Research Article

Semisynthetic Macrocyclic Lipo-lanthipeptides Display Antimicrobial Activity Against Bacterial Pathogens

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ABSTRACT: A large number of antimicrobial peptides depend on intramolecular disulfide bonds for their biological activity. However, the relative instability of disulfide bonds has limited the potential of some of these peptides to be developed into therapeutics. Conversely, peptides containing intramolecular (methyl)lanthionine-based bonds, lanthipeptides, are highly stable under a broader range of biological and physical conditions. Here, the class-II lanthipeptide synthetase CinM, from the cinnamycin gene cluster, was employed to create methyllanthionine stabilized analogues of disulfide-bond-containing antimicrobial peptides. The resulting analogues were subsequently modified *in vitro* by adding lipid tails of variable lengths through chemical addition. Finally, the created compounds were characterized by MIC tests against several relevant pathogens, killing assays, membrane permeability assays, and hemolysis assays. It was found that CinM could successfully install methyllanthionine bonds at the intended positions of the analogues and that the lipidated macrocyclic core peptides have bactericidal activity against tested Gram-positive and Gram-negative pathogenic bacteria. Additionally, fluorescence microscopy assays revealed that the lipidated compounds disrupt the bacterial membrane and lyse bacterial cells, hinting toward a potential mode of action. Notably, the semisynthesized macrocyclic lipo-lanthipeptides show low hemolytic activity. These results show that the methods developed here extend the toolbox for novel antimicrobial development and might enable the further development of novel compounds with killing activity against relevant pathogenic bacteria.

KEYWORDS: RiPPs, lanthipeptide, cyclic peptide, synthetic biology, antimicrobial, semisynthetic
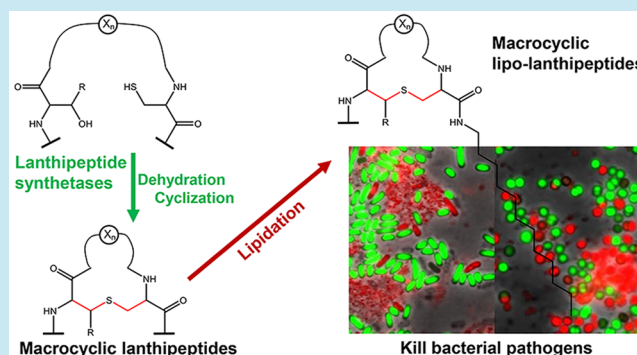
■ INTRODUCTION

Peptide-based drugs have shown extraordinarily high potencies and limited off-target side-effects because of their exquisite target selectivity.[1−3] One class of these peptide-based drugs is represented by lanthipeptides, which are (methyl)lanthionine ring-containing ribosomally synthesized and post-translationally modified peptides (RiPPs).[4,5] A vast number of lanthipeptides show antimicrobial activity against pathogenic bacteria, including known antibiotic-resistant strains.[6−11] Several lanthipeptides, including duramycin, mutacin 1140, NAI-107, and NVB-302,[8−13] have been tested in the clinic. Notably, these all have been demonstrated to have antimicrobial activity *in vivo*.[12−15] Because lanthipeptides are ribosomally synthesized and their modification enzymes have low substrate specificity,[16,17] novel variants can be made by modifying the precursor peptide at the genetic level and by the recombination of different compatible modification enzymes.[18,19] Thus, the lanthipeptide synthetic machinery provides opportunities to engineer a broad range of novel antimicrobial peptides.[16,17,20,21] Cinnamycin, a 19-residue lanthipeptide antibiotic, has a compact globular structure containing three (methyl)lanthionine rings formed by

lanthipeptide synthetase CinM and a lysinoalanine formed by Cinorf7 (Figure 1).[22−24] The capacity of CinM to form the overarching (methyl)lanthionine-based macro-ring makes this enzyme an attractive candidate tool for the engineering of novel macrocyclic lanthipeptides.

In the development of novel antimicrobial RiPPs, an increasingly used approach is the creation of hybrid peptides,[6,7,25] combining modifications from different RiPPs into a single molecule. As these different modification enzymes usually require respective recognition elements, a hybrid leader peptide is often needed. As CinM is a promising candidate for the creation of novel lanthipeptides, its crucial leader elements were identified through alanine scanning. Ala-scanning is a commonly used method for investigating the substrate recognition sites of modification enzymes. A library of mutants
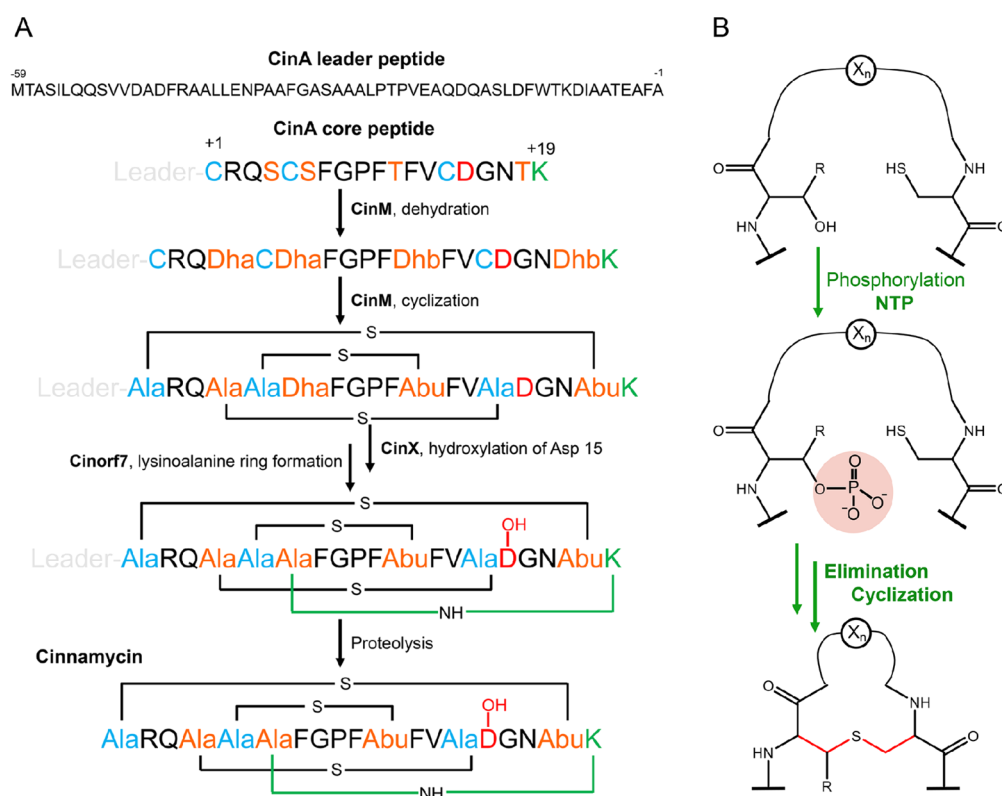
Figure 1. (A) Post-translational maturation of cinnamycin. The sequence of the CinA leader and core peptide is depicted, showing the stepwise installation of its lanthionine cross-links,[24] and other post-translational modifications. Dha, dehydroalanine; Dhb, dehydrobutyrine; Abu, aminobutyric acid. (B) Mechanisms of CinM processing. The mechanism used by CinM for serine and threonine dehydration, utilizing NTP for the activation of the Ser/Thr side chain hydroxyl groups prior to the phosphate elimination.[4]
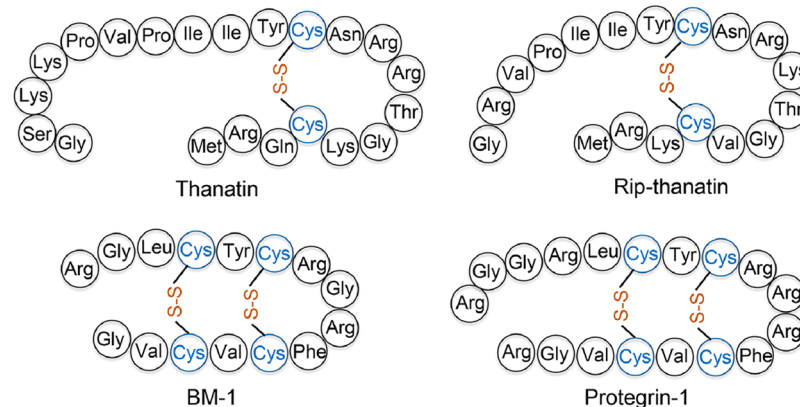

Figure 2. Examples of the structure of a selection of disulfide-bond-containing antimicrobial peptides. While having antimicrobial activity, peptides relying on intramolecular disulfide bonds suffer from stability issues in the presence of intra- and extracellular reducing agents.[33−37]

with "boxes" of residues, respectively, changed to alanines systematically scans the leader while monitoring modification efficiency, allowing for the identification of crucial leader elements.[26−30] For instance, the discovery of the NisB and NisC recognition region FNLD was made using this method.[29,30] The mutagenesis of the FNLD motif was found to significantly reduce the frequency of NisA modification, due to the decreased of binding affinity of NisB and NisC to the NisA leader.[28−32]

Interesting targets for modification by CinM are disulfide-bond-containing peptides, peptides that are stabilized by one or more intramolecular disulfide bonds.[33−37] Large numbers of disulfide-bond-containing peptides have antimicrobial activity,

such as thanatin, rip-thanatin, protegrin-1, and BM-1 (Figure 2).[33−37] In such antimicrobial peptides (AMPs), the disulfide bond plays a vital role in maintaining the molecule's biologically active conformation by reducing the entropic cost of target binding.[38,39] However, the disulfide bond's lability to intra- and extracellular reducing agents can reduce the biological activity of disulfide-containing AMPs and limit their potential for use as therapeutics.[40−42] In our previous study,[43] the nisin synthetic machinery was successfully employed to convert disulfide-containing AMPs into lanthipeptides. In addition, hybrid lanthipeptides were successfully engineered by using the lanthipeptide version of rip-thanatin and nisin (1−20). Interestingly, the hybrid lanthipeptides
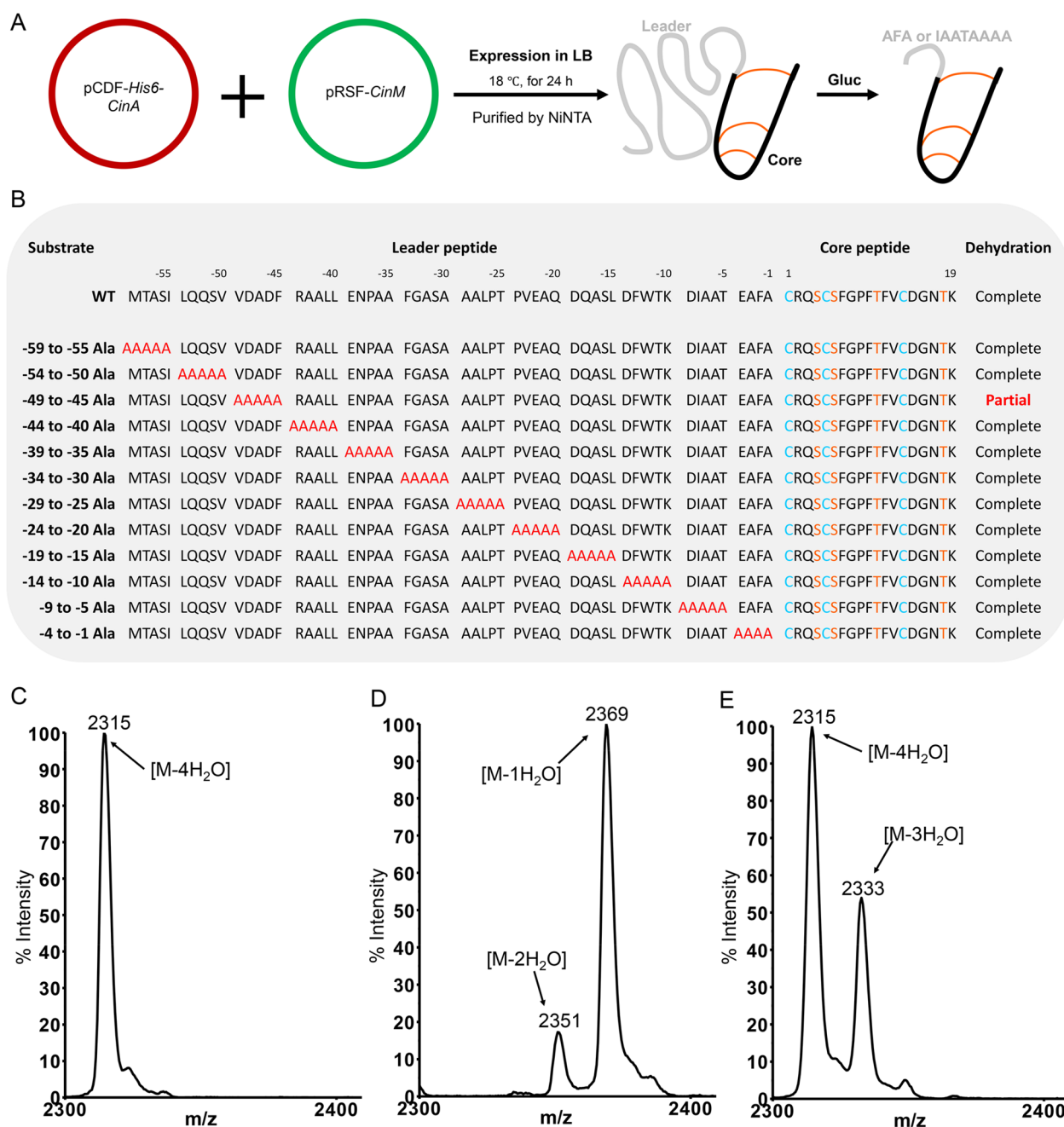
Figure 3. Determination of the CinM Binding Sites on the Substrate. (A) Schematic representation of the heterologous *E. coli* production system for His6-CinA in coexpression with CinM. After treatment with GluC, the cinA core peptide can be obtained, which increases the resolution of MALDI-TOF MS analysis due to its smaller size compared to the full CinA peptide. (B) Overview of all alanine-scanning variants of the His6-CinA leader, tested in the coexpression tests. (C) MALDI-TOF MS of the wild type CinA core peptide. (D) MALDI-TOF MS of the CinA (−49 to −45 Ala) core peptide. (E) MALDI-TOF MS of the CinA (−44 to −40 Ala) core peptide.

showed selective antimicrobial activity against *S. aureus*. Together, these findings suggested that converting disulfide bond-based AMPs into (methyl)lanthionine-based lanthipeptides can provide novel substrates for further modifications.

In this study, a class II lanthipeptide modification system (CinM/CinA) was employed to convert disulfide-bond-containing AMPs into macrocyclic (methyl)lanthionine analogues, and the resulting methyllanthionine analogues were used for the creation of macrocyclic lipo-lanthipeptides, synthesized through subsequent *in vitro* chemical addition. The created semisynthesized macrocyclic lipo-lanthipeptides were investigated on their antimicrobial activity against both Gram-

positive and Gram-negative bacterial pathogens, and some light is shed on the potential mode of action of these compounds. Finally, the hemolytic activity of the semisynthesized macrocyclic lipo-lanthipeptides was investigated.

■ **RESULTS AND DISCUSSION**

Determination of the CinM Binding Sites on the Substrate. To identify regions on the CinA leader peptide that are important for CinM recognition, an alanine-scanning analysis of the CinA leader peptide was performed. First, the CinA and CinM genes were cloned into pCDFDuet-1 and pRSFDuet-1, respectively, to generate pCDF-His6-CinA and

Table 1. Amino Acid Sequence and Dehydrations of Designed Peptides

		mass (Da)		
peptide[a]	amino acid sequence[b]	predicted	measured	dehydrations (observed/predicted)
lantha	GSKKPVPIIY**T**NRRTGKCQRM	**2379.93**	**2416**	1/3
lanrip	GRVPIIYTNRKTGVCKRM	2056.56	2092	0/2
lanbm-1	RGLSYCRGRFTVCVG	1637.98	1674	0/2
lanpro-1	RGGRLSYCRRRFTVCVGR	2106.55	2142	0/2

[a]The disulfide-containing template peptides of lantha, lanrip, lanbm-1, and lanpro-1 are thanatin, rip-thanatin, BM-1, and protegrin-1, respectively.
[b]The amino acid identified to be dehydrated is underlined.

pRSF-CinM (Figure S1). After verifying the plasmids by sequencing, they were used to transform *E. coli* BL21(DE3). After the induction and purification of the CinM-modified CinA, the modified CinA core peptide was obtained by treating CinA with the endoproteinase GluC (Figure 3A), which cleaves behind a Glu/Asp residue. By MALDI-TOF MS, it was verified that the CinA core peptide was fully dehydrated by CinM (Figure 3B,C), which is consistent with a previous study.[24] These results demonstrate full functionality of CinM on its natural substrate CinA in this expression system. Next, 12 plasmids encoding CinA leader mutants were constructed for alanine-scanning (Figure 3B), which were subsequently used to transform *E. coli* BL21(DE3) in combination with pRSF-CinM. After induction, the CinA core peptide of the leader mutants were obtained as previously described. MALDI-TOF MS was used to compare the dehydration rate of the different leader mutants to the wildtype CinA core peptide. The drastically reduced dehydration activity of CinM on the residue −49 to −45 alanine mutants indicates that this region is the most important for CinM recognition. The main product of this mutant was CinA with only one dehydration, with a minor product containing two dehydrations (Figure 3B,D). In addition, the residue −44 to −40 mutant also showed a slightly reduced CinM activity, indicating that this region is also involved in CinM recognition (Figure 3B,E). Finally, the other mutants did not show decreased modification efficiency, and their respective regions are thus assumed to not be directly involved in the recognition by CinM (Figure 3B and Figures S2 and S3). To fully modify CinA into its bioactive form, additional modification is required by the enzymes CinX, and Cinorf7 (Figure 1). It is not unlikely that these enzymes require different recognition sites, which would partially explain the length of the CinA leader. The results shown here confirm that CinM is a leader-dependent class II lanthipeptide dehydration and cyclization enzyme (Figure 1A,B) and show that the CinM recognition site lies within the −49 to −45 amino acid residues of CinA, VDADF (Figure 3B). These findings provide a guideline for the future engineering of macrocyclic lanthipeptide by employing CinM. Particularly, this knowledge should prove useful in the engineering of hybrid leaders,[18,19] allowing for modifications from other RiPP modification enzymes in addition to those of CinM.

Synthesis of Macrocyclic Lanthipeptides using Class II Lanthipeptide Enzyme CinM. To increase the stability of disulfide-bond-containing antimicrobial peptides, we attempted to replace their disulfide bonds with (methyl)lanthionine rings, installed by CinM. Several disulfide-bond-based macrocyclic antimicrobial peptides were chosen as templates, including thanatin, rip-thanatin, BM-1, and protegrin-1 (Figure 2). To allow for the formation of (methyl)lanthionine rings at the position of each disulfide bond of the templates, one of the

cysteines involved in the formation of each disulfide bond was replaced with a threonine or serine (Table 1). For the formation of the desired macro (methyl)lanthionine rings, the introduced threonines/serines should be dehydrated and subsequently cyclized with the corresponding cysteine sulfhydryl by CinM. For CinM recognition of the substrate, the CinA leader peptide was added to the N-terminus of each peptide, with a NisP enzyme cleavage site[44] in between (Figure 4A), for later release of the core peptide. Each of the designed genes was cloned into a pCDFDuet-1-derived plasmid and then used to transform competent *E. coli* BL21(DE3) in combination with pRSF-CinM. After induction and purification, the core peptides were obtained by treating the purified modified peptides with NisP. MALDI-TOF MS was used to determine the mass of the produced core peptides. Among the four designed peptides, a one-dehydration main product was observed for lantha (Figure 4B and Figure S4), which contains an aminobutyric acid at the desired position (Figure 4C,D; position 11; CinM dehydrates the Thr11 resulting in a dehydrobutyrine11 and subsequently forms the aminobutyric acid 11), evidenced by further studies (Figure 4D). Although dehydrated products were also observed in the other three designed peptides, the main products were nondehydrated (Figure S4).

After confirming that lantha was dehydrated by CinM, the next step was to confirm the subsequent cyclization of the dehydrated residues by the enzyme. Thus, to investigate if the macro-ring in lantha was correctly formed, a free cysteine assay was performed using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP). This compound should react with unmodified cysteines in the peptide, which would result in an increase of 25 Da in the peptide's molecular weight.[6,20,45] After the free cysteine assay, no adduct was observed for the CinM-modified lantha (Figure 4B), while the mass of the control, a known free Cys-containing peptide, was starkly shifted with a 25 Da increase (Figure S5). These results indicate that no unmodified Cys was present in the CinM-modified lantha and thus implies that the methyllanthionine ring in lantha was formed with a high efficiency. To further characterize the produced lantha molecule, LC−MS/MS analysis was performed. In the analysis, no fragmentation was observed between Thr11 and Cys18 of thanatin (Figure 4D), confirming the prior evidence for the correct formation of the macro methyllanthionine ring (Figure 4C). These results show that CinM selectively dehydrated the Thr11 of lantha, which indicates that CinM might have a similar substrate favorability as many other LanB and LanM enzymes have.[17,20] In general, Ser/Thr residues have a higher opportunity to be dehydrated when flanked by noncharged residues (YTN, in lantha) rather than by charged residues (GSK or RTG, in lantha).[17,20] The novel engineering system for the production of macrocyclic lanthipeptides constructed in this study can be used in the
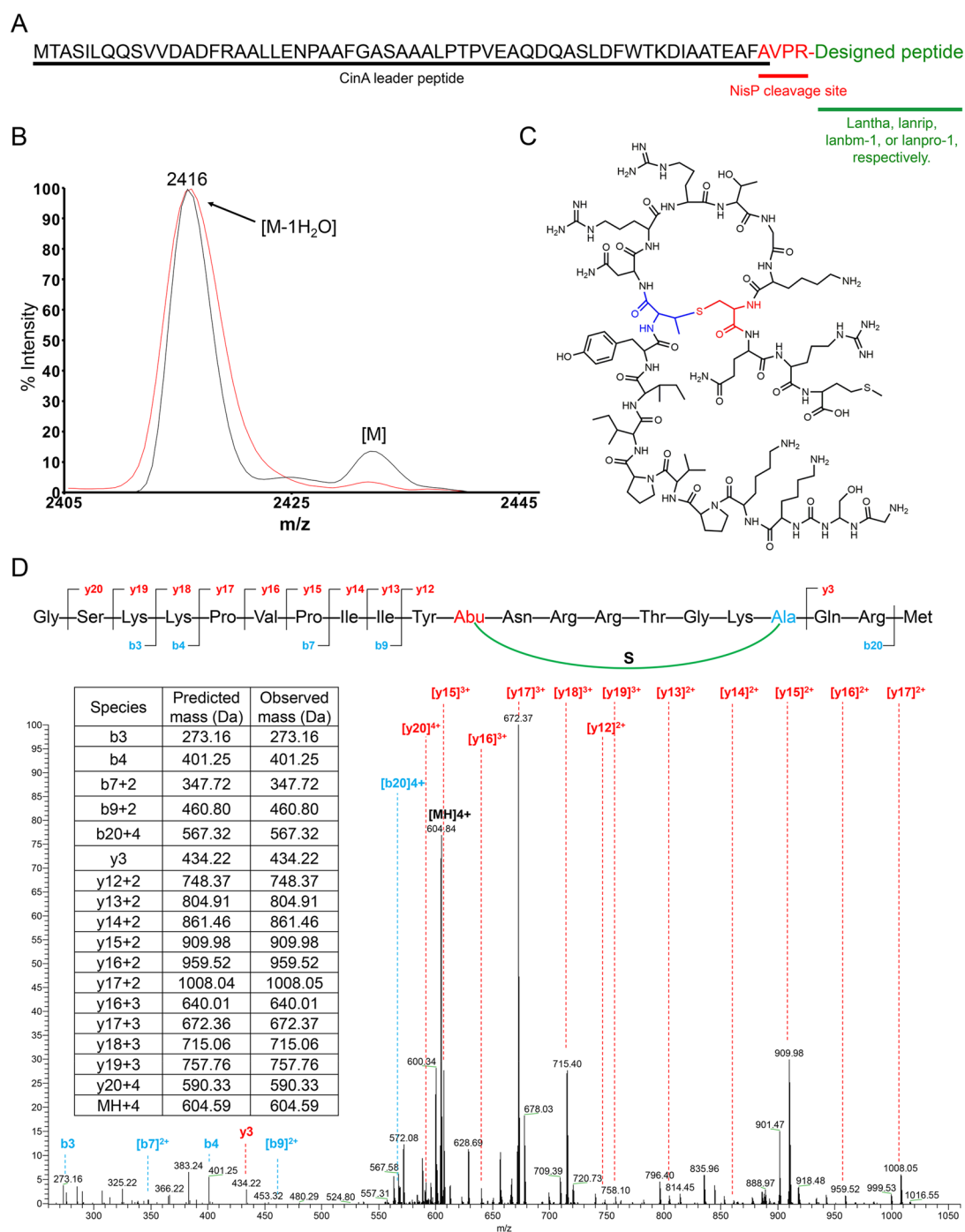
Figure 4. Characteristic of CinM-modified lantha. (A) Approach for the design of precursor peptides with a NisP enzyme cleavage site. The cleavage site was changed from the wild type ASPR to AVPR, as the serine downstream of the wild type leader may be dehydrated, causing problems in further workup and analysis of the core peptide. (B) MALDI-TOF MS of lantha before (black) and after (red) CDAP treatment. As no shift can be observed after CDAP treatment, all cysteines are likely involved in lanthionine ring formation. (C) Probable structure of lanthionine-containing lantha, as evidenced by CDAP reaction and LC–MS/MS analysis. (D) LC–MS/MS spectrum and proposed structure of lanthionine-containing lantha. Fragment ions are indicated. Abu: aminobutyric acid.

future for the production of novel antimicrobial compounds with a macro-ring with a higher stability compared to those with a disulfide bond. RiPPs have been shown to cover a variety of different bioactivities, *i.e.*, antibacterial, antitumor, and antiviral,[4,46−48] and an increasing number of engineered new-to-nature RiPP therapeutics is being reported.[4,6,21,25,48] The approach described here, where non-RiPP peptides are produced as RiPPs, combined with the identification of the

CinM leader recognition site described earlier in this study, could be an interesting approach to the developments of new antimicrobials by engineering hybrid peptides. The post-translational modification used here could, for example, be combined with those from other lanthipeptide systems. Lanthipeptides form one of the largest classes of RiPPs,[4,49] and their synthetic machineries are one the most extensively studied among RiPPs. Additionally, a vast number of new-to-
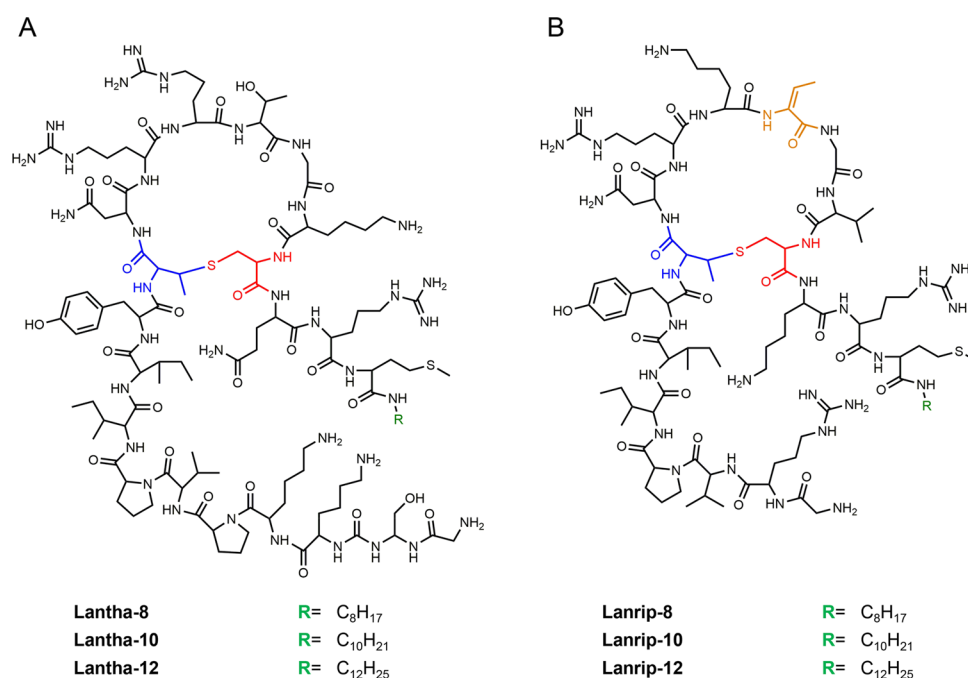
Figure 5. Structures of the lipidated lantha and lanrip analogues generated in this study.

Table 2. Antimicrobial Activity of Lanthas and Lanrips against Pathogenic Bacteria

organism and type[a]	MIC (μM)[b]									
	lantha-8	lantha-10	lantha-12	lanrip-8	lanrip-10	lanrip-12	lantha	lanrip	nisin	polyB
Gram-positive pathogens										
Staphylococcus aureus ATCC15975 (MRSA)	>32	8	8	>32	8	4	>256	>256	2	32
Enterococcus faecium LMG16003 (VRE)	>32	16	8	>32	16	4	>256	>256	2	16
Gram-negative pathogens										
Acinetobacter baumannii ATCC19606	16	4	4	8	2	2	>256	>256	4	1
Shigella flexneri ATCC29903	8	4	2	8	2	2	>256	>256	8	0.5
Escherichia coli ATCC25922	>32	32	8	>32	16	4	>256	>256	16	1
Klebsiella pneumoniae ATCC700603	>32	32	16	>32	16	8	>256	>256	64	2
Pseudomonas aeruginosa ATCC27853	>32	32	8	>32	16	8	>256	>256	64	1

[a]VRE, vancomycin-resistant enterococci; MRSA, oxacillin−methicillin-resistant *Staphylococcus aureus*. [b]The MIC was determined by broth microdilution. Nisin and polymyxin B were used as antibiotic controls. polyB, polymyxin B.

nature lanthipeptides with potential therapeutic activity have already been engineered by employing the synthetic machineries of various lanthipeptide systems.[6,21,48,50−56] Hence, by combining the vast knowledge of lanthipeptides that is already available with the knowledge acquired here, interesting new compounds can be conceived. In this study however, an additional line of novel molecule synthesis was pursued, namely, the production of semisynthetic lipo-lanthipeptides from the here-produced lanthipeptide lantha analogue.
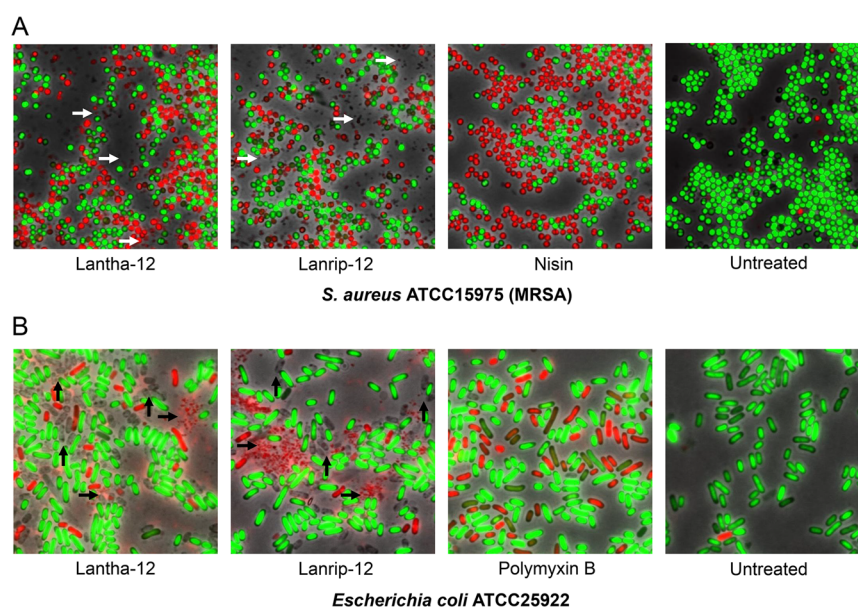
Semisynthetic Macrocyclic Lipo-lanthipeptides. After the successful production of the lanthipeptide lantha analogue, the peptide was subjected to an *in vitro* chemical lipidation process, following a previously reported method.[44] Concurrently, another lanthipeptide, lanrip, was subjected to the same protocol. The lanrip (originally called "ripcin") used in this study was purified from *Lactococcus lactis* NZ9000 (pIL3-BTC and pRipcin) as described previously[43] (Figures S7 and S8). The lanthipeptides lantha and lanrip were readily converted into the amide-coupled lipidated variants lantha-8, lantha-10, lantha-12, lanrip-8, lanrip-10, and lanrip-12 (Figure S). The coupling was done with a large excess of the selected

lipid-amine in the presence of BOP and DIPEA for 8 h, followed by HPLC purification of the desired products. Finally, MALDI-TOF MS was used to verify the obtained products. The correct mass was observed for all of the designed macrocyclic lipo-lanthipeptides (Figure S9), which shows that all the designed macrocyclic lipo-lanthipeptides were successfully synthesized and purified.

Synthesized Macrocyclic Lipo-lanthipeptides Show Antimicrobial Activity Against Human Bacterial Pathogens. To assess the antimicrobial activity of the lipidated lanthipeptide lantha and lanrip variants, minimum inhibitory concentration (MIC) assays were performed according to the standard guidelines.[57] Nisin and polymyxin B were used as standard antimicrobial activity controls. The results show that both the nonlipidated lantha and lanrip peptides lacked antimicrobial activity against all tested pathogenic bacteria (Table 2), which may be caused by the presence of the relatively shorter thioether cross-link and a likely changed conformation. Lantha-8 and lanrip-8, however, showed substantial antimicrobial activity against *Acinetobacter baumannii* ATCC19606 and *Shigella flexneri* ATCC29903 (Table 2).
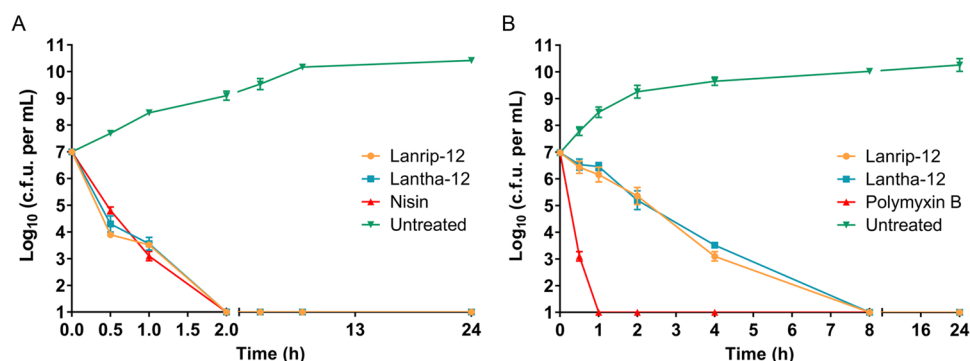
Figure 6. Lantha-12 and lanrip-12 act as bactericidal antibiotics against both Gram-positive and Gram-negative pathogens. (A) Time-killing assay of lantha-12 and lanrip-12 against *S. aureus* (MRSA). Lanrip-12 and lantha-12 kill *S. aureus* at the same rate as the control bactericidal compound nisin. (B) Time-killing assay of lantha-12 and lanrip-12 against *E. coli*. The tested analogues take much longer to kill *E. coli* than they do to kill *S. aureus*.

Figure 7. Synthesized macrocyclic lipo-lanthipeptides disrupt the cellular membrane. (A) Fluorescence microscopy images of *S. aureus* ATCC15975 (MRSA), challenged with both lipidated analogues and their respective controls at a concentration of 2 × MIC for 5 min. White right arrows (→) denote cell lysis fragments. (B) Fluorescence microscopy images of *E. coli*, challenged with both lipidated analogues and their respective controls at a concentration of 2 × MIC for 5 min. Black right arrows (→) denote cell lysis fragments, while black up arrows (↑) denote cells that lost both dyes (lysed cells). Green denotes a cell with an intact membrane, whereas red denotes a cell with a compromised membrane.

Surpassing this, lantha-10, lantha-12, lanrip-10, and lanrip-12 showed good antimicrobial activity to all tested bacterial pathogens (Table 2), with the activity of lantha-12 and lanrip-12 being the highest (Table 2). These results are consistent with those of a previous study, which showed that nisin-derived lipopeptides with a 10 or 14 hydrocarbon chain tail have a higher antimicrobial activity than shorter hydrocarbon chain-tail-containing products.[44] A previous study reported that nisin-derived lipopeptides showed antimicrobial activity against Gram-positive bacteria, but these lipopeptides lacked antimicrobial against Gram-negative bacteria.[44] Interestingly, the semisynthesized macrocyclic lipo-lanthipeptides produced here have antimicrobial activity against both Gram-positive and Gram-negative bacterial pathogens, including difficult to treat vancomycin-resistant *Enterococcus faecium* and oxacillin–methicillin-resistant *Staphylococcus aureus*. The group of cyclic lipopeptides forms a valuable source of antimicrobials, such as polymyxin B, enduracidin, and daptomycin,[58,59] and some recently discovered antimicrobials, including brevicidines and

relacidines.[60−62] A disadvantage of some of these peptides is a relatively high toxicity. Therefore, the here-described semisynthesis approach may offer great opportunities for developing lipo-lanthipeptides as antimicrobial candidates, enabling to engineer them further to reduce possible toxicity. Additionally, where possible, the ribosomal nature of this synthesis pathway allows for minor or major changes to be made on the genetic level to similar effects. The semisynthesis of (methyl)-lanthionine-stabilized macrocyclic lipo-lanthipeptides is thus a promising strategy for the development of novel antimicrobial candidates.

Synthesized Macrocyclic Lipo-lanthipeptides Act as Bactericidal Antimicrobials. To investigate whether lantha-12 and lanrip-12 act as either bacteriostatic or bactericidal agents, time-killing assays were performed on *S. aureus* ATCC15975 (MRSA) and *Escherichia coli* ATCC25922. The strains were inoculated in MHB and grown until the OD_{600} of the cell cultures reached 0.8. The cultures were then diluted to a concentration of $1 × 10^7$ CFUs per mL and challenged with

lantha-12 and lanrip-12 at a concentration of 10 × MIC. Nisin was used as a bactericidal antibiotic control against the Gram-positive *S. aureus,* whereas polymyxin B was used as the control for the Gram-negative *E. coli.*[63,64] Lantha-12 and lanrip-12 showed comparable killing capacities with the bactericidal antibiotic nisin against *S. aureus* (MRSA) (Figure 6A), demonstrating that lantha-12 and lanrip-12 act as bactericidal antibiotics against Gram-positive pathogens. Polymyxin B showed a faster killing capacity on *E. coli* ATCC25922 cells than lantha-12 and lanrip-12, killing all bacteria in 1 h (Figure 6B), compared to the 8 h of lantha-12 and lanrip-12 (Figure 6B). These results demonstrate that lantha-12 and lanrip-12 act as bactericidal antimicrobials against both Gram-positive and Gram-negative pathogens.

Synthesized Macrocyclic Lipo-lanthipeptides Disrupt the Bacterial Membrane and Lyse Bacteria. To assess the influence of lantha-12 and lanrip-12 on the bacterial membrane, fluorescence microscopy assays were performed using a commercial LIVE/DEAD Baclight Bacterial Viability Kit, which contains SYTO9 and propidium iodide. Cells with an intact membrane will stain green, whereas cells with a compromised membrane will stain red. After treatment with antibiotics at a concentration of 2 × MIC for 5 min, the cells were monitored by fluorescence microscopy. Green cells were observed for both untreated *S. aureus* (MRSA) and untreated *E. coli* (Figure 7A,B). The results show that both lantha-12 and lanrip-12 disrupted the membrane of *S. aureus* (MRSA) (Figure 7A). In addition, lantha-12 and lanrip-12 caused cell lysis on *S. aureus* (MRSA) (Figure 7A), indicating that they employ a different mode of action than the pore-forming antibiotic nisin. After lantha-12 and lanrip-12 treatment, gray *E. coli* cells, which were not stained with either dye, and red (nucleic-acid-containing) cell lysis fragments were observed, indicating lantha-12 and lanrip-12 caused the cell lysis of *E. coli* (Figure 7B). These results indicate that the synthesized macrocyclic lipo-lanthipeptides may exert their bactericidal activity by disrupting the cellular membrane and lysing bacterial cells (Figures 6A,B and 7A,B). However, previous studies reported that one of the template peptides of the synthesized macrocyclic lipo-lanthipeptides, thanatin, exerts its antimicrobial activity against Gram-negative bacteria pathogens by disrupting the bacterial outer membrane, targeting the intermembrane protein complex required for lipopolysaccharide transport and inactivating the NDM-1 metallo-β-lactamase.[65,66] Our results suggest that the synthesized macrocyclic lipo-lanthipeptides may employ a different mode of action than their mother peptides. The results presented in this study provide a partial understanding of the mode of action of the semisynthesized macrocyclic lipo-lanthipeptides, which in turn should aid the development of novel antimicrobial peptides in combination with the methods presented earlier in this paper.

Synthesized Macrocyclic Lipo-lanthipeptides Show Low Hemolytic Activity. As lantha-12 and lanrip-12 showed membrane disruption and bacteria lysis activity, a hemolytic activity assay was performed to assess in an initial test of their safety. Human blood cells were incubated in the presence of lantha, lantha-12, lanrip, or lanrip-12 concentrations ranging from 2 to 128 μM. After incubation at 37 °C for 1 h, the OD_{450} of the supernatants was measured, and the hemolytic activities of lantha, lantha-12, lanrip, and lanrip-12 were calculated as described in previous studies.[43,60,67] Lantha and lanrip showed no hemolytic activity at a high concentration of 128 μM

(Figure 8). In addition, lantha-12 and lanrip-12 showed very low hemolytic activity; lantha-12 and lanrip-12 only induced
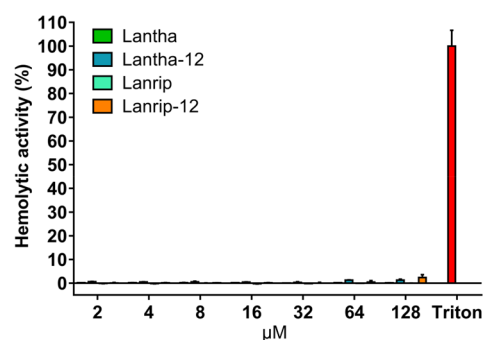
Figure 8. Synthesized macrocyclic lipo-lanthipeptides show low hemolytic activity. Human erythrocytes were incubated with compounds at concentrations ranging from 2 to 128 μM. Their hemolytic activity was assessed by the release of hemoglobin. Cells treated without a tested compound were used as no lysis control. Cells treated with 10% Triton X-114 were used as complete lysis control. The data are representative of three independent experiments.

1.3 ± 0.3 and 2.4 ± 0.9% of cell lysis, respectively, at a high concentration of 128 μM (Figure 8). These results indicate that thanatin- and rip-thanatin-derived macrocyclic lipo-lanthipeptides have a lower hemolytic activity than former reported nisin-derived lipo-lanthipeptides,[44] which makes the strategy described in this study more attractive. Together with their potential mode of action, these results suggest that lantha-12 and lanrip-12 may exert their antimicrobial activity *via* targeting bacterial-specific element(s) and lysing bacteria.

■ CONCLUSIONS

In this study, the disulfide-bond-containing antimicrobial peptide thanatin was successfully used as a template for the synthesis of macrocyclic lanthipeptide analogues, replacing its respective disulfide bond with a methyllanthionine ring employing the lanthipeptide synthetase CinM. These results show that CinM can be used for the creation of macrocyclic lanthipeptides of this nature. Furthermore, the alanine-scanning performed on the CinA leader region showed residues −49 to −45 VDADF (Figure 3B) to be crucial for recognition by CinM. This combined knowledge should prove useful in the creation of macrocyclic lanthipeptides with modification by other RiPP systems, as it is crucial for the design of hybrid leader peptides. The resulting macrocyclic analogues were subsequently lipidated through the chemical addition of a C-terminal hydrocarbon tail of lengths C-8, -10, or -12. MIC tests showed that all lipidated compounds were active against pathogenic bacteria, with activity increasing with tail length. Killing assays were performed with the two most active compounds, lantha-12 and lanrip-12, against *S. aureus* ATCC15975 and *E. coli* ATCC25922. These assays showed that both compounds were active against clinically relevant Gram-positive and Gram-negative strains. Additional assays showed that these compounds both disrupt the bacterial membrane and lyse bacterial cells, hinting toward the potential mode of action. Notably, lantha-12 and lanrip-12 showed low hemolytic activity against human erythrocytes. Taken together, this study provides a novel lanthipeptide engineering strategy, which can be used to engineer macrocyclic (lipo)-

lanthipeptides for the development of new classes of antimicrobials.

MATERIALS AND METHODS

Microbial Strains Used and Growth Conditions. Strains and plasmids used in this study are listed in Tables S1 and S2. *E. coli* TOP10 chemical competent cells were used as hosts in the construction of all plasmids. *E. coli* BL21(DE3) chemical competent cells were transformed with the verified plasmids and used for the subsequent expression of the plasmids encoding proteins. For plasmid selection, *E. coli* stains were grown in LB medium or on LB medium solidified with 1% (wt/vol) agar at 37 °C, when necessary, supplemented with 100 μg/mL spectinomycin and/or 20 μg/mL kanamycin for selection purposes. For protein expression, stationary-phase cultures, which were grown in LB, were inoculated (50-fold diluted) on LB and induced with IPTG (0.5 mM) at OD_{600}) = 0.6. All indicator strains were inoculated on LB and incubated at 37 °C with shaking at 220 rpm for preparing the overnight cultures.

Molecular Biology Techniques. Oligonucleotide primers used for cloning and sequencing in this study are listed in Tables S3, S4, and S5, and all the oligonucleotide primers and oligonucleotide inserts were purchased from Biolegio B.V. (Nijmegen, The Netherlands). CinM and CinA genes were inserted into pRSFDuet-1 and pCDFDuet-1, respectively, using GeneArt Gibson Assembly HiFi Cloning Kit (Thermo Fisher Scientific, Waltham, MA, A46624). Constructs coding for the designed peptides were made by amplifying template plasmid using downstream sense- and upstream antisense primers with a peptide-encoding tail and ca. 15 bp overlap on the 5′. DNA amplification was carried out using Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA). The designed plasmids were verified by sequencing at Macrogen Europe B.V.

Expression and Purification of His6-Tagged Peptides. *E. coli* BL21(DE3) cells containing pRSF-CinM for the expression of the CinM enzyme were transformed with the His6-peptide encoding plasmids (30 ng), plated on LB agar plates containing 100 μg/mL spectinomycin and 20 μg/mL kanamycin, and grown at 37 °C for 18 h with shaking at 220 rpm. A single colony of each of these plates was used to inoculate 20 mL of LB supplemented with 100 μg/mL spectinomycin and 20 μg/mL kanamycin and grown for 16−18 h at 37 °C. After that, the culture was used to inoculate 1 L (50-fold dilution) of LB supplemented with 100 μg/mL spectinomycin and 20 μg/mL kanamycin. Cultures were grown at 37 °C to an OD_{600} of 0.6. The cultures were chilled in ice water for 10 min, after which peptide expression was induced by the addition of IPTG to a final concentration of 0.5 mM, and the cultures were grown at 18 °C for 24 h with shaking at 220 rpm. After that, the cultures were centrifuged at 5000g for 10 min, and the cell pellets were collected. The pellets were resuspended in lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, and 0.5% Triton X-100, pH 8.5), and the suspension was sonicated for 30 min in total. The insoluble material was subsequently removed by centrifugation at 10 000g for 30 min, and supernatants were filtered through a 0.45 μm membrane. The supernatants were applied to Ni-NTA agarose columns (Qiagen) equilibrated with 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0. The flow-through was discarded, and the column was subsequently washed with 12 CV of wash buffer (50 mM NaH_2PO_4, 300

mM NaCl, and 20 mM imidazole, pH 8.0). The peptides were eluted with 8 CV elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 500 mM imidazole, pH 8.0). The obtained His-tag elution samples were desalted by using SIGMA-Aldrich C18 silica gel and subsequently lyophilized. For the analysis of the peptides' modification rate, the samples were treated with Gluc or nisP and thereafter desalted by C-18 ZipTip (Millipore) and analyzed by MALDI-TOF MS. For the purification of modified lantha, after treatment with nisP and filtration through a 0.2 μm membrane, lantha was purified on an Agilent 1260 Infinity HPLC system with a Phenomenex Aeris C18 column (250 mm × 4.6 mm, 3.6 μm particle size, 100 Å pore size). Acetonitrile was used as the mobile phase, and a gradient of 15−25% aq. MeCN over 40 min at 1 mL/min was used for separation. Lantha was eluted at 20−22% MeCN.

Mass Spectrometry. For MALDI-TOF analysis, a 0.5 μL sample (lyophilized sample dissolved in Milli-Q water) was spotted and dried on the target. Subsequently, 0.5 μL of matrix solution (5 mg/mL α-cyano-4-hydroxycinnamic acid from Sigma-Aldrich dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid) was spotted on top of the sample. Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer analysis was performed using a 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems) in the linear-positive mode.

Evaluation of Methyllanthionine Formation. After dissolving the freeze-dried samples in 18 μL of 0.5 M HCl (pH = 3), the samples were treated with 2 μL of 100 mg/mL tris[2-carboxyethyl]phosphine in 0.5 M HCl (pH = 3) for 30 min at room temperature. Subsequently, 4 μL of 100 mg/mL 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) in 0.5 M HCl (pH = 3) was added to the samples. After incubation at room temperature for 2 h, the samples were desalted by C-18 ZipTip (Millipore) and analyzed by MALDI-TOF MS.[20,68]

LC−MS/MS Analysis. To get deep insight into the lanthionine bridging pattern, an LC−MS/MS assay was performed. LC−MS was performed using a Q-Exactive mass spectrometer fitted with an Ultimate 3000 UPLC, an ACQUITY BEH C18 column (2.1 mm × 50 mm, 1.7 μm particle size, 200 Å; Waters), a HESI ion source and an Orbitrap detector. A gradient of 5−90% MeCN with 0.1% formic acid (v/v) at a flow rate of 0.35 mL/min over 60 min was used. MS/MS was performed in a separate run in PRM mode, selecting double, triple, and quadruple charged ions of the compound of interest.

Amide Coupled Lipid-lantha/lanrip. Lantha/lanrip was dissolved in DMF (1 mL), and the corresponding lipid-amine (50 equiv), BOP (2 equiv), and DiPEA (4 equiv) were added. The reaction was stirred for 8 h and subsequently quenched with 4 mL of 5% MeCN + 0.1% TFA. The solution was filtered through a 0.2 μm membrane to remove any insoluble material, and the synthesized macrocyclic lipopeptides were purified *via* HPLC. The final products were obtained through lyophilization.

Minimum Inhibitory Concentration (MIC) Assay. MIC values were determined by broth microdilution, according to the standard guidelines.[57] In short, tests were performed in cation-adjusted Mueller−Hinton broth (MHB). The starting concentration was adjusted to approximately 5×10^5 CFUs per mL. After 20 h of incubation at 37 °C, the MIC was defined as the lowest concentration of antibiotic with no visible growth. Each experiment was performed in triplicate.

Time-Killing Assay. This assay was performed according to a previously described procedure.[67] An overnight culture of either *Staphylococcus aureus* ATCC15975 (MRSA) or *Escherichia coli* ATCC25922 was diluted 50-fold in MHB and incubated at 37 °C with aeration at 220 rpm. Both strains were grown to an OD_{600} of 0.8, after which the cell concentration was adjusted to $\approx 1 \times 10^7$ cells per mL. Each strain was then challenged with $10 \times$ MIC antimicrobials in glass culture tubes at 37 °C and 220 rpm. Part of the culture was left untreated, functioning as negative controls. Aliquots (200 μL) were taken at desired time points, centrifuged at 6800g for 2 min, and resuspended in 200 μL of MHB. Ten-fold serially diluted samples were plated on MHA plates. After incubation at 37 °C overnight, colonies were counted and the CFUs per mL were calculated. Each experiment was performed in triplicate.

Fluorescence Microscopy Assay. *Staphylococcus aureus* ATCC15975 (MRSA) or *Escherichia coli* ATCC25922 was grown to an OD_{600} of 0.8. The cultures were pelleted at 5000g for 8 min and washed three times in MHB. After normalization of the cell density to an OD_{600} of 0.2 in MHB, a 2-fold MIC value concentration of each of the tested antibiotics was added to the cell suspension simultaneously with SYTO 9 and propidium iodide (LIVE/DEAD Baclight Bacterial Viability Kit, Invitrogen). After incubation at room temperature for 5 min, the tested compounds were removed by washing the cells three times with MHB. Subsequently, the cell suspensions were loaded on 1.5% agarose pads and analyzed by a DeltaVision Elite microscope (Applied Precision).

Hemolytic Activity. This assay was performed as described in previous studies.[43,60,67] In short, erythrocytes were isolated from a healthy human volunteer donor (Sanquin, The Netherlands) and washed with PBS three times. Subsequently, erythrocytes [2% (v/v) resuspended in PBS] were exposed to the tested compounds at final concentrations of 128, 64, 32, 16, 8, 4, 2, and 0 μM. After incubation at 37 °C for 1 h, the samples were centrifuged for 8 min at 8000g. The supernatant was transferred to a 96-well plate, and the absorbance was measured at a wavelength of 450 nm with a Thermo Scientific Varioskan LUX multimode microplate reader. The absorbance relative to the positive control, which was treated with 10% Triton X-114, was defined as the percentage of hemolysis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00161.

Figures of plasmid maps, MALDI-TOF MS data, LC−MS/MS spectrum, and structure image and tables of strains, plasmids, and primers used (PDF)

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Author Contributions

O.P.K. and X.Z. conceived the project and strategy. O.P.K. supervised and corrected the manuscript. X.Z. designed and carried out the experiments, analyzed data, and wrote the manuscript. Y.X. did experimental work on plasmid construction, peptide purification, and MALDI-TOF MS analysis. J.H.V. did experimental work on plasmid construction and manuscript revision work. All authors contributed to and commented on the manuscript text and approved its final version.

Notes

The authors declare no competing financial interest.

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