

Post-translational Modification of Therapeutic Peptides By NisB, the Dehydratase of the Lantibiotic Nisin

Leon D. Kluskens,^{‡,§} Anneke Kuipers,^{‡,§} Rick Rink,[‡] Esther de Boef,[‡] Susan Fekken,[‡] Arnold J. M. Driessen,^{||} Oscar P. Kuipers,[⊥] and Gert N. Moll^{*:‡}

BiOMaDe Technology Foundation, Nijenborgh 4, 9747 AG Groningen, The Netherlands, and Departments of Microbiology and Molecular Genetics, Kerlaan 30, 9751 NN Haren, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands

Received May 2, 2005; Revised Manuscript Received July 27, 2005

ABSTRACT: Post-translationally introduced dehydroamino acids often play an important role in the activity and receptor specificity of biologically active peptides. In addition, a dehydroamino acid can be coupled to a cysteine to yield a cyclized peptide with increased biostability and resistance against proteolytic degradation and/or modified specificity. The lantibiotic nisin is an antimicrobial peptide produced by *Lactococcus lactis*. Its post-translational enzymatic modification involves NisB-mediated dehydration of serines and threonines and NisC-catalyzed coupling of cysteines to dehydroresidues, followed by NisT-mediated secretion. Here, we demonstrate that a *L. lactis* strain containing the *nisBTC* genes effectively dehydrates and secretes a wide range of medically relevant nonlantibiotic peptides among which variants of adrenocorticotrophic hormone, vasopressin, an inhibitor of tripeptidyl peptidase II, enkephalin, luteinizing hormone-releasing hormone, angiotensin, and erythropoietin. For most of these peptides, ring formation was demonstrated. These data show that lantibiotic enzymes can be applied for the modification of peptides, thereby enabling the biotechnological production of dehydroresidue-containing and/or thioether-bridged therapeutic peptides with enhanced stability and/or modulated activities.

The presence of unusual dehydroamino acids in peptides can have a large effect on the biological activity. For instance, a synthesized pentapeptide containing a dehydroalanine acts as a potent inhibitor of a spider venom peptide epimerase (1). Dehydroalanines at position 5 in nisin and subtilin are responsible for the inhibition of the outgrowth of bacterial spores by reacting with sulfhydryl groups of membrane components (2). Besides their role in inhibitors, dehydroalanines can increase the efficiency by which (poly)peptides transmit signals by interacting with receptor or acceptor molecules; the synthesis of a dehydroalanine-containing neurokinin A receptor antagonist resulted in a more rigid and potent peptide (3). Dehydroamino acids are also important for the activity of, among others, thioestrepton, nosiheptide, and berninamycin (4). Dehydroresidues can furthermore be versatile starting points for the synthesis of unnatural amino acids, attachment sites for further modification, or serve as sites for peptide cyclization. Intramolecular coupling of a dehydroresidue to a cysteine has proven to be a valuable method to obtain biostable analogues with resistance against proteolytic degradation (5, 6) or modulated receptor interaction (7–9).

A well-known group of cyclized peptides is formed by the lantibiotics: antimicrobial peptides that contain the

thioether amino acids lanthionine and/or methylanthionine (10). A variety of lantibiotic activities is known, which all depend on the presence of thioether rings (11–14). The best studied lantibiotic is nisin, a widely applied food preservative that is produced by a number of *Lactococcus lactis* strains (15). Nisin biosynthesis involves the activity of four enzymes (5, 16). The nisin dehydratase (NisB)¹ dehydrates serines and threonines in the nisin propeptide, after which the formed dehydroresidues are stereo- and regiospecifically coupled to cysteines by the nisin cyclase (NisC) (Figure 1). The ABC transporter NisT then exports the fully modified prenisin, whereupon the extracellular peptidase NisP cleaves off the leader peptide, to liberate active nisin that contains four methylanthionines, one lanthionine, two dehydroalanines, and one dehydrobutyryne (17).

Chemical synthesis of dehydroamino acids in peptides (18–21) is costly, and chemical cyclization methods are cumbersome and primarily lack regio- and stereoselectivity. Chemical cyclization methods may also result in oligomerization (22). On the other hand, fermentative methods in which peptides are enzymatically modified would allow for a controlled formation of the desired product. In the past, only a few dehydroresidues in lantibiotics have been engineered (6, 15, 23) and only one new thioether ring in an existing lantibiotic has been generated (6). Here, we demonstrate that dehydroamino acids can be introduced in

* To whom correspondence should be addressed: BiOMaDe Technology Foundation, Nijenborgh 4, 9747 AG Groningen, The Netherlands. Telephone: +31-50-3638070. Fax: +31-50-3634429. E-mail: moll@biomade.nl.

[‡] BiOMaDe Technology Foundation.

[§] Contributed equally to this work.

^{||} Department of Microbiology, University of Groningen.

[⊥] Department of Molecular Genetics, University of Groningen.

¹ Abbreviations: NisB, nisin dehydratase; NisT, nisin transporter; NisC, nisin cyclase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; CDAP, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate.

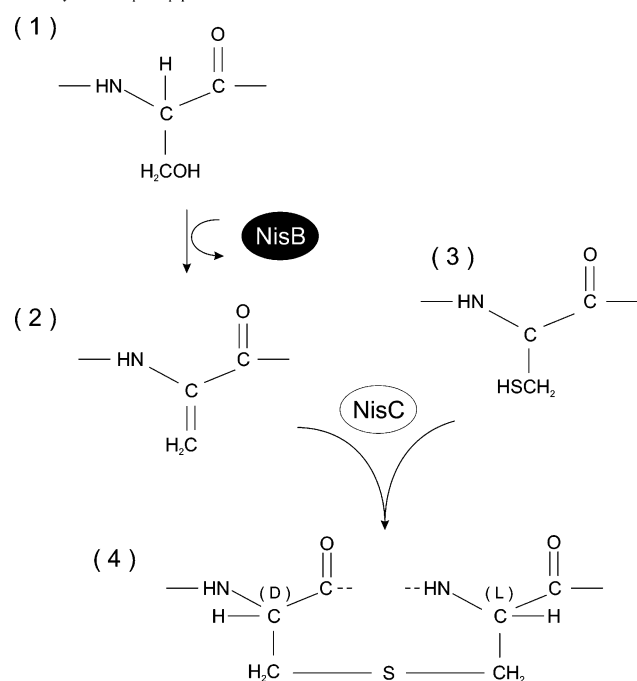


FIGURE 1: Intramolecular thioether bridge formation by the nisin biosynthesis enzymes. NisB dehydrates serine (1) (or threonine) and NisC couples the formed dehydroalanine (2) (or dehydrobutyryne) to cysteine (3), thus forming a lanthionine (4) (or methyl-lanthionine) ring.

a broad range of therapeutic peptides by exploiting the bacterial serine/threonine dehydratase, NisB.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The strains and plasmids used in this study are listed in Table 1. Peptides were encoded on pNZ8048-derived plasmids. Peptides were coexpressed with pIL253-based plasmids containing the *nisBTC* genes, except in combination with the peptide ACTH, for which the *nisBTC* genes were located on a pNZ8048-derived plasmid. Angiotensin (1–7) was encoded on the pILNisBTC plasmid.

Molecular Cloning. Nisin genes or combinations were amplified from chromosomal DNA of *L. lactis* NZ9700,

using Expand High Fidelity Polymerase (Roche, Mannheim, Germany) or *Pfu* polymerase (Invitrogen, CA). Plasmid DNA was isolated using the QIAGEN purification kit (Qiagen). DNA was restricted using restriction enzymes from New England BioLabs, Inc. Ligation was carried out with T4 DNA ligase (Roche). DNA fragments were isolated from agarose gel using the Zymoclean gel DNA recovery kit (Orange, CA) or from a PCR mix using the Roche PCR purification kit. Electrotransformation of *L. lactis* was carried out as previously described (24) using a Bio-Rad gene pulser (Biorad, Richmond, CA). Nucleotide sequence analyses were performed by BaseClear (Leiden, The Netherlands).

Growth Conditions. *L. lactis* was grown at 30 °C in MG17 broth, as described previously (25). Sample preparation for mass spectrometry or Western blot analysis was carried out as follows: overnight cultures of *L. lactis* were transferred to fresh MG17 medium. At an OD_{660 nm} of 0.4, cells were pelleted (2057g, 5 min, Eppendorf 5810 R) and the medium was replaced by minimal medium, adapted from Jensen and Hammer (26). Induction was carried out by adding 1/1000 volume of filtered (0.45 μm) medium from an overnight-grown culture of the nisin-producer *L. lactis* NZ9700. Incubation was continued overnight.

Sample Preparation. Samples were purified from the medium fraction by ziptip purification (C18 ziptip, Millipore) (27). Peptides from larger volumes were precipitated with 10% trichloroacetic acid (TCA) and kept on ice for 2 h. The sample was then pelleted by centrifugation at 18514g during 30 min at 4 °C, washed with acetone, and vacuum-dried. In the case of EPO, the medium fraction was freeze-dried (Labconco), desalted using PD-10 columns (Amersham Biosciences), and again freeze-dried. Dehydration was confirmed by ethanethiol treatment (28). A total of 40 μL of an ethanethiol mixture (80 μL of ethanol, 65 μL of 5 M NaOH, 60 μL of ethanethiol, and 400 μL of MQ) was added to vacuum-dried peptide and incubated at 50 °C for 2.5 h. The reaction was stopped by adding 10 μL of acetic acid. CDAP (1-cyano-4-dimethylaminopyridinium tetrafluoroborate) was used to react with free cysteine residues. Vacuum-dried sample was resuspended in 9 μL of 25 mM citrate buffer at pH 3.0 and reduced with 1 μL of Tris[2-

Table 1: *Lactococcus lactis* Strains and Plasmids

	characteristics	reference
strains		
NZ9700	<i>nisABTCIPRKEFG</i>	44
NZ9000	<i>nisRK</i> ⁺	44
plasmids		
pIL253-derived plasmids		
pIL2angBTC		45
pIL3BTC	<i>nisBTC</i> , derived from pIL2angBTC by deletion of <i>Em-r</i> gene and introducing stop codon at start <i>ang</i> gene	46
pIL4BTC	<i>nisBTC</i> , derived from pIL2angBTC by deletion of <i>Cm-r</i> gene and introducing stop codon at start <i>ang</i> gene	this paper
pNZ8048 derived plasmids		
pNGnisBT	<i>nisBT</i> , <i>Em-r</i> or <i>Cm-r</i>	44
pLPacth	leader peptide fused to SYSMECFRWG, <i>Cm-r</i>	27
pTPvp1	leader peptide fused to ATFQCAPRG, <i>Cm-r</i>	this paper
pTPvp2	leader peptide fused to AYTQNCPRG, <i>Cm-r</i>	this paper
pLPvp3	leader peptide fused to ITSYPQCTPRC, <i>Cm-r</i>	this paper
pTPtppII	leader peptide fused to ITSISRASVA, <i>Em-r</i>	this paper
pTPenkT4	leader peptide fused to ITSISKYTGFC, <i>Em-r</i>	this paper
pLPlhrh	leader peptide fused to ITSISLCTPGCKTGALMGCNKQHWSYGCRPG, <i>Em-r</i>	this paper
pLPepo	leader peptide fused to YASHFGPLGWVCK, <i>Cm-r</i>	this paper
pILang7BTC	leader peptide fused to DRVTIHC, <i>Cm-r</i> . Adaptation from pIL2angBTC	this paper

carboxyethyl]phosphine (TCEP). After a 10 min incubation at room temperature, 2 μ L of CDAP was added, followed by 15 min of incubation at room temperature. As positive controls, two chemically synthesized peptides (CRYTDPK-PHIRLRIK and MSTKDFNLDLVSVSKKDSGASPRITRICK) were used, both containing one cysteine. For tryptic digestion, TCA-precipitated LHRH was dissolved in 50 mM Tris at pH 6.8 (0.1 mg of LHRH/mL), 200 μ L of which was incubated with 20 μ L of trypsin (0.01 mg/mL) in the same buffer at 37 $^{\circ}$ C for 2 h.

Peptide Analysis. Mass spectra were recorded with a Bruker Biflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer. To maintain high sensitivity, an external calibration was applied. Trypsin-digested LHRH was separated on a reversed-phase high-performance liquid chromatography (HPLC) column (Alltima, C18, 5 μ m, Alltech chromatography). Separation was carried out at 1.0 mL/min, using acetonitrile (ACN) as a solvent in a gradient from 10 to 90%. Peak fractions (detection at 280 nm using a diode-array detector) were collected and analyzed by MALDI-TOF mass spectrometry. N-Terminal amino acid sequence analysis of LHRH was carried out by Eurosequence (Groningen, NL) using a Procise 494 sequencing system equipped with a 140C Microgradient System and a 785A Absorbance Detector (Applied Biosystems, Foster City, CA). Samples (1000 pmol) of LHRH were solubilized in 50% (v/v) acetic acid, and sequencing was performed using procedures and chemicals supplied by the manufacturer. Purified LHRH was modified by thiol addition, peroxidation using trifluoroacetic acid, and a second thiol addition step (29).

Western Blot Analysis. Polyclonal anti-leader peptide antibodies were raised in rabbits against the peptide H₂N-STKDFNLDLVSVSKKDC-CONH₂ coupled via the cysteine to keyhole limpet haemocyanin. TCA-precipitated peptides from 10 mL cultures were dissolved in 20 μ L of sample buffer and applied on a Tricine SDS-PAGE gel. Peptides were transferred to a PVDF Western blotting membrane (Roche) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). The membrane was blocked with 2% skim milk (Oxoid) in TBST (10 mM Tris-HCl at pH 8.0/150 mM NaCl/0.05% Tween-20) and 100 mM EDTA for 18 h at 4 $^{\circ}$ C and washed twice with TBST for 10 min. The membrane was incubated with anti-leader antibody (1:500) in TBST and 0.2% skim milk for 1 h at 25 $^{\circ}$ C, washed 3 times (10 min each) with 0.2% skim milk in TBST, and incubated with anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma) in TBST and 0.2% skim milk for 1 h at 25 $^{\circ}$ C (1:5000 dilution). The membrane was washed twice (10 min each) with TBST, followed by 6 times washing (10 min each) in alkaline phosphatase buffer (100 mM Tris-HCl at pH 9.5/100 mM NaCl/5 mM MgCl₂). Finally, the membrane was incubated with 10 mL of alkaline phosphatase, containing BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium), until bands appeared.

RESULTS AND DISCUSSION

We have recently shown that NisT of *L. lactis* excretes nonantibiotic peptides when fused to the nisin leader peptide. We examined here a range of medically relevant peptides with respect to their translocation and dehydration via

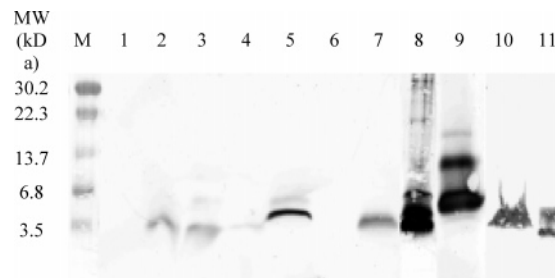


FIGURE 2: Extracellular production of nonantibiotic peptides in *L. lactis*. Samples were obtained by precipitating the medium fraction of an induced, overnight culture. Peptides were detected via Western blot analysis using anti-leader antibodies. *L. lactis* strain NZ9000 contained either pIL3BTC (lanes 6–9) or pIL4BTC (lanes 1–5 and 10), supplemented with the plasmids containing genes encoding the peptides to be modified. Maturation of angiotensin (1–7) was carried out on a one-plasmid system (pLang7BTC). M, marker; lane 1, pIL4BTC; lane 2, pIL4BTC/pLPacth; lane 3, pIL4BTC/pTPvp1; lane 4, pIL4BTC/pTPvp2; lane 5, pIL4BTC/pLPvp3; lane 6, pIL3BTC; lane 7, pIL3BTC/pTPpp II; lane 8, pIL3BTC/pTPenkT4; lane 9, pIL3BTC/pLPllrh, lane 10, pIL4BTC/pLPepo; and lane 11, pLang7BTC.

NisBTC. For that purpose, the nisin propeptide was partly or entirely replaced by a nonantibiotic peptide. In addition, serine/threonine and cysteine mutants of bioactive peptides were made, aiming at dehydration or thioether bridge formation. Peptide production and export was examined using *L. lactis* NZ9000 cells, containing one plasmid carrying the *nisBTC* genes and another one carrying the code for the fusion peptide (Table 1). Peptides, including mutants of ACTH, vasopressin, an inhibitor of tripeptidylpeptidase II, enkephalin, LHRH, angiotensin (1–7), and EPO, were isolated from the medium fraction and identified via immunodetection with anti-leader peptide antibodies (Figure 2). Clear expression was observed when peptides were either directly cloned behind the nisin leader (lanes 2–4 and 10–11) or fused with part of the N-terminal amino acids of nisin (lanes 5 and 7–9). In some cases, the latter strategy resulted in a significantly higher production level (data not shown). The absence of peptides in the medium fractions of NZ9000 with only pIL4BTC or pIL3BTC (Figure 2, lanes 1 and 6, respectively) proved that the detected peptides are encoded on the cotransformed fusion peptide constructs. Without nisin induction, no peptides were observed (not shown).

To investigate whether the exported fusion peptides were dehydrated, ziptip-treated culture medium (27) was subjected to MALDI-TOF mass spectrometry (Table 2). Measured values consistently refer to fusion peptides with and without the N-terminal Met of the leader peptide. For clarity, Table 2 only shows values belonging to fusion peptides without the initial Met. The corresponding mass spectra are electronically available as Supporting Information (Figures S1–S11).

The adrenocorticotrophic hormone (ACTH) acts upon the adrenal cortex by stimulating the secretion of glucocorticoids, such as cortisol. An ACTH fragment (1–10), with a H6C mutation (SYSMECFRWG), was fused to the leader peptide. Mass spectrometry analysis of the produced peptide yielded peaks corresponding to one (3581.8 Da) and no (3600.0 Da) dehydration (Table 2), suggesting dehydration of one of the two serine residues. To establish which one was dehydrated, the serines were independently changed into glycines by site-directed mutagenesis and the peptides were matured and exported via NisBTC. Mass spectrometry analysis of the new

Table 2: Dehydration of Therapeutic Peptides and Analogues by NisB, Analyzed by MALDI–TOF Mass Spectrometry^a

peptide ^b	sequence	dehydration extent	mass (M + H ⁺) w/o Met1 (Da)			
			dehydration		+ ethanethiol	
			observed	calculated	observed	calculated
ACTH(1–10)	<u>SYSM</u> ECFRWG	1	3581.8	<i>3582.1</i>	3643.9	<i>3644.2</i>
		0	3600.0	<i>3600.1</i>		
ACTH(S1G)	<u>GYSME</u> CFRWG	1	3552.3	<i>3552.0</i>	3613.8	<i>3614.1</i>
		0	3570.7	<i>3570.0</i>		
ACTH(S3G)	<u>SYGME</u> CFRWG	1		<i>3552.0</i>		<i>3614.1</i>
		0	3570.6	<i>3570.0</i>		
VP1	<u>ATFQC</u> APRG	1	3264.8	<i>3266.7</i>	3329.4	<i>3328.8</i>
		0		<i>3284.7</i>		
VP2	<u>AYTQN</u> CPRG	1	3327.6	<i>3325.7</i>	3390.6	<i>3387.8</i>
		0	3345.3	<i>3343.7</i>		
VP3	<u>ITSYFQCT</u> PRC	3	3625.3	<i>3625.2^c</i>	3814.0	<i>3813.3</i>
		2	3643.0	<i>3643.2</i>	3769.7	<i>3768.3</i>
		1	3662.4	<i>3661.2</i>	3727.2	<i>3723.3</i>
		0		<i>3679.2</i>		
TPP II	ITSIS <u>RAS</u> VA	4	3265.5	<i>3266.7</i>	3514.9	<i>3517.8</i>
		3	3283.8	<i>3284.7</i>	3470.9	<i>3472.8</i>
		2	3302.4	<i>3302.7</i>	3427.3	<i>3427.8</i>
		1	3320.1	<i>3320.7</i>	3383.5	<i>3382.8</i>
		0	3338.7	<i>3338.7</i>		
EnkT4	ITSISKY <u>TGFC</u>	4	3482.0	<i>3482.0</i>	3734.6	<i>3733.1</i>
		3	3500.4	<i>3500.0</i>	3690.1	<i>3688.1</i>
		2	3518.5	<i>3518.0</i>	3646.0	<i>3643.1</i>
		1	3536.5	<i>3536.0</i>	3601.9	<i>3598.1</i>
		0	3554.1	<i>3554.0</i>		
Enk.A	IAAIARY <u>TGFC</u>	1	3500.3	<i>3502.0</i>	3564.1	<i>3564.0</i>
		0	3518.2	<i>3520.0</i>		
Enk.B	IAAIARY <u>SGFC</u>	1	3488.9	<i>3488.0</i>	3550.1	<i>3549.8</i>
		0	3506.8	<i>3506.0</i>		
LHRH	ITSISLCTPGCK TGALMGCKNK <u>QHWSYGCRPG</u>	6	5499.8	<i>5497.4</i>	5871.0	<i>5874.5</i>
		5		<i>5515.4</i>		
Ang(1–7)	<u>DRV</u> TIHC	1	3157.9	<i>3159.6</i>	3219.5	<i>3221.7</i>
		0	3176.0	<i>3177.6</i>		
EPO(1–13)	<u>YASHFG</u> PLGWVCK	1	3784.1	<i>3781.3</i>	3843.9	<i>3843.4</i>
		0		<i>3799.3</i>		

^a The dehydration of serines/threonines was verified by ethanethiol modification. Amino acids part of the potentially active part of the peptide are underlined. Amino acids in bold correspond to mutations. Calculated values are displayed in italics. ^b VP, vasopressin; ACTH, adrenocorticotrophic hormone; TPP II, tripeptidylpeptidase II; Enk, enkephalin; LHRH, luteinizing hormone-releasing hormone; Ang, angiotensin; EPO, erythropoietin. ^c Leader peptide contains a S10L mutation.

ACTH variants (S1G and S3G, respectively) showed that dehydration only occurred when the serine at position 3 was left intact (Table 2). These data prove that in the original ACTH (SYSMECFRWG) the serine at position 1 is not dehydrated but the serine at position 3 is.

Vasopressin is an antidiuretic hormone with the sequence CYFQNCPRG-NH₂ and has a disulfide bond that bridges the two cysteines. We genetically made three fusion peptides of the nisin leader with vasopressin variants, termed VP1 (leader-ATFQCAPRG), VP2 (leader-AYTQNCPRG), and VP3 (leader-ITSYFQCTPRC). For both VP1 and VP2, mass spectrometry showed peaks corresponding to a single dehydration in each peptide. Because both peptides contain one threonine and no serines, the threonines at position 2 and 3, respectively, were dehydrated by NisB. VP3 contains the first two residues of nisin, IT, between the nisin leader and the vasopressin variant. Figure 3a depicts a MALDI–TOF mass spectrum of VP3 after dehydration. The spectra show peak patterns corresponding to 3, 2, and 1 time dehydrated VP3, with and without the N-terminal methionine attached.

The phosphopentapeptide RAS(P)VA inhibits tripeptidylpeptidase II (TPP II), after processing of the octapeptide

precursor VALRAS(P)VA. Chemical replacement of the serine by a dehydroalanine (RADhaVA) reduced the *K_i* of this already potent inhibitor 45 times (30). TPP II degrades, among others, cholecystokinin, responsible for satiety feeling, and inhibition of this peptidase may therefore contribute to battling obesity. The peptide termed TPP II (leader peptide fused to ITSISRASVA) was matured and exported via NisBTC. Mass spectrometry of the secreted peptide fused to the leader peptide resulted in peaks corresponding to 1–4 times dehydration of the peptide.

Leu-enkephalin (YGGFL) and Met-enkephalin (YGGFM) have a morphine-like effect. The potential impact of cyclization by insertion of a thioether bridge in peptides is well-demonstrated by the observation that thioether-enkephalin is resistant to enzymatic degradation, whereas noncyclized Leu-enkephalin had a *t*_{1/2} of less than 7 min (31). Furthermore, thioether-enkephalin is *in vivo* 10 000 times more potent than morphine and 1 000 000 times more potent than enkephalin cyclized via a disulfide bridge (32). A fusion of a prenisin fragment and a peptide containing a pentapeptide enkephalin variant (YTGFC) was termed EnkT4 (leader-ITSISKYTGFC). After maturation and export via NisBTC, mass spectrometry revealed mass peaks for EnkT4 corre-

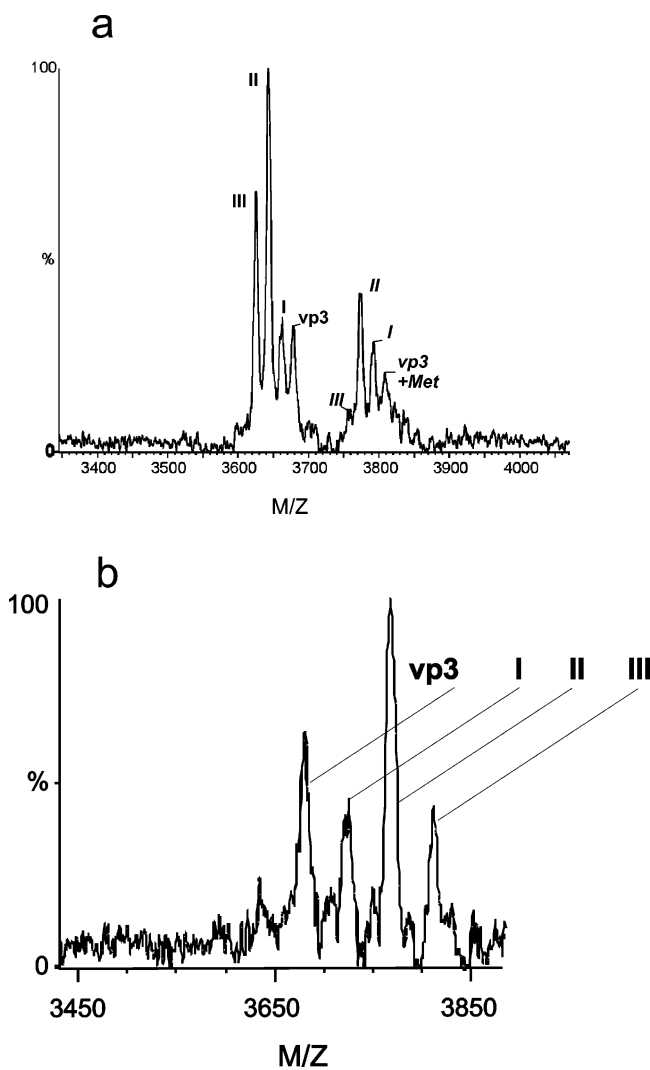


FIGURE 3: Detection of multiple forms of dehydration of the vasopressin analogue VP3 via MALDI-TOF mass spectrometry. (a) Extent of dehydration is indicated in roman letters, corresponding to 1 (I), 2 (II), and 3 (III) times dehydration. VP3 peaks representing vasopressin including the N-terminal methionine are indicated in italics. (b) Mass shift of 62 Da as a result of ethanethiol addition to VP3 without methionine. The data corresponding to this graph are shown in Table 2.

sponding to a stepwise dehydration of all four serines/threonines present. Two enkephalin variants were made to prevent undesired ring formation of the cysteine with the dehydroamino acids upstream of the ultimate threonine. Enk.A (IAAIKTYTGFC) and Enk.B (IAAIKYSGFC) were both dehydrated, although the extent of dehydration for Enk.B (serine variant) was much lower than for Enk.A.

The luteinizing hormone-releasing hormone (LHRH) has the sequence pGluHWSYGLRPG-NH₂. Its functionality with respect to receptor binding and activation of each amino acid is known (33). Recent studies have shown that mono- and dicyclic versions of this hormone can act as potent antagonists (34, 35). The leucine in position 7 can be replaced by another uncharged amino acid. In addition to a L7C-LHRH mutation, we also changed the pGlu into a glutamine, which can spontaneously form a pGlu group. A plasmid-encoded peptide was composed of the first N-terminal 44 amino acids of prenisin fused to the L7C-LHRH variant (QHWSYGCRPG). Mass spectrometry showed peaks corresponding to 5

and 6 times dehydration. A 6 times dehydration implied that also the serine in the LHRH-coding part was dehydrated.

Angiotensin is a cardiovascular peptide, which occurs in several forms. The angiotensin (1–7) variant (DRVYIHP) plays a crucial role in the preservation of the cardiac function (36). A 2-fold mutant of this peptide was made (Y4T and P7C), fused to the leader peptide, and subjected to dehydration. The observed mass peaks were related to the nondehydrated and 1 time dehydrated peptide.

EPO (erythropoietin) is a glycoprotein hormone, responsible for the regulation of the production of red blood cells. We selected an EPO peptide mimetic (EMP1) of 13 amino acids, EPO(1–13) (37), to serve as a substrate for dehydration. Aromatic amino acids at positions essential for mimetic action were kept in tact, and one serine for dehydration (C3S) was introduced (YASHFGPLGWCK). Mass spectrometry gave peaks corresponding to a 1 time dehydrated peptide.

To confirm that the observed mass decreases of 18 Da were the result of a dehydration step, the peptides were submitted to ethanethiol treatment. Ethanethiol couples to dehydrated residues and under the applied conditions also to subsequently formed thioether rings. In both cases, this results in a mass increase of 62 Da. For all peptides described above, dehydration could be confirmed by ethanethiol addition, even for those containing multiple dehydrations (Table 2). As an illustration, Figure 3b demonstrates the mass shift of dehydrated VP3 as a result of ethanethiol addition. Additions for all three dehydrated amino acids could be identified. Cyclization of the generated dehydroresidue with the downstream cysteine by NisC does not result in a mass shift. The applied conditions for ethanethiol treatment do not allow us to discriminate between the absence or presence of a thioether ring. However, ethanethiol addition proves that NisB-mediated dehydration has taken place in a wide variety of unrelated therapeutic peptides.

CDAP was used to examine whether the cysteines present in most of the peptides are accessible or have undergone a thioether linkage with a dehydroresidue. Formation of the isothiocyanate part will result in a mass increase of 25 Da, whereas the absence of a mass shift indicates that the cysteine is involved in ring formation. Thioethers typically do not react with CDAP (van der Donk, personal communication) and neither do addition reactions with other residues, as long as large excesses of CDAP are avoided (38). Two cysteine-containing, chemically synthesized, peptides were used as positive controls. In addition, peaks corresponding to nondehydrated peptides served as an internal control. Figure 4a shows the mass spectrum of the Ang(1–7) peptide before and after CDAP treatment. The dehydration peak (3159.7 Da) remains present, whereas the nondehydration peak (3177.6 Da) is completely replaced by a +25 Da adduct (3202.9 Da). The presence of a minor peak representing dehydrated Ang(1–7) and CDAP (3185.2 Da) shows that most likely a minor fraction of the peptide has escaped ring formation. As a positive control, the cysteine-containing peptide NisB2 was used (Figure 4b). A total mass shift with +25 Da showed complete CDAP addition under the conditions used. Analysis of the other therapeutic peptides after treatment with CDAP showed that, when fully dehydrated, no additions were found. This indicates that the cysteine is not available for CDAP and thus points at the presence of a thioether ring. Simultaneously, peaks corresponding to non-

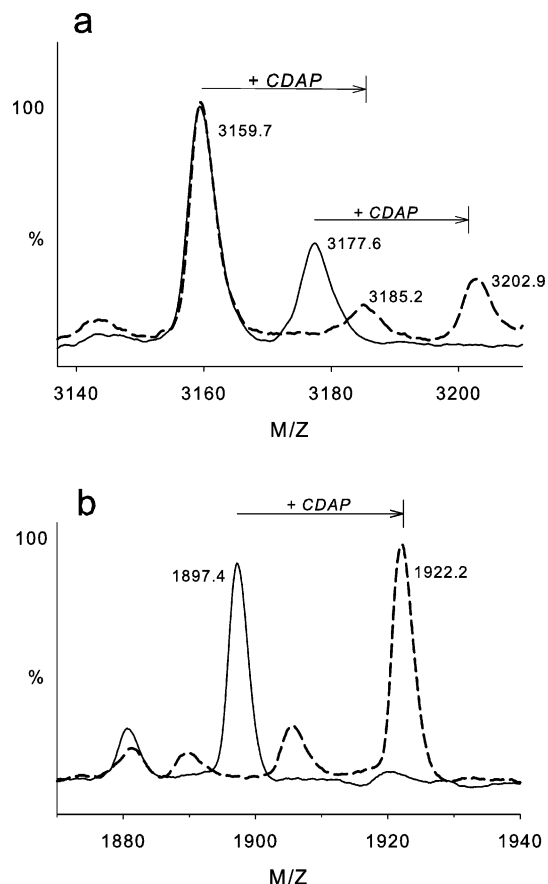


FIGURE 4: CDAP addition of Ang(1–7) shown by MALDI–TOF mass spectrometry. Nontreated peptide is indicated by a solid line, and CDAP-treated peptide is presented by a dashed line. Mass shifts of 25 Da are indicated with an arrow. (a) Leader-Ang(1–7) peptide (DRVTHC). (b) Control peptide NisB2 (CRYTDPKPHIRLRIK).

dehydrated peptides shifted 25 Da, which points at the accessibility of the cysteine for CDAP. This indicates that the cysteine is not involved in ring formation. The peptides for which total CDAP addition was observed and hence lack ring formation are the enkephalin variants starting with IAAIA. No conclusive results were obtained from CDAP-treated EPO.

When the ACTH variants were analyzed under nonreduced conditions, cysteine additions were observed for only the

nondehydrated peptide. Additional peaks of +119 Da were observed for the peptides with (3821.5 Da) and without (3689.8 Da) the initial methionine (see also Figure S3 in the Supporting Information). For the mass peaks corresponding to the dehydrated peptide ACTH(S1G) (3552.0 Da), no equivalent peaks, pointing at cysteine addition, were detected. Cysteinylation can only occur when the cysteine of the peptide is not involved in ring formation. When these data are taken together, they are compatible with ring formation in the dehydrated ACTH variants.

LHRH was chosen for more detailed studies to confirm the presence of a lanthionine ring. The LHRH fusion peptide was obtained in larger quantities by TCA precipitation from a 1 L culture, after which LHRH was released from the rest of the fusion peptide by tryptic digestion. Cleavage of the arginine in LHRH was either hampered by the presence of a proline at position P1' or protected by the introduced lanthionine ring (Figure 5). The trypsin digest was separated by reversed-phase HPLC, where the LHRH peptide fraction eluted at 28% ACN (see Figure S12 in the Supporting Information). The identity of the LHRH peptide in the fraction was confirmed by mass spectrometry (1172.6 Da). N-Terminal sequencing was carried out on both the ethanethiol-modified and nontreated LHRH. The results are shown in Figure 5. Ethanethiol modification results in the formation of *S*-ethylcysteine at position 4, which confirms that dehydration at position 4 has taken place. The presumed Cys at position 7 could not be determined. It is known that when becoming N-terminally exposed (e.g., during Edman degradation) dehydroamino acids spontaneously deaminate and become unstable, thereby blocking the sequence reaction (29). However, N-terminal sequencing of the nontreated LHRH peptide did not result in a sequence block, which can only mean that a thioether bridge is present.

Membrane-associated enzyme complexes have been described for nisin- and subtilin-modifying and exporting enzymes (39, 40). Karakas Sen et al. (41) demonstrated that additional NisB in a nisin-producing strain resulted in a higher extent of dehydration in nisin. The latter observation suggests that no strict stoichiometry is required for the enzyme complex to function. Recently, we found that the NisBT complex remains active in the absence of NisC.

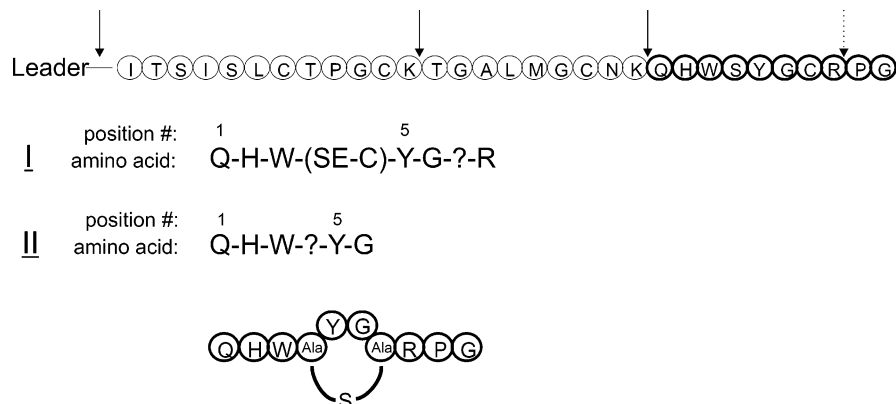


FIGURE 5: Amino acid sequence determination of the lanthionine-containing LHRH peptide. The primary sequence of LHRH is shown, in which Leader represents the leader peptide (MSTKDFNLDLVSVKKDSGASPR). Arrows indicate trypsin-sensitive sites, and the dotted arrow designates the arginine excluded from cleavage. The effective LHRH peptide is highlighted in bold. The N-terminal sequence of both ethanethiol (I) and untreated (II) LHRH is shown. SE-C denotes *S*-ethylcysteine, and a question mark indicates a nonidentified amino acid. Numbers indicate the position of the amino acids. A schematic picture of thioether-linked LHRH is shown, in which the lanthionine ring (–S–) is formed between the N-terminal Ala (derived from Dha) and the C-terminal Ala (derived from Cys).

Because the angiotensin (1–7) peptide, used in this study, is completely dissimilar to nisin, dehydration is likely directed by the N-terminal leader peptide. For LctM, Xie et al. found that in the absence of the leader peptide no modification occurred (42). Hence, the presence of the leader sequence is an effective means to target peptides to the lantibiotic enzymes.

Eventually, removal of the leader peptide is a prerequisite. Recently, we demonstrated that the extracellular leader peptidase NisP is highly specific and only recognizes thioether ring-containing prenisin (27). In addition, cleavage of fully modified prenisin by trypsin has proven to be an efficient method to obtain active nisin. Trypsin can be used to liberate therapeutic peptides that are free of trypsin-sensitive cleavage sites (arginine or lysine) or when these sites are protected by thioether rings. Alternatively, the introduction of appropriate cleavage sites would allow the removal of the leader peptide in other fusion peptides.

The generation of dehydroresidues can serve as a step toward the biochemical synthesis of thioether linkages in peptides. Monosulfide bridges are more stable than disulfide bridges. They enhance the peptide stability and protect against proteolytic degradation. Furthermore, thioether bridges may generate enhanced activities, as mentioned for thioether enkephalin. Thioether variants of oxytocin (43), somatostatin (7), and RGD peptides (9) with modulated receptor interaction have been described. Using lantibiotic enzymes, a large number of analogues of therapeutic peptides can be easily achieved by varying the amino acids that are not involved in dehydration and/or ring formation. Biological synthesis of thioether peptides allows the generation of cyclopeptide libraries by using semirandomized primers and might eventually lead to cost-effective production of dehydroresidues and/or thioether ring-containing larger peptides. Altogether, the data presented here indicate that a broad range of therapeutic and other bioactive peptides can serve as a substrate for lantibiotic enzyme-mediated insertion of dehydroresidues and/or thioether rings.

ACKNOWLEDGMENT

We are grateful to Wilfred van der Donk for advice on the use of CDAP and to Kees Leenhouts, Karin Scholtmeijer, and George T. Robillard for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

MALDI–TOF mass spectrometry of NisB-dehydrated therapeutic peptides that are attached to the nisin leader peptide without the starting methionine (Figures S1–S11). Reversed-phase HPLC UV absorbance chromatogram of trypsin-digested leader-LHRH, measured at 280 nm (Figure S12). This material is available free of charge via the Internet at <http://pubs.acs.org>.

REFERENCES

- Murkin, A. S., and Tanner, M. E. (2002) Dehydroalanine-based inhibition of a peptide epimerase from spider venom, *J. Org. Chem.* **67**, 8389–8394.
- Morris, S. L., Walsh, R. C., and Hansen, J. N. (1984) Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action, *J. Biol. Chem.* **259**, 13590–13594.
- Lombardi, A., D'Agostino, B., Nastri, F., D'Andrea, L. D., Filippelli, A., Falciani, M., Rossi, F., and Pavone, V. (1998) A novel super-potent neurokinin A receptor antagonist containing dehydroalanine, *Bioorg. Med. Chem. Lett.* **8**, 1153–1156.
- Palmer, D. E., Pattaroni, C., Nunami, K., Chadha, R. K., Goodman, M., Wakamiya, T., Fukase, K., Horimoto, S., Kitazawa, M., Fujita, H., Kubo, A., and Shiba, T. (1992) Effects of dehydroalanine on peptide conformations, *J. Am. Chem. Soc.* **114**, 5634–5642.
- van der Meer, J. R., Polman, J., Beerthuyzen, M. M., Siezen, R. J., Kuipers, O. P., and de Vos, W. M. (1993) Characterization of the *Lactococcus lactis* nisin A operon genes *nisP*, encoding a subtilisin-like serine protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis, *J. Bacteriol.* **175**, 2578–2588.
- Bierbaum, G., Szekat, C., Josten, M., Heidrich, C., Kemper, C., Jung, G., and Sahl, H.-G. (1996) Engineering of a novel thioether bridge and role of modified residues in the lantibiotic Pep5, *Appl. Environ. Microbiol.* **62**, 385–392.
- Ósabay, G., Prokai, L., Kim, H. S., Medzihradsky, K. F., Coy, D. H., Liapakis, G., Reisine, T., Melacini, G., Zhu, Q., Wang, S. H., Mattern, R. H., and Goodman, M. (1997) Lanthionine-somatostatin analogs: Synthesis, characterization, biological activity, and enzymatic stability studies, *J. Med. Chem.* **40**, 2241–2251.
- Li, P., and Roller, P. P. (2002) Cyclization strategies in peptide derived drug design, *Curr. Top. Med. Chem.* **2**, 325–341.
- Kelleman, A., Mattern, R.-H., Pierschbacher, M. D., and Goodman, M. (2003) Incorporation of thioether building blocks into an α,β -specific RGD peptide: Synthesis and biological activity, *Biopolymers* **71**, 686–695.
- Bierbaum, G. (1999) Genetics of lantibiotic biosynthesis, in *Amino Acids, Peptides, Porphyrins, Alkaloids* (Kelly, J. W., Barton, D., Nakanishi, K., and Meth-Cohn, O., Eds.) pp 275–301, Elsevier Science Publishers B.V., Amsterdam, The Netherlands.
- Xie, L., and van der Donk, W. A. (2004) Post-translational modifications during lantibiotic biosynthesis, *Curr. Opin. Chem. Biol.* **8**, 498–507.
- Sahl, H.-G., and Bierbaum, G. (1998) Lantibiotics: Biosynthesis and biological activities of uniquely modified peptides from Gram-positive bacteria, *Annu. Rev. Microbiol.* **52**, 41–79.
- McAuliffe, O., Ross, R. P., and Hill, C. (2001) Lantibiotics: Structure, biosynthesis, and mode of action, *FEMS Microbiol. Rev.* **25**, 285–308.
- Chatterjee, C., Paul, M., Xie, L., and van der Donk, W. A. (2005) Biosynthesis and mode of action of lantibiotics, *Chem. Rev.* **105**, 633–684.
- Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O. P., Sahl, H.-G., and de Kruijff, B. (1999) Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic, *Science* **286**, 2361–2364.
- Kuipers, O. P., Beerthuyzen, M. M., Siezen, R. J., and de Vos, W. M. (1993) Characterization of the nisin gene cluster *nis-ABTCIPR* of *Lactococcus lactis*. Requirement of expression of the *nisA* and *nisI* genes for development of immunity, *Eur. J. Biochem.* **216**, 281–291.
- Gross, E., and Morell, J. L. (1971) The structure of nisin, *J. Am. Chem. Soc.* **93**, 4634–4635.
- Schmidt, U., Lieberknecht, A., and Wild, J. (1988) Didehydroamino acids (DDAA) and didehydropeptides (DDP), *Synthesis* **1988**, 159–172.
- Stohlmeyer, M. M., Tanaka, H., and Wandless, T. J. (1999) A stereospecific elimination to form dehydroamino acids: Synthesis of the phomopsin tripeptide side chain, *J. Am. Chem. Soc.* **121**, 6100–6101.
- Coghlan, P. A., and Easton, C. J. (1999) β -Nitro- α -amino acids as latent α,β -dehydro- α -amino acid residues in peptides, *Tetrahedron Lett.* **40**, 4745–4748.
- Okeley, N. M., Zhu, Y., and van der Donk, W. A. (2000) Facile chemoselective synthesis of dehydroalanine-containing peptides, *Org. Lett.* **2**, 3603–3606.
- Kohli, R. M., Trauger, J. W., Schwarzer, D., Marahiel, M. A., and Walsh, C. T. (2001) Generality of peptide cyclization catalyzed by isolated thioesterase domains of nonribosomal peptide synthetases, *Biochemistry* **40**, 7099–7108.
- Kuipers, O. P., Rollema, H. S., Yap, W. M. G. J., Boot, H. J., Siezen, R. J., and de Vos, W. M. (1992) Engineering dehydrated amino acid residues in the antimicrobial peptide nisin, *J. Biol. Chem.* **267**, 24340–24346.

24. Holo, H., and Nes, I. F. (1995) Transformation of *Lactococcus* by electroporation, *Methods Mol. Biol.* 47, 195–199.
25. Terzaghi, B. E., and Sandine, W. E. (1975) Improved medium for lactic streptococci and their bacteriophages, *Appl. Microbiol.* 29, 807–813.
26. Jensen, P. R., and Hammer, K. (1993) Minimal requirements for exponential growth of *Lactococcus lactis*, *Appl. Environ. Microbiol.* 59, 4363–4366.
27. Kuipers, A., de Boef, E., Rink, R., Fekken, S., Kluskens, L. D., Driessen, A. J. M., Leenhouts, K., Kuipers, O. P., and Moll, G. N. (2004) NisT, the transporter of the lantibiotic nisin, can transport fully modified, dehydrated, and unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides, *J. Biol. Chem.* 279, 22176–22182.
28. Novak, J., Kirk, M., Caufield, P. W., Barnes, S., Morrison, K., and Baker, J. (1996) Detection of modified amino acids in lantibiotic peptide mutacin II by chemical derivation and electrospray ionization-mass spectroscopic analysis, *Anal. Biochem.* 236, 358–360.
29. 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.
30. Tomkinson, B., Grehn, L., Fransson, B., and Zetterqvist, O. (1994) Use of a dehydroalanine-containing peptide as an efficient inhibitor of tripeptidyl peptidase II, *Arch. Biochem. Biophys.* 314, 276–279.
31. Svensson, C. I., Rew, Y., Malkmus, S., Schiller, P. W., Taulane, J. P., Goodman, M., and Yaksh, T. L. (2003) Systemic and spinal analgesic activity of a δ -opioid-selective lanthionine enkephalin analog, *J. Pharmacol. Exp. Ther.* 304, 827–832.
32. Rew, Y., Malkmus, S., Svensson, C., Yaksh, T. L., Chung, N. N., Schiller, P. W., Cassel, J. A., DeHaven, R. N., and Goodman, M. (2002) Synthesis and biological activities of cyclic lanthionine enkephalin analogues: δ -Opioid receptor selective ligands, *J. Med. Chem.* 45, 3746–3754.
33. Sealfon, S. C., Weinstein, H., and Millar, R. P. (1997) Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor, *Endocr. Rev.* 18, 180–205.
34. Rivier, J. E., Jiang, G., Struthers, R. S., Koerber, S. C., Porter, J., Cervini, L. A., Kirby, D. A., Craig, A. G., and Rivier, C. L. (2000) Design of potent dicyclic (1–5/4–10) gonadotropin releasing hormone (GnRH) antagonists, *J. Med. Chem.* 43, 807–818.
35. Rivier, J. E., Porter, J., Cervini, L. A., Lahrichi, S. L., Kirby, D. A., Struthers, R. S., Koerber, S. C., and Rivier, C. L. (2000) Design of monocyclic (1–3) and dicyclic (1–3/4–10) gonadotropin releasing hormone (GnRH) antagonists, *J. Med. Chem.* 43, 797–806.
36. Loot, A. E., Roks, A. J. M., Henning, R. H., Tio, R. A., Suurmeijer, A. J. H., Boomsma, F., and van Gilst, W. H. (2002) Angiotensin-(1–7) attenuates the development of heart failure after myocardial infarction in rats, *Circulation* 105, 1548–1550.
37. Johnson, D. L., Farrell, F. X., Barbone, F. P., McMahon, F. J., Tullai, J., Hoey, K., Livnah, O., Wrighton, N. C., Middleton, S. A., Loughney, D. A., Stura, E. A., Dower, W. J., Mulcahy, L. S., Wilson, I. A., and Jolliffe, L. K. (1998) Identification of a 13 amino acid peptide mimetic of erythropoietin and description of amino acids critical for the mimetic activity of EMP1, *Biochemistry* 37, 3699–3710.
38. Wu, J., and Watson, J. T. (1997) A novel methodology for assignment of disulfide bond pairings in proteins, *Protein Sci.* 6, 391–398.
39. Kiesau, P., Eikmanns, U., Gutowski-Eckel, Z., Weber, S., Hammelmann, M., and Entian, K. D. (1997) Evidence for a multimeric subtilin synthetase complex, *J. Bacteriol.* 179, 1475–1481.
40. Siegers, K., Heinzmann, S., and Entian, K. D. (1996) Biosynthesis of lantibiotic nisin. Posttranslational modification of its prepeptide occurs at a multimeric membrane-associated lanthionine synthetase complex, *J. Biol. Chem.* 271, 12294–12301.
41. Karakas Sen, A., Narbad, A., Horn, N., Dodd, H. M., Parr, A. J., Colquhoun, I., and Gasson, M. J. (1999) Post-translational modification of nisin. The involvement of NisB in the dehydration process, *Eur. J. Biochem.* 261, 524–532.
42. Xie, L., Miller, L. M., Chatterjee, C., Averin, O., Kelleher, N. L., and van der Donk, W. A. (2004) Lacticin 481: *In vitro* reconstitution of lantibiotic synthetase activity, *Science* 303, 679–681.
43. Jošt, K. (1987) Modifications in the disulfide bridge (Carba-analogs), in *Handbook of Neurohypophyseal Hormone Analogs* (Jošt, K., Lebl, M., and Brtnik, F., Eds.) pp 144–156, CRC Press, Boca Raton, FL.
44. Kuipers, O. P., de Ruyter, P. G., Kleerebezem, M., and de Vos, W. M. (1997) Controlled overproduction of proteins by lactic acid bacteria, *Trends Biotechnol.* 15, 135–140.
45. Simon, D., and Chopin, A. (1988) Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*, *Biochimie* 70, 559–566.
46. Rink, R., Kuipers, A., de Boef, E., Leenhouts, K. J., Driessen, A. J. M., Moll, G. N., and Kuipers, O. P. (2005) Lantibiotic structures as guidelines for the design of peptides that can be modified by lantibiotic enzymes, *Biochemistry* 44, 8873–8882.

BI050805P