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On the Regioselectivity of Thioether Formation by Lacticin 481 Synthetase

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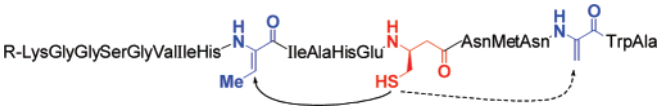
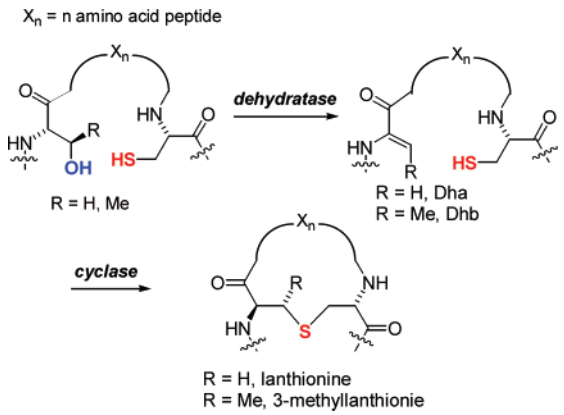
ABSTRACT



Lantibiotic synthetases generate intramolecular thioether cross-links within peptides through the Michael-type addition of cysteines onto dehydroamino acids originating from Ser and Thr. Presented here is an assay that readily distinguishes between enzymatic and nonenzymatic formation of these cross-links. The results demonstrate unequivocally that lacticin 481 synthetase can generate non-native thioether cross-links.

Lantibiotics are a class of ribosomally synthesized and post-translationally modified peptide antimicrobials.[1] The first step in these modifications features dehydration of Ser and Thr residues in the prepeptide precursors to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. This process is then followed by the intramolecular addition of cysteines to the dehydroamino acids to produce thioether cross-links called lanthionines (from Ser) and methyllanthionines (from Thr) (Scheme 1). The recent successful in vitro reconstitution of enzymes that catalyze these steps[2] has opened the door to investigate their reaction mechanisms and analyze their utility for lantibiotic engineering. In the case of the biosynthesis of lacticin 481, the bifunctional synthetase LctM catalyzes both the dehydration and cyclization steps. Previous studies have demonstrated that the dehydratase domain of LctM carries out the dehydration of nonproteinogenic Thr analogues as well as Ser/Thr residues installed at non-native positions in the peptide substrates.[3] This relaxed substrate specificity was also found in the cyclization activity of LctM as several Cys analogues were converted to the corresponding thioether rings.[3a] Whereas these studies show the potential of this class of enzymes for lantibiotic engineering as well as protein engineering,[4] a more stringent test of the substrate specificity would not only focus on the ability to achieve cyclization but also to control the *regio-* and *chemoselectivity* of thioether ring formation. This issue is particularly relevant because biomimetic studies have shown that nonenzymatic cyclization of Cys residues onto Dha residues is much faster

Scheme 1



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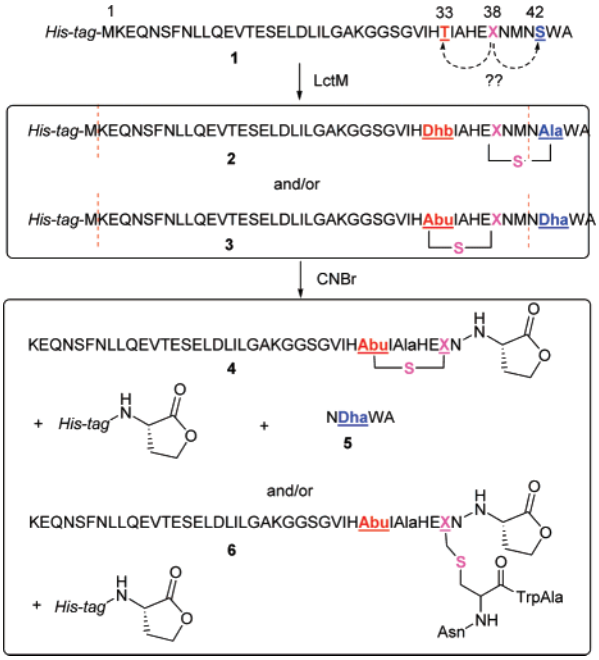
than the corresponding reaction with Dhb residues.[5] Therefore, formation of a methyllanthionine in preference to generation of a lanthionine requires enzymatic catalysis.

The key question is then how to design a relatively rapid assay that allows the evaluation of the substrate specificity of the enzymatic cyclization reaction that simultaneously tests for cyclization as well as its chemo- and regioselectivity. The challenges for such a test are 2-fold. First, cyclization does not result in a change in molecular mass, and as a result, previous tests for cyclization have relied predominantly on the disappearance of free thiols from the substrate or substrate analogues.[3a,4,6] Such assays, however, analyze the remaining substrate and do not report on the regioselectivity of cyclization if more than one reactive dehydro amino acid is available. A second complication is that any observed cyclized product can be the result of enzymatic or nonenzymatic cyclization, and these different origins for cyclized products have not been distinguished in previous studies.

Our design to address which Cys analogues are accepted as substrates for cyclization catalyzed by LctM is depicted in Scheme 2. It relies on a truncated analogue **1** of the LctA substrate for lacticin 481 synthetase. After LctM dehydrates this substrate, it contains two dehydro amino acids, a Dhb at position 33 and a Dha at position 42, as well as one Cys (or Cys analogue) at residue 38. On the basis of our previous studies, we anticipated that after dehydration of this substrate, any nonenzymatic cyclization would occur between Cys38 and Dha42 to give product **2** given the much higher reactivity of Dha compared to Dhb.[5c] On the other hand, the enzyme was expected to overcome the inherent kinetic bias against formation of a methyllanthionine and catalyze the addition of Cys38 to Dhb33 to provide peptide **3**, containing the A-ring of lacticin 481. These two reaction products can be distinguished by treatment with cyanogen bromide. This reagent fragments peptides C-terminal to Met residues, converting the Met into a homoserine (Hse) lactone. In the case of peptides **2** and **3**, CNBr treatment should result in cleavage of the amide bonds between Met1 and Lys2 as well as Met40 and Asn41. For product **3**, in which Cys38 is engaged in a methyllanthionine linkage to residue 33, this cleavage would result in two peptide products, **4** and **5**, in addition to a fragment originating from the His-tag. On the other hand, in peptide **2**, in which Cys38 is linked to residue 42 through a lanthionine linkage, CNBr treatment would result in peptide **6** in addition to the His-tag peptide fragment. The sulfur atom of the (methyl)lanthionines can also react with CNBr[7] but the expected products of such a reaction were not observed.

Scheme 2



Validation of the Assay with LctM and LctM-C781A. To test the experimental design, LctM was first incubated with ATP, Mg^{2+}, and substrate **1** in which position 38 was occupied by Cys, resulting in the anticipated two dehydrations of Thr33 and Ser42. Incubation with the thiol reactive agent *p*-hydroxymercuribenzoic acid (PMBA)[2b,6,8] indicated that the product did not contain free thiols,[9] consistent with a cyclization reaction involving Cys38. Treatment of the enzyme assay product with CNBr in formic acid (70%) followed by analysis by MALDI-MS showed the expected production of peptide **4** (4163 Da, calcd 4161 Da, Figure 1A). Note that in addition to fragmentation at Met40, the conditions used result in oxidized (M + 16) and formylated species (M + 28). Formylation of amine and hydroxyl side chains has been reported previously following treatment of peptides with formic acid.[10] We tentatively assign the oxidation products seen in fragment **6** to Trp43.[11] The observation of fragment **4** as the major cyclization product confirmed that LctM still forms the A-ring of lacticin 481 and controls the chemoselectivity of the cyclization. To verify our supposition that nonenzymatic cyclization should display preference for the formation of a lanthionine linkage to Dha42, the reaction sequence was repeated with a LctM mutant in which one of the zinc ligands (Cys781) is replaced by an Ala. The zinc is believed to be critical for the

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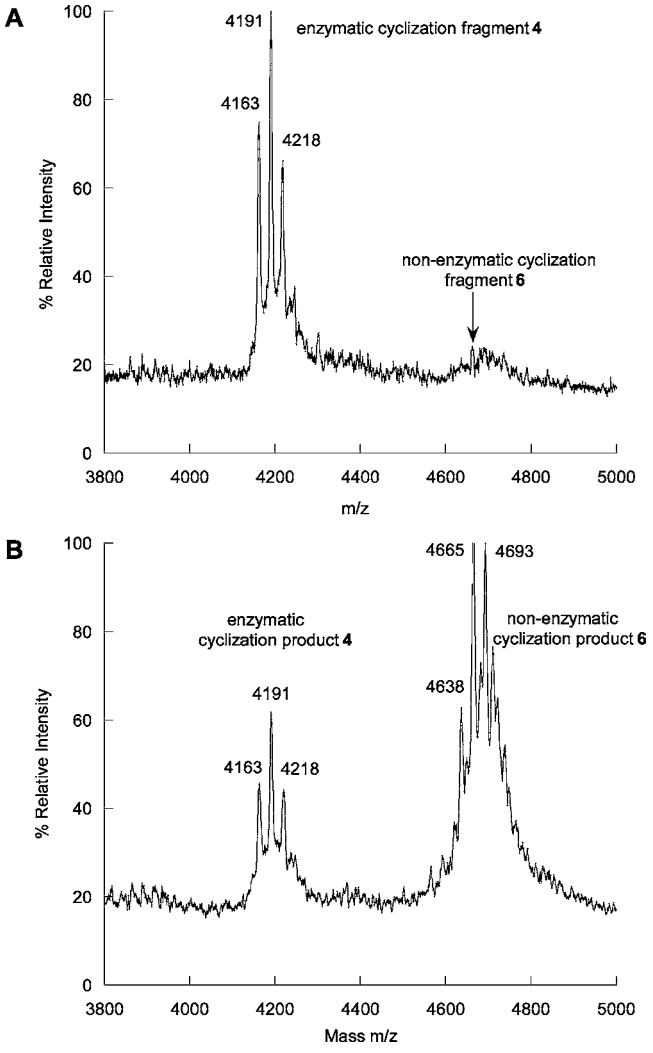

Figure 1. MALDI-MS data of LctA(1−43) **1** incubated with LctM (A) or LctM-C781A (B) followed by CNBr treatment in formic acid.

cyclization reaction by activation of the thiol of cysteines in the substrate.[2b,12] Indeed, this mutant retains dehydration activity but not cyclization activity.[6] As envisioned, LctM-Cys781Ala catalyzed the dehydration of Thr33 and Ser42 and no free thiol was detected in the assay product suggesting

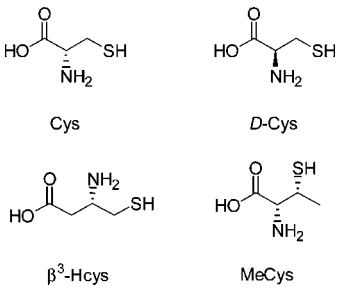

Figure 2. Structure of Cys and Cys analogues used in this study.

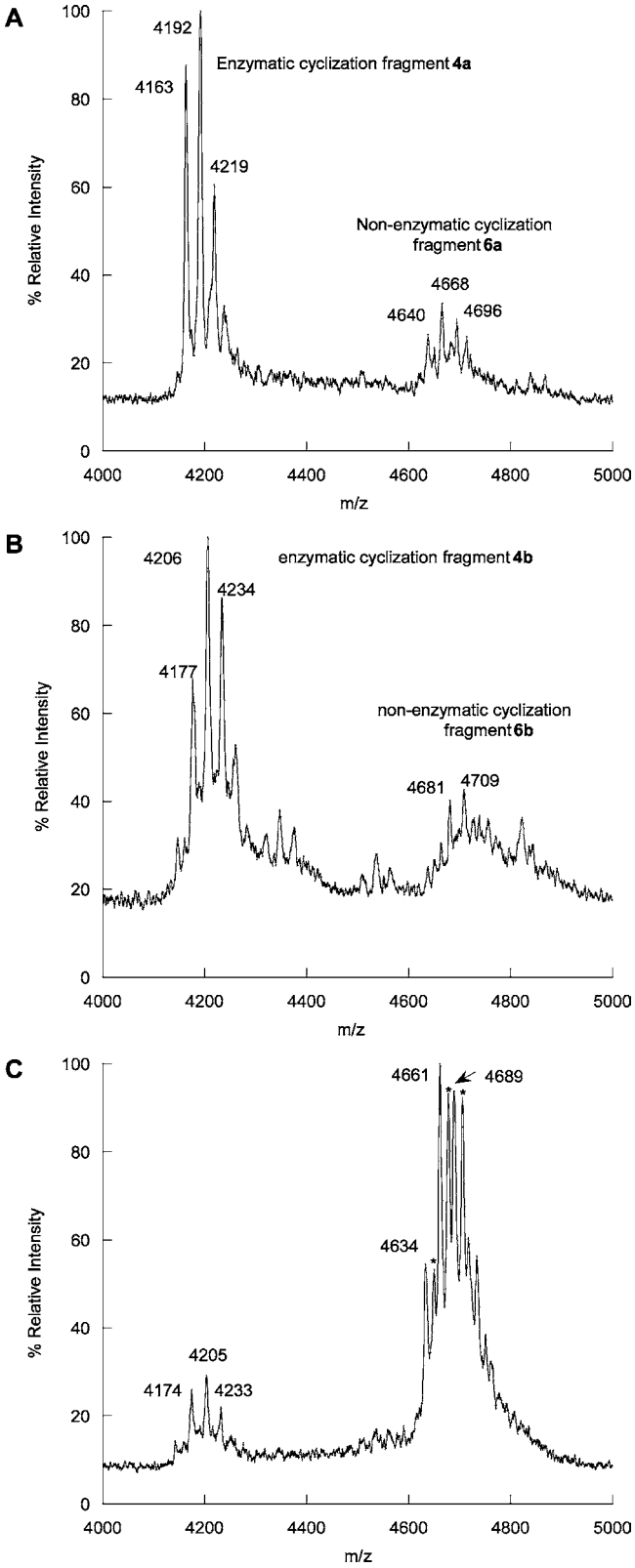

Figure 3. MALDI-MS data of (A) LctA(1−43)Cys38D-Cys, (B) LctA(1−43)Cys38β^3-Hcys, and (C) LctA(1−43)Cys38βMeCys incubated first with LctM followed by CNBr treatment in formic acid.

cyclization had taken place. Subsequent treatment with CNBr resulted in a small peak of product **4** (Figure 1B) with the major peak consistent with peptide **6** resulting from nonenzymatic reaction of Cys38 and Dha42 and subsequent Hse lactone formation. Thus, the designed assay could readily distinguish between enzymatic and nonenzymatic cyclization products.

Cyclization Assays with Cys Analogues. Previous studies have shown that substrate variants containing C-terminal Cys analogues were dehydrated by LctM and the assay products contained lanthionine cross-links.[3a] Because only a single dehydrated amino acid was available for cyclization, those studies did not report on enzymatic control over the chemoselectivity of cyclization. The assay described above was used to address this issue. First, D-Cys was investigated to probe the stereospecificity of LctM. D-Cys was incorporated at the N-terminus of a synthetic heptapeptide corresponding to residues 38−43 of peptide **1**. This peptide was then ligated to the thioester of a bacterially expressed peptide corresponding to LctA(1−37) using the expressed protein ligation protocol reported previously.[3b,13] Incubation of the resulting LctA(1−43) peptide **1a** in which Cys38 was replaced by D-Cys with wild-type LctM afforded two dehydrations and analysis with PMBA showed the absence of a free thiol in the product(s).[9] Subsequent treatment with CNBr in formic acid and analysis by MALDI-TOF MS showed the enzymatic cyclization product **4a** as the major product (Figure 3A). The chemoselectivity of the nonenzymatic cyclization reaction was again confirmed by incubation of **1a** with LctM-C781A and subsequent cleavage with CNBr. MALDI MS analysis showed that peptide **6a** (4623 Da, calcd 4619 Da) was the major product of these control experiments as well as a small amount of peptide **4a** (see the Supporting Information). The data demonstrate that the stereochemistry of the cysteine is not important for LctM to catalyze the cyclization. As a result, the enzyme can be used to produce a diastereomer of the naturally occurring isomer of (methyl)lanthionine. Although the stereochemistry at carbons 2 and 3 of the resulting methyllanthionine is not rigorously determined in this work, the fact that cyclization is enzyme catalyzed combined with the known stereoselectivity of the Michael-type addition catalyzed by LctM strongly suggests that the stereochemistry of the product is (2*S*,3*S*).

Next two cysteine analogues, (*S*)-β^3-homocysteine (β^3-Hcys) and (2*R*,3*R*)-methylcysteine (β-MeCys, Figure 2) appropriately protected for Fmoc solid-phase peptide synthesis,[14] were incorporated at position 38 of LctA(1−43) by using the same protocol as described for **1a.** The resulting substrates **1b** (X = β^3-Hcys) and **1c** (X = β-MeCys) were then incubated with wild-type LctM resulting in two dehydrations. Analysis by PMBA showed that both products were almost entirely devoid of free thiols.[9] Subsequent treatment with CNBr and MALDI-MS analysis indicated that the majority of the product obtained with substrate **1b** corresponded to the enzymatic cyclization product **4b** with a minor amount of nonenzymatic cyclization product **6b** (Figure 3B). The production of **6b** by nonenzymatic means was again confirmed with the mutant LctM-C781A (Supporting Information). On the other hand, repeating the protocol with substrate analogue **1c** resulted in product **6c** as the major product (4634 Da, Figure 3C).[9] Thus, the enzyme cannot tolerate substitution at the β-carbon of cysteine but a peptide containing a β-homocysteine is enzymatically cyclized.

In summary, an assay has been developed that can relatively rapidly assess whether thioether formation by a lantibiotic cyclase is the result of enzymatic cyclization or nonenzymatic cyclization. The assay has been used to demonstrate that lacticin 481 synthetase catalyzes the cyclization of peptides containing D-Cys and β^3-homocysteine, but that methyl substitution at the β-carbon of Cys was not accepted. With this tool in hand, the potential of lantibiotic biosynthetic enzymes to alter the structure of naturally occurring lantibiotics or to introduce thioether rings in nonlantibiotic peptides can be probed in greater detail.

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Supporting Information Available: Detailed experimental procedures and mass spectra of semisynthetic peptides and assay products. This material is available free of charge via the Internet at http://pubs.acs.org.

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