Structural Characterization of Lacticin 3147, a Two-Peptide Lantibiotic with Synergistic Activity†

Nathaniel I. Martin,‡ Tara Sprules,‡ Michael R. Carpenter,§ Paul D. Cotter,|| Colin Hill,|| R. Paul Ross,⊥ and John C. Vederas*,‡

Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada, Alberta Peptide Institute, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada, Department of Microbiology, University College, Cork, Ireland, and Dairy Products Research Centre, Teagasc, Moorepark, Fermoy, County Cork, Ireland

Received December 8, 2003; Revised Manuscript Received January 16, 2004

ABSTRACT: Lantibiotics are antibacterial peptides isolated from bacterial sources that exhibit activity toward Gram-positive organisms and are usually several orders of magnitude more potent than traditional antibiotics such as penicillin. They contain a number of unique structural features including dehydro amino acid and lanthionine (thioether) residues. Introduced following ribosomal translation of the parent peptide, these moieties render conventional methods of peptide analysis ineffective. We report herein a new method using nickel boride (Ni_2B), in the presence of deuterium gas, to reduce dehydro side chains and reductively desulfurize lanthionine bridges found in lantibiotics. Using this approach, it is possible to identify and distinguish the original locations of dehydro side chains and lanthionine bridges by traditional peptide sequencing (Edman degradation) followed by mass spectrometry. The strategy was initially verified using nisin A, a structurally well characterized lantibiotic, and subsequently extended to the novel two-component lantibiotic, lacticin 3147, produced by *Lactococcus lactis* subspecies *lactis* DPC3147. The primary structures of both lacticin 3147 peptides were then fully assigned by use of multidimensional NMR spectroscopy, showing that lacticin 3147 A1 has a specific lanthionine bridging pattern which resembles the globular type-B lantibiotic mersacidin, whereas the A2 peptide is a member of the elongated type-A lantibiotic class. Also obtained by NMR were solution conformations of both lacticin 3147 peptides, indicating that A1 may adopt a conformation similar to that of mersacidin and that the A2 peptide adopts α-helical structure. These results are the first of their kind for a synergistic lantibiotic pair (only four such pairs have been reported to date).

Bacteriocins are bacterially produced peptides that display antimicrobial properties against other bacteria, often closely related to the producer strain (*1*, *2*). The first bacteriocin was discovered in 1925 (*3*), and in the past 20 years there has been an increasing interest in such compounds as preservative agents for food and as potential supplements or replacements for currently used antibiotics. The ribosomal production of these small (2−6 kDa) antimicrobial peptides by Gram-positive bacteria, especially lactic acid bacteria, as a defense mechanism against other organisms is well documented and represents an intensive area of research (*4*, *5*). Among the known bacteriocins, two distinct families have emerged: the nonlantibiotics and the lantibiotics (*6*, *7*). The latter contain the unusual amino acids lanthionine and β-methyllanthionine present as intramolecular (thioether) rings. Many lantibiotics also possess other modified residues such as dehydro amino acids, D-alanine centers, N-terminal α-keto amides (*8*), and C-terminal rings formed by oxidative decarboxylation. Lantibiotics are ribosomally produced as prepeptides, which are enzymatically modified (*9*) to yield the structurally unique active species. Nisin A (Figure 1) is the most highly studied lantibiotic (*10*−*14*) and illustrates some of these structural features.

Both lantibiotic and nonlantibiotic bacteriocins are most often found as single active peptides, but a unique class of two-peptide systems, wherein both components act synergistically and are required for full activity, is now known. Two-component lantibiotic systems are rarer than their nonlantibiotic counterparts (the first two-component lantibiotic was reported in 1996 (*15*)), and to date only four such systems are described (*16*). Our efforts in this area have focused on the structural characterization of a new two-peptide lantibiotic, lacticin 3147, identified in the fermentation supernatant of *Lactococcus lactis* subspecies *lactis* DPC3147 (*17*). When lacticin 3147 is applied at nanomolar concentrations (1:1 stoichiometry), its synergistic peptides display potent activity against *Listeria monocytogenes*, a food-borne pathogen, and have proved to be effective as a protective culture in cheese production (*18*). Lacticin 3147

† These investigations were supported by the Natural Sciences and Engineering Research Council of Canada, the Alberta Heritage Foundation for Medical Research, CanBiocin Ltd. (Edmonton AB), the Irish Government under the National Development Programme (2000−2006), and Science Foundation Ireland.

* Corresponding author. Tel.: (780) 492-5475. Fax: (780) 492-8231. E-mail: john.vederas@ualberta.ca.

‡ Department of Chemistry, University of Alberta.

§ Alberta Peptide Institute, Department of Biochemistry, University of Alberta.

|| University College, Cork.

⊥ Dairy Products Research Centre, Teagasc.

[1] Abbreviations: Abu, α-aminobutyric acid; Dhb, dehydrobutyrine; Dha, dehydroalanine; Lan, (2*S*,6*R*)-lanthionine; MeLan, (2*S*,3*S*,6*R*)-3-methyllanthionine; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; DQF−COSY, double-quantum-filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HSQC heteronuclear single-quantum correlation.

10.1021/bi0362065 CCC: $27.50
Published on Web 02/26/2004

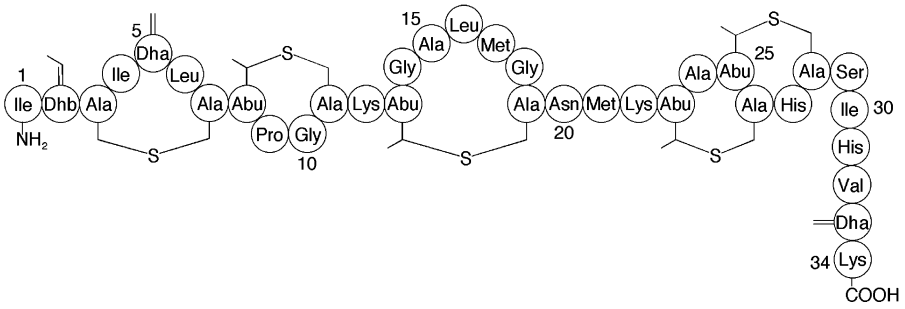


FIGURE 1: Nisin.

Scheme 1 : Formation of Nickel Boride in Methanol

$$4\,NiCl_2 + 8\,NaBH_4 + 18\,CH_3OH \xrightarrow[0\,^{\circ}C]{MeOH} 2\,Ni_2B + 25\,H_2 + 6\,B(OCH_3)_3 + 8\,NaCl$$

is also highly active against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), penicillin-resistant *Pneumococcus* (PRP), *Propionibacterium*, and *Streptococcus mutans* (*19*).

The extensive modifications present in lantibiotics as well as limited production of the active species by most producing organisms present significant challenges to the determination of primary structures. Standard analytical techniques, such as Edman degradation, are often unsuccessful when investigating lantibiotics. Lanthionine bridges sequence poorly, and dehydro amino acid residues within the peptide prevent sequencing once exposed at the N-terminus (presumably due to rapid deamination leading to an N-terminally blocked peptide). Also, the N-terminal α-keto amide moiety found in some lantibiotics prevents any N-terminal sequencing. No two-peptide lantibiotics and only a small number of single-peptide lantibiotics discovered to date have had their primary structures assigned. With the exception of nisin A and its derivatives, in all cases the final structural assignments have relied on multidimensional NMR (*14*, *20*−*26*). In situations where the producing organism does not generate the milligram quantities of peptide required for NMR analysis, structure determination can be very difficult.

Early work on the structure elucidation of nisin A and other lantibiotic peptides relied upon enzymatic or chemical cleavage strategies (*11*, *27*−*29*). Such approaches are often specific for the particular peptide and lack generality. A very limited number of general methods have been developed (*30*), most often relying on thiol addition to convert both dehydro amino acid and lanthionine moieties to sequencer-friendly amino acids for Edman degradation. Although such techniques are valuable, they are unable to give specific insights into the exact location of dehydro amino acid residues and the specific bridging patterns of lanthionine linkages. The chemical modification strategies developed in the present work make use of nickel boride (Ni_2B), a reducing and desulfurizing agent when used in the presence of hydrogen or deuterium gas. Forming rapidly in situ when nickel chloride is treated with sodium borohydride in protic solvent (Scheme 1), nickel boride has been used previously for thioether desulfurization (*31*) and olefin reduction (*32*). Although a detailed structure of nickel boride is unknown, its elemental composition is consistent with the formula Ni_2B and contains hydrogen that is gradually released upon heating (*33*).

We now report that application of a deuterating variant of the process, using $NaBD_4$ in deuterated solvent, to lantibiotics leads to lanthionine desulfurization with the concomitant incorporation of a single deuterium atom at each of the residues participating in the sulfur bridge. Conversely, dehydro amino acid residues are reduced with incorporation of two deuterium atoms. Using this approach, it is possible to identify and distinguish the original locations of dehydro side chains and lanthionine bridges by use of peptide sequencing (Edman degradation) followed by mass spectrometry. This methodology has been successfully applied on minute quantities (micrograms) of nisin and both of the lacticin 3147 peptides and should make partial structure elucidation on the microscale viable for most lantibiotics.

The utility of the nickel boride modification strategy was confirmed by determination of the primary structures of the lacticin 3147 peptides by multidimensional NMR spectroscopy. After amassing quantities of material suitable for NMR analysis, we determined the primary structures, including the specific lanthionine bridging patterns, of both lacticin 3147 peptides. As well, solution conformations of both peptides were attained by NMR and shed further light on their proposed synergistic mode of action.

EXPERIMENTAL PROCEDURES

Material and Methods. All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. Nisin was purchased (Sigma) as a 2.5% purity preparation requiring HPLC purification prior to use (see Supporting Information for isolation protocol). After isolation, the identity of nisin A was confirmed by MALDI-TOF mass spectrometry (see Supporting Information). The isolation of the lacticin 3147 peptides and growth conditions for the producing organism are also included in the Supporting Information.

Amino Acid Analysis. Peptides for amino acid analysis were hydrolyzed in an inert gas atmosphere (N_2) in 6 M HCl at 110 °C for 16 h in sealed glass vials. Following subsequent removal of HCl under vacuum, the amino acid composition of the samples was determined after *o*-phthaldialdehyde derivatization by a previously described method specifically optimized for the identification of Lan and MeLan (*34*). The presence and quantitation of D-alanine was determined by gas chromatography (GC)−MS following a previously described method (*35*).

Mass Spectrometry. Samples for MALDI mass analysis were prepared using sinnapinic acid as matrix. Solutions containing the sample peptide were mixed in even part with a stock solution of sinnapinic acid (10 mg/mL) in 60% acetonitrile (0.1% TFA). A thin layer of sinnapinic acid was

deposited on the surface of the gold target plate by delivery of a small droplet (0.7 μL) of a solution containing sinnapinic acid (4 mg/mL) in 50% acetone/50% methanol. After evaporation of the acetone/methanol, a 0.3 μL droplet of the solution containing the sample peptide−matrix mixture was deposited on top of the fresh matrix layer on the plate. The solvent was evaporated at 1 atm prior to analysis. Mass spectra were recorded with a single-stage reflectron, MALDI-TOF mass spectrometer (Applied BioSystems, Foster City, CA; API QSTAR Pulsar with an oMALDI source) (*36*).

Lacticin A2 N-Terminal Deblocking. Unlike the other lantibiotics to be desulfurized and reduced, the lacticin A2 peptide has its N-terminus blocked by an α-keto amide moiety. It was therefore necessary to chemically remove this group prior to treatment with nickel boride. This was accomplished via the method of Dixon and co-workers (*37*, *38*). Briefly, purified A2 peptide (100 μg, 0.04 μmol) was dissolved in 3.5 mL of a strong acetic acid/sodium acetate buffer (4.0 M, pH 4.8) containing 1,2-diaminobenzene at a concentration of 40 mM (3500-fold excess relative to the peptide). The mixture was stirred at 38 °C for 12 h, after which MALDI-TOF mass analysis revealed the desired deblocked species as the major product (MW = 2680; see Supporting Information). The peptide was then isolated by reverse-phase HPLC using a steel-walled column (Vydac, 10 × 250 mm, 5 μm). A method employing a 1.0 mL injection loop was used with a gradient of water and 2-propanol (0.1% TFA): 24% 2-propanol for 5 min, climbing to 44% in 20 min, returning to 24% in 0.5 min, and remaining at this concentration for an additional 5 min (flow rate 2.25 mL/min, detection at 220 nm). The desired peptide was isolated as a single broad peak (t_R = 23.0 min).

Ni_2B Desulfurization/Reduction of Lantibiotic Peptides. The lantibiotic peptide (0.5 mg) and nickel chloride (1 mg) were dissolved/suspended in 2.0 mL of CD_3OD/D_2O (1:1) This mixture was then transferred via pipet into a screw-cap flask containing $NaBD_4$ (1.0 mg) with rapid resealing of the reaction vial. A black precipitate (Ni_2B) formed immediately with the evolution of deuterium gas. The mixture was then stirred for 1 h at 50 °C, followed by removal of the Ni_2B by centrifugation. After removal and retention of the supernatant, fresh CD_3OD/D_2O (2.0 mL) was added to the Ni_2B precipitate, and the mixture sonicated and recentrifuged to recover any residual peptide. After the supernatants were combined, the methanol was removed by rotary evaporation and the aqueous solution, containing the modified peptide, submitted for automated Edman degradation analysis.

Peptide Sequencing and LCMS Characterization of PTH Amino Acids. Peptide sequencing was performed using a Hewlett-Packard G1000A protein sequencer coupled to a Hewlett-Packard series II 1090 HPLC instrument for online PTH identification. Hewlett-Packard routine 3.0 was utilized in the automated Edman chemistry involving phenylisothiocyanate derivatization and TFA cleavage. HPLC characterization of the PTHs made use of a steel-walled C_{18} analytical column (Agilent, Hypersil C_{18}, 2.1 × 250 mm, 5 μm) at 40 °C. The method employed a two-component gradient, component A (15% acetonitrile buffered to pH 5.2; Agilent Technologies, Palo Alto, CA) and component B (31% 2-propanol, 0.2% buffer; Agilent Technologies). The gradient was 100% A, changing to 100% B over 16 min (flow rate 300 μL/min, detection at 269 nm). PTH alanine was isolated as a single peak (t_R = 10.7 min), as was PTH aminobutyric acid (t_R = 12.9 min). For each cycle of the Edman degradation generating a PTH derivative incorporating an unknown amount of deuterium, a manual collection of the PTH species was performed (a reliable and practical automated system could not be achieved). Deuterium incorporation was quantified using an 1100MSD single-quadrupole instrument from Agilent Technologies (formerly Hewlett-Packard). This low-resolution ESI system was equipped with an HPLC interface and variable-wavelength detector for LCMS analysis. Standards of PTH alanine and PTH α-aminobutyric acid were used for establishing an appropriate HPLC method (see Supporting Information) using a steel-walled column (Phenomenex Luna, 2.1 × 50 mm, 5 μm, at 50 °C). In the method employed, a 5-μL injection was applied and a gradient of water and methanol (0.1% TFA) was used to isolate each PTH amino acid: 10% methanol, climbing to 95% in 5 min, remaining at this concentration for an additional 5 min (flow rate 0.25 mL/min).

NMR Spectroscopy. NMR spectra were acquired on a Varian Inova 600 spectrometer at 27 °C; data matrixes of 2048 detected and 1024 indirect data points with 64 scans were recorded and processed using a 90°-shifted sine bell window function (unshifted for DQF−COSY). Solvent signal suppression was achieved by transmitter presaturation. Samples of the A1 peptide were prepared in either 90% H_2O−10% D_2O (for amide analysis) or 100% D_2O at a peptide concentration of 2 mM. All experiments with the A1 peptide were performed at pH 2 (H_2O containing 0.1% TFA). Under these conditions, the solubility of the peptide was greatly improved, likely due to reduced peptide aggregation via protonation of cationic side chains. Samples of the more hydrophobic A2 peptide were prepared in either 100% CD_3OH (for amide analysis) or 100% CD_3OD at a peptide concentration of 2 mM. The assignment of ^1H resonances was performed using standard two-dimensional DQF−COSY (*39*), TOCSY (*40*) (mixing time 75 ms), and NOESY (*41*) (mixing time 200 ms) experiments.

Structure Calculations. Structures for lacticin A1 and A2 peptides were calculated from 301 and 381 unique NOEs, respectively, using CNS 1.1 (*42*). For preliminary structure determinations, the stereochemistry of the dehydrobutyrine residues and lanthionine and β-methyllanthionine bridges was assigned to be *Z*, (2*S*,6*R*), and (2*S*,3*S*,6*R*), respectively, in accord with those reported for all known lantibiotics. Topology and parameter files for these nonstandard amino acids were graciously supplied by Shang-Te Hsu (*43*).

RESULTS AND DISCUSSION

Preliminary Characterization of Lacticin 3147 and Amino Acid Composition. Early attempts at sequencing the lacticin peptides by Edman degradation (*44*) gave limited information about the A1 peptide (12 of 30 residues) and no data for the A2 peptide (N-terminally blocked). The preliminary sequence data obtained did, however, provide a tentative confirmation of the structural genes identified earlier (*45*). Amino acid analysis revealed that, in both peptides, many of the hydroxy side-chain residues (serine and threonine) predicted were absent in the mature peptides. Also quantified in both peptides was the presence of D-alanine, lanthionine, and β-methyllanthionine (Table 1). On the basis of the number

Table 1: Amino Acid Analysis of A1 and A2 Peptides

	A1 peptide		A2 peptide	
amino acid	predicted[a]	detected	predicted[a]	detected
Ser	3	0.7	3	0.1
Thr	4	0.0	8	2.3
L-Ala[c]	2	2.3	4	4.3
D-Ala[c]	0	1.1	0	2.1
Cys	4	nd[b]	3	nd[b]
Lan/MeLan	0	3.2	0	2.9

[a] Predicted from gene sequences. [b] Not detected. [c] D/L-alanine ratio by chiral GC−MS of hydrosylate.

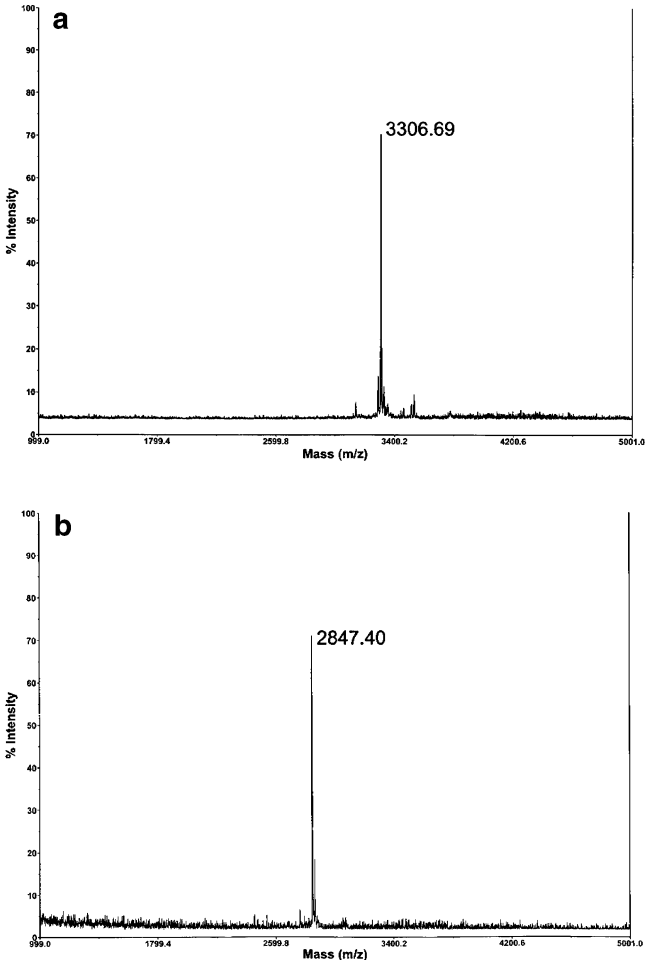
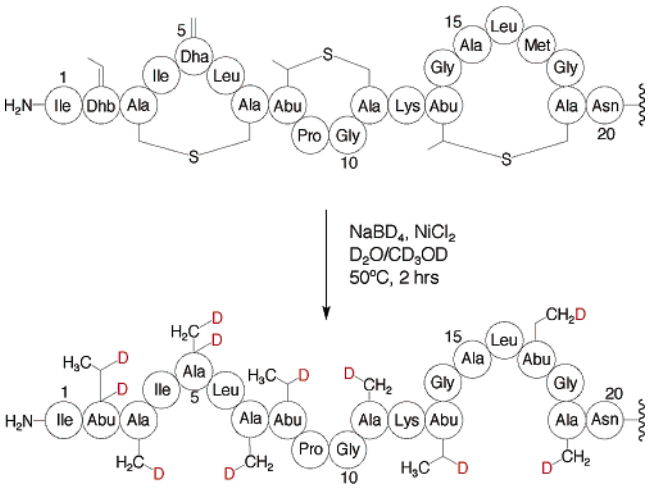


FIGURE 2: (a) MALDI-TOF mass spectrum of lacticin 3147 A1. (b) MALDI-TOF mass spectrum of lacticin 3147 A2.

of dehydrations required and the number of lanthionine or *β*-methyllanthionine residues present in each peptide, predictions for the masses of the mature peptides were made. In the case of A1, the ribosomal peptide predicted by the structural gene has a mass of 3430.88 Da. Invoking seven dehydrations (−126.14 Da) and one reduction, leading to a new D-alanine center (+2.02 Da), a mass of 3306.76 Da is suggested, agreeing well with the experimental value of 3306.69 ± 0.5 Da (Figure 2a). The A2 ribosomal peptide predicted by the structural gene has a mass of 2986.44 Da. In this case, eight dehydrations (−144.16 Da), two reductions leading to new D-alanine centers (+4.04 Da), and a N-terminal deamination to an α-keto amide (+1.01 Da) suggests a mass of 2847.33 Da, also agreeing well with the experimental value of 2847.40 ± 0.5 Da (Figure 2b). These preliminary calculations and the number of lanthionine or *β*-methyllanthionine residues detected suggest the presence

Scheme 2 : Deuterating Nickel Boride Modification of Nisin[a]



[a] Deuterium incorporation (in red) identifies original location of dehydro and lanthionine residues. Also, methionine 17 desulfurized to yield aminobutyric acid (Abu) with indicated deuterium incorporation.

of two dehydro amino acid side chains in each mature lantibiotic peptide.

Deuterating Desulfurization/Reduction of Lantibiotics with Ni_2B. Prior to our work with the lacticin 3147 peptides, nisin A was used as a model lantibiotic and treated with nickel boride under deuterating conditions. The product peptide was subjected to Edman sequencing, and those residues (within the first 20) known to be dehydrated, or to be part of the lanthionine bridges in the native peptide, were collected for mass analysis. The results clearly allow for distinction of those residues involved in lanthionine bridges and those that are dehydrated in the native peptide (Scheme 2). With the successful deuteration of nisin A, the focus became the modification of the lacticin 3147 peptides. The A2 peptide was not immediately amenable to the nickel boride modification strategy. Initial sequencing work with the native peptide returned no data, suggesting an N-terminal α-keto amide moiety (blocking N-terminal sequencing (*13*)). Using the dinucleophile 1,2-diaminobenzene in strong acetic acid/sodium acetate buffer, the two N-terminal residues were removed to provide a free amino terminus. Nickel boride treatment of the lacticin 3147 peptides, followed by the same analysis protocol used with nisin, provided similar deuterium incorporation results (Figure 3).

While this methodology does not reveal all aspects of a lantibiotic peptide's structure (precise lanthionine bridging patterns in particular), it remains a valuable tool. The microscale capability of this approach is especially valuable when dealing with quantities of peptide too small for conventional approaches such as multidimensional NMR and crystallography. Nickel boride chemistry as well as Edman sequencing are reliably used with submilligram quantities of peptide, and mass spectrometric analysis of the PTH derivatives is routinely performed on the nanogram scale.

NMR Spectroscopy. After milligram quantities of both peptides were amassed, the structure elucidation of the lacticin 3147 peptides turned to the use of NMR spectroscopy. The most important structural challenge still remaining was that of the lanthionine bridging patterns in both peptides. A number of two-dimensional NMR experiments were

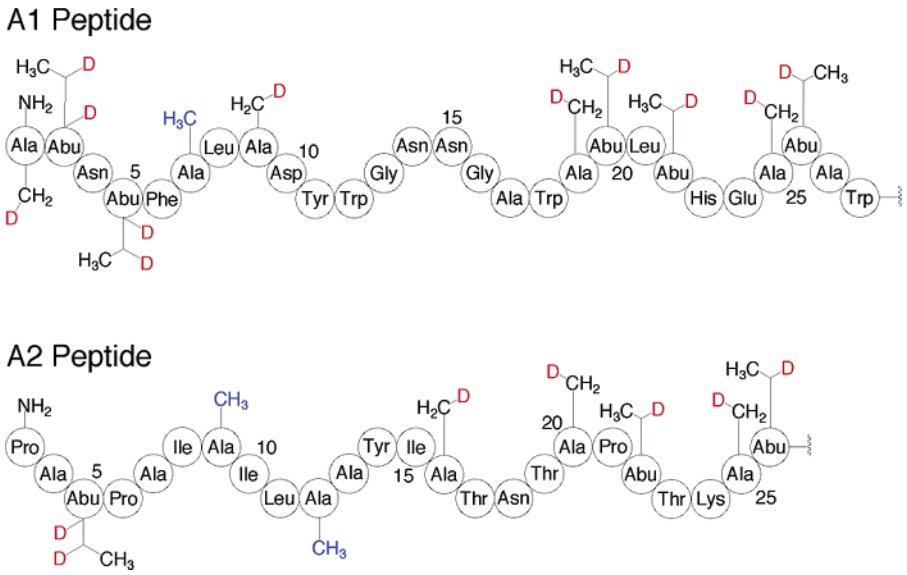


FIGURE 3: Dehydro residue and lanthionine bridge location in lacticin A1 and A2 peptides. Deuterium incorporation, indicated in red, shows likely positions of lanthionine linkages and dehydro side chains in the mature lantibiotic peptide. Alanines in blue indicate likely site(s) of D-alanine residues produced by enzymatic reduction of dehydroalanine. Note that the A1 peptide is clipped by one residue at the N-terminus; for the A2 peptide two residues are removed by 1,2-diaminobenzene N-terminal deblocking.

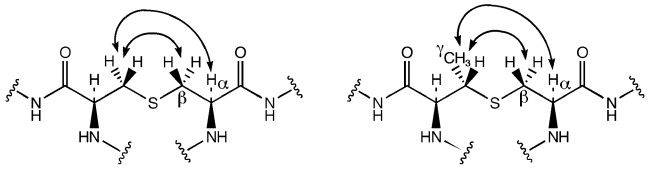


FIGURE 4: Intrabridge NOEs used in Lan/MeLan bridging pattern assignment.

performed with each of the lacticin 3147 peptides. The spin systems in the TOCSY spectra of lacticin A1 and A2 (see Supporting Information) were assigned to the respective residues by taking into consideration the characteristic frequencies and numbers of resonances. Sequential assignment of the peptides agreed completely with results from the Edman degradation and was achieved using the $d_{\alpha N}(i,i+1)$ and $d_{NN}(i,i+1)$ connectivities in the NOESY spectra. Finally, intrabridge NOEs were used to establish the connectivities of the lanthionine and β-methyllanthionine linkages in each peptide (see Supporting Information). The most useful correlations detected in this analysis were the α/β and β/β interactions in all the bridges as well as the α/γ and β/γ interactions present for β-methyllanthionine moieties (Figure 4).

The NMR spectra acquired for the lacticin 3147 peptides not only allowed for identification of the lanthionine bridges, they also indicated that the peptides were structured. Broad spectral dispersion, with very little degeneracy of chemical shifts, was observed for the amide protons in both the A1 and A2 peptides (see Supporting Information), and a number of medium- and long-range NOEs were present in the NOESY spectra of each peptide. Therefore, full structure determination for both lacticin A1 and A2 has been initiated.

In methanol, residues Ala7 to Ile15 of the A2 peptide form an α-helix. The C-terminal half of the peptide, containing the three lanthionine bridges, is in an extended conformation. Future studies will determine whether formation of the α-helix is induced by membrane-mimetic solvent, as is the case for many antibacterial peptides (*43*, *46*−*48*). Lacticin A1 was found to be unstructured and aggregated in methanol, as evidenced by broad line widths and poor spectral dispersion of the amide protons. In water, however, the structure of the polypeptide is well-defined. Many NOEs are observed between hydrophobic side chains in the C-terminal half of the molecule, indicating it forms a globular domain similar to that observed for mersacidin (*43*). The N-terminus, from residues Lan1 to Leu8, is in an extended conformation.

Solved Structures. The structures and bridging patterns suggested by the NMR spectroscopy results are in complete agreement with all data obtained for the peptides via the nickel boride desulfurization/reduction and Edman degradation (Figure 5). Comparison with the structures of known lantibiotics indicates that the lanthionine bridging pattern of the A1 peptide is very similar to that of mersacidin (*22*). Mersacidin (Figure 6) is a small (20 residue), globular type-B lantibiotic with a lipid II-mediated mode of action (*43*, *49*). The A2 peptide is a member of the elongated type-A family of lantibiotics and may have similarity to lactocin S. Lactosin S is the only other lantibiotic proven to contain D-alanine (*50*), although a full structure elucidation has yet to be reported. Refinement of the preliminary 3D structures obtained for each of the lacticin peptides is underway, including solvent effects, conformational mobility, and chemical studies to determine structure−activity relationships. Future studies will focus on the interaction of the two peptides in the presence and in the absence of lipid II.

Proposed Mode of Action. The solution conformations of the lacticin 3147 peptides obtained by NMR (Figure 7) support a proposed dual mode of action for the synergistic peptide pair (*51*). The globular A1 peptide could be expected to adopt a conformation similar to that of mersacidin (*43*) upon contact with lipid II. It is noteworthy that many of the residues common to the A1 peptide and mersacidin have been shown to undergo conformational changes in NMR experiments with mersacidin in the presence of DPC micelles (used to mimic cell membranes) with and without lipid II. Specifically, two-step $^{15}N-^{1}H$ HSQC titration revealed that

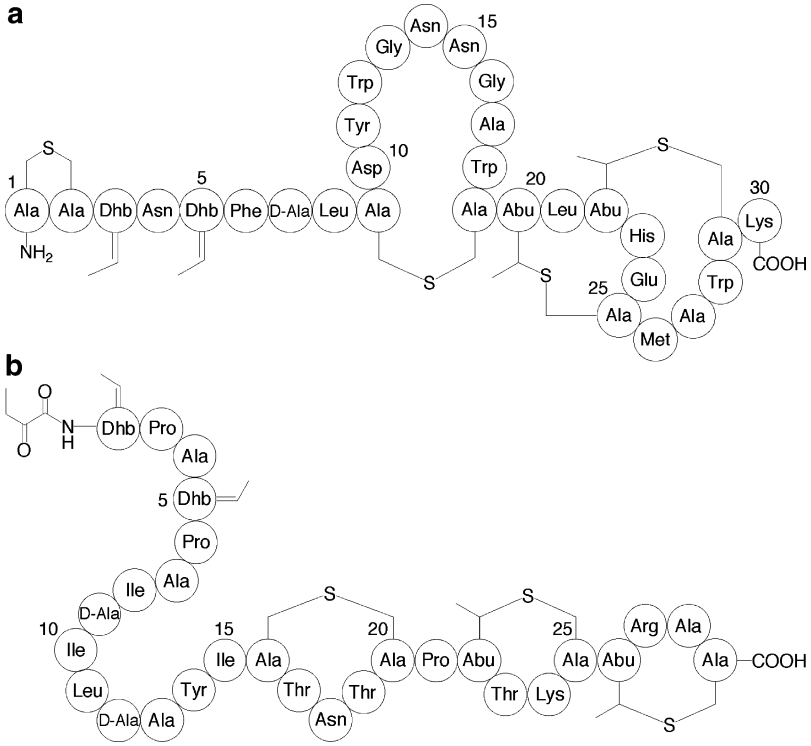


FIGURE 5: (a) Lacticin 3147 A1. (b) Lacticin 3147 A2.

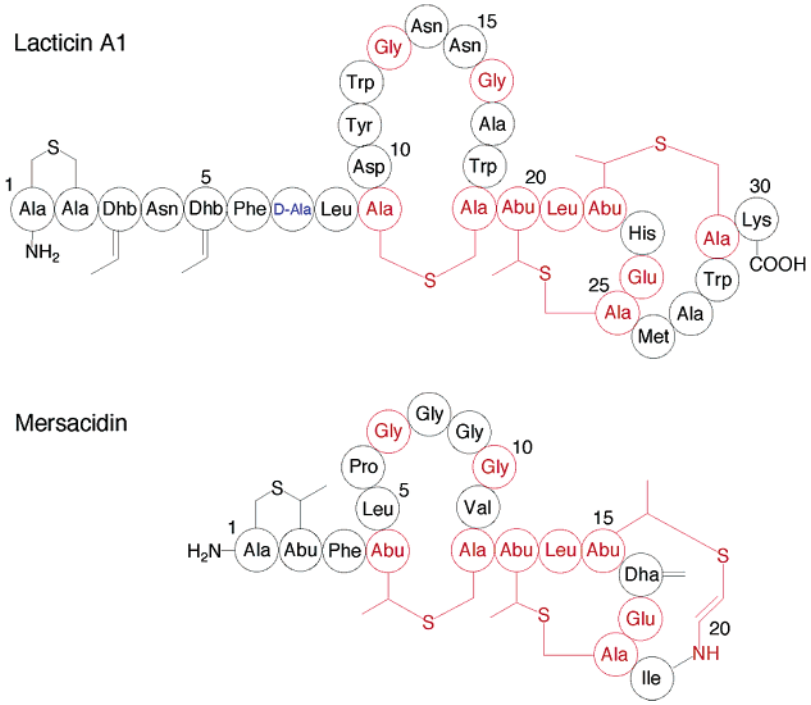


FIGURE 6: Comparison of mersacidin and lacticin 3147 A1. Highlighted in red are the structural similarities between the two globular type-B peptides.

the addition of DPC micelles strongly affects Gly7, Abu 13, Abu15, and Glu17 in the ^{1}H dimension and indeed most of the C-terminal residues in the ^{15}N dimension. Subsequent addition of lipid II is associated with perturbation of Gly7, Abu13, and Glu17 in both dimensions (*43*). The important nature of this conserved region was further demonstrated with the observation that a Glu17Ala alteration in mersacidin abolishes antimicrobial activity (*52*). It is thus likely that the corresponding amino acids in lacticin A1 play a role in its interaction with the cell membrane and suggest interaction with lipid II. It may also emerge that the junction between Ala19 and Abu20 in the A1 peptide serves as a hinge for the opening and closing of ring structures, as is the case with the corresponding region in mersacidin (*43*). The A2 peptide is clearly a more elongated species potentially capable of pore formation in target membranes.

Earlier specific activity investigations (*51*) demonstrated that it is necessary for the A1 peptide to be bound to cells prior to the A2 peptide in order to induce cell death. This observation, in combination with the solution conformations of both peptides, suggests a model for the mode of action of lacticin 3147 in which the A1 peptide may initially bind to

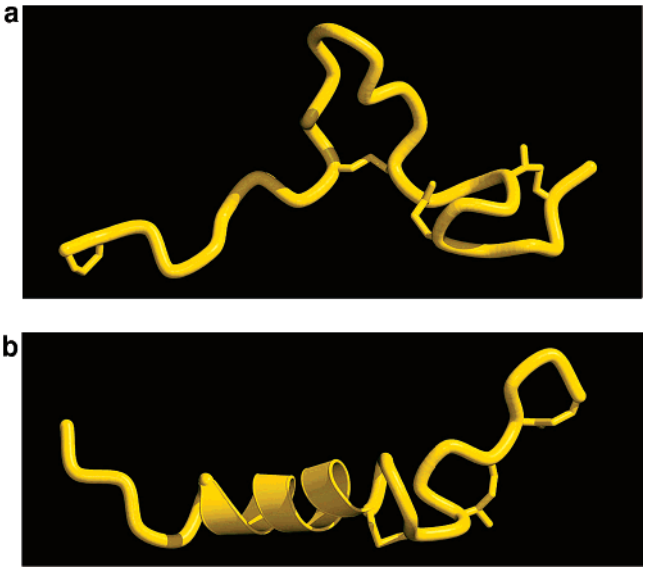


FIGURE 7: (a) NMR solution conformation of lacticin 3147 A1 in water. (b) NMR solution conformation of lacticin 3147 A2 in methanol.

lipid II (possibly also inhibiting cell wall biosynthesis through the prevention of transglycosylation). The killing activity of the A1 peptide is significantly enhanced by the presence of the A2 peptide, which we propose may elicit or stabilize possible pore formation and depolarization of the cell membrane (substantiated by earlier potassium ion release experiments). The partial activity of the A1 peptide alone and the fact that the A1 peptide combined with the A2 peptide causes cell death at single nanomolar concentration support this suggestion (*51*).

Thus, lacticin 3147 may have a mode of action similar to that of nisin; however, in the case of nisin the killing effect is elicited by a single bifunctional peptide, whereas in the case of lacticin 3147, cell wall inhibition and pore-forming functions may be separated across two peptides (*51*). The dual functionality of nisin, and potentially lacticin 3147, seems to be the basis of the very high specific activity relative to eukaryotic antimicrobial peptides, many of which exert their effect at higher concentrations without the involvement of a docking molecule. Thus, the dual functioning peptides may provide better candidates for the treatment of human pathogens of clinical significance.

Summary and Conclusions. Most often, structure elucidations of lantibiotic peptides are plagued by incompatibility with standard analysis methods such as the Edman degradation. The combined use of nickel boride desulfurization/reduction chemistry using deuterium followed with Edman degradation and mass spectrometry represents a novel and general method that should work with any lantibiotic peptide. With the successful determination of the primary structures of both lacticin 3147 peptides, further NMR studies are possible.

Following the recent work of others with the lantibiotics nisin A (*10*, *53*, *54*), mersacidin (*43*), and mutacin 1140 (*55*), the interaction of the lacticin peptides with their proposed cellular target (lipid II) will next be examined. Such studies should help shed further light on the mechanism of action of lacticin 3147. It is likely that the A1 peptide is responsible for interaction with lipid II in a manner similar to that of mersacidin, and that the A2 peptide then recognizes the A1−lipid II association complex, causing or enhancing pore formation in the membrane of the target bacteria. No other two-component lantibiotic has been fully characterized. The structural characterization of lacticin 3147 presents a unique opportunity to expand understanding of its potential pore-forming abilities and antimicrobial effects.

ACKNOWLEDGMENT

The authors are grateful to Randy Whittal for assistance with mass spectrometry, to Paula O'Connor for advice and instruction in the isolation of the lacticin 3147 peptides, and to Glen Armstrong for assistance with peptide isolation.

SUPPORTING INFORMATION AVAILABLE

Peptide isolation procedures, mass spectrometry, and TOCSY and NOESY NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Klaenhammer, T. R. (1988) Bacteriocins of lactic acid bacteria, *Biochimie 70*, 337−349.
2. Tagg, J. R., Dajani, A. S., and Wannamaker, L. W. (1976) Bacteriocins of gram-positive bacteria, *Bacteriol. Rev. 40*, 722−756.
3. Gratia, A. (1925) Sur un remarquable example d'antagonisme entre deux souches de colibacille, *C. R. Seances Soc. Biol. Fil. 93*, 1040−1041.
4. Hansen, J. N., Banerjee, S., and Buchman, L. W. (1989) Potential of small ribosomally synthesized bacteriocins in the design of new food preservatives, *J. Food Safety 10*, 119−130.
5. Nissen-Meyer, J., and Nes, I. F. (1997) Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action, *Arch. Microbiol. 167*, 67−77.
6. Guder, A., Wiedemann, I., and Sahl, H.-G. (2000) Posttranslationally modified bacteriocins−the lantibiotics, *Biopolymers 55*, 62−73.
7. van Belkum, M. J., and Stiles, M. E. (2000) Nonlantibiotic antibacterial peptides from lactic acid bacteria, *Nat. Prod. Rep. 17*, 323−335.
8. Kellner, R., Jung, G., and Sahl, H.-G. (1991) Structure elucidation of the tricyclic lantibiotic Pep5 containing eight positively charged amino acids, in *Nisin and Novel Lantibiotics* (Jung, G., and Sahl, H.-G., Eds.) pp 141−158, ESCOM, Leiden.
9. Kaletta, C., Entian, K. D., Kellner, R., Jung, G., Reis, M., and Sahl, H.-G. (1989) Pep5, a new lantibiotic: structural gene isolation and prepeptide sequence, *Arch. Microbiol. 152*, 16−19.
10. Breukink, E., van Heusden, H. E., Vollmerhaus, P. J., Swiezewska, E., Brunner, L., Walker, S., Heck, A. J., and de Kruijff, B. (2003) Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes, *J. Biol. Chem. 278*, 19898−19903.
11. Gross, E., and Morell, J. L. (1971) Structure of Nisin, *J. Am. Chem. Soc. 93*, 4634−4635.
12. Hansen, J. N. (1994) Nisin as a model food preservative, *Crit. Rev. Food Sci. Nutr. 34*, 69−93.
13. Kellner, R., Jung, G., and Sahl, H.-G. (1991) *Nisin and novel lantibiotics*, ESCOM, Leiden.
14. Lian, L. Y., Chan, W. C., Morley, S. D., Roberts, G. C., Bycroft, B. W., and Jackson, D. (1992) Solution structures of nisin A and its two major degradation products determined by NMR, *Biochem. J. 283 (Pt. 2)*, 413−420.
15. Booth, M. C., Bogie, C. P., Sahl, H.-G., Siezen, R. J., Hatter, K. L., and Gilmore, M. S. (1996) Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic, *Mol. Microbiol. 21*, 1175−1184.
16. Garneau, S., Martin, N. I., and Vederas, J. C. (2002) Two-peptide bacteriocins produced by lactic acid bacteria, *Biochimie 84*, 577−592.
17. Ryan, M. P., Rea, M. C., Hill, C., and Ross, R. P. (1996) An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147, *Appl. Environ. Microbiol. 62*, 612−619.

18. McAuliffe, O., Hill, C., and Ross, R. P. (1999) Inhibition of *Listeria monocytogenes* in cottage cheese manufactured with a lacticin 3147-producing starter culture, *J. Appl. Microbiol. 86*, 251−256.
19. Ross, R. P. Assessment and Control of Foodborne Pathogens in Ireland, http://www.teagasc.ie/research/reports/dairyproduction/4541/eopr-4541.htm (August 2003).
20. Kettenring, J. K., Malabarba, A., Vekey, K., and Cavalleri, B. (1990) Sequence determination of actagardine, a novel lantibiotic, by homonuclear 2D NMR spectroscopy, *J. Antibiot. (Tokyo) 43*, 1082−1088.
21. Kogler, H., Bauch, M., Fehlhaber, H.-W., Griesinger, C., Schubert, W., and Teetz, V. (1991) NMR-spectroscopic investigations on mersacidin. in *Nisin and novel lantibiotics* (Jung, G., and Sahl, H.-G., Eds.) pp 159−170, ESCOM, Leiden.
22. Prasch, T., Naumann, T., Markert, R. L., Sattler, M., Schubert, W., Schaal, S., Bauch, M., Kogler, H., and Griesinger, C. (1997) Constitution and solution conformation of the antibiotic mersacidin determined by NMR and molecular dynamics. *Eur. J. Biochem. 244*, 501−512.
23. van de Kamp, M., van den Hooven, H. W., Konings, R. N., Bierbaum, G., Sahl, H.-G., Kuipers, O. P., Siezen, R. J., de Vos, W. M., Hilbers, C. W., and van de Ven, F. J. (1995) Elucidation of the primary structure of the lantibiotic epilancin K7 from *Staphylococcus epidermidis* K7. Cloning and characterisation of the epilancin-K7-encoding gene and NMR analysis of mature epilancin K7, *Eur. J. Biochem. 230*, 587−600.
24. Van de Ven, F. J., Van den Hooven, H. W., Konings, R. N., and Hilbers, C. W. (1991) NMR studies of lantibiotics. The structure of nisin in aqueous solution, *Eur. J. Biochem. 202*, 1181−1188.
25. Zimmermann, N., and Jung, G. (1997) The three-dimensional solution structure of the lantibiotic murein-biosynthesis-inhibitor actagardine determined by NMR, *Eur. J. Biochem. 246*, 809−819.
26. Zimmermann, N., Metzger, J. W., and Jung, G. (1995) The tetracyclic lantibiotic actagardine. ^{1}H-NMR and ^{13}C-NMR assignments and revised primary structure, *Eur. J. Biochem. 228*, 786−797.
27. Shiba, T., Wakamiya, T., Fukase, K., Ueki, Y., Teshima, T., and Nishikawa, M. (1991) Structure of the lanthionine peptides nisin, ancovenin and lanthiopeptin, in *Nisin and novel lantibiotics* (Jung, G., and Sahl, H.-G., Eds.) ESCOM, Leiden.
28. Morell, J. L., and Gross, E. (1973) Configuration of Beta-Carbon Atoms of Beta-Methyllanthionine Residues in Nisin, *J. Am. Chem. Soc. 95*, 6480−6481.
29. Gross, E., and Morell, J. L. (1970) Nisin−Assignment of Sulfide Bridges of Beta-Methyllanthionine to a Novel Bicyclic Structure of Identical Ring Size, *J. Am. Chem. Soc. 92*, 2919−2920.
30. Meyer, H. E., Heber, M., Eisermann, B., Korte, H., Metzger, J. W., and Jung, G. (1994) Sequence analysis of lantibiotics: chemical derivatization procedures allow a fast access to complete Edman degradation, *Anal. Biochem. 223*, 185−190.
31. Back, T. G., Yang, K. X., and Krouse, H. R. (1992) Desulfurization of Benzothiophenes and Dibenzothiophenes with Nickel Boride, *J. Org. Chem. 57*, 1986−1990.
32. Jung, M., Elsohly, H. N., Croom, E. M., McPhail, A. T., and McPhail, D. R. (1986) Practical Conversion of Artemisinic Acid into Desoxyartemisinin, *J. Org. Chem. 51*, 5417−5419.
33. Back, T. G., Baron, D. L., and Yang, K. X. (1993) Desulfurization with Nickel and Cobalt Boride−Scope, Selectivity, Stereochemistry, and Deuterium-Labeling Studies, *J. Org. Chem. 58*, 2407−2413.
34. Sahl, H.-G., Grossgarten, M., Widger, W. R., Cramer, W. A., and Brandis, H. (1985) Structural similarities of the staphylococcin-like peptide Pep-5 to the peptide antibiotic nisin, *Antimicrob. Agents Chemother. 27*, 836−840.
35. Gerharolt, J., and Nicholson, G. J. (1994) in *Peptides: Chemistry, Structure, and Biology* (Hodges, R. S., and Smith, J. A., Eds.) pp 241−243, ESCOM, Leiden.
36. Baldwin, M. A., Medzihradszky, K. F., Lock, C. M., Fisher, B., Settineri, T. A., and Burlingame, A. L. (2001) Matrix-assisted laser desorption/ionization coupled with quadrupole/orthogonal acceleration time-of-flight mass spectrometry for protein discovery, identification, and structural analysis, *Anal. Chem. 73*, 1707−1720.
37. Sunde, M., Sparkes, M. J., and Dixon, H. B. (1998) Improved conditions for the removal of 2-oxoacyl groups from the N-terminus of proteins, *Biochim. Biophys. Acta 1388*, 45−52.
38. Stevens, J., and Dixon, H. B. (1995) The removal of 2-oxoacyl residues from the N-terminus of peptides and cystatin in non-denaturing conditions, *Biochim. Biophys. Acta 1252*, 195−202.
39. Piantini, U., Sorensen, O. W., and Ernst, R. R. (1982) Multiple Quantum Filters for Elucidating NMR Coupling Networks, *J. Am. Chem. Soc. 104*, 6800−6801.
40. Braunschweiler, L., and Ernst, R. R. (1983) Coherence Transfer by Isotropic Mixing−Application to Proton Correlation Spectroscopy, *J. Magn. Reson. 53*, 521−528.
41. Jeener, J., Meier, B. H., Bachmann, P., and Ernst, R. R. (1979) Investigation of Exchange Processes by 2-Dimensional NMR-Spectroscopy, *J. Chem. Phys. 71*, 4546−4553.
42. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination, *Acta Crystallogr. D: Biol. Crystallogr. 54 (Pt. 5)*, 905−921.
43. Hsu, S. T., Breukink, E., Bierbaum, G., Sahl, H.-G., de Kruijff, B., Kaptein, R., van Nuland, N. A., and Bonvin, A. M. (2003) NMR study of mersacidin and lipid II interaction in dodecylphosphocholine micelles. Conformational changes are a key to antimicrobial activity, *J. Biol. Chem. 278*, 13110−13117.
44. Ryan, M. P., Jack, R. W., Josten, M., Sahl, H.-G., Jung, G., Ross, R. P., and Hill, C. (1999) Extensive post-translational modification, including serine to D-alanine conversion, in the two-component lantibiotic, lacticin 3147, *J. Biol. Chem. 274*, 37544−37550.
45. Dougherty, B. A., Hill, C., Weidman, J. F., Richardson, D. R., Venter, J. C., and Ross, R. P. (1998) Sequence and analysis of the 60 kb conjugative, bacteriocin-producing plasmid pMRC01 from *Lactococcus lactis* DPC3147, *Mol. Microbiol. 29*, 1029−1038.
46. Wang, Y., Henz, M. E., Gallagher, N. L., Chai, S., Gibbs, A. C., Yan, L. Z., Stiles, M. E., Wishart, D. S., and Vederas, J. C. (1999) Solution structure of carnobacteriocin B2 and implications for structure−activity relationships among type IIa bacteriocins from lactic acid bacteria, *Biochemistry 38*, 15438−15447.
47. Fregeau-Gallagher, N. L., Sailer, M., Niemczura, W. P., Nakashima, T. T., Stiles, M. E., and Vederas, J. C. (1997) Three-dimensional structure of leucocin A in trifluoroethanol and dodecylphosphocholine micelles: spatial location of residues critical for biological activity in type IIa bacteriocins from lactic acid bacteria, *Biochemistry 36*, 15062−15072.
48. Uteng, M., Hauge, H. H., Markwick, P. R., Fimland, G., Mantzilas, D., Nissen-Meyer, J., and Muhle-Goll, C. (2003) Three-dimensional structure in lipid micelles of the pediocin-like antimicrobial peptide sakacin P and a sakacin P variant that is structurally stabilized by an inserted C-terminal disulfide bridge, *Biochemistry 42*, 11417−11426.
49. Brotz, H., Bierbaum, G., Leopold, K., Reynolds, P. E., and Sahl, H.-G. (1998) The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II, *Antimicrob. Agents Chemother. 42*, 154−160.
50. Skaugen, M., Nissen-Meyer, J., Jung, G., Stevanovic, S., Sletten, K., Inger, C., Abildgaard, M., and Nes, I. F. (1994) In vivo conversion of L-serine to D-alanine in a ribosomally synthesized polypeptide, *J. Biol. Chem. 269*, 27183−27185.
51. Morgan, S. M., O'Conner, P. M., Cotter, P. D., Ross, R. P., and Hill, C., unpublished results.
52. Szekat, C., Jack, R. W., Skutlarek, D., Farber, H., and Bierbaum, G. (2003) Construction of an expression system for site-directed mutagenesis of the lantibiotic mersacidin, *Appl. Environ. Microbiol. 69*, 3777−3783.
53. Hsu, S. T., Breukink, E., de Kruijff, B., Kaptein, R., Bonvin, A. M., and van Nuland, N. A. (2002) Mapping the targeted membrane pore formation mechanism by solution NMR: the nisin Z and lipid II interaction in SDS micelles, *Biochemistry 41*, 7670−7676.
54. van Heusden, H. E., de Kruijff, B., and Breukink, E. (2002) Lipid II induces a transmembrane orientation of the pore-forming peptide lantibiotic nisin, *Biochemistry 41*, 12171−12178.
55. Smith, L., Zachariah, C., Thirumoorthy, R., Rocca, J., Novak, J., Hillman, J. D., and Edison, A. S. (2003) Structure and dynamics of the lantibiotic mutacin 1140, *Biochemistry 42*, 10372−10384.

BI0362065