Nine Post-translational Modifications during the Biosynthesis of Cinnamycin

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 S *Supporting Information*

ABSTRACT: Lantibiotics are ribosomally synthesized and post-translationally modified antimicrobial peptides that are characterized by the thioether cross-linked amino acids lanthionine (Lan) and methyllanthionine (MeLan). Cinnamycin is a 19 amino acid lantibiotic that contains one Lan and two MeLan. Cinnamycin also contains an unusual lysinoalanine (Lal) bridge

formed from the ε-amino group of lysine 19 and a serine residue at position 6, and an *erythro*-3-hydroxy-L-aspartic acid resulting from the hydroxylation of L-aspartate at position 15. These modifications are critical in mediating the interactions of cinnamycin with its target, phosphatidylethanolamine. Recently, the cinnamycin biosynthetic gene cluster (*cin*) from *Streptomyces cinnamoneus cinnamoneus* DSM 40005 was reported. Herein, we investigated the biosynthetic machinery using both in vitro studies and heterologous expression in *Escherichia coli*. CinX is an α-ketoglutarate/iron(II)-dependent hydroxylase that carries out the hydroxylation of aspartate 15 of the precursor peptide CinA. In addition, CinM catalyzes dehydration of four Ser and Thr residues and subsequent cyclization of Cys residues to form the three (Me)Lan bridges. The order of the post-translational modifications catalyzed by CinM and CinX is interchangeable in vitro. CinX did not require the leader sequence at the N-terminus of CinA for activity, but the leader peptide was necessary for CinM function. Although CinM dehydrated serine 6, it did not catalyze the formation of Lal. A small protein encoded by *cinorf7* is critical for the formation of the cross-link between Lys19 and dehydroalanine 6 as shown by coexpression studies of CinA, CinM, CinX, and Cinorf7 in *E. coli*.
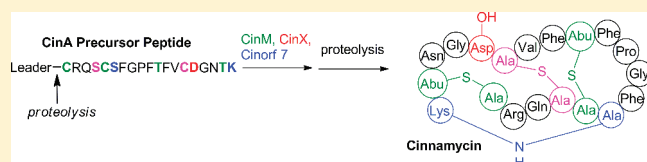
■ INTRODUCTION

Lantibiotics are ribosomally synthesized antimicrobial peptides that undergo extensive post-translational modifications to attain their active antimicrobial forms.[1] They are characterized by the presence of thioether cross-linked amino acids called lanthionine (Lan) and methyllanthionine (MeLan), which are formed by intramolecular addition of cysteine thiols to dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively. The dehydroalanines are generated by dehydration of Ser residues, whereas the dehydrobutyrines are formed by dehydration of threonines.[1] Cinnamycin is a 19 amino acid lantibiotic with antimicrobial activity against gram-positive rods such as bacilli, *Clostridium botilinum*, and *Mycobacterium*.[2] The compound also causes transbilayer phospholipid movement in the cell membrane of mammalian cells to access phosphotidylethanolamine (PE) residing predominantly in the inner leaflet of the membrane.[3] The interaction of cinnamycin with PE has provided researchers with tools to monitor PE[4] and also renders the compound pharmacologically valuable. For example, duramycin, a close structural analogue of cinnamycin, promotes chloride secretion in lung epithelial cells by binding to PE; this activity in turn promotes mucus clearance from the lungs. As a result, the compound is in phase II clinical trials for the treatment of cystic fibrosis,[5−7] a common genetic disorder characterized by abnormal chloride ion transport.[6] Cinnamycin has also been suggested as an alternative treatment for atherosclerosis through its ability to inhibit phospolipase A2 by binding to its substrate PE.[8,9]

Cinnamycin has a compact globular structure with one Lan and two MeLan residues in addition to an unusual lysinoalanine (Lal) formed from lysine 19 and serine 6 (Figure 1).[10,11] Unlike most lantibiotics, the rings are generated in a bidirectional manner such that the MeLan bridges are formed by cysteines that are located N-terminal to the dehydrobutyrine residues with which they react, and the Lan bridge is formed from a cysteine located C-terminal to the dehydroalanine with which it reacts. Similarly, the Lal is generated from a Lys located C-terminal to the serine with which it forms a cross-link. Unlike the Lan and MeLan structures, which have D-stereochemistry at the α-carbon originating from Dha/Dhb, the Lal has the L-configuration at the α-carbon originating from Ser,[12,13] raising the question whether it is formed with the intermediacy of a Dha or not.

In addition to the macrocyclic structures, cinnamycin also contains an *erythro*-3-hydroxy-L-aspartic acid resulting from the hydroxylation of L-aspartate at position 15 (Figure 1).[10,12] The binding of cinnamycin to its target PE is mediated by interaction of this post-translational modification with the ammonium group of PE.[8,14] The duramycins (duramycin, duramycin B and C) are natural analogues of cinnamycin with the same number of amino acids and very high sequence similarity including identical positions of the four cross-links and the hydroxylated Asp.[13]
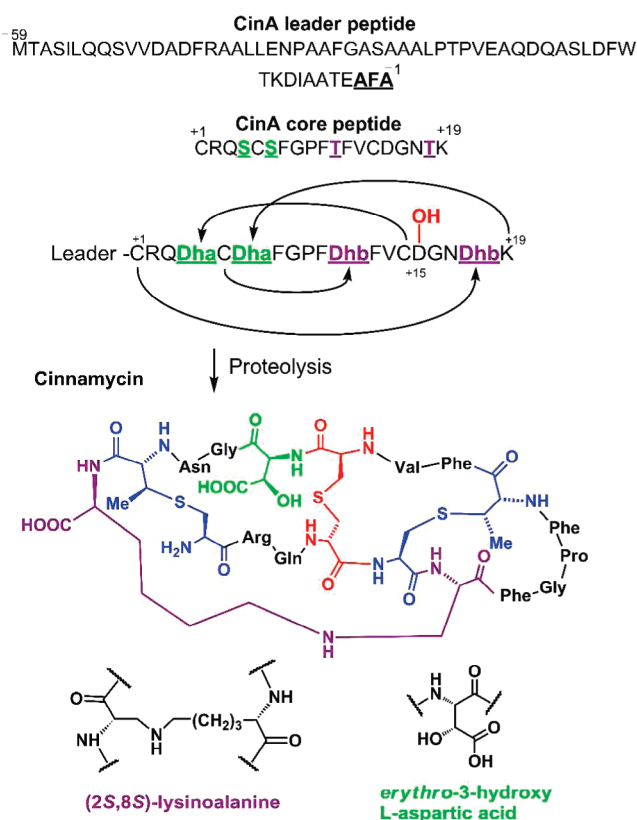
Figure 1. The structure of cinnamycin. Lan and MeLan are shown in red and blue, respectively, Lal is depicted in purple, and the hydroxylated Asp is shown in green. The sequence of the CinA core and leader peptides is depicted as well as the residues that form the cross-links. The *sec*-cleavage sequence at the end of the leader peptide is shown in bold underlined font.

Recently, the cinnamycin biosynthetic gene cluster (*cin*) from *Streptomyces cinnamoneus cinnamoneus* DSM 40005 was reported,[15] providing opportunities to investigate the biochemistry of its biosynthetic pathway. Lantibiotics are produced from precursor peptides termed LanA that have an N-terminal sequence called the leader peptide that is recognized by various enzymes to process the C-terminal core peptide.[16] (Me)Lan intramolecular bridges are formed by enzymatic dehydration of specific Ser and Thr residues to form Dha and Dhb residues followed by subsequent stereoselective intramolecular Michael-type addition of cysteine thiols to these dehydroamino acids. Lanthionine synthetases are divided into four classes based on sequence homology.[17−19] In class II lantibiotics to which cinnamycin belongs, both dehydration and cyclization are catalyzed by a bifunctional enzyme called LanM.[20] After the core peptide is processed, the leader peptide is removed proteolytically to afford the mature, biologically active compound.[21]

The cinnamycin gene cluster contains 21 open reading frames (17 083 bp), which include the genes for cinnamycin production, regulation, and self-resistance (Figure S1).[15] The cinnamycin precursor peptide, CinA, consists of a C-terminal core region that is 19 residues long and that will be transformed into cinnamycin through post-translational modifications. Appended to the N-terminus of the core peptide is an unusually long 59 amino acid leader sequence that is not modified. Instead of a GG or GA protease cleavage motif seen in most class II lantibiotics, an AXA

motif is present between the leader sequence and the core region of CinA (Figure 1). This sequence is recognized by type I signal peptidases of the general secretory (*sec*) pathway,[22] which explains the absence of a cinnamycin-specific protease in the gene cluster.[15] The CinM sequence shows high homology with other class II LanM enzymes.[15] However, the enzymes responsible for Asp hydroxylation and Lal bridge formation have not been explored.

Understanding the biosynthetic pathway of cinnamycin may be used to generate analogues with potentially improved properties for treatment of cystic fibrosis or clostridium infections. Herein, we investigated the biosynthetic machinery of cinnamycin in vitro and characterized the enzymes involved in the formation of the thioether cross-links and the β-hydroxylation of aspartate 15. In addition, we developed a coexpression system in *E. coli* that resulted in fully modified CinA and identified *cinorf7*, an open reading frame of unknown function, as a critical determinant for lysinoalanine formation.

■ RESULTS

In Vitro Reconstitution of the Enzymatic Activity of the Lanthionine Synthetase, CinM. The gene for the precursor peptide CinA was cloned into a pET15b vector, and the gene encoding CinM was cloned into a pET16b vector to generate N-terminal histidine-tagged fusion proteins upon heterologous expression. Site-directed mutagenesis was used to mutate the alanine residue at the last position of the leader peptide of CinA (position−1) to lysine. The resulting His_6-CinA(A−1K) mutant enables removal of the leader peptide using the commercial endoproteinase LysC for bioactivity assays. This substrate analogue was used as a precursor peptide throughout this study. Both His_6-CinA(A−1K) and His_{10}-CinM were heterologously expressed in *E. coli* BL21 (DE3) cells and purified by immobilized metal ion affinity chromatography (IMAC). Approximately 6−10 mg of His_{10}-CinM was obtained per liter of cell culture. The His_6-CinA(A−1K) peptide was further purified after IMAC by reverse-phase high-performance liquid chromatography (RP-HPLC) resulting in 40 mg of purified peptide per liter of cell culture that was detected as the Na$^+$ adduct after LysC protease cleavage (Figure 2A). The enzyme CinM migrated as a protein of approximately 120 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration analysis, close to the predicted monomeric molecular weight of His_{10}-CinM (118.8 kDa) (Figure S2).

To evaluate dehydration activity, His_6-CinA(A−1K) (20 μM) was incubated with His_{10}-CinM (5 μM) at 25 °C for 6 h in the presence of adenosine triphosphate (ATP), MgCl$_2$, and immobilized tris(2-carboxyethyl)phosphine (TCEP). Use of immobilized reducing agent was required to prevent unwanted additions of reducing agents (TCEP/dithiothreitol) to the dehydrated precursor peptide. Analysis of the core peptide by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) after LysC endoproteinase treatment revealed a mass corresponding to loss of four water molecules (Figure 2B). This finding demonstrates that CinM is responsible for the dehydration of the two threonine (Thr11 and Thr18) and two serine residues (Ser4 and Ser6) in the core region of CinA. Addition of cysteine thiols to the dehydroamino acids does not cause a change in mass, which prevents observation of cyclization by MALDI-ToF MS. To investigate the cyclization activity of CinM, iodoacetamide (IAA), a thiol alkylating agent that induces
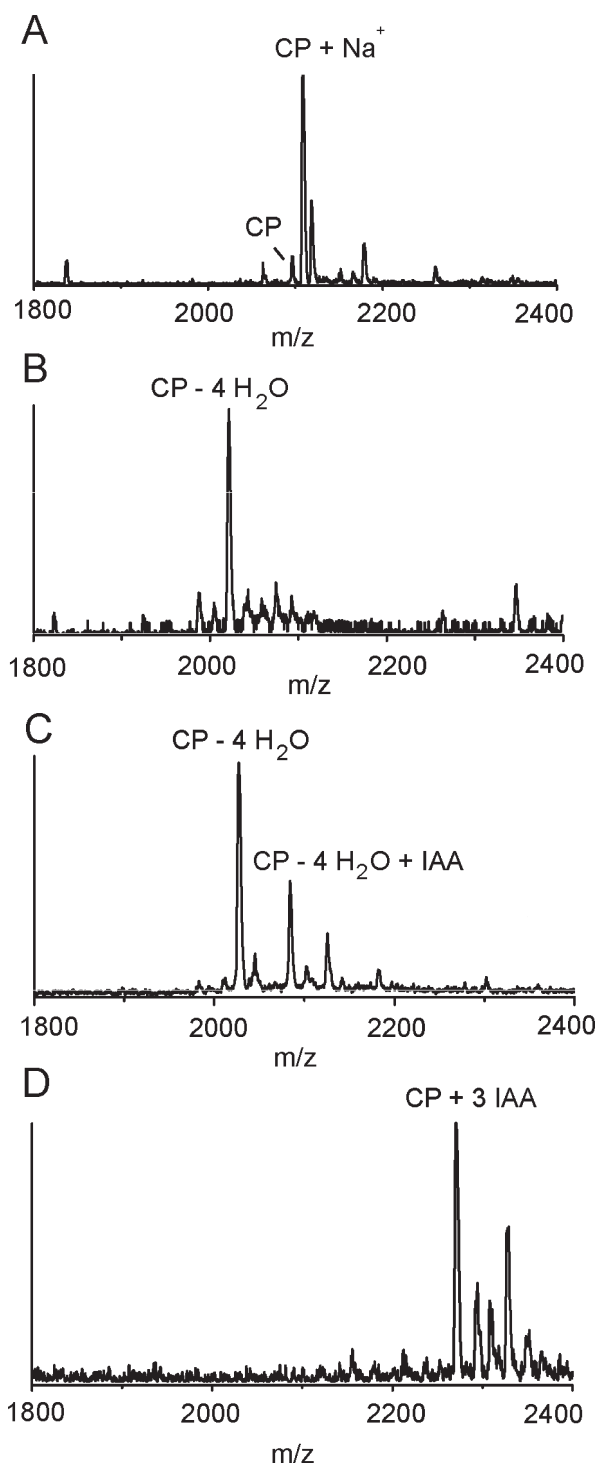
Figure 2. MALDI-ToF MS analysis of IAA modification assay of CinA modified in vitro by CinM. (A) MALDI ToF MS of His$_6$-CinA(A−1K) treated with LysC, (B) MALDI ToF MS of His$_6$-CinA(A−1K) after incubation with His$_{10}$-CinM followed by LysC treatment, (C) MALDI mass spectrum of His$_6$-CinA(A−1K) after incubation with His$_{10}$-CinM followed by IAA and LysC treatment, and (D) MALDI mass spectrum of His$_6$-CinA(A−1K) after IAA and LysC treatment. CP = CinA core peptide.

a mass increase upon reaction with cysteine thiols, was used. His$_6$-CinA(A−1K) and His$_6$-CinA(A−1K) modified by His$_{10}$-CinM were treated with IAA and LysC protease and then subjected

to MALDI-ToF MS analysis. As expected, His$_6$-CinA(A−1K) that had not been treated with His$_{10}$-CinM showed a mass increase corresponding to alkylation of the three cysteines in the core peptide (Figure 2D). In contrast, only minor alkylation products were observed in the His$_{10}$-CinM treated sample (Figure 2C). The data strongly support conversion of the majority of the free thiols to thioether rings by His$_{10}$-CinM, although one Cys^{23} appears to be less efficiently cyclized resulting in some peptide with one IAA adduct (Figure 2C). Taken together, these results demonstrate that CinM functions as a bifunctional LanM enzyme that catalyzes both dehydration and cyclization reactions to form the thioether cross-links. CinM did not, however, form the lysinoalanine (vide infra).

Quantitative Analysis of Zinc Bound to CinM. Metal analysis of the cyclase enzymes involved in subtilin (SpaC) and nisin (NisC) biosynthesis demonstrated that these proteins contained 1 equiv of zinc.[24,25] These results were confirmed with the crystal structure of NisC.[26] The zinc has been proposed to activate the Cys thiols of the peptide substrate for intramolecular addition to the dehydro amino acids in the substrate. To date, no structural information is available for LanM enzymes. However, because LanM enzymes share sequence homology at the C-terminus with LanC cyclases (20−30% identity), including two Cys and one His that coordinate to the Zn^{2+} in LanC proteins, it was hypothesized that the LanM cyclase domains might act through a similar mechanism of zinc-mediated activation of the Cys thiols in LanA substrates.[27]

To test if CinM contained zinc, a spectrophotometric assay was performed using the metallochromic indicator 4-(2-pyridylazo)-resorcinol (PAR).[28] Treatment of His$_{10}$-CinM with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) under denaturing conditions (6 M guanidine hydrochloride) converted Cys residues in CinM to disulfides including any metal ligands. As a result, DTNB facilitates release of the active site zinc,[24] which subsequently formed the orange Zn−(PAR)$_2$ complex that was monitored by absorbance at 500 nm. Corrections for DTNB absorbance levels were applied, and a linear standard plot determined from zinc standards was used to calculate the CinM:zinc ratio. On the basis of this experiment, His$_{10}$-CinM was found to bind 1.05 equiv of zinc.

In Vitro Reconstitution of CinX. The *cinX* gene was cloned into pET28b, and the encoded protein was heterologously expressed in *E. coli* with an N-terminal His$_6$-tag and was purified by IMAC. His$_6$-CinX migrated as a monomer of approximately 38 kDa in gel filtration and SDS-PAGE analysis (Figure S2). Sequence analysis suggested that CinX may be a member of the nonheme Fe(II)-dependent family of enzymes that utilizes α-ketoglutarate (α-KG) as cofactor.

His$_6$-CinA(A−1K) was modified with His$_{10}$-CinM followed by His$_6$-CinX treatment in the presence of Fe(II) and α-KG for 2 h. After subsequent digestion with LysC, four dehydrations and a hydroxylation were observed in the core region of CinA by MALDI-MS (Figure 3). In the absence of either Fe(II) or α-KG, no mass change corresponding to hydroxylation was observed, which supports the hypothesis from its sequence that CinX is an α-ketoglutarate/Fe(II)-dependent hydroxylase.

The order in which CinM and CinX may carry out their posttranslational modifications was also investigated. In vitro these two enzymes did not have a compulsory order of action as His$_{10}$-CinM was able to dehydrate and cyclize His$_6$-CinA that had been hydroxylated by His$_6$-CinX (Figure S3), and His$_6$-CinX was able to hydroxylate His$_6$-CinA that had been dehydrated and cyclized
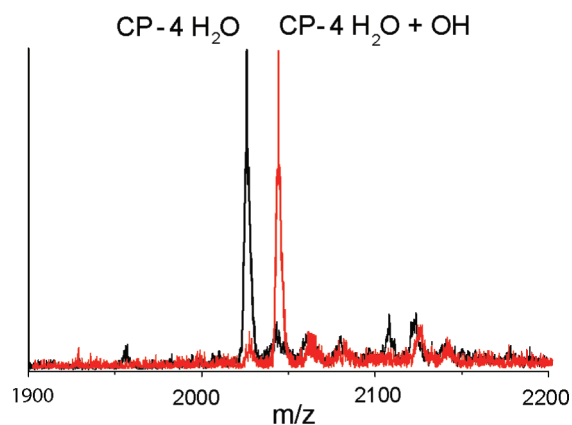
Figure 3. MALDI-ToF MS analysis of hydroxylation by CinX. His_6-CinA(A−1K) modified by His_{10}-CinM and subsequently digested with LysC is shown in black. His_6-CinA(A−1K) modified by His_{10}-CinM and then His_6-CinX and subsequent digestion with LysC is shown in red. Changing the order of modification by His_{10}-CinM and His_6-CinX resulted in the same product as shown in Figure S3. CP = CinA core peptide.

(Figure 3). To validate the site of hydroxylation, aspartate 15 was mutated to alanine, and the mutant CinA peptide was incubated with His_6-CinX. MALDI ToF MS analysis showed no change in the mass of the mutant peptide (data not shown). In lantibiotics, the leader peptide is required for activity of most of the biosynthetic enzymes.[21] To evaluate if this requirement was also the case for His_{10}-CinM and His_6-CinX, the core peptide of CinA (CinA1−19) was synthesized by solid-phase peptide synthesis. As expected, His_{10}-CinM showed no activity in the absence of the leader peptide (Figure S4A). In contrast, His_6-CinX was capable of hydroxylating CinA(1−19) (Figure S4B). LC−MS/MS analysis of the product further verified that the hydroxylation occurs on Asp15 (Figure S5).

Lysinoalanine Formation and Bioactivity Assays. Genetic studies in the producing organism have shown that *cinA*, *cinM*, *cinX*, and *cinorf7* are required for formation of cinnamycin.[29] Having assigned the function of the first two enzymes, we investigated whether Cinorf7 was responsible for Lal formation. Cinorf7 was expressed in *E. coli*, purified as an N-terminal hexa histidine fusion protein, and added to various assays containing His_6-CinA and His_{10}-CinM and/or His_6-CinX, with various orders of addition and supplementation with cofactors. Unfortunately, Lal was not formed under any conditions as determined using a variety of assays described below. Nonenzymatic formation of Lal, which results in both L and D configurations, has been observed in food products under alkaline conditions by chemical dehydration of Ser or elimination from Cys/cystine and subsequent nonselective conjugate addition of lysine to the resulting Dha.[30] On the basis of these observations, we investigated the nonenzymatic in vitro formation of the Lal ring under mild alkaline conditions (pH 9.5) after the precursor peptide His_6-CinA(A−1K) had been processed with His_{10}-CinM and His_6-CinX. The leader peptide was removed with LysC endoproteinase, and the resulting product was analyzed for Lal formation. The product was first hydrolyzed in strongly acidic conditions, and the resulting amino acids were derivatized to the corresponding methyl esters and pentafluoropropionyl amides.[31,32] Authentic cinnamycin was subjected to the same treatment to provide a standard. Subsequent analysis by gas chromatography−tandem

mass spectrometry (GC−MS/MS) demonstrated that derivatized Lal from authentic cinnamycin eluted as two peaks, probably due to epimerization during the acidic hydrolysis.[30] The same retention times for these two peaks were observed for Lal derived from both authentic cinnamycin and the in vitro assay sample (Figure S6A and B). The fragmentation pattern of the peaks in the GC−MS was also identical to and consistent with those in the literature for lysinoalanine derivatives (Figure S6C and D).[33] Thus, these experiments verify formation of the lysinoalanine ring at increased pH, but because the Lal residue originating from authentic cinnamycin was epimerized, we were unable to establish whether nonenzymatic Lal formation was stereoselective.

To investigate if the nonenzymatic Lal cyclization product has the correct stereochemistry, an antimicrobial assay was employed, under the assumption that cyclization with the incorrect stereochemistry would give an inactive product. A well diffusion assay against the indicator strain *B. subtilis* LH45 demonstrated clear antimicrobial activity of cinnamycin synthesized in vitro. A sample that was not treated under alkaline conditions did not produce a zone of growth inhibition, which demonstrates the requirement of Lal for antimicrobial activity of cinnamycin. Unfortunately, this in vitro process to produce cinnamycin did not allow comparison of its bioactivity with authentic cinnamycin because the sample contained unknown amounts of material derived from the biosynthetic enzymes, the leader peptide, and LysC. Therefore, the concentration of cinnamycin within the sample was not known. Attempts to purify the cyclized core peptide by reverse phase high-performance liquid chromatography (RP-HPLC) produced very low quantities of cinnamycin, preventing comparison with authentic cinnamycin.

Coexpression of CinA, CinM, and CinX in *E. coli*. Quantification of Lal formation after alkaline treatment by GC−MS has been challenging because of the large background resulting from the leader peptide and the biosynthetic enzymes. In an effort to improve both the amounts of material and their purity, we turned to coexpression of the CinA peptide and the CinM and CinX modification enzymes in *E. coli*. Recent studies in several laboratories have demonstrated the feasibility of conducting the post-translational modifications that generate lantibiotics in this heterologous host.[34−37] In our laboratory, pRSFDuet-1 and pACYCDuet-1 plasmids were used for these studies, which offer convenient purification of the post-translationally modified peptide using IMAC because of a His_6-tag at the N-terminus of the leader peptide of the substrate peptide.[35] A similar approach was employed here in an attempt to obtain higher quantities of peptide processed by CinM and CinX.

The gene encoding CinA(A−1K) with an N-terminal flag tag[38] was inserted into multiple cloning site I (MCSI) of the pRSFDuet-1 vector to generate His_6-Flag-CinA(A−1K). The gene encoding CinM was inserted into MCSII of the same plasmid resulting in a protein without a His-tag, thus preventing copurification of the CinM enzyme, which complicated the in vitro experiments. Similarly, *cinX* was cloned into MCSII of the pACYCDuet-1 vector, which also produces CinX without a His-tag. Coexpression of CinM and CinX together with His_6-Flag-CinA(A−1K) yielded 2 mg of His_6-Flag-CinA(A−1K) peptide per liter of cell culture after IMAC purification, and subsequent RP-HPLC. MALDI-ToF MS analysis of the LysC-treated peptide showed a mass difference with respect to unmodified core peptide corresponding to four dehydrations and one hydroxylation (Figure S7A). In vivo thioether cyclization activity was investigated by IAA treatment and analysis by
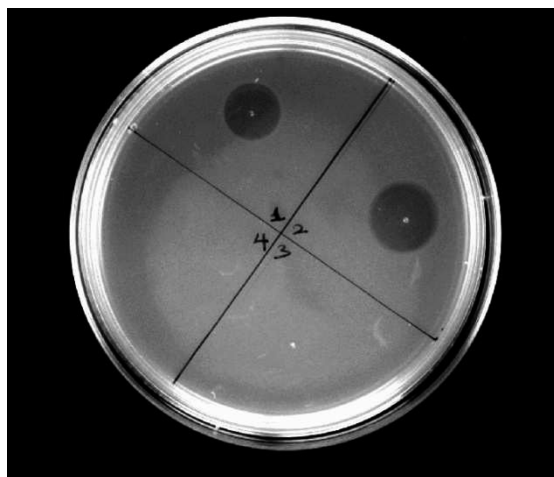
Figure 4. Bioassay with the indicator strain *B. subtilis* LH45. Sector 1, authentic cinnamycin; sector 2, His$_6$-CinA(A−1K) modified by CinM and CinX in *E. coli* and treated with LysC followed by incubation at pH 9.5; sector 3, His$_6$-CinA(A−1K) modified by CinM and CinX in *E. coli* treated with LysC (no alkaline treatment); sector 4 was not used.

MALDI-ToF MS as previously described. Similar to the in vitro results, CinM formed predominantly three thioether rings in the CinA core region (Figure S7B).

The peptide obtained from coexpression did not have antimicrobial activity toward the indicator strain *B. subtilis* LH45 similar to the observations for the product of CinM and CinX treatment in vitro. However, when Lal cyclization was induced by mild alkaline treatment, the peptide showed strong antimicrobial activity comparable to authentic cinnamycin (Figure 4).

With larger quantities of material in hand, the peptide produced in *E. coli* was used to investigate nonenzymatic Lal formation in more detail. *o*-Phthalaldehyde (OPA) is a reagent that reacts selectively with primary amines in the presence of a thiol such as β-mercaptoethanol but does not react with secondary amines.[39] Thus, OPA was used to monitor conversion of the primary ε-amine of Lys to the secondary amine in Lal. CinA that had been modified by CinM and CinX in *E. coli* and then incubated with LysC was treated with OPA, causing a mass increase corresponding to two OPA additions in the core peptide. These two OPA adducts originate from the N-terminal amino group and the ε-amino group of Lys. Furthermore, one β-mercaptoethanol addition was observed as a consequence of Michael-type addition to dehydroalanine 6 (Figure 5A). Collectively, these data clearly show the absence of a lysinoalanine. In contrast, the OPA assay of the same sample after alkaline treatment resulted in only one OPA addition and no β-mercaptoethanol adducts, which was also observed when authentic cinnamycin was used as a positive control (Figure 5B,C). The absence of the peak corresponding to the formation of two OPA and one β-mercaptoethanol adducts in this sample suggests that the cyclization occurred with near quantitative efficiency.

Coexpression of CinA, CinM, CinX, and Cinorf7 in *E. coli*. The in vitro studies did not provide any insights into the possible role of Cinorf7 in cinnamycin biosynthesis. In an alternative approach, the coexpression system in *E. coli* was used to investigate whether Cinorf7 is involved in formation of the Lal in vivo. For this purpose, the *cinorf7* gene was cloned into MCSI of pACYCDuet-1 that already contained the gene encoding CinX in MCSII. The resulting plasmid was used with the
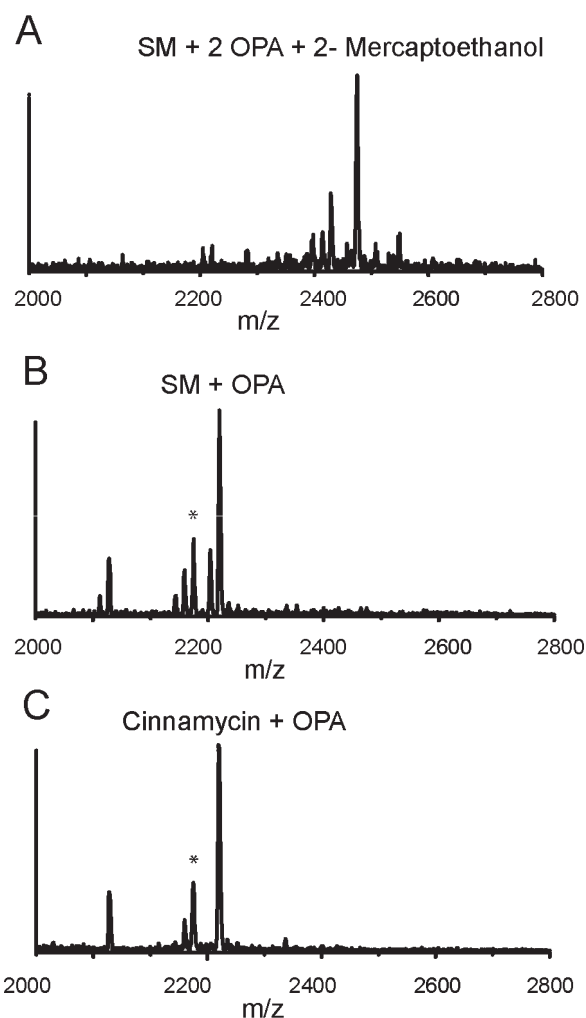

Figure 5. Use of *o*-phthalaldehyde (OPA) to monitor lysinoalanine formation. (A) CinA(A−1K) modified by CinM and CinX and treated with LysC was incubated with OPA and β-mercaptoethanol. (B) CinA(A−1K) modified by CinM and CinX followed by alkaline treatment and removal of the leader peptide with LysC was incubated with OPA and β-mercaptoethanol. (C) Authentic cinnamycin was incubated with OPA and β-mercaptoethanol. SM = mass of core peptide after four dehydrations and one hydroxylation. Asterisk indicates 4-fold dehydrated core peptide that did not react with OPA.

pRSFDuet-1 vector containing the genes for CinA(A−1K) and CinM for coexpression in *E. coli*. The modified CinA(A−1K) peptide was purified by IMAC, treated with LysC, and analyzed with the OPA assay. The resulting mass spectrum showed that the Lal structure was formed without the need of raising the pH (Figure S8A). Also, an antimicrobial assay toward the indicator strain *B. subtilis* LH45 verified that the activity of cinnamycin synthesized heterologously in *E. coli* is similar to the antimicrobial activity of authentic cinnamycin (Figure S8B). Because in the absence of Cinorf7 both the OPA assay and the antimicrobial assay were negative for the peptide produced in *E. coli*, these results reveal that Cinorf7 is required for the formation of the Lal.

■ DISCUSSION

The in vitro biosynthesis of cinnamycin was achieved in this work by reconstitution of the activities of two enzymes in the

biosynthetic pathway, CinM and CinX. His_{10}-CinM dehydrated two threonine and two serine residues in the core region of His_6-CinA and catalyzed the subsequent cyclization by cysteine thiols to form three thioether rings. The observation that CinM dehydrated Ser6 confirms that the Lal is formed with the intermediacy of a dehydroalanine despite the L-stereochemistry at the α-carbon of the former Ser residue. Although His_{10}-CinM dehydrated Ser6, it did not catalyze the formation of the Lal ring. Considering that LanC/LanM enzymes typically stereoselectively protonate the enolate formed during the Michael-type addition to provide D-stereochemistry at the α-carbon, it is not surprising that Lal formation, which results in the L-configuration at the newly formed stereogenic center,[12,13] is not catalyzed by CinM. In addition, CinM is much better suited to activate a thiol nucleophile with the Zn^{2+} in its active site than to activate a primary amine.

CinX is shown to be an Fe(II)/α-ketoglutarate-dependent hydroxylase that catalyzes the β-hydroxylation of Asp15 in the CinA precursor peptide. Interestingly, His_6-CinX accepted the core region of CinA as a substrate without the need of the leader sequence. Similar observations were made for the oxidative decarboxylase EpiD,[40] suggesting that tailoring enzymes that introduce post-translational modifications beyond dehydration and cyclization may not require the leader peptide. However, as observed for other lanthionine synthetases,[41–43] His_{10}-CinM required the leader peptide for efficient processing. Changing the order of incubation of the precursor peptide with His_{10}-CinM or His_6-CinX resulted in the same product.

In vitro biosynthesis of cinnamycin was accomplished by inducing nonenzymatic formation of the Lal by raising the pH after treatment of CinA with His_{10}-CinM and His_6-CinX in vitro or after heterologous coexpression of CinM and CinX with the precursor peptide in *E. coli*. The high bioactivity of the resulting peptide suggests that the nonenzymatic cyclization occurs with high stereoselectivity, assuming that cinnamycin with a Lal residue with incorrect stereochemistry has no or low antimicrobial activity. At present, we cannot test this assumption, but high stereoselectivity for nonenzymatic formation of Lan and MeLan has been demonstrated previously.[44–49] No such investigations have been conducted for Lal formation, but a previous study on the structure elucidation of cinnamycin concluded that the alternative configuration of Lal results in high strain energy.[12] The Lan and MeLan residues generated by CinM may preorganize the conformation of the peptide to favor stereoselective formation of Lal, similar to a recent proposal for a lanthionine synthetase that generates a large number of thioether cross-links of high structural diversity.[32]

Genetic studies have identified the *cinorf7* gene to be essential in the biosynthesis pathway of cinnamycin.[29] This gene, composed of just 360 nucleotides, is located immediately upstream of *cinA* and the two genes are cotranscribed.[15] We show here that Cinorf7 is required for lysinoalanine formation. Unfortunately, in vitro reconstitution of its activity has been unsuccessful to date. Sequence analysis of Cinorf7 shows that it does not have significant homology to other functionally characterized proteins. Two other genes encoding proteins with high sequence similarity to Cinorf7 were identified in the protein databases (Figure S9A). One of these, found in the genome of *Lyngbya majuscule*, has two nearby likely precursor peptides, whereas the second, found in the genome of *Frankia Sp.*, has one gene encoding a possible precursor peptide nearby (Figure S9B). Both *cinorf7* orthologues also are near LanM-like genes. Although the

sequences of the predicted leader peptides of these putative precursor peptides vary, their core regions showed very high sequence identity to the cinnamycin group of lantibiotics (Figure S9B), suggesting these gene clusters have the genetic capacity to generate cinnamycin-like peptides.

In summary, we demonstrate here that CinM is responsible for dehydration of two Ser and two Thr residues and also generates the three thioether cross-links in cinnamycin. Furthermore, we show that CinX hydroxylates a single Asp in a 78-residue substrate and that it also accepts just the core peptide as substrate. Finally, our data indicate that Cinorf7 is critical for the formation of the unique cross-linked lysinoalanine. These studies set the stage for the generation of cinnamycin analogues and investigations of the mechanism by which Cinorf7 achieves Lal formation.

■ EXPERIMENTAL SECTION

General. All sources of materials, molecular biology procedures, and protein purification procedures are provided in the Supporting Information.

CinM Dehydration and Cyclization Activities in Vitro. CinM assay components consisted of 50 mM MOPS, pH 7.5, 10 mM $MgCl_2$, 20 μM CinA(A−1K) peptide, 2.5 mM ATP, 5 μM enzyme, and 1 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP) or dithiothreitol (DTT). MALDI-ToF analysis after LysC treatment showed that CinM is responsible for four dehydrations in the core region. However, assays using TCEP and DTT formed side products of +180 or +250 Da for TCEP and +154 Da for DTT (data not shown). To prevent the formation of these adducts, enzyme assays were conducted in the presence of TCEP-immobilized gel (20 μL, Thermo Scientific) in 50 mM MOPS, pH 7.5, 10 mM $MgCl_2$, and 2.5 mM ATP (60 μL final volume) for 6 h while gently rocking the suspension.

Procedure for LysC Cleavage. Enzymatic assay products were separated from the immobilized TCEP gel by centrifugation and dried on a centrivap concentrator. The products were resuspended in 19 μL of 100 mM Tris, pH 8.3, and 2 μL of LysC (0.4 μg/mL in 100 mM Tris, pH 8.3) was added. The cleavage reaction was incubated at 37 °C for 3 h and then desalted using a ZipTip (Millipore). The product was eluted in 5 μL of sinapinic acid and analyzed by MALDI-ToF MS.

CinM Cyclization Activity Assay. After incubation of His_6-CinA(A−1K) with His_{10}-CinM, the assay components were separated from the immobilized TCEP resin by centrifugation, and products were dried in an eppendorf tube on a centrivap concentrator. The assay products were resuspended in 100 mM Tris, pH 8.3, 1 mM TCEP, and 5 mM iodoacetamide and then incubated at 25 °C for 90 min in the dark, desalted using a C18 ZipTip, and subjected to MALDI-ToF MS.

Hydroxylation Activity Assay with CinX. A solution with the final concentrations of 100 mM MOPS pH 7.5, 1 mM α-ketoglutaric acid sodium salt (Sigma), 2 mM of L-(+)-ascorbic acid (Acros), 0.1 mM ferrous sulfate 7-hydrate (Mallinckrodt Chemicals), 20 μM peptide, and 25 μM of CinX was prepared in a final volume of 25 μL and incubated at room temperature for 1 h. The assay was quenched by the addition of TFA to a final concentration of 0.1%, and the assay mixture was desalted using a C18 ZipTip and eluted with 5 μL of sinapinic acid. The samples were then analyzed by MALDI-ToF MS analysis.

Tandem MS Analysis To Determine the Hydroxylation Site. CinA(1−19) was modified by CinX in vitro and treated with 5 mM TCEP to reduce any disulfides that might have formed. The peptide was then analyzed by LC-ESI-MSMS using a Synapt ESI quadrupole ToF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters) (Figure S5).

Coexpression of His₆-CinA(A−1K), CinM, and CinX with and without Cinorf7 in *E. coli*. To coexpress CinA(A−1K), CinM, and CinX, *E. coli* BL21 (DE3) cells were transformed with a pRSFDuet-1 vector containing *cinA(A−1K)* and *cinM* and a pACYCDuet-1 vector containing *cinX*. Similarly, for coexpression of His₆-CinA(A−1K), CinM, and CinX together with Cinorf7, *E. coli* BL21 (DE3) cells were transformed with a pRSFDuet-1 vector containing *cinA(A−1K)* and *cinM* and a pACYCDuet-1 vector containing *cinX* and *cinorf7*. Single colony transformants were grown in a 37 °C shaker for 12−15 h in 50 mL of Luria−Bertani (LB) medium supplemented with 25 µg/mL kanamycin/12.5 µg/mL chloramphenicol. A 20 mL aliquot was centrifuged at 5000g for 10 min, the spent LB medium was discarded, and the cell pellet was resuspended in fresh LB medium. The resuspended cells (20 mL) were added to 2 L of LB supplemented with the appropriate antibiotics, and the culture was grown aerobically at 37 °C until the A_{600} was ∼0.6−0.8. IPTG was added to a final concentration of 0.5 mM, and the culture was transferred to 18 °C for aerobic growth for an additional 20 h. Cells were harvested by centrifugation at 5000g for 20 min at 4 °C. The cell paste (∼3−4 g) was stored at −80 °C until use.

Peptides were purified from *E. coli* BL21 (DE3) cultures as described for other His₆-LanA peptides[50] by use of 5 mL HiTrap chelating HP nickel affinity column (GE Healthcare) followed by HPLC.

Bioactivity Assays with *B. subtilis* LH45. CinM (5 µM) was incubated with 50 mM MOPS, pH 7.5, 10 mM MgCl₂, 20 µM CinA-(A−1K) peptide, 2.5 mM ATP, and 150 µL of immobilized TCEP (600 µL total volume) at 25 °C for 6 h. Enzymatic assay products were separated from the TCEP immobilized resin by centrifugation and dried via on a centrivap concentrator. The CinM-modified peptide was resuspended in CinX assay components, and hydroxylation was carried out for 2 h as described for the CinX assay. The leader peptide was then cleaved with 15 µL of LysC (0.4 µg/mL in 100 mM Tris, pH 8.3) at 37 °C for 12 h. The pH of the solution was carefully increased to pH 9.5 by addition of 5 M NaOH, and the solution was incubated at room temperature for 12 h. A solid-phase extraction column (SPE, 1 mL, Discovery DSC-18) was used to partially purify the peptide from the assay components. The C18 column was first washed with 2 mL of 80% acetonitrile (ACN)/water and equilibrated with 2 mL of water, and then the sample was applied. The column was washed with 4 mL of water, and the sample was eluted with 1 mL 80% ACN/water solution. The sample was dried on a centrivap concentrator, and the peptide was dissolved in 10 µL of 100 mM Tris pH 7.5, analyzed by MALDI-MS, and used for the bioassay.

Similarly, 0.5 mg of modified peptide obtained from the coexpression of CinM and CinX (sample A) and from coexpression of CinM, CinX, and Cinorf7 (sample B) was separately dissolved in 1 mL of 100 mM Tris-HCl pH 8.3 buffer and digested with 50 µL of LysC (0.4 µg/mL in 100 mM Tris, pH 8.3) at 37 °C for 12 h. Next, the pH of sample A was carefully increased to pH 9.5 by addition of 5 M NaOH, and the solution was incubated at room temperature for 12 h for nonenzymatic formation of Lal. Both samples were then further purified by SPE as described above.

B. subtilis LH45 was grown at 37 °C for 15 h in 5 mL of LB supplemented with 0.1% D-(+)-glucose (Calbiochem). LB medium containing 1.5% agar was autoclaved and cooled to 42 °C in a water bath, and 15 mL of this solution was mixed with 375 µL of *B. subtilis* LH45 culture and with a D-(+)-glucose solution to give a 0.1% final glucose concentration. The agar was poured into sterile plates, and shallow wells were generated after the agar solidified. The peptide samples (10 µL in 50 mM Tris buffer) were spotted, and the plates were incubated for 10 h at 25 °C.

ASSOCIATED CONTENT

Ⓢ Supporting Information. Description of all molecular biology procedures, protein purifications, PAR assay, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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