Nisin- and Ripcin-Derived Hybrid Lanthipeptides Display Selective Antimicrobial Activity against *Staphylococcus aureus*

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ABSTRACT: Lanthipeptides are (methyl)lanthionine ring-containing ribosomally synthesized and post-translationally modified peptides (RiPPs). Many lanthipeptides show strong antimicrobial activity against bacterial pathogens, including antibiotic-resistant bacterial pathogens. The group of disulfide-bond-containing antimicrobial peptides (AMPs) is well-known in nature and forms a rich source of templates for the production of novel peptides with corresponding (methyl)lanthionine analogues instead of disulfides. Here, we show that novel macrocyclic lanthipeptides (termed thanacin and ripcin) can be synthesized using the known antimicrobials thanatin and rip-thanatin as templates. Notably, the synthesized nisin(1−20)−ripcin hybrid lanthipeptides (ripcin B−G) showed selective antimicrobial activity against *S. aureus*, including an antibiotic-resistant MRSA strain. Interestingly, ripcin B−G, which are hybrid peptides of nisin(1−20) and ripcin that are each inactive against Gram-negative pathogens, showed substantial antimicrobial activity against the tested Gram-negative pathogens. Moreover, ripcin B−G was highly resistant against the nisin resistance protein (NSR; a peptidase that removes the C-terminal 6 amino acids of nisin and strongly reduces its antimicrobial activity), opposed to nisin itself. This study provides an example of converting disulfide-bond-based AMPs into (methyl)lanthionine-based macrocyclic hybrid lanthipeptides and can yield antimicrobial peptides with selective antimicrobial activity against *S. aureus*.

KEYWORDS: lanthipeptide, nisin, antimicrobial activity, Lactococcus, Staphylococcus, thanatin
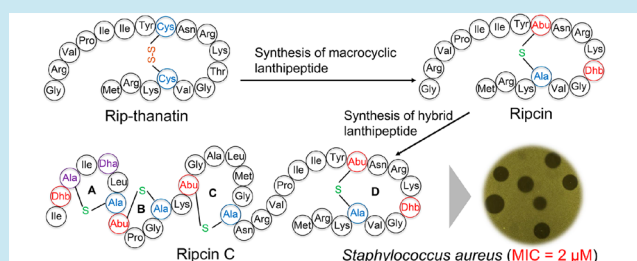
INTRODUCTION

Lanthipeptides are (methyl)lanthionine ring-containing ribosomally synthesized and post-translationally modified peptides (RiPPs).[1] Many lanthipeptides show potent antimicrobial activity against pathogens and/or even against antibiotic-resistant pathogens.[2−7] Notably, several lanthipeptides, including duramycin, NVB-302, mutacin 1140, and NAI-107,[3−6,8,9] have been tested in the clinic. These all have been demonstrated to display potent antimicrobial activity *in vivo*.[8−11] The ribosomal synthesis and low substrate specificity of some of the lanthipeptide modification enzymes provide an opportunity to engineer large numbers of novel antimicrobials.[12]

Nisin, the best-studied lantibiotic, is a 34 amino acid (or 29 amino acids, if one considers a (methyl)lanthionine as a single amino acid) cationic lanthipeptide produced by various *Lactococcus lactis* strains. Because of its potent antimicrobial activity and safety, it has been used as a food preservative for many years. The N-terminal A/B-rings of nisin form a "pyrophosphate cage" that physically interacts with the pyrophosphate of lipid II, resulting in the formation of nisin−lipid II hybrid pores in the target membrane and inhibition of cell wall synthesis *via* lipid II abduction.[13,14] Three essential post-translational modifications of the

ribosomally synthesized nisin precursor take place to yield active nisin (Figure 1).[12,15,16] First, NisB catalyzes the dehydration of Ser and Thr residues in the precursor core peptide to dehydroalanine and dehydrobutyrine, respectively. Subsequently, NisC catalyzes the intramolecular (methyl)-lanthionine ring formation of dehydroalanine/dehydrobutyrine with Cys residues. Finally, NisP cleaves the nisin leader peptide, releasing the mature core peptide. These modification enzymes constitute the nisin biosynthetic machinery, which has been widely applied to engineer lanthipeptide drug candidates.[2,17−22]

Thanatin (Figure 2A), a 21-residue inducible defense peptide from the hemipteran insect *Podisus maculiventris*, was first reported to exert potent antimicrobial activity against bacteria and fungi in 1996.[23] Later studies found that thanatin also has good antimicrobial activity against Gram-negative bacterial pathogens,[24,25] and mode of action studies show that
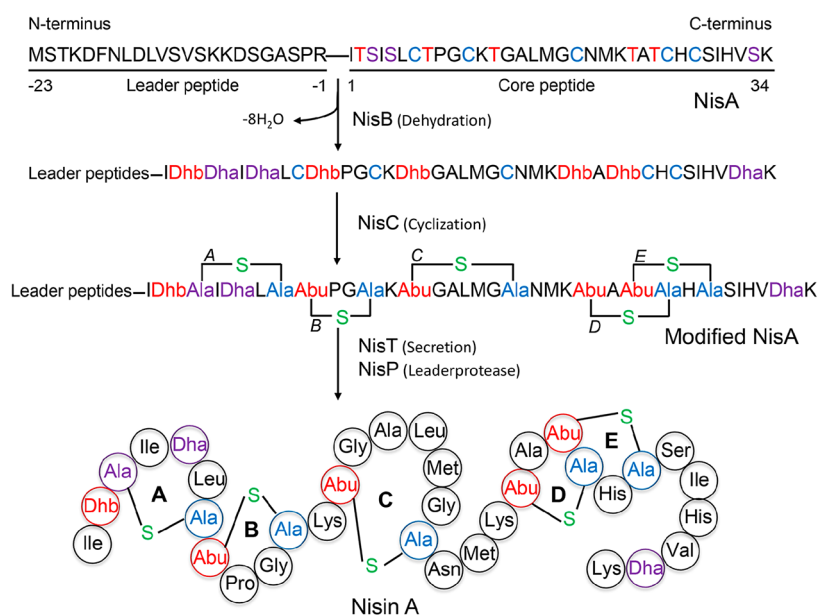
Figure 1. Schematic representation of the biosynthetic route of the model lantibiotic nisin. Dha, dehydroalanine; Dhb, dehydrobutyrine; Abu, aminobutyric acid.

the antimicrobial activity against Gram-negative bacteria pathogens of thanatin is related to targeting the intermembrane protein complex required for lipopolysaccharide transport.[26] Rip-thanatin (Figure 2B), an 18-residue insect defense peptide from *Riptortus pedestris*, has also been reported to exert antimicrobial activity against Gram-negative bacteria.[27] Both rip-thanatin and thanatin are intramolecular disulfide-bond-containing antimicrobial peptides (AMPs). A vast number of such disulfide-bond-containing AMPs are known in nature,[28−30] which form a rich source of templates for producing hybrid peptides with the corresponding (methyl)lanthionine analogues.

In this study, we describe a strategy in which thanatin and rip-thanatin were used as templates for producing the corresponding (methyl)lanthionine analogues. To this end, the nisin synthetic machinery was used for producing methyllanthionine-stabilized thanatin and rip-thanatin analogues (termed thanacin and ripcin, Figure 2C,D). Methyllanthionine-stabilized peptide macrocycles were successfully introduced into thanacin and ripcin, corroborated by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and liquid chromatography tandem mass spectrometry (LC−MS/MS) analysis. However, the first generation of peptides showed insufficient antimicrobial activity against the pathogens tested and showed only antimicrobial activity against *Micrococcus flavus* [minimal inhibitory concentration (MIC), 4 μM] among the bacterial strains tested. Subsequently, either ripcin or a part of ripcin was genetically fused to the C-terminal end of nisin(1−20) to generate the second generation of six macrocyclic peptides that we called ripcin B−G. Ripcin B−G showed a stronger antimicrobial activity than either nisin(1−20) or ripcin alone against the Gram-positive pathogens tested. Notably, ripcin B−G showed selective antimicrobial activity against *S. aureus*, including an antibiotic-resistant MRSA strain. Interestingly, the fusion of two inactive peptides, nisin(1−20) and ripcin (or part of ripcin), also yielded active lanthipeptides against Gram-negative bacterial pathogens. Ripcin C showed the highest

antimicrobial activity against the tested Gram-negative and Gram-positive pathogens among all designed peptides. Moreover, ripcin B−G was not sensitive to the nisin resistance protein (NSR; a peptidase that removes the C-terminal 6 amino acids of nisin and strongly reduces its antimicrobial activity), opposed to nisin itself. Finally, we give information on the potential mechanism of action of ripcin C against Gram-negative and Gram-positive pathogens. This study provides an example of converting disulfide-bond-based AMPs into hybrid (methyl)lanthionine-based macrocyclic lanthipeptides, and some candidates with selective antimicrobial activity against *S. aureus* (MRSA) were obtained.

■ RESULTS AND DISCUSSION

Synthesis of Macrocyclic Lanthipeptides by Using Thanatin and Rip-thanatin as Templates. Considering that Thr has a higher potential of being dehydrated by the dehydratase NisB than Ser,[31] a methyllanthionine linkage was designed to replace the disulfide linkage of thanatin and rip-thanatin. To introduce a methyllanthionine-based peptide macrocycle into thanatin and rip-thanatin,[23,27] the Cys11 of thanatin and Cys8 of rip-thanatin were designed to be replaced by Thr (Table 1), which can be potentially dehydrated by the NisB dehydratase and subsequently form a methyllanthionine-based peptide macrocycle with the Cys18 of thanatin and the Cys15 of rip-thanatin by the NisC cyclase. The genes encoding the designed peptides were constructed into a pNZ8048-derived plasmid, a commonly used expression vector for *L. lactis*, fused to the *nisA* leader peptide gene (Figure S1).[32] After verifying the plasmids by sequencing, *L. lactis* NZ9000[33] with pIL3 BTC,[34] a plasmid encoding the NisB dehydratase gene, the NisC cyclase gene, and the gene of the transporter NisT (Figure 1), was transformed with these designed plasmids. After induction and purification, MALDI-TOF MS was used to check the mass of the produced peptides. Ripcin was fully dehydrated as predicted (Table 1 and Figure 2E), while only one dehydration was observed for thanacin (Table 1 and Figure 2F). We found out this is caused by the substrate
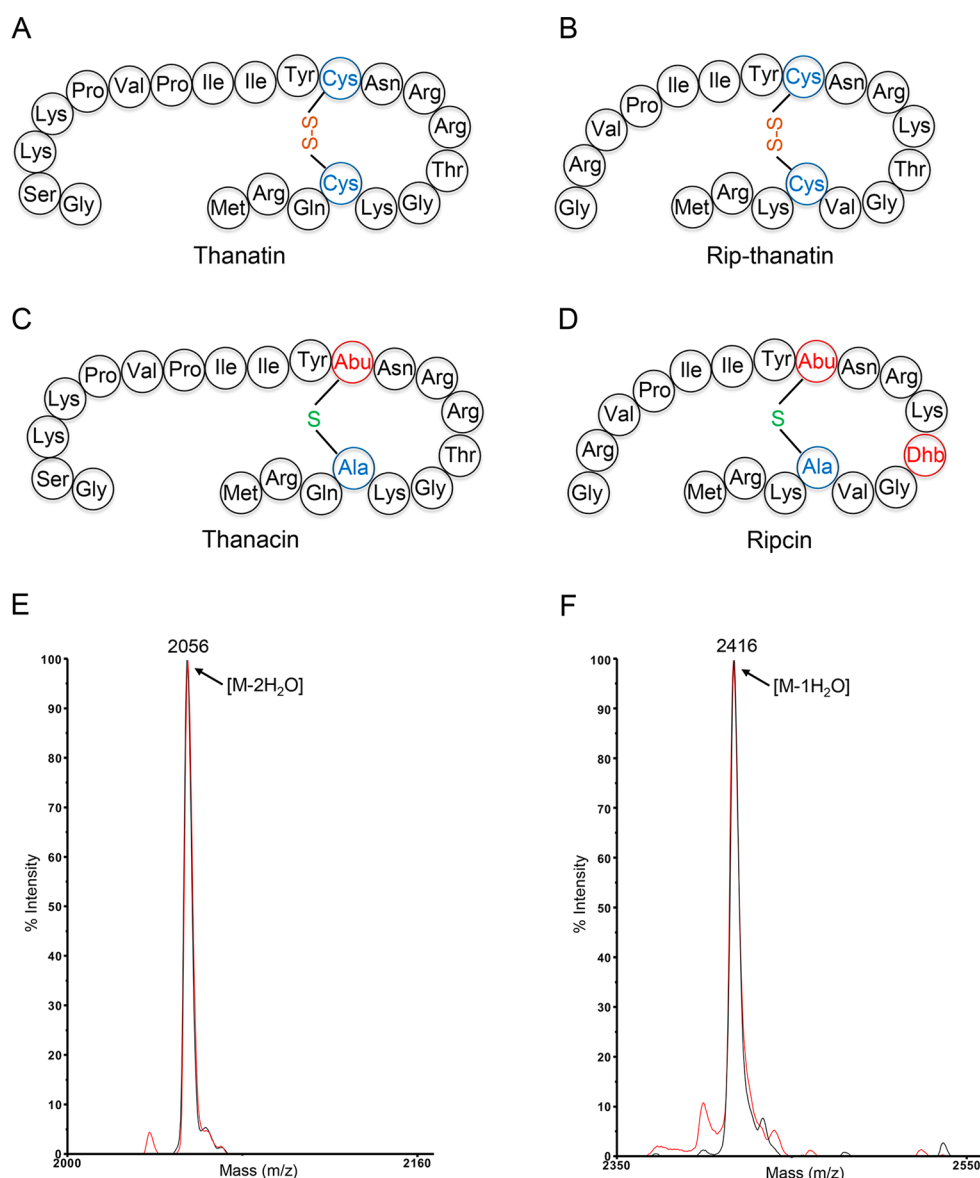
Figure 2. Structures of (A) thanatin and (B) rip-thanatin. Structures of designed (C) thanacin and (D) ripcin. MALDI-TOF MS of (E) ripcin and (F) thanacin before (black) and after (red) CDAP treatment. Dhb, dehydrobutyrine; Abu, aminobutyric acid.

Table 1. Amino Acid Sequence, Dehydrations, and Yield of Designed Peptides

peptide	amino acid sequence[a]	mass (Da) predicted	mass (Da) measured	dehydrations (observed/predicted)	yield[b] (mg/L)
thanacin	GSKKPVPIIY**T**NRRTGKCQRM	2379.93	2416	1/3	0.1
ripcin	GRVPIIY**T**NRK**T**GVCKRM	2056.56	2056	2/2	5.2
ripcin B	ITS*IS*LC**T**PGCK**T**GALMGCN*GRVPIIYTNRKTGVCKRM*	3918.52	3919	7/7	7.4
ripcin C	ITS*IS*LC**T**PGCK**T**GALMGCN*RVPIIYTNRKTGVCKRM*	3861.47	3861	7/7	5.8
ripcin D	ITS*IS*LC**T**PGCK**T**GALMGCN*VPIIYTNRKTGVCKRM*	3705.28	3705	7/7	6.7
ripcin E	ITS*IS*LC**T**PGCK**T**GALMGCN*PIIYTNRKTGVCKRM*	3606.15	3607	7/7	6.3
ripcin F	ITS*IS*LC**T**PGCK**T**GALMGCN*IIYTNRKTGVCKRM*	3509.03	3509	7/7	6.4
ripcin G	ITS*IS*LC**T**PGCK**T**GALMGCN*IYTNRKTGVCKRM*	3395.87	3396	7/7	7.6

[a]For peptides ripcin B to ripcin G, the amino acids from the N-terminus of nisin are underlined; the amino acids from the C-terminus of ripcin are italicized; and the amino acids identified with dehydration are in boldface font. [b]The yields displayed were calculated post-HPLC purification, and the modification rates of products are as displayed in Figures 2 and 4.

specificity of NisB[31] and the difference in sequences in ripcin and thanatin: Thr12(KTG) in ripcin is modified, while Thr15(RTG) and Ser2(GSK) in thanatin are not modified.

Interestingly, further studies evidenced that the dehydrated amino acid residue in thanacin was the Thr11, the desired position. The formation of the potentially NisC-induced
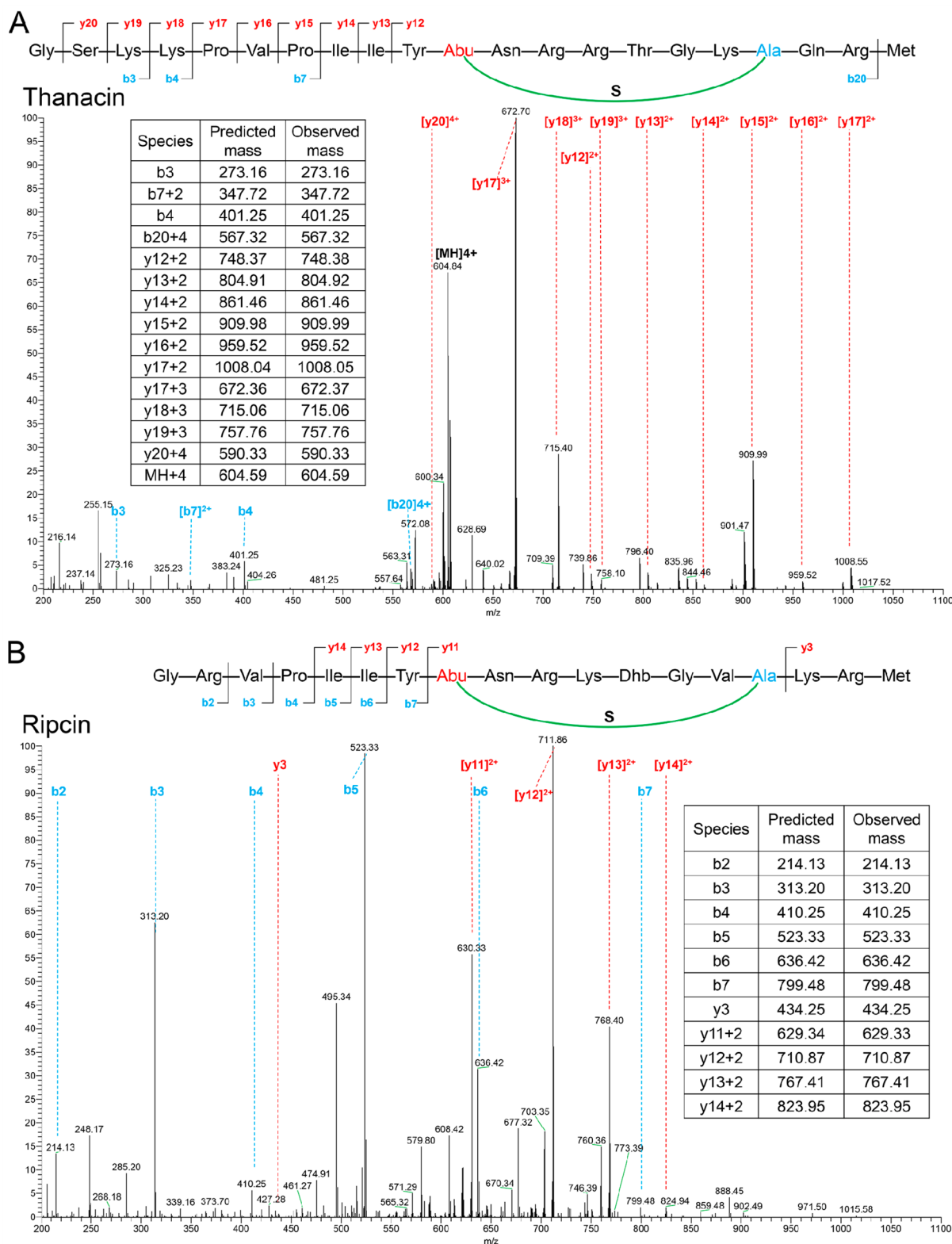
Figure 3. LC−MS/MS spectrum and the proposed structures of thanacin and ripcin. Fragment ions are indicated (also see Figure S3).

thioether cross-link-based ring was investigated by using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), a compound that reacts with unmodified cysteines in peptides and results in an increase of 25 Da in the peptide's molecular weight.[7,32,35] No adduct was observed for either of the designed thanacin or ripcin (Figure 2E,F), while the mass of a free Cys-containing peptide was shifted entirely with a 25 Da

increase (Figure S2), indicating that no unmodified cysteines were present in either thanacin or ripcin. These results imply that thioether cross-links in both thanacin and ripcin were formed. To further characterize the produced thanacin and ripcin molecules, LC−MS/MS analysis was performed. No fragmentation was observed between Thr11 and Cys18 of thanatin and between Thr8 and Cys15 of ripcin (Figure 3A,B),

Table 2. Antimicrobial Activity of Designed Peptides against Microorganisms

organism and type[a]	MIC (μM)									
	nisin(1−20)	ripcin	ripcin B	ripcin C	ripcin D	ripcin E	ripcin F	ripcin G	nisin	polymyxin B
Gram-positive bacteria										
Staphylococcus aureus ATCC15975 (MRSA)	32	>256	*4*	*2*	*8*	*4*	*4*	*4*	2	32
Staphylococcus aureus LMG10147	32	>256	*2*	*2*	*4*	*4*	*4*	*4*	4	32
Enterococcus faecium LMG16003 (VRE)	64	>256	16	8	32	32	16	16	2	16
Enterococcus faecalis LMG16216 (VRE)	128	>256	32	16	32	32	32	32	2	16
Bacillus cereus ATCC 14579	64	>256	32	16	16	16	16	32	2	8
Lactococcus lactis NZ9000 (pNZ8048-Em^r)	4	>256	0.5	0.25	0.5	0.5	0.5	0.5	**0.02**	>32
Lactococcus lactis NZ9000 (pNZ-SV- SaNSR)	4	>256	0.5	0.25	0.5	0.5	0.5	0.5	**2**	>32
Gram-negative bacteria										
Acinetobacter baumannii LMG01041	>256	>256	8	4	32	32	16	32	4	1
Acinetobacter baumannii LMG17978	>256	>256	64	32	>128	>128	>128	>128	32	4
Shigella flexneri ATCC29903	>256	>256	16	8	32	32	32	32	16	0.5
Escherichia coli ATCC25922	>256	>256	16	16	32	64	32	64	16	2
Klebsiella pneumoniae LMG20218	>256	>256	64	32	>128	>128	>128	>128	64	2
Pseudomonas aeruginosa LMG6395	>256	>256	>128	64	>128	>128	>128	>128	64	2
Enterobacter cloacae LMG02783	>256	>256	64	32	>128	>128	>128	>128	32	>128

[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 well-known antibiotic controls. The low micromolar MIC values of ripcins against bacterial pathogens are italicized. The MIC values obtained for nisin against *Lactococcus lactis* and *Lactococcus lactis* (NSR) are shown in boldface font.

demonstrating that a methyllanthionine-based ring was correctly formed for either thanacin or ripcin (Figure 2C,D). Although the nisin biosynthetic machinery has been widely used in lanthipeptide engineering, this is the first time it was used for the successful synthesis of macrocyclic lanthipeptides with six residues in between the β-methyl-lanthionine forming residues.

Synthesis of Nisin- and Ripcin-Derived Hybrid Lanthipeptides. After high-performance LC (HPLC) purification, the antimicrobial activities of modified thanacin and ripcin were determined by minimum inhibitory concentration (MIC) assays. The results show that both thanacin and ripcin had substantial antimicrobial activity against *M. flavus* (MIC, 4 μM), while both of them had insufficient antimicrobial activity against bacterial pathogens (Table 2, data not shown for thanacin). Considering that ripcin is a large ring and many positively charged amino-acids-containing cyclic peptide and thanacin had a much lower yield than ripcin (Table 1), we chose ripcin for further studies. We envisioned that a nisin lipid-II-binding moiety, together with a fused ripcin, might display strong antimicrobial activity against bacterial pathogens. Considering the fact that nisin(1−20) and ripcin-fused peptide (ripcin B) would contain 38 amino acids, which might be too long to be modified by the NisB dehydratase and NisC cyclase, five shorter peptides (ripcin C− G) were designed by using nisin(1−20) and a selected part of ripcin (Table 1). The genes encoding the designed peptides were constructed into a pNZ8048-derived plasmid, in which they were fused to the *nisA* leader peptide gene (Figures S4 and S5).[32] After verification of the plasmids by sequencing, *L. lactis* NZ9000[33] with pIL3 BTC[34] was transformed with these plasmids. After induction and purification, MALDI-TOF MS was used to measure the mass of the produced peptides. All of the six designed peptides were dehydrated as predicted (Table 1 and Figure 4), and the yields of the designed peptides were between 5.8 and 7.6 mg/L (Table 1 and Figure S6), which is a relatively high yield compared to previously reported nisin-derived peptides.[36] A CDAP assay showed that no adduct was observed for all of the six designed peptides (Figure 4),

indicating that the peptides were modified as predicted (Figure 5). In a previous study, the nisin synthetic machinery was successfully applied to modify a peptide with nine dehydrations and seven rings.[7] Van Heel *et al.*[19] reported that flavucin, which was discovered by genome mining, was successfully modified by the nisin synthetic machinery. Overall, these studies on the production and secretion of modified peptides show that the nisin synthetic machinery provides an efficient lanthipeptide engineering system.

Ripcin B−G Show Selective Antimicrobial Activity Against S. aureus. To determine the antimicrobial activity of ripcin B−G against bacterial pathogens, a MIC assay was performed according to the standard guidelines.[37] Nisin and polymyxin B were used as antibiotic controls. Ripcin showed antimicrobial activity against neither the tested Gram-positive nor Gram-negative bacterial pathogens (Table 2). Nisin(1− 20) was obtained using chymotrypsin to digest full nisin (Figures S7 and S8).[38] Nisin(1−20) showed insufficient antimicrobial activity against all tested Gram-positive pathogens, while it showed no antimicrobial activity against the tested Gram-negative pathogens (Table 2). Ripcin B−G showed stronger antimicrobial activity against the tested Gram-positive pathogens than nisin(1−20). Notably, ripcin B−G showed selective antimicrobial activity against *S. aureus* (Table 2), including an antibiotic-resistant MRSA strain. The antimicrobial activity of ripcin B−G against *S. aureus* is comparable with that of the well-known antimicrobial RiPP nisin.

Many studies have shown that the recombination of antimicrobial peptides forms an alternative strategy for the synthesis of antibiotics with specific antimicrobial activity.[7,39,40] Taken together, these studies and our results suggest that additional hybrid antimicrobials could be synthesized for developing antibiotics with a specific antimicrobial activity. Interestingly, ripcin B−G, which are recombinant peptides of two inactive (against Gram-negative pathogens) peptides nisin(1−20) and ripcin, showed substantial antimicrobial activity against the tested Gram-negative bacteria (Table 2). Consistently, a previous study showed that the recombination
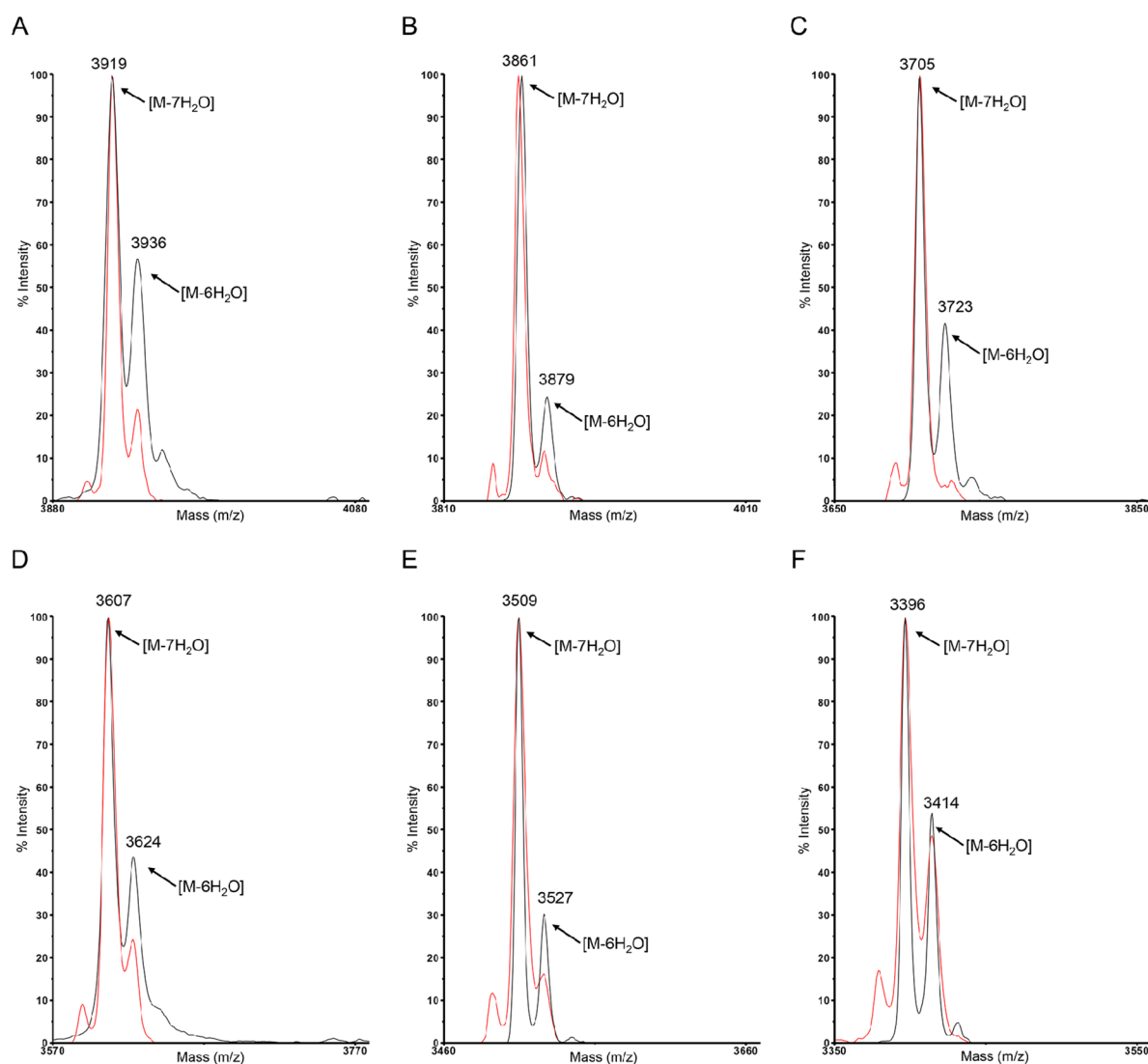
Figure 4. MALDI-TOF MS of ripcins before (black) and after (red) CDAP treatment: (A) ripcin B; (B) ripcin C; (C) ripcin D; (D) ripcin E; (E) ripcin F; (F) ripcin G.

of the nisin N-terminus part with cationic antimicrobial peptides could increase the antimicrobial activity of nisin against Gram-negative pathogens.[36]

To investigate the stability of ripcin B−G against the nisin resistance protein (NSR),[41,42] a peptidase that removes the C-terminal 6 amino acids of nisin, a MIC test was performed by using NSR producing strain *L. lactis* NZ9000 (pNZ-SV-SaNSR, Em^r)[42] and non-NSR producing strain *L. lactis* NZ9000 (pNZ8048- Em^r, Em^r).[43] Due to removal of the C-terminal 6 amino acids of nisin by NSR peptidase, the antimicrobial activity of nisin against *L. lactis* NZ9000 was decreased 100-fold in the presence of the NSR peptidase (Table 2). Interestingly, ripcin B−G showed no reduction of antimicrobial activity against *L. lactis* NZ9000 in the presence of the NSR peptidase (Table 2). These results demonstrate that the hybrid macrocyclic lanthipeptides, ripcin B−G, bypassed the NSR resistance mechanism toward nisin. Together, the here-synthesized lanthipeptides with a large C-terminal ring, named ripcin B−G, have shown selectively antimicrobial activity against *S. aureus* without the concern of NSR. Among the designed peptides, ripcin C showed the

highest antimicrobial activity against the tested bacterial pathogens. Therefore, ripcin C was selected for further studies.

Ripcin C Acts as a Bacteriostatic Antibiotic Against Gram-Positive Pathogens, While it Shows Bactericidal Activity Against Gram-Negative Pathogens. To investigate whether ripcin C acts as a bacteriostatic or bactericidal antibiotic against bacteria pathogens, time-killing assays were performed. *S. aureus* (MRSA) and *A. baumannii* LMG01041 were inoculated in MHB and grown until the OD$_{600}$ of cell cultures reached 0.8. Cell cultures were then diluted to a concentration of 5×10^6 c.f.u. per mL and thereafter challenged with antibiotics at a concentration of 10× MIC. Nisin was used as a bactericidal antibiotic control against Gram-positive bacteria.[13] Nisin killed all of the *S. aureus* (MRSA) in 2 h, while ripcin C did not reduce the population of *S. aureus* (MRSA) during 8 h after treatment (Figure 6A). These results demonstrate that ripcin C acts as a bacteriostatic antibiotic against Gram-positive bacteria. These results are in line with the later membrane permeability assay and lipid-II-binding assay, which showed that ripcin C binds to lipid II (Lys) but does not disrupt the cell membrane (Figures 6B and 7B). Polymyxin B was used as a bactericidal antibiotic control
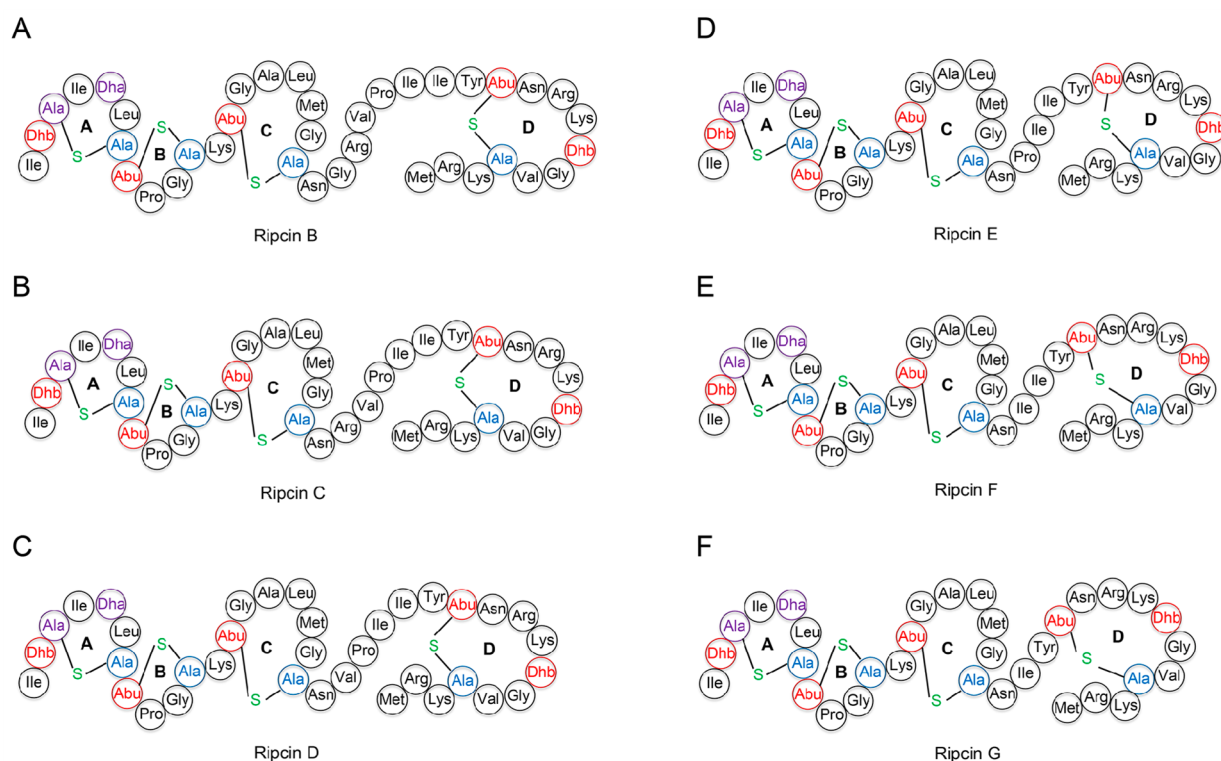
Figure 5. Hypothetical structures of ripcins: (A) ripcin B; (B) ripcin C; (C) ripcin D; (D) ripcin E; (E) ripcin F; (F) ripcin (G) Dha, dehydroalanine; Dhb, dehydrobutyrine; Abu, aminobutyric acid.
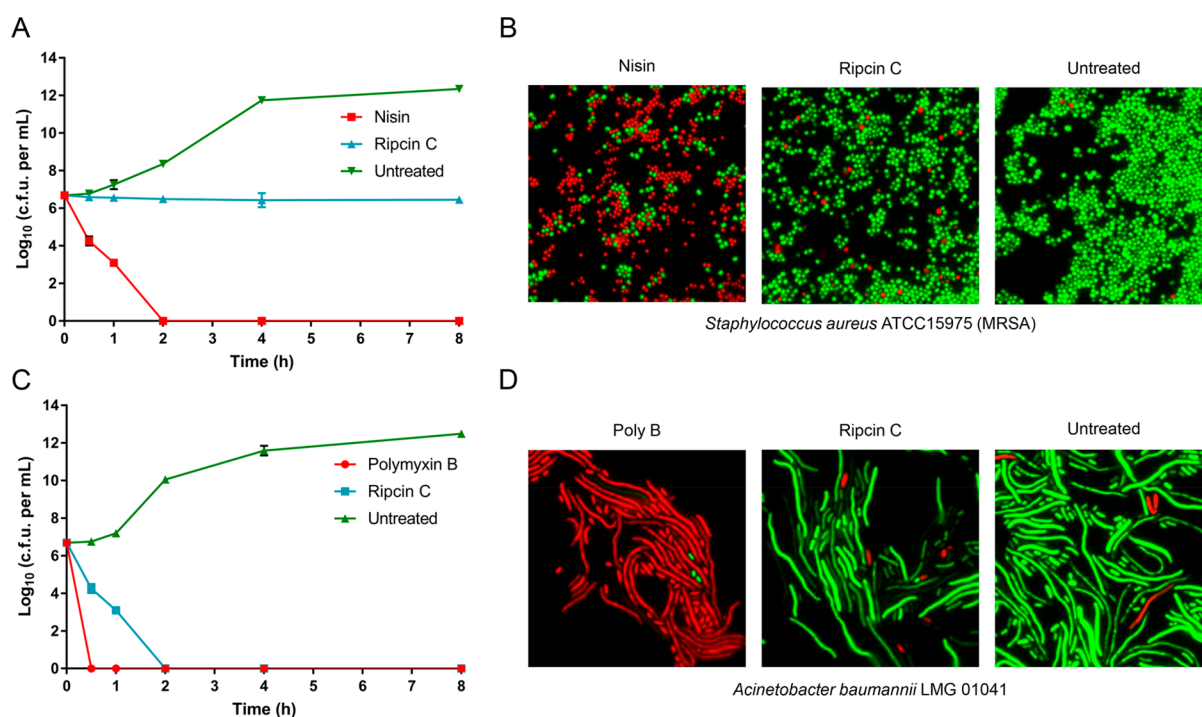

Figure 6. Ripcin C acts as a bacteriostatic antibiotic against Gram-positive pathogens and shows bactericidal activity against Gram-negative pathogens. (A) Time-killing assay of ripcin C, *S. aureus* (MRSA), was challenged with lantibiotics (10× MIC). Data are representative of three independent experiments. (B) Fluorescence microscopy images of *S. aureus* (MRSA), which was challenged with different antibiotics at a concentration of 2× MIC. (C) Time-killing assay of ripcin C, *A. baumannii*, was challenged with lantibiotics (10× MIC). Data are representative of three independent experiments. (D) Fluorescence microscopy images of *A. baumannii*, which was challenged with different antibiotics at a concentration of 2× MIC. In B and D, green denotes a cell with an intact membrane, whereas red denotes a cell with a compromised membrane.

against Gram-negative bacteria.[44] Polymyxin B showed a quick killing capacity on *A. baumannii* cells, which killed all bacteria

in half an hour (Figure 6C). Ripcin C showed slower bactericidal activity than polymyxin B against *A. baumannii*
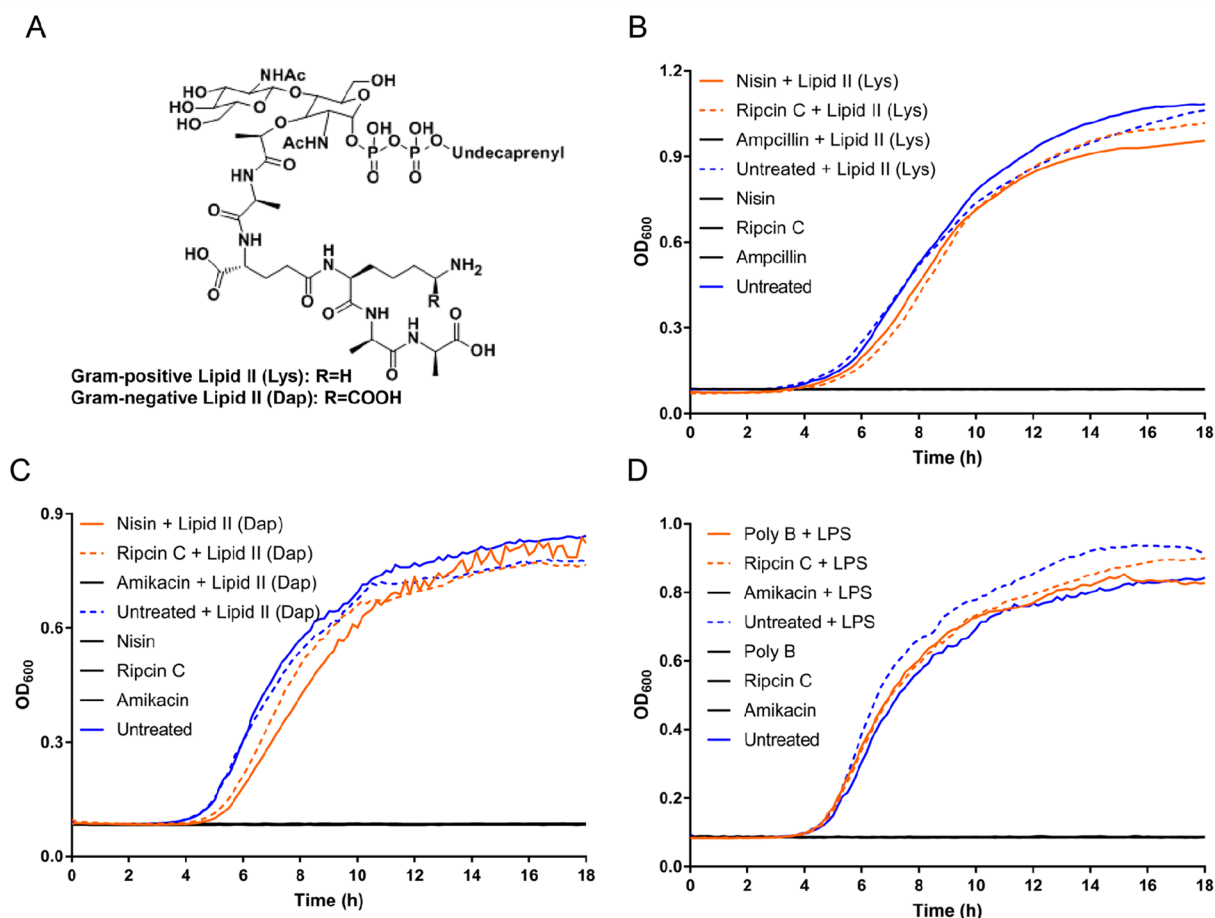
Figure 7. Ripcin C binds with the cell wall synthesis precursor lipid II and LPS. (A) Structure of Gram-positive lipid II (Lys) and Gram-negative lipid II (Dap). (B) Addition of purified lipid II (Lys) (10 μM) inhibited the antimicrobial activity of ripcin C (2× MIC) against *Staphylococcus aureus* ATCC15975 (MRSA), indicating the binding of ripcin C and lipid II (Lys). (C) Addition of purified lipid II (Dap) (10 μM) inhibited the antimicrobial activity of ripcin C (2× MIC) against *Acinetobacter baumannii* LMG01041, indicating the binding of ripcin C and LPS. (D) Addition of purified LPS (100 μg/mL) inhibited the antimicrobial activity of ripcin C (2× MIC) against *Acinetobacter baumannii* LMG01041, indicating the binding of ripcin C and lipid II (Dap). In B–D, black lines denote all groups of bacteria that did not grow.

cells, which killed all Gram-negative bacteria in 2 h (Figure 6C).

Ripcin C Does Not Disrupt the Membrane of Bacteria. To assess the influence of ripcin C on the bacterial membrane, membrane permeability assays were performed by 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 ripcin C at a concentration of 2× MIC for 15 min, the cells were monitored by fluorescence microscopy. Green cells were observed for both *S. aureus* (MRSA) and *A. baumannii* (Figure 6B,D), indicating that ripcin C exerts its antimicrobial activity not *via* the disruption of the cellular membrane.

Ripcin C Binds to the Cell Wall Synthesis Precursor Lipid II and LPS. To assess the functionality of the lipid-II-binding motive of ripcin C, bacterial growth assays were performed with or without purified lipid II (Figure 7A) in the presence of ripcin C at a concentration of 2× MIC. For the Gram-positive lipid-II (Lys)-binding assay, nisin was used as a lipid-II-binding antibiotic control.[45] In addition, ampicillin was used as a nonlipid-II-binding antibiotic control, which exerts its antimicrobial activity *via* inhibiting bacterial transpeptidase enzyme.[46] Ripcin C inhibited the growth of *S. aureus* (MRSA)

cells, while *S. aureus* (MRSA) cells treated with ripcin C were grown in the presence of 10 μM Gram-positive lipid II (Lys) (Figure 7B), indicating ripcin C still keeps the lipid-II-binding capacity of nisin. For the Gram-negative lipid-II (Dap)-binding assay, nisin was used as a lipid-II-binding antibiotic control. Amikacin, an aminoglycoside antibiotic that exerts its antimicrobial activity by inhibiting bacterial protein synthesis,[46] was used as a nonlipid-II-binding antibiotic control. In the presence of 10 μM Gram-negative lipid II (Dap), ripcin C lost its antimicrobial activity against *A. baumannii* LMG01041 cells (Figure 7C), indicating that ripcin C also binds with the Gram-negative lipid II (Dap). For the lipopolysaccharide (LPS)-binding assay, polymyxin B was used as an LPS-binding antibiotic control, whereas amikacin was used as a non-LPS-binding antibiotic control. Interestingly, in the presence of 100 μg/mL LPS, ripcin C lost its antimicrobial activity against *A. baumannii* cells (Figure 7D), indicating that ripcin C has a LPS-binding capacity. The LPS-binding capacity of ripcin C maybe one of the primary reasons that ripcin C exerts its antimicrobial activity against Gram-negative pathogens, since nisin(1−20) lacks antimicrobial activity against Gram-negative pathogens. In addition, the C-terminus of ripcin C may target the intermembrane protein complex required for lipopolysaccharide transport as thanatin

does,[26] which is probably the reason it shows bactericidal activity against Gram-negative bacteria since binding to lipid II can only arrest bacterial growth. These results provide preliminary data for the mode of action of the synthesized hybrid lanthipeptides.

■ CONCLUSIONS

Here, we show that macrocyclic lanthipeptides (thanacin and ripcin) can be synthesized by using thanatin and rip-thanatin as templates. This is the first time that the nisin synthetic machinery was used successfully to synthesize such a macrocyclic lanthipeptide (six residues within the ring). In addition, nisin(1−20) and ripcin hybrid lanthipeptides showed stronger antimicrobial activity than either nisin(1−20) or ripcin alone against the tested bacterial pathogens. Notably, ripcin B−G showed selective antimicrobial activity against *S. aureus*, including an antibiotic-resistant MRSA strain. Interestingly, ripcin B−G, which are hybrid peptides of two inactive (against Gram-negative pathogens) peptides, *i.e.*, nisin(1−20) and ripcin, showed substantial antimicrobial activity against the tested Gram-negative pathogens. Moreover, ripcin B−G were fully resistant against the NSR, while efficient degradation takes place with full nisin,[41,42] making our hybrid peptides more attractive for potential application in complex microbial environments like the gut or skin, also in view of their higher target specificity. Mode of action studies show that ripcin C binds to lipid II and acts as bacteriostatic antimicrobial. In addition, the bactericidal antimicrobial activity of ripcin C against Gram-negative pathogens is probably due to its LPS-binding capacity. Together, this study shows a convenient and effective approach for converting disulfide-bond-based AMPs into (methyl)lanthionine-based macrocyclic lanthipeptides, yielding hybrid macrocyclic lanthipeptides with selective antimicrobial activity against *S. aureus*.

■ MATERIALS AND METHODS

Microbial Strains Used and Growth Conditions. Strains and plasmids used in the present study are listed in Table S1. *L. lactis* NZ9000 was used for plasmid construction, plasmid maintenance, and peptide expression. For plasmid selection, *L. lactis* was grown at 30 °C in M17 broth (Oxoid) or M17 broth solidified with 1% (wt/vol) agar, containing 0.5% (wt/vol) glucose (GM17), when necessary, supplemented with chloramphenicol (5 μg/mL) and/or erythromycin (5 μg/mL). For protein expression, stationary-phase cultures, which were gown in GM17, were inoculated (20-fold diluted) on minimal expression medium (MEM) and induced with nisin (5 ng/mL) at an optical density at 600 nm (OD_{600}) of about 0.35.

Molecular Biology Techniques. Oligonucleotide primers used for cloning and sequencing in this study are listed in Table S2, and all the oligonucleotide primers were purchased from Biolegio B.V. (Nijmegen, The Netherlands). Constructs coding for designed peptides were made by amplifying template plasmid using a phosphorylated downstream sense- (or upstream antisense) primer and an upstream antisense (or downstream sense) primer with a peptide-encoding tail. The DNA amplification was carried out by using phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA). Self-ligation of the resulting plasmid was carried out with T4 DNA ligase (Thermo Fisher Scientific, Waltham, MA). The electrotransformation of *L. lactis* was carried out as previously described using a Bio-Rad gene pulser (Bio-Rad, Richmond, CA).[47] The designed peptide mutations were verified by sequencing using the primer PrXZ12 at Macrogen Europe B.V.

Small Scale Expression and Trichloroacetic Acid (TCA) Precipitation of Peptides for Tricine-SDS Protein Gel Assay. *L. lactis* NZ9000 cells with pIL3 BTC were transformed with peptide plasmids (50 ng), plated on GM17 agar plates supplemented with chloramphenicol (5 μg/mL) and erythromycin (5 μg/mL) (GM17CmEm), and grown at 30 °C for 20 h. A single colony was used to inoculate 5 mL of GM17CmEm broth. The culture was grown overnight at 30 °C and then used to inoculate 45 mL (20-fold dilution) of MEM. After induction at 30 °C for 3 h, the supernatant of cultures were harvested by centrifugation at 10 000g for 30 min. Ice-cold 100% TCA was added to the ice-cold supernatants to a final concentration of 10%, and samples were subsequently kept on ice for 2 h to precipitate peptides. Samples were then centrifuged at 10 000g at 4 °C for 30 min. The precipitate was washed three times with 20 mL of ice-cold acetone to remove any residual TCA. Samples were dried in the fume hood and resuspended in 0.5 mL of 0.1 M PBS buffer. Subsequently, peptides were separated by Tricine-SDS gel (16%) electrophoresis and visualized by Coomassie Blue staining.

Large Scale Expression and Purification of Designed Peptides. *L. lactis* with pIL3 BTC and the corresponding peptide was inoculated on 50 mL of GM17CmEm. After being grown overnight at 30 °C, cultures were inoculated on 1 L (20-fold dilution) of MEM. After induction, cultures were grown at 30 °C overnight. After centrifugation of the overnight expressed cultures ($OD_{600} \approx 1.2$), the supernatants were collected, and the pH was adjusted to 7.0. After that, the cultures were applied to a CM Sephadex C-25 column (GE Healthcare) equilibrated with distilled water. The flow-through was discarded, and the column was subsequently washed with 12 column volumes (CVs) of distilled water. The peptide was eluted with 6 CVs of 2 M NaCl. The eluted peptide was then applied to a SIGMA-Aldrich C18 silica gel spherically equilibrated with 10 CVs of 5% aqueous MeCN containing 0.1% trifluoroacetic acid. After washing with 10 CVs of 5% aqueous MeCN containing 0.1% trifluoroacetic acid, the peptide was eluted from the column using up to 10 CVs of 50% aqueous MeCN containing 0.1% trifluoroacetic acid. Fractions containing the eluted peptide were freeze-dried, and the peptide was subsequently dissolved in Tris-HCl (pH = 6.5) containing an appropriate amount of NisP leader protease and incubated at 37 °C for 3 h to cleave off the leader peptide. After filtration through a 0.2 μm filter, the core peptide 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% aqueous MeCN for thanacin and ripcin and 35−45% aqueous MeCN for ripcin B−G over 25 min at 1 mL per min was used for separation. Peptides were eluted at 21−23% MeCN for thanacin and ripcin and 38−41% MeCN for ripcin B−G. After lyophilization, peptides were dissolved in sterilized water and stored at −80 °C. The expression levels for designed peptides are listed in Table 1.

Mass Spectrometry. The sample (0.5 μL) 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 (methyl)Lanthionine 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 treated 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.[48]

LC−MS/MS Analysis. To gain deep insight into the lanthionine bridging pattern, we performed LC−MS/MS assay. 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 a 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 the doubly and triply charged ions of the compound of interest.

Minimum Inhibitory Concentration (MIC) Assay. MIC values were determined by broth microdilution, according to the standard guidelines.[37] Briefly, the test medium was cation-adjusted Mueller−Hinton broth (MHB). Cell concentration was adjusted to approximately 5×10^5 cells 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.

For the nisin resistance protein-producing strain *L. lactis* NZ9000 (pNZ-SV-SaNSR, Em^r)[42] and non-NSR-producing strain *L. lactis* NZ9000 (pNZ8048- Em^r, Em^r),[43] the MIC tests were performed in GM17, supplemented with erythromycin (5 μg/mL) and nisin (1 ng/mL, for maintenance of the induction of NSR). *L. lactis* NZ9000 strains were induced by nisin at a concentration of 5 ng/mL for 3 h before being exposed to antibiotics.

Time-Killing Assay. This assay was performed according to a previously described procedure.[49] An overnight culture of either *Staphylococcus aureus* ATCC15975 (MRSA) or *Acinetobacter baumannii* LMG01041 was diluted 50-fold in MHB and incubated at 37 °C with aeration at 220 r.p.m. Bacteria were grown to an OD$_{600}$ of 0.6, and then, the concentration of the cells was adjusted to ≈5×10^6 cells per mL for both strains. Bacteria were then challenged with 10× MIC antimicrobials in glass culture tubes at 37 °C and 220 r.p.m. Bacteria not treated with peptides were used as a negative control. At desired time points, 200 μL aliquots were taken, centrifuged at 8000g 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 c.f.u. per mL was calculated. Each experiment was performed in triplicate.

Fluorescence Microscopy Assay. *Staphylococcus aureus* ATCC15975 (MRSA) or *Acinetobacter baumannii* LMG01041 was grown to an OD$_{600}$ of 0.8. The culture was pelleted at 4000g for 5 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 antimicrobials was added.

Simultaneously, SYTO 9 and propidium iodide (LIVE/DEAD Baclight Bacterial Viability kit, Invitrogen) were added to the above cell suspensions. After incubation at room temperature for 15 min, peptides were removed and washed three times with MHB. Then, the cell suspensions were loaded on 1.5% agarose pads and analyzed by a DeltaVision Elite microscope (applied precision).[50]

Bacterial Growth Assay to Monitor Lipid-II- and Lipopolysaccharide (LPS)-Binding of Antibiotics. Briefly, the test medium was cation-adjusted Mueller−Hinton broth (MHB). The cell concentration was adjusted to approximately 5×10^5 cells per mL, and then, the cells were transferred to a 96-well plate and thereafter challenged with antibiotics at a concentration of 2× MIC either with or without lipid II[51] (10 μM; provided by Prof. Eefjan Breukink)/LPS (100 μg/mL; Merck, L2880-100MG). The absorbance values were measured by using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific) at 600 nm. Each experiment was performed in triplicate.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00080.

> Figure of critical area of plasmids encoding the designed peptide genes, MALDI-TOF MS data, full LC−MS/MS spectra, expression of peptides measured by SDS-tricine gel, and digestion of nisin by chymotrypsin for the production of nisin(1−20) 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. 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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