The lantibiotics are characterized by the presence of lanthionine (Lan) and methyllanthionine (MeLan), amino acids containing thioether bridges (Figure 1a and b).[1,3] Biosynthetically, these residues originate from cysteine, serine and threonine residues. First, selected serine and threonine residues are enzymatically dehydrated to form dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively. Then, the nucleophilic addition of a cysteine thiol onto a Dha or Dhb residue in Michael manner forms a Lan or MeLan residue, respectively. These modifications establish a covalent linkage between a cysteine thiol and the β-carbon of another residue, thereby forming a thioether bridge. Dha and Dhb residues not involved in Lan/MeLan formation are sometimes found in the mature peptide.

The sactibiotics are another group of sulfur-bridged bacteriocins.[1] Sactibiotics are characterized by the presence of a linkage between a cysteine thiol and the α-carbon of another residue (Figure 1c). This too serves to form a thioether bridge, thereby conformationally constraining the peptide. Some aspects of sactibiotic biosynthesis were recently elucidated, wherein a radical SAM (S-adenosylmethionine) enzyme was shown to be responsible for thioether bridge formation.[4] Thioether formation in sactibiotics is believed to be a radical process, requiring the abstraction of a hydrogen atom from the α-position of an amino acid.

Despite the large number of bacteriocins reported in the literature, relatively few of them have been fully structurally characterized. However, knowledge regarding their structures is an important prerequisite for understanding how they exert their antimicrobial effects. Comparison of their structures to previously characterized bacteriocins may indicate their probable mode of action. Knowledge of the structure also allows for structure–activity relationship studies to be performed, which may permit the rational development of novel bacteriocins with increased activity. Furthermore, knowledge regarding the structures of bacteriocins provides information about their biosynthetic machinery, which may prove useful for future bioengineering efforts.[5]

Edman degradation and tandem MS/MS are techniques that are commonly applied to the study of bacteriocins. The post-translational

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modifications that typify the lantibiotics and sactibiotics complicate their characterization by these techniques. This review will describe the structural information that these analytical techniques, in addition to NMR spectroscopy, can obtain for the lantibiotics and sactibiotics. Chemical and enzymatic transformations that have been used to modify bacteriocins to make them more amenable to analysis will also be discussed.

LANTIBIOTICS

Background and challenges

Some early studies of nisin, the most well-studied lantibiotic, were published in the mid-1940s.[6,7] It was not until more than 25 years later that the structure of nisin was fully elucidated.[8] Over the following decades, the primary structures of several other lantibiotics were solved, such as subtilin,[9] gallidermin[10] and epidermin.[11] More recently, the number of solved lantibiotic structures has quickly increased, partly coincident with improved capabilities in NMR spectroscopy and MS.[12]

Earlier structural elucidations of lantibiotics were highly reliant upon protease- and chemical-mediated cleavages. These were used to cut a lantibiotic into smaller, more easily characterized fragments.

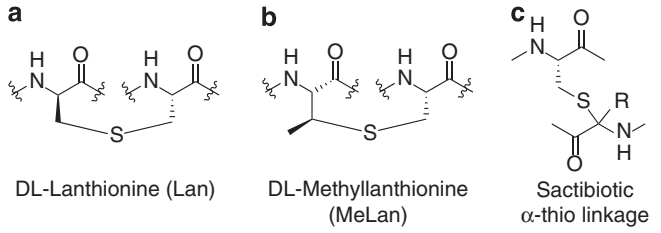

Figure 1 Structures of (**a**) lanthionine, (**b**) methyllanthionine and (**c**) a sactibiotic linkage.

Treatment of nisin with cyanogen bromide, cleaving the peptide bond C terminal to methionine residues, generated an N-terminal fragment of 21 amino acids and a C-terminal fragment of 13 amino acids (Figure 2).[8] The N-terminal fragment was further cleaved with trypsin, generating peptides of 12 and 9 amino acids. Similarly, the C-terminal nisin fragment was digested with chymotrypsin, the products of which were further digested with aminopeptidase and carboxypeptidases. Structural information was gathered through the analyses of these fragments, using techniques such as Edman degradation, analysis of peptide hydrolysates, desulfurization and other chemical modifications. Finally, the structural information obtained for these fragments was pieced together, providing the structure of nisin. This kind of approach was used for other early structural characterization efforts, for such lantibiotics as subtilin[9] and epidermin.[11]

The drawback of such an approach is the requirements it places on the lantibiotic amino-acid sequence. Protease and chemical cleavage sites must be distributed at convenient positions throughout the lantibiotic. For this reason, such an approach is not easily generalized toward novel lantibiotics with unknown amino-acid sequences. More recent approaches, based largely on NMR spectroscopy and MS, are less sequence dependent and are more versatile for this purpose.

The post-translational modifications typical of the lantibiotics pose challenges for typical peptide analysis techniques. Lan and MeLan residues are generally not observed by Edman degradation analysis, resulting in a gap in the observed peptide sequence. Dehydrated residues Dha and Dhb are more detrimental to Edman-based analysis, as no sequence information following these residues can be obtained. Once a dehydro residue becomes the N-terminal amino acid, it tautomerizes to the corresponding imine, which reacts with water to form an α-ketoamide. Without an amino group, the peptide can no longer undergo Edman degradation. Furthermore, some lantibiotics are produced with an N-terminal blockage (e.g., Pep5[13] and lacticin

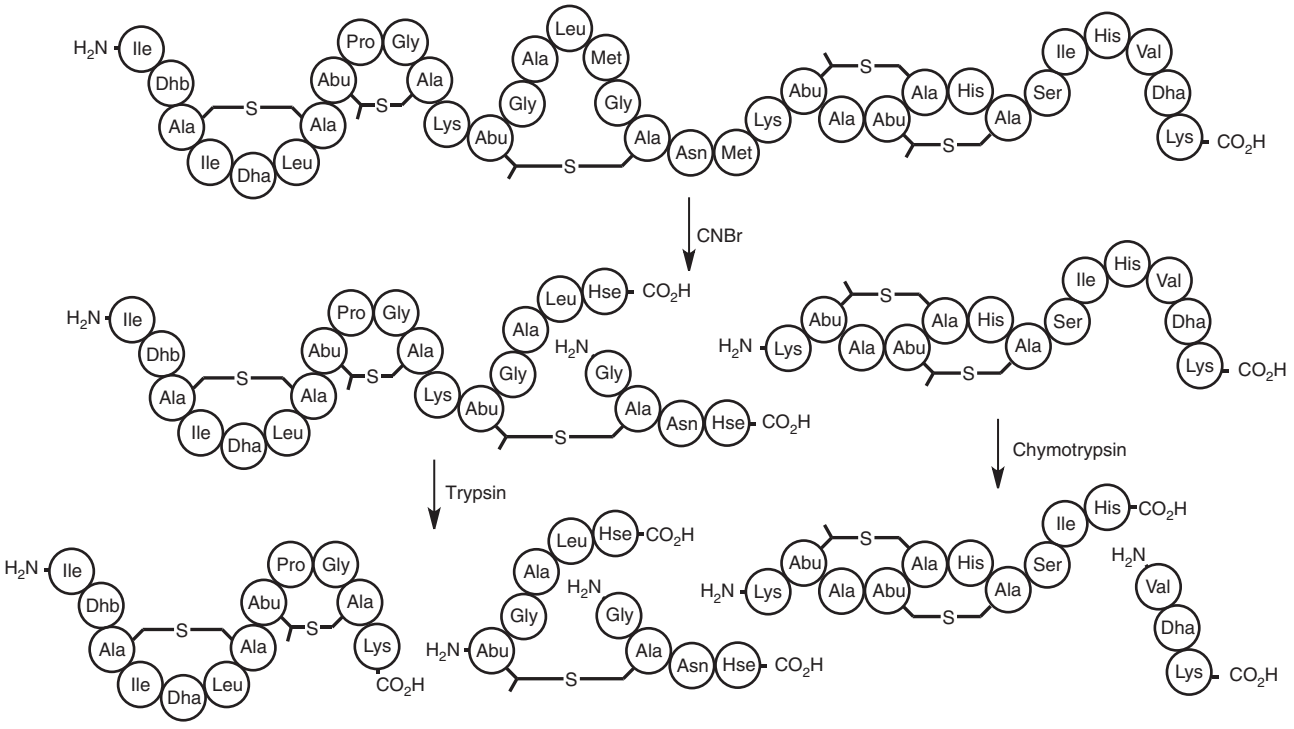

Figure 2 Degradation analysis of nisin, using cyanogen bromide, trypsin and chymotrypsin.

3147 A2[14] bear a 2-oxobutyryl group, whereas lactocin S has a 2-oxopropionyl group),[15] preventing Edman analysis without its removal. Although MS/MS is not impeded by N-terminal blockages and dehydro residues, difficulties are encountered because of Lan and MeLan rings. Fragmentation between residues contained within a Lan/MeLan ring is not typically observed, which prevents sequence information from being obtained for stretches of the peptide sequence. Many other post-translational modifications have been found in the lantibiotics, with their own inherent challenges for structural characterization. The focus of this review is on the strategies and techniques that have been applied to characterizing the post-translational modifications most commonly found in the lantibiotics: Lan and MeLan rings, and dehydro residues Dha and Dhb. Special attention will be placed on the determination of the bridging patterns of Lan and MeLan rings, one of the more challenging aspects of lantibiotic structural characterization.

NMR spectroscopy

With the advances in peptide and protein analysis through NMR spectroscopy, this technique has proven to be powerful for the purpose of lantibiotic structural characterization. First, chemical shift assignments are made on the basis of TOCSY and NOESY data.[16] This often provides an indication regarding which residues are post-translationally modified. The chemical shifts of the β-protons of Lan and MeLan residues are quite different from those of unmodified serines and threonines. Dha and Dhb residues are distinctive, providing signals in the olefinic region of an NMR spectrum. Importantly, NMR spectroscopy has proven invaluable for determining the connectivity of Lan and MeLan residues. NOESY experiments reveal NOE correlations between the protons on either side of a Lan or MeLan thioether bridge (Figure 3). Association of these protons with the rest of their spin system, and establishment of the location of these spin system within the amino-acid sequence provides the connectivity of the Lan or MeLan residue. Another approach involves the use of heteronuclear multiple bond correlation experiments, wherein α- and β-protons and carbons on one side of the Lan/MeLan thioether can be correlated with α- and β-protons and carbons on the other side.[17]

NMR spectroscopy has been used to determine the primary structures of many lantibiotics. One of the earlier structures characterized through the use of NMR was actagardine.[17] The structures of both components of lacticin 3147 were elucidated through the use of homonuclear NMR experiments.[14] NMR is still commonly used for the study of lantibiotic structures, having been recently applied to the characterization of lichenicidin,[18] geobacillin I[19] and paenibacillin.[20]

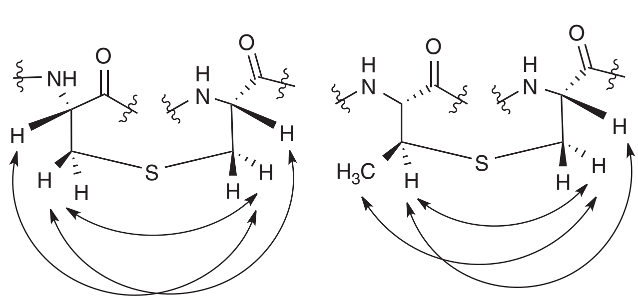

Figure 3 NOE correlations used for determining the connectivity of lanthionine (Lan) and methyllanthionine (MeLan) residues.

Despite its utility, there are some drawbacks to the study of lantibiotics using NMR spectroscopy. First, such analysis requires low milligram quantities of peptide, which may be difficult to obtain for some lantibiotics. Furthermore, a relatively concentrated lantibiotic solution is required, which may not be possible because of solubility. This requirement is especially important to obtain adequate NOESY and heteronuclear multiple bond correlation data to establish Lan or MeLan connectivity. The symmetry of Lan residues can also be a problem for determining connectivity. Sometimes, the β-protons from different Lan residues, or different β-protons within the same Lan residue have the same chemical shift.[21,22] Finally, the presence of an NOE correlation between residues thought to be involved in a single Lan/MeLan ring may actually result from the three-dimensional structure of the lantibiotic situating two separate Lan/MeLan residues in close proximity. These factors can make it difficult, if not impossible, to unequivocally determine Lan residue connectivity.

MS of unmodified lantibiotics

The combination of the monoisotopic mass of a mature lantibiotic and its known structural gene is useful for structural elucidation. The difference between the monoisotopic masses of the putative unmodified amino-acid sequence and the mature bacteriocin provides insight into the number and the nature of the post-translational modifications. As most typical lantibiotic post-translational modifications (Lan, MeLan, Dha and Dhb) involve the loss of water, the monoisotopic mass can be related to the number of dehydrations.

The positions of the modified residues can often be obtained with MS/MS analysis. Fragmentation data, in combination with the lantibiotic amino acid sequence, can allow for the identification of the serines and threonines involved in Lan, MeLan, Dha and Dhb formation. Fragmentations between residues contained within a Lan/MeLan ring are not typically observed, so the extent of modification of these residues may be unclear. Still, the lack of fragmentation in certain regions of a lantibiotic can be informative. If no fragmentation is observed within an amino-acid sequence bordered by a cysteine and a serine or threonine, it is suggestive that these residues were modified into a Lan or MeLan residue. The Lan and MeLan connectivities of some lantibiotics, such as geobacillin II,[19] have been solved on the basis of the lack of fragmentation observed in certain regions when compared with the predicted amino-acid sequence. However, the absence of fragmentation in a region must be carefully considered, as it is not necessarily diagnostic of the presence of a Lan/MeLan ring. Lantibiotics containing interlocking Lan/MeLan rings pose an even greater challenge to MS/MS analysis. In such interlocking ring systems, different connectivities exist that are indistinguishable to typical MS/MS. These interlocking rings require further chemical modifications, which are discussed below.

Chemical modifications

Treatment with thiols. As indicated above, Dha and Dhb residues are problematic for the Edman degradation analysis of a lantibiotic. Using the reactivity of these dehydro residues as Michael acceptors, they can be modified to make them amenable to Edman analysis. Treatment of a lantibiotic with an excess of a substituted thiol converts Dha and Dhb residues into S-alkylated cysteine and β-methylcysteine derivatives, respectively (Figure 4a). These cysteine derivatives are compatible with Edman degradation, which allows for further sequence information to be obtained. Such an approach has been used for the structural characterization of many lantibiotics, including Pep5,[13] mutacin B-Ny266[23] and mutacin II.[24] A variety of thiols have been

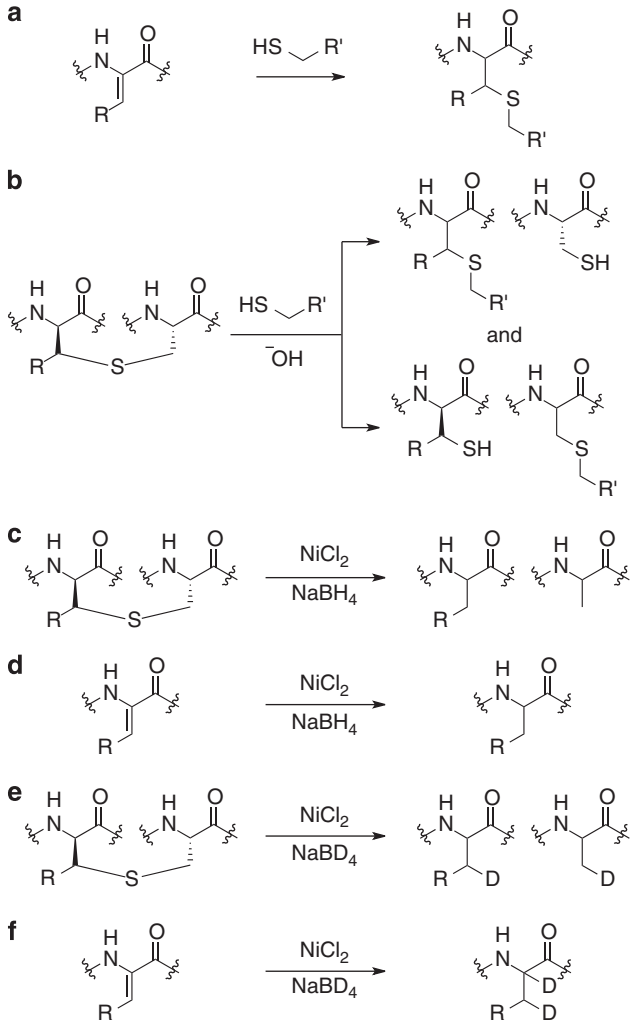

Figure 4 Chemical modifications of dehydroalanine (R = H), dehydrobutyrine (R = CH_3), lanthionine (R = H) and methyllanthionine (R = CH_3). (**a**) Addition of thiols to dehydroalanine (Dha) and dehydrobutyrine (Dhb). (**b**) Treatment of lanthionine (Lan) and methyllanthionine (MeLan) with alkaline thiols. Desulfurization of (**c**) Lan and MeLan, and (**d**) Dha and Dhb. Desulfurization under deuterated conditions of (**e**) Lan and MeLan, and (**f**) Dha and Dhb.

used for this purpose, such as mercaptoethanol,[17] ethanethiol[23] and benzyl mercaptan.[13]

During the analyses of lantibiotics using Edman degradation, Lan and MeLan residues are not observable under standard conditions. Lan and MeLan residues can be chemically modified using thiols in alkaline conditions, generating linear lantibiotic derivatives (Figure 4b).[25] These conditions modify Dha and Dhb as described above, but also cleave Lan and MeLan rings. This generates a free cysteine and an alkylated cysteine (or β-methylcysteine) derivative. The resulting linearized peptide can then be analyzed by Edman degradation without the complications caused by dehydro residues, and the derivatives from Lan and MeLan residues can also be observed. However, the regiochemistry of this process is not fixed, as this cleavage reaction can occur on either side of the thioether group. Although these peptides can still be analyzed by Edman degradation, MS/MS analysis would be complicated by this variability. Furthermore, these data do not indicate whether an alkylated cysteine residue is derived from a dehydro residue or from a Lan/MeLan ring.

Desulfurization and reduction. Another approach wherein the Lan and MeLan residues are linearized is through the use of desulfurization/reduction conditions. Treatment of a lantibiotic with nickel boride[14] or Raney nickel[26] desulfurizes Lan and MeLan residues, and reduces Dha and Dhb. Following desulfurization, Lan residues are converted into two alanine residues, whereas MeLan residues are converted into an alanine and a 2-aminobutyrate (Abu) residue (Figure 4c). Dha and Dhb are converted into alanine and Abu, respectively (Figure 4d). Once the Lan and MeLan residues are desulfurized, the resulting linear peptide is amenable to analysis by Edman degradation and MS/MS. Dha and Dhb are no longer a problem for Edman degradation, and fragmentations can be observed between residues formerly contained in a Lan or MeLan ring using MS/MS. Desulfurization was recently used in the structural characterization of lantibiotics lacticin 3147,[14] paenibacillin,[27] entianin[26] and paenicidin A.[22]

Using such an approach, the alanine residues generated from a Lan residue are indistinguishable from an alanine residue produced by the reduction of Dha. Similarly, the Abu residue from MeLan desulfurization is identical to that from Dhb reduction. This ambiguity can be addressed by performing the desulfurization under fully deuterated conditions.[14] Using deuterated reagents, an alanine generated from a Lan residue is labeled with one deuterium atom, whereas an alanine produced by Dha reduction is labeled with two deuterium atoms (Figure 4e and f). Similarly, Abu derived from MeLan is singly deuterated, whereas Abu derived from Dhb is doubly deuterated. Therefore, using this alternate approach, residues involved in Lan and MeLan residues can be distinguished from dehydro residues.

These deuterated conditions were applied to structural characterization of the two-component lantibiotic lacticin 3147.[14] Edman degradation analyses of the A1 and A2 peptides were hindered by the presence of Dha and Dhb residues, as well as a 2-oxobutyryl group blocking the N terminus of the A2 peptide. Following the removal of the 2-oxobutyryl group using 1,2-diaminobenzene, the A1 and A2 peptides were fully desulfurized in deuterated conditions. Edman analysis of the modified peptides allowed for the extent of post-translational modifications to be determined for all of the amino acids. Although the residues involved in Lan and MeLan rings were differentiable from Dha and Dhb, it was not possible to determine the connectivity of the Lan/MeLan rings based on these data. Instead, the connectivities of lacticin 3147 A1 and A2 were resolved using NMR spectroscopy, based on NOE crosspeaks bridging the thioether bridges.[14]

Paenibacillin was incompatible to analysis using Edman degradation due to an N-terminal acetyl group.[27] However, only limited sequence information could be obtained using MS/MS. The absence of fragmentation in certain regions of the peptide was indicative of the presence of Lan and/or MeLan residues. Without the structural gene, further interpretation of the fragmentation data was hampered. To obtain more sequence information, paenibacillin was desulfurized and reduced. On the basis of the MS/MS data obtained for the fully desulfurized peptide, a full tentative amino-acid sequence was determined. Further, desulfurization experiments in deuterated conditions allowed for the extent of post-translational modifications of all residues to be determined. As the Lan and MeLan rings are necessarily interlocking, the connectivity could not be determined based on the data obtained for the fully desulfurized peptide. The full

connectivity and structure of paenibacillin was later reported based on NMR spectroscopic characterization.[20]

Lantibiotic structural elucidation based on full desulfurization experiments has often relied either on NMR spectroscopy[14,20] or on analogy to lantibiotics of known structure.[26] However, NMR-based characterization is not always possible, nor will a novel lantibiotic necessarily resemble previously characterized lantibiotics. As described above, analysis of a fully desulfurized lantibiotic indicates the post-translational modification for every residue, but it does not provide information about the connectivity of Lan and MeLan residues. Although MS/MS data for an unmodified lantibiotic may suggest the regions contained within Lan and MeLan rings, it does not provide further structural information if there are interlocking Lan and MeLan rings. Furthermore, any information about this connectivity is lost if the lantibiotic is fully desulfurized.

If the lantibiotic is only partially desulfurized, additional MS/MS fragmentation may be observed because of the desulfurization of some Lan and MeLan residues, whereas fragmentation will still not be observed in regions contained within Lan or MeLan rings. Through the analysis of the lantibiotic in several different partially desulfurized states, it becomes possible to map out the locations and connectivities of Lan and MeLan rings, even if they are interlocking. Such an approach was used for the structural elucidation of the hexacyclic lantibiotic, paenicidin A.[22] MS/MS data for mature paenicidin A provided the approximate Lan and MeLan ring topology, and indicated that three of the rings were necessarily interlocking (Figure 5a). Analysis of the fully desulfurized peptide provided little structural information (Figure 5b). To elucidate the connectivity in the interlocking region, MS/MS data was analyzed for several partially desulfurized states. One partially desulfurized state revealed new fragmentations in the middle of the interlocking region (Figure 5c). On the basis of these fragmentations, it was possible to map the connectivity of the remaining intact Lan and MeLan rings in the interlocking region.

Structural elucidation through biosynthesis of lantibiotic variants

Following advances in the heterologous expression of lantibiotics,[28] a radically different means of lantibiotic structural elucidation may be used. Coexpression of a lantibiotic precursor peptide (unmodified amino-acid sequence and its associated leader peptide sequence) with the enzyme(s) involved in dehydration and cyclization allows for the heterologous production of lantibiotics. Alternately, chemically synthesized precursor peptides can be provided to purified lantibiotic modification enzymes to achieve the same result. This has proven useful for the structural characterization of lantibiotics, as it allows for lantibiotic precursor peptides with amino-acid substitutions to be fed to the biosynthetic machinery, thereby producing variants of the natural peptide.

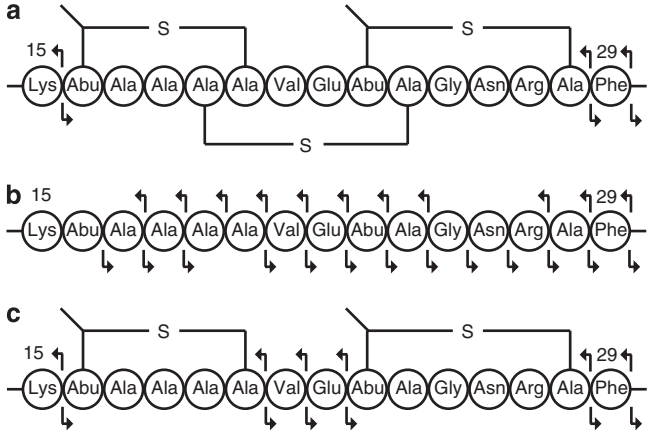

Figure 5 MS/MS data obtained for (**a**) natural paenicidin A, (**b**) fully desulfurized paenicidin A and (**c**) partially desulfurized paenicidin A.

In the structural characterization of enterococcal cytolysin, such an approach was used to clarify the structure of $CylL_L''$ (Figure 6a).[29] The central region of the peptide features two adjacent serines (Ser-14 and Ser-15), both of which are judged to be dehydrated on the basis of MS/MS. One of these residues is further modified, becoming part of a Lan residue, whereas the other remains a Dha residue. On the basis of MS/MS data, it was not possible to unequivocally determine which position was involved in Lan formation. To determine the connectivity of the Lan ring, two variants were prepared wherein one of the two serines was replaced with a threonine. Following the heterologous expression of the mutated genes with the lantibiotic biosynthetic machinery, the encoded variant peptides were purified. The lantibiotic variants were then hydrolyzed, derivatized and analyzed by chiral GC–MS. Comparison of the relative amounts of the derivatized Lan and MeLan residues between the two peptides thus revealed that the Lan residue was formed from Ser-14. A similar approach has been used for other lantibiotics, such as bovicin HJ50.[30]

On the basis of its structural gene, the B-ring of curvopeptin contains a serine and a threonine residue (Ser-15, Thr-16; Figure 6b).[31] MS/MS suggests that only one of these two residues is dehydrated. As these residues are contained within a Lan ring, it was not possible to determine which was modified. To resolve this ambiguity, a chemically synthesized variant of the precursor peptide was incubated with heterologously expressed and purified CurKC, the enzyme responsible for Lan and MeLan biosynthesis in curvopeptin. Both cysteines found in the wild-type precursor peptide were replaced with alanines in this variant. CurKC processed this precursor peptide variant, resulting in the expected number of dehydrations. MS/MS analysis of the dehydrated peptide, lacking Lan residues, revealed additional fragmentations, which indicated that Ser-15 was the dehydrated residue.

Summary and outlook

As described above, early efforts toward the elucidation of lantibiotic structures relied heavily on selective chemical and proteolytic cleavages, generating fragments that could be more easily studied. Such an approach is heavily dependent on the lantibiotic amino-acid sequence, and is not generalizable toward the study of novel lantibiotics. Edman degradation has been commonly applied toward the earlier structural characterizations of lantibiotics. Chemical modifications, such as the addition of thiols to Dha and Dhb, and the desulfurization of Lan and MeLan residues, have been used to

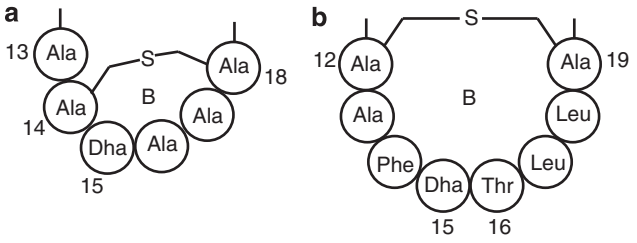

Figure 6 (**a**) The B-ring of $CylL_L''$. (**b**) The B-ring of curvopeptin.

make lantibiotics more amenable to Edman degradation, providing additional structural information.

NMR spectroscopy has proven to be a powerful tool for lantibiotic structural characterization. NMR data may be used to determine not only the positions that have been post-translationally modified but also the topology of Lan and MeLan bridges. However, some lantibiotics are not amenable to analysis through NMR because of spectral overlap, low production levels and limited solubility. The use of MS/MS has proven to be an increasingly powerful tool for the structural characterization of lantibiotics. Although Lan and MeLan residues complicate MS/MS analyses of unmodified lantibiotics, desulfurization experiments allow for the residues formerly contained within these rings to be examined. Furthermore, the use of a partial desulfurization approach allows critical information to be obtained with regard to the connectivities of Lan and MeLan residues. MS-based approaches, which are relatively less demanding for sample quantity and concentration, are likely to be used to a much greater extent in the future. Perhaps, the most definitive means of structural identification is through chemical synthesis. The structures of several lantibiotics have been confirmed through total syntheses,[32,33] clearly demonstrating connectivity and stereochemistry. Nonetheless, such syntheses are labor-intensive and time-consuming, largely limiting them to a confirmatory role for structures elucidated through other techniques.

SACTIBIOTICS

Background and challenges

Compared with the lantibiotics, the number of sactibiotics that have been isolated and structurally characterized is relatively small. However, genome mining based on sactibiotic biosynthetic genes suggests that genetic determinants for sactibiotics are widespread.[34] Subtilosin A was the first peptide of this class for which the primary structure was solved (Figure 7).[35,36] More recently, the structures of the two-component sactibiotic thuricin CD,[37,38] sactipeptide sporulation killing factor (SKF) from *Bacillus subtilis*[39] and sactibiotic thurincin H[40] have been elucidated. Sactibiotics are amenable to some of the techniques commonly used for the structural characterization of lantibiotics, excluding Edman analysis, which has been largely unsuccessful for these peptides. Both subtilosin A and SKF feature cyclization of the N and C termini, and cannot be analyzed by Edman degradation. Edman analyses of the two components of thuricin CD, which do not have cyclized N and C termini, yielded incomplete peptide sequences.[37] Still, NMR spectroscopy and MS/MS have proven to be useful towards the structural characterization of sactibiotics. Furthermore, these techniques have allowed for the connectivities of sactibiotic thioether bridges to be determined. As with lantibiotics, desulfurization results in linearization, which produces derivatives amenable to typical peptide-sequencing techniques.[39]

NMR spectroscopy

NMR studies have been used to fully or partially elucidate the structures of all five sactibiotics whose structures have been characterized. ^{13}C-,^{15}N-labeled subtilosin A was analyzed with a suite of two- and three-dimensional NMR experiments.[35,36] On the basis of previous reports, Phe-22, Thr-28 and Phe-31 were presumed to be modified in subtilosin A, although the nature of these modifications was unknown. From the NMR data, it was noted that the chemical shift values of the α-carbons of these three residues were higher than those of the natural amino acids. Furthermore, no α-protons were observed for these residues. Evidence for the nature of the modifications was obtained through analysis of the NOESY data for the three cysteine residues in subtilosin A. The β-protons of Cys-7 showed NOEs to the modified spin system of Thr-28. Similar interactions were observed implicating Cys-4 with Phe-31, and Cys-13 with Phe-22. On the basis of these data, the overall pattern of the subtilosin A thioether bridges was established (Figure 7).

The NMR spectroscopic characterization of isotopically labeled peptides was also used for the structural elucidation of the two-component sactibiotic thuricin CD[37] and for thurincin H.[40] As with subtilosin A, the α-carbons of all of the modified residues in these peptides displayed distinctive chemical shifts in HNCACB and CBCACONH experiments. Furthermore, no α-protons were observed at these positions, and the side chains of these modified residues showed up as spin systems lacking an α-proton. Finally, as before, NOE correlations between cysteine β-protons and the protons of the modified residue were used to determine the connectivity of the thioether bridges. SKF, which only contains one thioether bridge, was analyzed with homonuclear NMR experiments to confirm both the residues involved in this bridge, as well as the carbon atom to which the thiol is attached.[39] TOCSY data revealed a spin system consistent with a methionine lacking an α-proton. Furthermore, the chemical shift of the α-carbon was determined to be higher than the standard amino acid using heteronuclear multiple bond correlation data.

Mass spectrometry

As with the lantibiotics, the monoisotopic mass of a sactibiotic provides an indication of the number and the nature of the post-translational modifications. The monoisotopic mass of subtilosin A, as obtained by MALDI-TOF MS, was suggestive of the loss of a water molecule (due to the cyclization of the N and C termini), as well as the loss of six hydrogen atoms.[36] This is consistent with the presence of three thioether bridges, the formation of which requires the loss of two hydrogen atoms. This difference in mass is also observed as a result of disulfide formation. Regardless, the presence of a disulfide bridge can be confirmed through reductive alkylation methods, or via tandem MS as was carried out for SKF.[39]

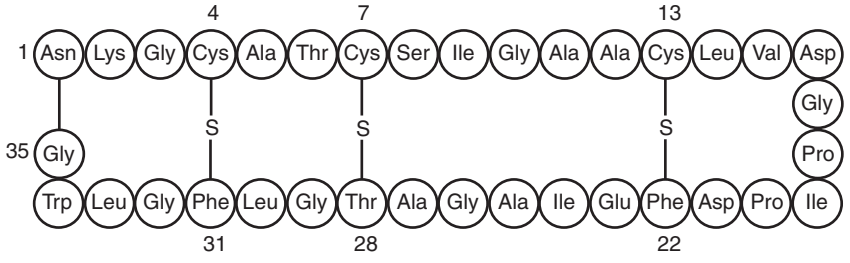

Figure 7 The structure of subtilosin A, with the thioether bridges indicated.

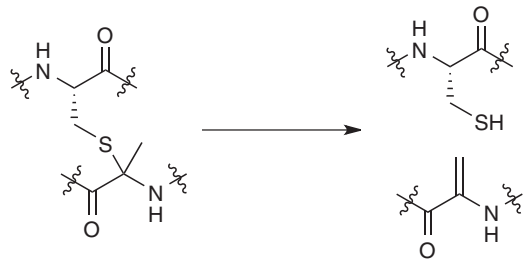

Figure 8 Cleavage of sactibiotic thioether bridges observed during MS/MS analyses.

Unlike the Lan and MeLan rings in the lantibiotics, sactibiotic thioether bridges are prone to opening during MS/MS (Figure 8). This is likely due to the presence of an amide nitrogen on the carbon bearing the thioether, allowing for an elimination-based ring opening to occur. Although this may provide less information for backbone cyclized sactibiotics (i.e., subtilosin A, SKF), the opening of thioether bridges allows the residues involved to be identified. MS/MS data obtained for thuricin CD indicated that the residues with modified α-positions show up with a mass two Daltons smaller than what would be expected based on the unmodified amino acid.[37] This mass difference can be rationalized because of the opening of the thioether, forming the corresponding dehydro residue. Therefore, identification of residues with masses smaller than predicted through the use of MS/MS may provide initial evidence of the thioether bridge connectivity patterns.

Chemical modifications

Desulfurization. The desulfurization of sactibiotic thioether bridges was first applied to subtilosin A.[36] Previous attempts to hydrolyze subtilosin A for amino-acid analysis were problematic. Upon treatment of subtilosin A with desulfurization/reduction conditions, the three thioether bridges were successfully cleaved. The three cysteines involved in these residues were modified into alanines, whereas the residues to which the cysteine sulfurs were attached were converted into their unmodified parent amino acids. This desulfurized peptide could then be hydrolyzed, derivatized and analyzed by chiral GC–MS. Further investigation suggested that the stereochemistries of the desulfurized α-thio residues are dependent on the geometry of the peptide, and do not occur with predictable retention or inversion of configuration.

In an attempt to characterize SKF, this peptide was treated with desulfurization reagents.[39] This allowed more sequence information to be obtained through MS/MS, and allowed for the confirmation of an N- to C-terminal backbone cyclization. However, these data did not indicate the residue to which the cysteine thiol was bonded. Desulfurization replaced the α-thio linkage with a proton, essentially reforming the unmodified amino acid (of uncertain stereochemistry). Desulfurization of SKF using deuterated conditions labeled the modified residue with a deuterium atom on the α-position. MS/MS data obtained for the deuterated desulfurized peptide allowed for the identification of the modified residue, and consequently the topology of the thioether bridge.

Summary and outlook

So far, the structures of sactibiotics have been primarily elucidated through the use of NMR spectroscopy. The characteristics of the thioether linkages make the modified residues easily identified using NMR spectroscopic data. Furthermore, NOE correlations have proven to be very useful for determining the connectivities of the thioether bridges. Compared with lantibiotics, issues of spectral overlap are much less problematic. Although MS/MS has been used to identify modified residues, an approach that is entirely MS-based has not yet been used to elucidate the connectivities of a sactibiotic containing more than one thioether bridge. It is likely that novel sactibiotics will be discovered, which are not amenable to NMR analysis, because of issues with low production or solubility. Although desulfurization experiments have already shown utility for the structural analysis of sactibiotics, they have the potential to be used for the full determination of thioether bridge topology of novel sactibiotics.

DEDICATION

This paper is dedicated to Professor Christopher Walsh, who has been a leader and mentor to generations of scientists.

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