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Engineering Dehydro Amino Acids and Thioethers into Peptides Using Lacticin 481 Synthetase

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Summary

Lantibiotics are peptide antimicrobials containing the thioether-bridged amino acids lanthionine (Lan) and methyllanthionine (MeLan) and often the dehydrated residues dehydroalanine (Dha) and dehydrobutyrine (Dhb). While biologically advantageous, the incorporation of these residues into peptides is synthetically daunting, and their production in vivo is limited to peptides containing proteinogenic amino acids. The lacticin 481 synthetase LctM offers versatile control over the installation of dehydro amino acids and thioether rings into peptides. In vitro processing of semisynthetic substrates unrelated to the prelacticin 481 peptide demonstrated the broad substrate tolerance of LctM. Furthermore, a chemoenzymatic strategy was employed to generate novel thioether linkages by cyclization of peptidic substrates containing the nonproteinogenic cysteine analogs homocysteine and β-homocysteine. These findings are promising with respect to the utility of LctM toward preparation of conformationally constrained peptide therapeutics.

Introduction

The prevalence of drug resistance in clinical settings as a consequence of bacterial ingenuity in altering metabolic pathways and/or cell morphology poses a persistent threat to human health [1]. Surprisingly, bacterial resistance to the lantibiotic nisin has been limited during its prolonged and ubiquitous usage in the dairy industry [2]. Lantibiotics are a class of posttranslationally modified antimicrobial peptides containing thioether crosslinks called lanthionines [3]. The conformational constraints induced by lanthionines are essential for nisin's antimicrobial activity through binding to the cell wall biosynthetic intermediate lipid II [4, 5] and forming pores in the bacterial cell membrane [6]. These multiple modes of bactericidal action are believed to account for the observed high efficacy of nisin (nM MICs) as well as the slow emergence of resistance [7]. Other lantibiotics with entirely different primary and three-dimensional structures such as mersacidin and cinnamycin also recognize with high affinity their targets, lipid II [8] and phosphatidyl ethanolamine [9], respectively, suggesting that the lanthionine motif is a naturally privileged architecture for constraining peptides into a bioactive conformation [3]. In fact, lanthionines have very recently been found in morphogenetic peptides involved in sporulation in the streptomycetes [10, 11], demonstrating that their use extends beyond antimicrobial peptides.

Synthetic lanthionine containing peptides have found use as mimics of disulfides or as structures that limit the conformational flexibility of bioactive compounds [12]. The lanthionine moiety also provides higher chemical, proteolytic, and metabolic stability for such analogs [13–15]. The biologically advantageous properties conferred by cyclic lanthionine containing peptides may therefore provide a framework for the design of a range of therapeutic peptides containing these structures [16–18]. Given the difficulty of installing lanthionine synthetically [12], realization of this goal will be greatly aided by a detailed understanding of the enzymes involved in posttranslational modification.

Lantibiotics are ribosomally synthesized as precursor peptides termed LanA (Lan is used as a generic label for lantibiotic biosynthetic proteins). They consist of a leader sequence and a propeptide region, which undergoes the posttranslational modifications. The cyclic thioethers are produced by a two-step process involving dehydration of Ser and Thr residues in the propeptide portion of the LanA substrates to generate dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively (Figure 1A). The intramolecular thioether crosslinks are then introduced by nucleophilic attack of cysteine residues within the peptides onto the dehydro amino acids. In class I lantibiotics like nisin, the dehydration is performed by a LanB dehydratase, and the cyclization by a LanC enzyme. In contrast, for class II lantibiotics like lacticin 481, a single LanM protein (e.g., LctM) catalyzes both reactions (Figure 1B). Until recently, the lack of a functional in vitro assay for the lantibiotic dehydratase and cyclase enzymes restricted tests of their substrate selectivity to in vivo mutagenesis experiments with the 20 proteinogenic amino acids. Investigations of mutations in the propeptide region of the *lanA* genes on lantibiotic production have been conducted in engineered expression systems for nisin, subtilin, mutacin II, cinnamycin, and mersacidin [3, 18], and more recently lacticin 3147 [19]. Tests of substrate specificity have also involved the expression of fusions of the nisin leader peptide to short nonlantibiotic peptides in *Lactococcus lactis* strains bearing plasmid-encoded dehydratase and cyclase enzymes [17, 20, 21]. Collectively, these studies have shown that lantibiotic biosynthetic enzymes have substantial tolerance with respect to the sequence of their peptide substrates; however, several factors can limit the information gained from in vivo experiments. In particular, when negative results are obtained regarding an alteration of the structure of the substrate peptide, this may be due to failure of the biosynthetic proteins, or alternatively to poor expression of the mutant substrates, inability of the wild-type transport proteins to secrete the

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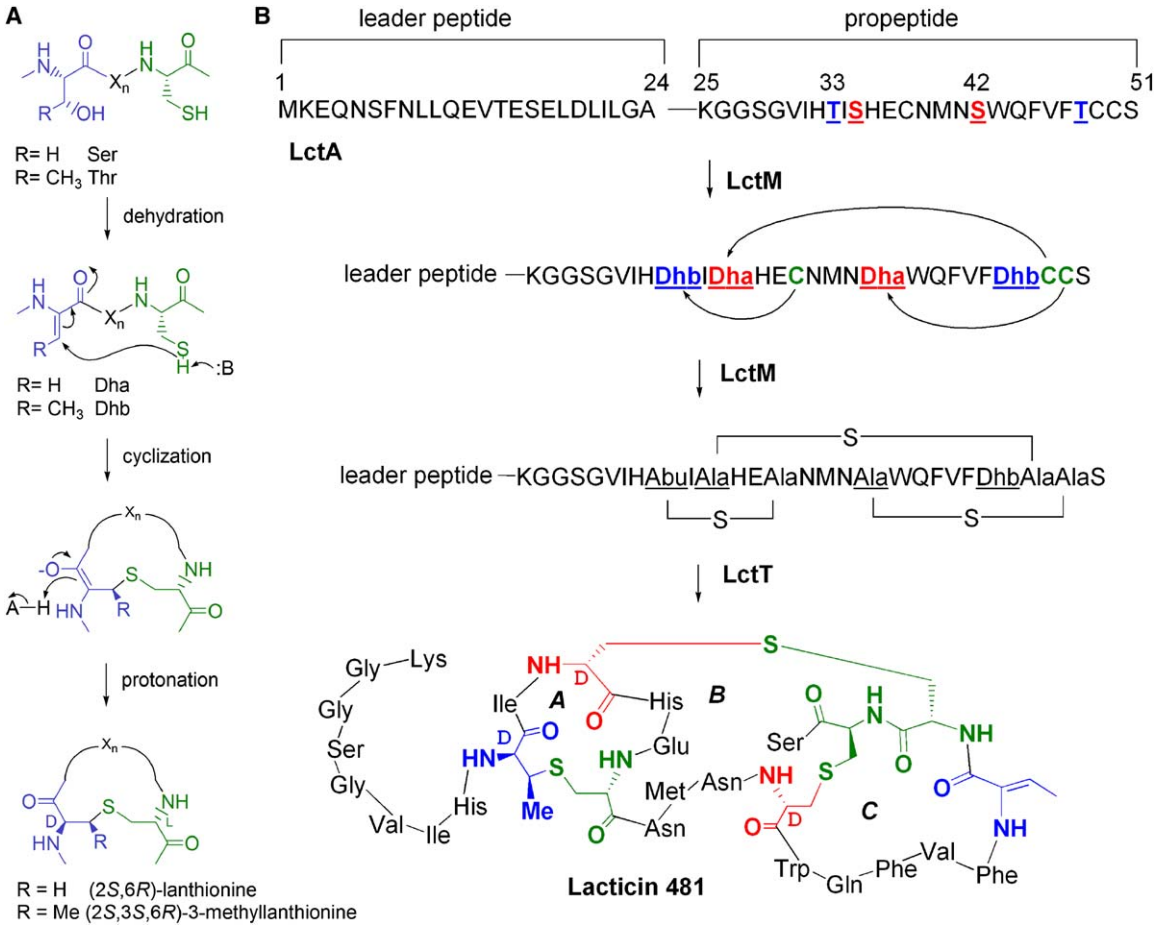


Figure 1. Posttranslational Modifications in Lantibiotics

(A) Formation of lanthionine (Lan) and methyllanthionine (MeLan) crosslinks from Ser and Thr residues by dehydration to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, and subsequent stereoselective addition of Cys thiol groups to the Dha and Dhb residues.

(B) Biosynthesis of lacticin 481 [23, 44]. The dehydration of Ser/Thr residues and subsequent regio- and chemoselective cyclization of Cys residues is catalyzed by a single enzyme, LctM. Subsequent proteolytic removal of the unmodified leader sequence from the modified propeptide region by the bifunctional protease/transporter LctT leads to export of mature lacticin 481 to the extracellular milieu [45].

processed mutants, breakdown of the analog products, or potential toxicity of the nonnatural products to the producing strain due to nonrecognition of the mutants by the innate immunity proteins [22]. These limitations associated with in vivo methods to determine substrate specificity may be overcome by in vitro assays of peptide substrates.

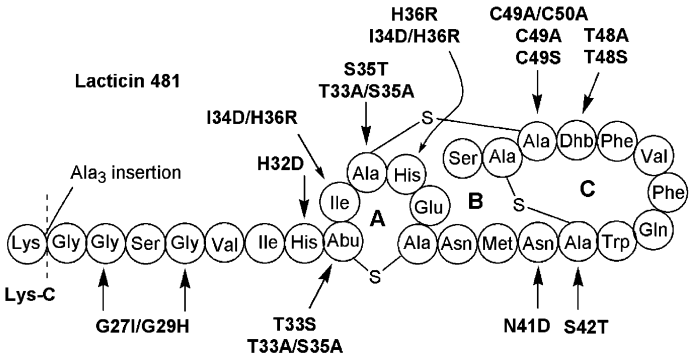
In this article, we discuss the in vitro substrate specificity of lacticin 481 synthetase (LctM) and document its utility for peptide engineering. This enzyme performs both the dehydration of a defined set of Ser and Thr residues in the C-terminal region of the LctA substrate peptide as well as the subsequent cyclization steps (Figure 1B) [23–26]. Employing both site-directed mutagenesis and expressed protein ligation techniques, we demonstrate that LctM can dehydrate and cyclize a wide spectrum of peptides containing both natural and nonproteinogenic amino acids, allowing precise control over the size of lanthionine rings. Furthermore, a predictive model is presented for the sequence and positional specificity of dehydration by LctM that allows for rational design of modified peptides.

Results and Discussion

Specificity of LctM Action and Biological Activity of Lacticin Analogs

In our initial in vitro characterization of LctM activity, the enzyme was shown to be tolerant of several single-point mutants of its substrate LctA fused at the N terminus to a hexa-His tag (His_6-LctA) [23]. To extend the evaluation of LctM substrate selectivity, an expanded set of site-directed mutants of His_6-LctA were tested as substrates (Figure 2A). Mutations that lead to replacement of lanthionines with methyllanthionines and vice versa were all tolerated (entries 2–4, Figure 2B) as was substitution of Dhb at position 48 with a Dha (entry 5). One difference in comparison with the physiological substrate involves the accumulation of more substantial amounts of partially processed substrates (M − x H_2O, x = 1–3), although the fully dehydrated product (M − 4 H_2O) generally remained the major product (e.g., Figure S1A; see the Supplemental Data available with this article online). After modification by LctM, the products were treated with the protease Lys-C to remove the leader peptide

A



B

Entry	His_6-Substrate	Major assay product	Structural Change after Proteolysis	Antimicrobial Activity
1	wt	-4 H_2O	*Wild type*	+
2	T33S	-4 H_2O	MeLan to Lan, A-ring	+
3	S35T	-4 H_2O	Lan to MeLan, B-ring	+
4	S42T	-4 H_2O	Lan to MeLan, C-ring	+
5	T48S	-4 H_2O	Dhb24 to Dha	+
6	C49A	-4 H_2O	B-ring absent	-
7	T33A/S35A	-2 H_2O	A & B-rings absent	-
8	C49A/C50A	-4 H_2O	B & C-rings absent	-
9	C49S	-5 H_2O	B-ring absent, Cys25Dha	-
10	T48A	-3 H_2O	Dhb24Ala	+
11	H32D	-4 H_2O	His8Asp	+
12	N41D	-4 H_2O	Asn17Asp	+
13	I34D/H36R	-4 H_2O	Ile10Asp/His12Arg	+
14	H36R	-4 H_2O	His12Arg	+

Figure 2. Mutants of Lacticin 481 and Their Biological Activity

(A) The structure of wild-type lacticin 481 and the mutants tested in this study. The ring designations are shown in bold capital letters. Residue numbering refers to the full-length wild-type LctA substrate that includes the leader sequence but not the His_6-tag.

(B) Products of incubation of His_6-tagged LctA mutants with LctM. Biological activity of lacticin 481 mutants (after proteolytic processing with Lys-C) toward the indicator strain *L. lactis* CNRZ 117 is indicated as + (active) or − (inactive).

as well as the Lys that normally occupies position 1 of lacticin 481. This Lys is not essential for antimicrobial activity [23]. The lacticin 481 analogs produced were evaluated for their biological activity by a standard agar diffusion assay versus a known susceptible indicator strain, *Lactococcus lactis* CNRZ 117. The partially processed substrates (e.g., Figure S1A) could not be removed during HPLC purification preventing accurate quantitation of the bactericidal activity of the analogs; a qualitative assessment is presented in Figure 2B.

Mutation of one or more Cys residues or their respective Ser/Thr partners results in deletion of thioether rings (entries 6–9). The removal of one or more of the rings does not affect the dehydration of the Ser/Thr residues, showing that the dehydratase activity of LctM is not coupled to the cyclization activity. The antimicrobial assays, on the other hand, clearly indicate the requirement for all three Lan/MeLan rings in lacticin 481 for bactericidal activity under these assay conditions. The requirement for the A ring that is highly conserved in the lacticin 481 subgroup of lantibiotics (Figure 3A) may reflect its similarity with the C ring of mersacidin, which is critical for its mode of action [27], binding to lipid II [8, 28]. The exact nature of the thioether rings in lacticin 481, whether Lan or MeLan, appears less important (entries 2–4), indicating that it is the constraint imposed by the crosslink that is critical. Indeed, several lantibiotics closely related to lacticin 481 contain a methyllanthionine rather than a lanthionine in the C ring (Figure 3A) [29, 30]. However, the electrophilic Dhb24 in the mature product (derived from Thr48 in the LctA prepeptide) that is conserved in these closest relatives is not absolutely necessary for biological activity of lacticin 481 as demonstrated by the antimicrobial activity of the lacticin analogs produced with the His_6-LctA(T48S) and His_6-LctA(T48A) mutants (entries 5 and 10, Figure 2B). The deleterious effects of removing Lan and/or MeLan rings on the biological activity of lantibiotics has also been demonstrated for Pep5 [14], mutacin II [30], and nisin [22].

Sequence and Position Dependence of Dehydration by LctM

LctM acts upon the His_6-LctA substrate in a site-specific manner such that only four of the 14 possible reactive serine and threonine residues undergo dehydration. Nevertheless, introduction of an additional Ser residue in the propeptide region of His_6-LctA at position 49 resulted in incorporation of a fifth dehydro amino acid (entry 9, Figure 2B) suggesting that the selectivity for four dehydrations is not strict. A recent comprehensive analysis of all known lantibiotic structures identified a weak consensus regarding the sequence context of Ser/Thr residues that are dehydrated in the peptide substrates [21]. In most cases, the dehydrated residues were found to be flanked by at least one hydrophobic residue with carboxylate side chains rarely present at these positions. Especially uncommon is the combination of an Asp immediately N-terminal to Ser/Thr with

A

Lacticin 481	KGGSGVIHTISHECNMNSWQFVFTCCS
Streptococcin A-FF22	NGVFKTISHECHLNTWAFLATCCS
Variacin	GSGVIPTISHECHMNSFQFVFTCCS
Butyrivibriocin OR79	GNGVIKTISHECHMNTWQFIFTCCS
Mutacin II	NRWWQGVVPTVSYECRMNSWQHVFTCC
Salivaricin A	KRGSGWIATITDDCP-NS-VFV--CC
Salivaricin A1	KKGSGWFATITDDCP-NS-VFV--CC
Mersacidin	CTFTLPGGGGVCTLTSECIC
Cinnamycin	CRQSCSFGPFTFVCDGNTK

B

Entry	Designation	Sequence
1	wt-LctA	His_6-tag-Leader-KGGSGVIHTISHECNMNSWQFVFTCCS
2	LctA-H32D	His_6-tag-Leader-KGGSGVI**D**TISHECNMNSWQFVFTCCS
3	LctA-N41D	His_6-tag-Leader-KGGSGVIHTISHECNM**D**SWQFVFTCCS
4	LctA-I34D/H36R	His_6-tag-Leader-KGGSGVIHT**D**S**R**ECNMNSWQFVFTCCS
5	LctA-H36R	His_6-tag-Leader-KGGSGVIHTIS**R**ECNMNSWQFVFTCCS
6	LctA(1-38)VISHEA	His_6-tag-Leader-KGGSGVIHTISHEC**VISHEA**
7	LctA(1-38)V$(ISH)_3$A	His_6-tag-Leader-KGGSGVIHTISHEC**VISHISHISHA**
8	LctA(1-38)VGSGEA	His_6-tag-Leader-KGGSGVIHTISHEC**VGSGEA**
9	LctA-G27I/G29H	His_6-tag-Leader-KG**ISH**VIHTISHECNMNSWQFVFTCCS
10	LctA(K25-G26insAAA)G27I/G29H	His_6-tag-Leader-K**AAA**G**ISH**VIHTISHECNMNSWQFVFTCCS
11	LctA(K25-G26insAAA)	His_6-tag-Leader-K**AAA**G**GSG**VIHTISHECNMNSWQFVFTCCS

Figure 3. Mutants of the Substrate for Posttranslational Modification

(A) Primary sequence of the propeptide regions of several class II lantibiotics; for a more extensive list, see [29].
(B) Primary sequence of His_6-wt-LctA and mutants thereof that were tested for dehydration by LctM. Mutations are highlighted in bold and dehydrated residues are underlined. For nomenclature, see [46]. The sequence of the His_6-tag leader peptide is GSSHHHHHHSSGL VPRGSH-MKEQNSFNLLQEVTESELDLILGA.

a C-terminally positioned arginine (D-S/T-R) [21]. These results may in part reflect the generally high hydrophobicity and often net positive charge of lantibiotics, which all target membranes or membrane-bound molecules [3]. An analysis of the class II lantibiotic propeptides to which lacticin 481 belongs [29] does not demonstrate any clear recognition motifs (Figure 3A), although indeed at least one flanking hydrophobic residue is typically present and few Asp/Glu residues are found (4%). Given the advantages of an in vitro system for evaluating a possible recognition motif, LctM was used toward this goal.

The effect of installing acidic residues adjacent to Ser/Thr residues on the dehydration activity of LctM was investigated with a series of site-directed mutants (Figure 3B, entries 2–4). Aspartates were introduced N-terminal to Thr33, Ser35, and Ser42 as well as C-terminal to Thr33, and the mutant substrates were incubated with LctM. Interestingly, all proved to be good substrates for LctM (Figure 2B, entries 11–13). Furthermore, conversion of the ISH sequence at positions 34–36 to ISR and DSR provided mutant substrates that were still dehydrated at Ser35 (Figure 2B, entries 13 and 14). These results demonstrate that at least for lacticin 481 and its producing strain, the paucity of acidic flanking residues reflects the desired biological activity of the product and not the substrate specificity of the synthetase LctM. These results are opposite those found for the dehydratase NisB involved in nisin biosynthesis for which it was shown that serines flanked by Asp and Arg were not substrates in nonlantibiotic test peptides [21]. The dehydratase domain of LctM has no sequence homology with NisB, thus different substrate specificity is not unexpected. Interestingly all analogs showed antimicrobial activity after removal of the leader peptide (Figure 2B, entries 11–14).

To explore the tolerance of LctM for sequences unrelated to its natural substrate, analogs were prepared by using intein technology [31, 32], introducing synthetic peptides behind the first 38 residues of LctA. The substrate analogs were prepared from a MES (2-mercaptoethanesulfonic acid) thioester of a truncated peptide, His_6-LctA(1–37), expressed in *E. coli*. This thioester was ligated to several synthetic peptides (Figure 3B, entries 6–8). Ligation with the heptamer CVISHEA yielded His_6-LctA(1–38)-VISHEA. In the appended sequence, this peptide contains 6 simultaneous mutations of the residues present in His_6-LctA, and a new Ser is introduced at position 41 where none is found in prelacticin 481 (Figure 3B). This peptide was chosen as it contains the sequence ISH that undergoes dehydration in the wild-type substrate. The peptide was subjected to LctM and a triply dehydrated species was detected by MALDI-TOF analysis of the assay products (Figure 4A). Since two dehydrations occur at Ser33 and Thr35, the presence of a peak in the mass spectrum corresponding to three dehydrations indicates dehydration of Ser41. Next, a substrate analog containing multiple ISH repeats, His_6-LctA(1–38)-VISHISHISHA, was synthesized by ligation of the His_6-LctA(1–37) thioester with the synthetic dodecamer CVISHISHISHA and subjected to LctM under standard assay conditions. The MALDI-TOF spectrum of

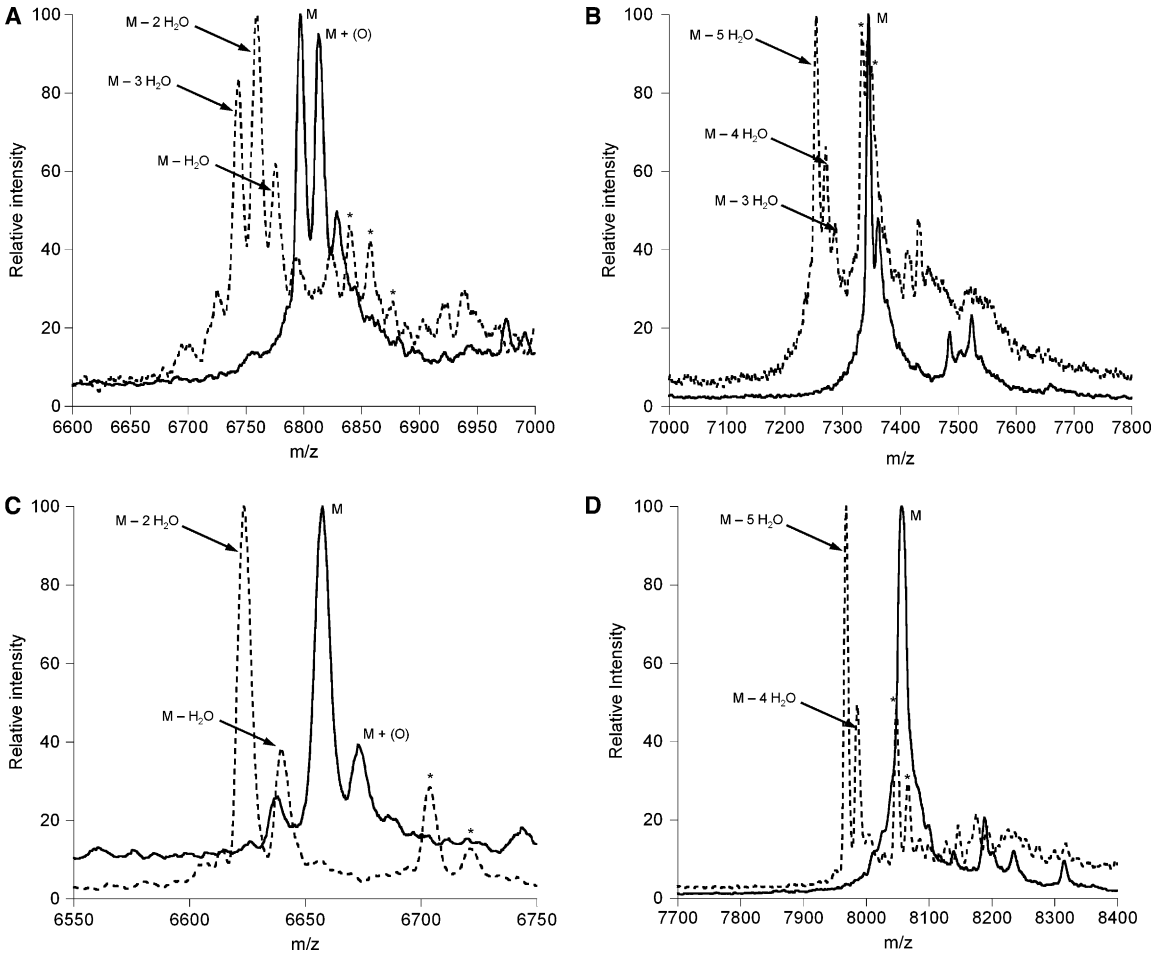


Figure 4. Mass Spectra of LctA Mutants Designed to Test the Positional Specificity of Dehydration

MALDI-TOF mass spectra of (A) assay product of His_6-LctA(1–38)-VISHEA with LctM (dashed) and a control assay without LctM (solid), (B) assay product with His_6-LctA(1–38)-VISHISHISHA (dashed) and control assay (solid), (C) assay product (dashed) and a control assay (solid) for His_6-LctA(1–38)-VGSGEA, and (D) assay product (dashed) and control (solid) for LctA(K25-G26insAAA)G27I/G29H. Phosphorylated peptides are indicated by asterisks [25].

the assay product clearly indicates formation of a species corresponding to five dehydrations (Figure 4B). These results, in conjunction with the dehydration of Ser49 in the His_6-LctA(C49S) mutant (Figure 2B, entry 9), suggest that LctM action is not position dependent, a finding that has also been reported from recent in vivo studies of the dehydratase NisB involved in nisin biosynthesis [17, 33]. The ability to site-specifically introduce Dha residues in this manner by both class I and class II lantibiotic dehydratases holds promise for future lantibiotic engineering [3, 22] as well as for introducing electrophilic handles into proteins that can be site-selectively modified via chemoselective reactions [34–36].

Based on these results, it is surprising that Ser28 escapes dehydration in the propeptide region of wt LctA. Two possible explanations can be postulated. Either Ser28 is too close to the leader peptide, or a Ser flanked by two glycines is not a good substrate for LctM. A series of experiments were carried out to distinguish between these possibilities. First, the mutant His_6-LctA(1–38)-VGSGEA was generated by ligation of the His_6-LctA(1–37)-MES thioester with the synthetic heptapeptide CVGSGEA (Figure 3B, entry 8). Upon subjecting the purified ligation product to LctM, just two dehydrations were observed (Figure 4C). This finding, in conjunction with the three dehydrations observed with His_6-LctA(1–38)-VISHEA (Figure 4A), suggests that two Gly residues flanking a Ser indeed deactivate LctM for dehydration.

These results, however, do not rule out that the distance of Ser/Thr residues to the leader peptide is also critical for dehydration by LctM. Hence, the genetic mutant His_6-LctA(G27I/G29H) was constructed in which the ISH sequence motif that resulted in dehydration at three positions unrelated to lacticin 481 was installed in place of the GSG sequence of wild-type LctA (Figure 3B, entry 9). When submitted to LctM, Ser28 remained unmodified in this peptide (Figure S1B). On the other hand, when the sequence was moved more distal to the leader sequence by insertion of three alanines between Lys25 and Gly26 (Figure 3B, entry 10), five dehydrations were observed, and Ser28 was dehydrated (Figure 4D). An identical strategy was employed to move the GSG sequence more distant to the leader sequence (Figure 3B, entry 11), and the insertion mutant underwent only four

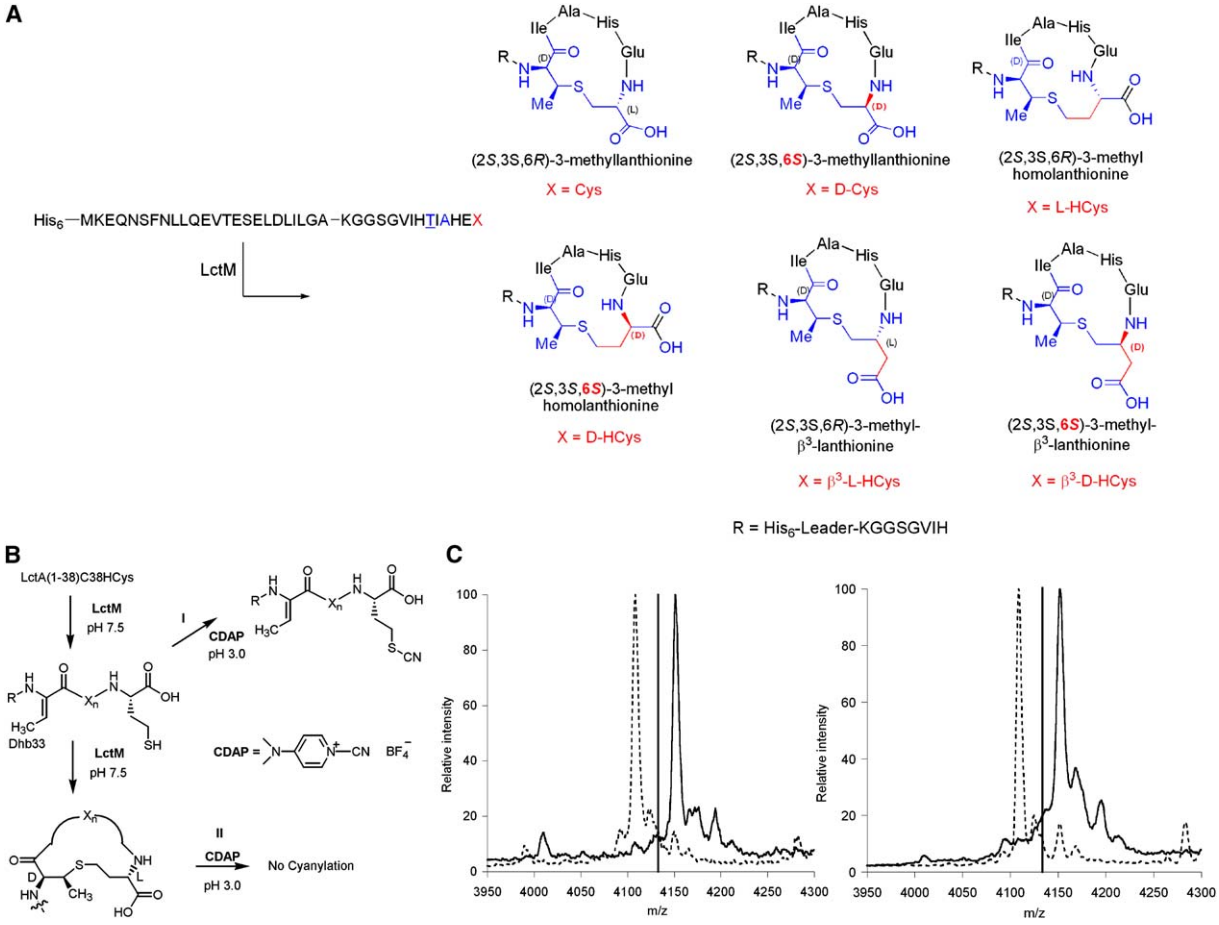


Figure 5. Lanthionine Analogs Generated with Lacticin 481 Synthetase

(A) Structures of methyllanthionine and analogs formed by the action of LctM on truncated substrates containing cysteine derivatives X as discussed in the text. The changes compared to the parent structure (X = Cys) are highlighted in red. The stereochemistry at carbons 2 and 3 of the analogs is inferred from the known stereochemistry of enzymatic cyclization by LctM. The crystal structure of the NisC cyclase [37] with which the cyclization domain of LctM has clear homology strongly suggests that this stereochemistry will be retained.

(B) Test for a free thiol as illustrated for LctA(1–38)S35A/C38Hcys via cyanylation of the LctM reaction product. Uncyclized assay product would be cyanylated by CDAP (path I), whereas in the cyclized product no free thiols are available for cyanylation (path II).

(C) MALDI-TOF mass spectra of cyanylation assays of the reactions of LctA(1–38)S35A/C38β^3-*R*-HCys (left) and LctA(1–38)S35A/C38D-Hcys (right) with LctM. Control cyanylations of the substrates are shown as solids lines (M + 25) and assay products after treatment with CDAP as dashed lines (M − H_2O). Cyanylated products that would be formed if a free thiol were present in the assay product are indicated by a black bar (M − H_2O − H + CN).

dehydrations when incubated with LctM (Figure S2). Collectively, these results demonstrate that efficient dehydration by LctM requires the Ser to be at a certain distance C-terminal to the last amino acid of the leader sequence and that Gly residues flanking a Ser deactivate its dehydration. These findings also provide further evidence that the leader sequence is important for binding and directing posttranslational modification of the propeptide region [23, 37]. Understanding of the molecular logic underlying these results requires structural information and such studies are currently in progress.

Generation of Lanthionine Analogs

As discussed in the introduction, the lanthionine structural motif is a powerful and stable conformational constraint for bioactive peptides. Access to such structures with the lantibiotic biosynthetic machinery is attractive for both lantibiotic engineering and constraining other bioactive peptides [16, 17], and precise control over the size and stereochemistry of the rings at atomic resolution would be a powerful extension of current possibilities. The ability of LctM to catalyze the formation of (Me)Lan analogs was tested with truncated substrates containing D-cysteine, L-homo, and D-homocysteine (HCys), as well as the synthetic cysteine homologs β^3-(*R*)-homocysteine and β^3-(*S*)-homocysteine [38] (Figure 5A). We chose for our tests a truncated substrate in which Ser35 was replaced by Ala to minimize nonenzymatic processes since biomimetic studies show that nonenzymatic cyclization of thiols onto dehydroalanines can occur rapidly whereas the corresponding reactions with dehydrobutyrines are slow [39–41]. Thus, the truncated mutant LctA(1–37)S35A was purified as the MES thioester and ligated to the Cys analogs (X) to generate the corresponding mutants LctA(1–38)S35A/C38X (Figure 5A). These mutants were then subjected to LctM under standard assay conditions and underwent the expected single dehydration (Dhb33) by MALDI-TOF

MS (Figure S3). Clearly, the presence of nonproteinogenic amino acids in the substrates did not affect the dehydratase activity of LctM.

As both the uncyclized and cyclized assay products have identical masses, they are indistinguishable by linear mode MALDI-TOF mass spectrometry. With this in mind, a chemical approach for the detection of free thiols was adopted (Figure 5B) [42]. Cyanylation of free thiols with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) readily takes place under acidic conditions (pH 3–5) [42] minimizing risk of nonenzymatic addition of the thiol side chains of the cysteine analogs to Dhb33. The enzymatic assay products were purified by HPLC under acidic conditions and reacted with CDAP after reduction with tris(2-carboxyethyl)phosphine (TCEP) to ensure that all uncyclized side-chain thiols were present in the reduced reactive form. Representative results from these experiments are presented in Figure 5C (for all data see Figure S4). Cyanylation of a free thiol is observed as a change in mass of +25 Da (M − H + CN) by MALDI-TOF mass spectrometry. Control experiments with the starting peptides indicated these were completely cyanylated (Figure 5C and Figure S4). In contrast, cyanylation is not observed in the MALDI-TOF spectrum of the enzymatic cyclization assay products indicating that the enzyme is capable of generating the noncanonical ring structures depicted in Figure 5A. The products thus formed include stereoisomers of MeLan found in natural lantibiotics as well as homologated products that have one more atom in the ring or peptide backbone.

Although much effort was made to prevent complications of nonenzymatic cyclization, an unambiguous control experiment was sought in which a dehydrated substrate could be incubated under the same reaction conditions in the absence of enzyme. For this purpose, a LctM mutant was needed that would retain dehydration activity but lacked any cyclization activity. The C terminus of LctM has sequence homology with NisC, the cyclase involved in nisin biosynthesis. NisC is a Zn protein [43], and its structure was recently solved revealing the Zn ligands that are also conserved in LctM [37]. The zinc is believed to activate the thiol of the substrate for nucleophilic attack onto the dehydro amino acid. Therefore, the conserved Zn ligand Cys836 in LctM was mutated to Ala. As intended, LctM(C836A) retained dehydration activity but lacked full cyclization activity because a free thiol was detected in the dehydrated LctA(1–38)S35A product (M.P., G.C.P., and W.A.v.d.D., unpublished data). With this cyclization-deficient LctM mutant in hand, nonenzymatic cyclization could be ruled out for the experiments discussed above, which unequivocally demonstrates that LctM has relaxed specificity with respect to the nucleophilic thiol.

Significance

We report a highly adaptable in vitro chemoenzymatic route to access diverse structurally constrained molecules that incorporate nonproteinogenic amino acids. The bifunctional enzyme lacticin 481 synthetase is demonstrated to have relaxed substrate specificity for both its dehydratase and cyclase activities, allowing access to lacticin 481 variants. Importantly, nonlantibiotic peptides were also dehydrated by LctM, thereby installing a convenient electrophilic handle for further derivatization. The only requirement appears to be the presence of the leader peptide and a minimum distance between the residue to be modified and this leader peptide. This permits the utilization of semisynthetic substrates that possess the lacticin 481 leader sequence and nonlantibiotic C termini. The exact position of Ser/Thr residues was less important as demonstrated by both the insertion and extension mutants. While an analysis of lantibiotic sequences and studies on NisB [21] suggest that Asp and Glu residues flanking Ser/Thr may not be conducive to dehydration, LctM is tolerant toward these amino acids. On the other hand, Gly adjacent to Ser is clearly demonstrated to impair the dehydration reaction. The cyclization reaction tolerates a variety of thiol substrates, which may be utilized to prepare conformationally constrained bioactive peptides with increased biostability. The use of nonproteinogenic Cys analogs greatly expands the arsenal of accessible cyclic thioether structures by this mild and convenient methodology.

Experimental Procedures

For detailed descriptions of the construction of genetic mutants, see the online Supplemental Data.

General Protocol for LctM Activity Assays

RP-HPLC purified wt His_6-LctA and mutant peptides were dissolved in Millipore water and incubated with assay buffer containing 25 mM Tris (pH 7.5), 5 mM $MgCl_2$, 12.5 μg/ml BSA, and 2.5 mM ATP. Assays were typically performed in the presence of 0.2 mM or 1 mM TCEP. The mixtures were adjusted to pH 7.5 prior to addition of LctM and then incubated at 25°C for 2–4 hr. Control assays were under identical conditions without LctM. An assay aliquot was purified by C18 Zip-tip and analyzed by MALDI-TOF mass spectrometry.

General Procedure for Bioassays

The RP-HPLC-purified wt His_6-LctA and mutant peptides were dissolved in Millipore water to a final concentration >2 mg/ml. The peptides (0.6–1.0 mg) were added to 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 25 μg/ml BSA, 5 mM ATP, and 5 μM $ZnCl_2$. To drive the reactions to completion as much as possible, His_6-LctM (~40 μg) was added every hour for 4 hr at 25°C (total assay volume of 6 ml). Assay mixtures were concentrated to 1 ml on a Labconco Centrivap Concentrator. The assay products were purified by C4 analytical RP-HPLC with monitoring at 220 nm. Fractions containing product were identified by MALDI-TOF MS, lyophilized, and resuspended in 30 μl Millipore water. Commercially available protease Lys-C (Roche) was resuspended in 50 μl 100 mM Tris (pH 8.3) to 0.4 μg/μl final concentration. The purified assay product (~0.3 μg) was resuspended in 16 μl 100 mM Tris (pH 8.3) with 4 μl LysC and incubated for 3 hr at 37°C. Removal of the leader sequence from the mature propeptide was confirmed by MALDI-TOF MS, and the mixture of cleaved products evaporated to dryness. The products were redissolved in 5 μl sterile Millipore water. Freshly autoclaved GM17 agar (4 % M17, 0.5 % glucose, and 1.5 % agar) was cooled to 50°C, and 750 μl of an overnight culture of *L. lactis* CNRZ 117 (Centre National de Recherches Zootechniques, Jouy-enJosas, France) in GM17 broth (4 % M17 and 0.5 % glucose) was added prior to pouring plates. Wells were made in these agar plates with a sterile pipet tip, and the cleavage products were applied. Autoclaved water (5 μl) was applied as a negative control, while ampicillin (1 μl 100 mg/ml) or kanamycin (1 μl 50 mg/ml) served as a positive control. Plates were incubated overnight at 25°C and monitored for a zone of clearance, which indicated lantibiotic activity.

General Peptide Synthesis

Peptides were synthesized on a 0.1 mmol or 0.05 mmol scale employing Fmoc-based solid-phase peptide synthesis protocols.

Coupling and deprotection steps were undertaken at room temperature in DMF. Deprotection of the Fmoc group was achieved with 20% piperidine in DMF for 4 × 5 min. Coupling of amino acids was carried out for at least 1 hr with 0.4–0.8 mmol (four to eight equivalents) of each Fmoc-AA and HBTU in the presence of 0.4 M *N*-methylmorpholine (NMM) in DMF. Couplings were monitored by the ninhydrin test. To minimize racemization Fmoc-L-Cys(Trt)-OH was manually coupled in a base-free manner employing equimolar amounts of DIC and HOBt. After completion of synthesis, the resin was washed with DMF, ethanol, and DCM and dried in vacuo. Peptide cleavage from the resin and global deprotection was achieved with a mixture of TFA, water, and ethanedithiol (EDT, 95:2.5:2.5) with the drop-wise addition of triisopropylsilane until a colorless suspension was obtained. Crude peptides were precipitated and washed with cold diethyl ether and lyophilized from ~10% aqueous acetic acid after filtration through a 0.45 μm filter.

Lyophilized crude peptides were purified by C18 preparative RP-HPLC, and the pure products were characterized by MALDI-TOF MS. The calculated mass of the CVISHEA peptide was 758 Da, and the observed mass was 759 Da. The calculated mass of the CVGSGEA peptide was 622 Da, and the observed mass was 623 Da. The calculated mass of the CVISHISHISHA peptide was 1303 Da, and the observed mass was 1304 Da.

General Expressed Protein Ligation Protocol

The RP-HPLC purified and lyophilized synthetic peptides were redissolved in a minimum volume of ligation buffer (200–500 μl) containing 100 mM HEPES (pH 7.75), 200 mM NaCl, 1 mM EDTA, and 50 mM MESNa to a final concentration of 32 mM (CVISHEA), 51 mM (CVGSGEA), or 14 mM (CVISHISHISHA). The peptide solution was directly added to the lyophilized peptide thioester [25] to obtain a final concentration of ~1 mM of the thioester. The pH was adjusted to 7.6–7.8, and the reaction was kept at 4°C for 12–16 hr. The crude products were analyzed by MALDI-TOF MS for completeness of the reaction prior to acidification with 5% TFA. The acidified ligation mixture was incubated with 60 mM TCEP for 30 min at 25°C prior to purification by C4 analytical RP-HPLC.

Fractions containing the ligation products were analyzed by MALDI-TOF MS. The calculated and the observed mass of *His_6-LctA(1–38)-VISHEA* was 6797 Da. The calculated and the observed mass of *His_6-LctA(1–38)-VGSGEA* was 6661 Da. The calculated mass of *His_6-LctA(1–38)-V(ISH)$_3$A* was 7343 Da, and the observed mass was 7344 Da.

Cyanylation Protocol

The LctM assay products were purified by C4 analytical RP-HPLC. Fractions containing the assay products were evaporated to dryness, and to each 1.7 ml Eppendorf tube containing assay product, or an equivalent amount of the unmodified substrate, 5 μl denaturation buffer (100 mM sodium citrate [pH 3.0], 6 M guanidine hydrochloride) and 10 μl reducing buffer (100 mM sodium citrate [pH 3.0], 100 mM TCEP) was added. The substrate and assay product were redissolved by vortexing briefly and incubated at 25°C for 15 min to reduce disulfide bonds. Then, 20 μl freshly prepared cyanylation solution (100 mM sodium citrate [pH 3.0], 100 mM CDAP) was added to each tube. After an additional 15 min incubation at 25°C, the samples were acidified with 3 μl 5% aqueous TFA. The acidified samples were purified by means of C18 Zip-tip and eluted with 3 μl α-hydroxycinnamic acid matrix (prepared in 50% MeCN, containing 0.1% TFA) of which 1 μl was spotted directly on the MALDI target and analyzed by MALDI-TOF.

Supplemental Data

Supplemental Data include procedures for the construction of LctA mutant peptides as well as mass-spectrometry data for all peptides and assays conducted and are available at http://www.chembiol.com/cgi/content/full/13/10/1109/DC1/.

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