Mimicry of a Non-ribosomally Produced Antimicrobial, Brevicidine, by Ribosomal Synthesis and Post-translational Modification
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Mimicry of a Non-ribosomally Produced Antimicrobial, Brevicidine, by Ribosomal Synthesis and Post-translational Modification

Graphical Abstract

Highlights

- Mimicry of NRPs by RiPP biosynthesis is a feasible strategy for drug discovery
- The acyl chain of brevicidine can be mimicked by hydrophobic amino acid residues
- The lactone ring of brevicidine can be functionally mimicked by a thioether ring
- Engineered RiPPs display a similar antimicrobial mode of action as brevicidine

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In Brief

Zhao et al. describe a strategy to synthesize mimics of the recently discovered antimicrobial non-ribosomal peptide, brevicidine. The engineered mimics show antimicrobial activities against pathogens susceptible to brevicidine, which demonstrate that conversion of NRPs to RiPPs is feasible and offer great opportunities for engineering a wide range of effective antibiotics.

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Mimicry of a Non-ribosomally Produced Antimicrobial, Brevicidine, by Ribosomal Synthesis and Post-translational Modification

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SUMMARY

The group of bacterial non-ribosomally produced peptides (NRPs) forms a rich source of antibiotics, such as daptomycin, vancomycin, and teixobactin. The difficulty of functionally expressing and engineering the corresponding large biosynthetic complexes is a bottleneck in developing variants of such peptides. Here, we apply a strategy to synthesize mimics of the recently discovered antimicrobial NRP brevicidine. We mimicked the molecular structure of brevicidine by ribosomally synthesized, post-translationally modified peptide (RiPP) synthesis, introducing several relevant modifications, such as dehydration and thioether ring formation. Following this strategy, in two rounds peptides were engineered in vivo, which showed antibacterial activity against Gram-negative pathogenic bacteria susceptible to wild-type brevicidine. This study demonstrates the feasibility of a strategy to structurally and functionally mimic NRPs by employing the synthesis and post-translational modifications typical for RiPPs. This enables the future generation of large genetically encoded peptide libraries of NRP-mimicking structures to screen for antimicrobial activity against relevant pathogens.

INTRODUCTION

The increasing antibiotic resistance in pathogenic bacteria is a serious threat to healthcare worldwide (O’Neill, 2014). Moreover, the number of approved antibiotics has been decreasing in the past decades, especially those for treating infections by Gram-negative pathogenic bacteria (Butler et al., 2013, 2017; Roemer and Boone, 2013). Therefore, alternative sources of antibiotics are urgently required. Non-ribosomally produced peptides (NRPs) constitute an important source of antibiotics. These peptide antibiotics include more than 20 marketed antibacterial drugs, such as vancomycin, penicillin G, and daptomycin (Sussmuth and Mainz, 2017). The synthesis of NRPs requires NRP synthetases (NRPSs) that act in modular assembly-line logic (Hur et al., 2012; Weissman, 2015). NRPSs are large proteins of about 220 kDa to 2.2 MDa mass range, with a high substrate specificity and encoded by very large gene clusters (Challis et al., 2000; Reimer et al., 2018; Stachelhaus et al., 1999; Villiers and Hollfelder, 2009). Importantly, the combination of the large size of their gene clusters and the high substrate specificity of NRPSs makes it difficult to efficiently engineer libraries of structural and functional mimics of NRP to screen for activity against antibiotic-resistant pathogens.

Ribosomally synthesized and post-translationally modified peptides (RiPPs) form a diverse class of natural products that are produced by a variety of organisms, including more than 20 subclasses, such as lanthipeptides, lasso peptides, microcins, microviridins, linaridins, and many others (Arnison et al., 2013). Notably, RiPPs have been proved to have a wide range of biological activities (Arnison et al., 2013). Lantibiotics belong to the lanthipeptides and are lanthionine-ring-containing peptides that possess strong antimicrobial activity (Montalbán-López et al., 2017). The lanthipeptide biosynthetic dehydratase LanB and the cyclase LanC are enzymes that introduce hydrated residues and (methyl)lanthionine rings into ribosomally synthesized precursor peptides (Figure 1) (Ortega et al., 2015; Repka et al., 2017). LanT transporters act as rafts for secreting precursor lanthipeptides (Arnison et al., 2013). Some of these enzymes and transporters have low substrate specificity, allowing modification and secretion of substrates with both proteinogenic and non-proteinogenic amino acids (Arnison et al., 2013). The dehydratase NisB, the cyclase NisC, and the transporter NisT constitute one of the best-studied lanthipeptide modification and secretion machineries, the nisin biosynthetic machinery (Figure S1) (Kuipers et al., 1995, 1998; De Ruyter et al., 1996; Zhao et al., 2020a). This biosynthetic machinery has been widely and successfully used in producing designed lanthipeptides and for screening potent genetically encoded libraries of...
In this study, we aimed to structurally and functionally mimic antimicrobial NRPs by peptides that are ribosomally produced. The rationale for such endeavor is the resulting opportunity to screen for a large number of variants via high-throughput mutagenesis or combinatorial DNA synthesis of the peptide-encoding gene. There are several steps required for the production of NRPs, such as acylation, introduction of D-amino acids, substitution of non-canonical amino acid residues, introduction of other (ring-forming) modifications, and secretion. Here, we employ a strategy to mimic a recently found antimicrobial NRP, brevicidine (Li et al., 2018b) (Figure 2A), by synthesis of mimics of this molecule by RiPP biosynthesis, i.e., by using lanthipeptide synthetic machineries. We explored whether the methyllanthionine ring can be used to mimic the lactone ring, whether the D-amino acids can be replaced by L-amino acids, whether ornithine can be mimicked by lysine, and whether a hydrophobic amino acid chain can be used to mimic a relatively short acyl chain. After two rounds of engineering, RiPPs with antibacterial activity against Gram-negative pathogenic bacteria were successfully obtained. This work shows that mimicking NRPs by RiPP synthesis provides a valuable strategy for the discovery of effective antimicrobials.

RESULTS

Using NRPs as Scaffolds for RiPP Biosynthesis Yields Modified Peptides with Antimicrobial Activity against Gram-Negative Bacteria

Brevicidine and laterocidine are recently described NRPs with potent antibacterial activities against Gram-negative pathogenic bacteria, which were found through global genome mining (Li et al., 2018b). Brevicidine and laterocidine are produced by *Brevibacillus laterosporus* DSM 25 and *Brevibacillus laterosporus* ATCC9141, respectively. Moreover, laterocidine and brevicidine resemble each other in molecular structure and function; they are cationic NRPs with a hydrophobic acyl chain at the N terminus, positively charged non-canonical amino acid residues in the central part, and a hydrophobic lactone ring at the C terminus (Figure 2A). In this study, we developed a strategy to mimic laterocidine and brevicidine by synthesizing structurally resembling RiPPs by the following strategy: (1) all five D-amino acids were replaced by L-amino acids; (2) Ser, which in laterocidine might perturb the intended ring formation, was replaced by Asn; (3) positively charged amino acid residues D-Orn and L-Orn were replaced by the structurally closest proteinogenic amino acid residue L-Lys; and (4) the C-terminal amino acid was replaced by Cys to enable the formation of a methyllanthionine ring between dehydro-amino acid and Cys. Dha, dehydroalanine; Dhb, dehydrobutyryrle; Abu, α-aminobutyric acid. See also Figure S1.
Plasmids encoding the designed peptides, which were fused to the nisin leader peptide (a leader peptide is needed for the nisin synthetic and transport enzyme recognition; Figure S2 A), were constructed. Lactococcus lactis NZ9000 with pIL3-BTC, harboring the nisB, nisC, and nisT genes, was transformed with the constructed plasmids, respectively (Kuipers et al., 1997; Rink et al., 2005). Subsequently, designed peptides were expressed using the nisin modification and export machinery (Figure S1) and purified after production. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis demonstrated that the main product of RiPep2 (Figure S3 B) was fully dehydrated; however, the main product of RiPep1 was non-dehydrated, and only very little dehydrated product was observed for RiPep1 (Figure S3A). After removal of the leader peptide by the NisA leader protease NisP (Figure S1) (Montalbán-López et al., 2018), the antimicrobial activities of RiPep1 and RiPep2 were evaluated using Gram-negative Xanthomonas campestris (pv. campestris NCCB92058) and Gram-positive Micrococcus flavus as indicator strains. Both of the two designed peptides lacked antimicrobial activity against M. flavus under the experimental conditions used (data not shown). Surprisingly, RiPep2 showed clear antimicrobial activity against X. campestris (Figure S3 C), while RiPep1 showed no antimicrobial activity against X. campestris. Therefore, RiPep2, which mimicked the structure of brevicidine, was selected for further characterization and engineering studies.

An N-Terminal Hydrophobic Tail Increases the Activity of RiPep2

The N-terminal acyl chain, in this case a C10 extension, has been proved to be important for the antibacterial activity of NRPs (Winn et al., 2016). To mimic the N-terminal acyl chain of...
NRPs, we introduced a hydrophobic tail of either one or two isoleucines or a tail composed of isoleucine-alanine-isoleucine, at the N terminus of RiPep2, respectively (Figures 2A and S2B). All of the designed peptides were fully dehydrated as evidenced by MALDI-TOF MS (Figures 2B and S4). After removal of the leader peptide by the NisA leader protease NisP (Figure 3), the antimicrobial activity of RiPep3, RiPep4, and RiPep5 against X. campestris was evaluated by an agar diffusion assay (Figure 4). All three hydrophobic N-terminal tails increased the antibacterial activity of RiPep2. Both RiPep4 and RiPep5 showed strong antimicrobial activity against X. campestris, and both RiPep4 and RiPep5 showed higher antimicrobial activity than RiPep2 and RiPep3 against X. campestris.

RiPep2, RiPep3, RiPep4, and RiPep5 Contain a Methyllanthionine Ring
Liquid chromatography-tandem mass spectrometry (LC-MS/MS) can be used to yield b and y ions for peptides, and the methyllanthionine ring can prevent the fragmentation in the ring. Therefore, LC-MS/MS is widely used to analyze the (methyllanthionine ring information of lantibiotics (van Heel et al., 2016; McCleren et al., 2006; Zhang et al., 2014; Zhao et al., 2020b). To obtain deeper insight into the methyllanthionine ring information of RiPep2, RiPep3, RiPep4, and RiPep5, we applied LC-MS/MS analysis. No fragmentation in the ring was observed, demonstrating that the methyllanthionine rings were correctly formed in all four peptides (Figures 5 and S5).

The Engineered RiPeps Show Antimicrobial Activity against Several Gram-Negative Pathogenic Bacteria
The antibacterial activity of RiPep2, RiPep3, RiPep4, and RiPep5 against X. campestris, and the important human pathogens Acinetobacter baumannii (LMG01041), Escherichia coli (LMG8223), and Pseudomonas aeruginosa (PA01), was evaluated by minimum inhibitory concentration (MIC) assays. Brevicidine was used as the wild-type NRP for benchmarking. MALDI-TOF MS data and LC-MS/MS data of high-performance liquid chromatography (HPLC)-purified brevicidine are shown in Figure S6. The MIC assay results are shown in Table 1. RiPep4 and RiPep5 showed strong antimicrobial activity against X. campestris, i.e., a MIC value of 4 μM. RiPep3, having a less hydrophobic tail, exerted less antimicrobial activity against X. campestris than RiPep4 and RiPep5, i.e., a MIC value of 8 μM. Consistently, RiPep2, lacking any hydrophobic tail, was the least active compound against X. campestris. The RiPeps furthermore showed antimicrobial activity against A. baumannii, E. coli, and P. aeruginosa, again in line with the notion that a hydrophobic tail increases the antimicrobial activity. Overall, RiPep4 and RiPep5 showed only 4-fold less antibacterial activity against X. campestris, 4-fold less antibacterial activity against A. baumannii, 8-fold less antibacterial activity against E. coli, and 16-fold less antibacterial activity against P. aeruginosa than the wild-type NRP brevicidine.

The C-Terminal Ring of RiPep2 Plays a Vital Role in Its Antibacterial Activity
As RiPep1 and RiPep2 have highly conserved amino acid sequences and the ringless RiPep1 showed no activity against X. campestris (Figure S3C), we hypothesized that perhaps the formed C-terminal ring plays a vital role in the antimicrobial activity of RiPep2. To investigate the role of the C-terminal ring of RiPep2, we co-expressed RiPep2 with only NisBT in L. lactis NZ9000, yielding linear Thr9-dehydrated RiPep2 (RiPep2-L) (Khusainov et al., 2011). The antimicrobial activity of RiPep2-L was evaluated by MIC assays. RiPep2-L showed no antimicrobial activity against all tested Gram-negative pathogenic bacteria (Table 1). These results demonstrate that the C-terminal ring of RiPep2 is essential for its antibacterial activity.

RiPep3, RiPep4, and RiPep5 Exhibit the Capacity of Pore Formation, Similar to Brevicidine
Investigating the mode of action of cationic peptides can be challenging. Although brevicidine was first reported in 2018, very little is known about its antimicrobial mode of action. Li et al. (2018b) Li et al. reported that brevicidine could disrupt the morphology of Gram-negative bacteria, likely by LPS binding and pore formation. To investigate the influence of brevicidine and RiPeps on the membrane permeability, we performed a pore-formation assay using the dye propidium iodide, a cell-membrane-impermeable nucleic-acid-intercalating dye. Due to the formation of pores in the cell membrane, this dye enters the cell and binds to nucleic acids in the cytoplasm, causing an increase in fluorescence (Stoddard, 2011; Zhao et al., 2020a). Immediate pore formation was observed when X. campestris cells pretreated with propidium iodide were exposed to polymyxin B (Figure 6), which is known as a membrane that disrupts antimicrobial peptide (Hancock and Wong, 1984), whereas addition of the C-terminally truncated nisin peptide, nisin(1-22), which is known as a non-pore-forming peptide due to the lack of rings D and E and the C-terminal tail of nisin, did not cause any fluorescence increase (Figure 6) (Rink et al., 2007; Zhao et al., 2020a). There was no fluorescence increase observed by addition of RiPep2. In contrast, immediate pore formations were observed by addition of RiPep3, RiPep4, RiPep5, and brevicidine (Figure 6). These results demonstrate that RiPep3, RiPep4, and RiPep5 show an antimicrobial mode of action similar to that of brevicidine, and the N-terminal hydrophobic amino acid residues of RiPep3, RiPep4, and RiPep5 are important for their pore-formation ability, since RiPep2 showed no pore-formation ability.

DISCUSSION
A large number of NRPs have potent antibacterial activity, including some marketed antibiotics such as vancomycin, penicillin, and daptomycin (Sussmuth and Mainz, 2017). NRPs show antibacterial activity against Gram-positive and/or Gram-negative pathogenic bacteria. However, it is not feasible to screen NRP antimicrobials on the basis of genetic libraries because the peptide sequences are not gene encoded (Challis et al., 2000; Reimer et al., 2018; Stachelhaus et al., 1999; Viliers and Holfelder, 2009). Here, we introduce a strategy that circumvents the difficulty in engineering NRP libraries. We mimicked the molecular structure of the NRP brevicidine by ribosomal synthesis and subsequent in vivo post-translational modification. To this end, we employed a dehydration and cyclization enzyme from a lantibiotic biosynthetic system to mimic the brevicidine lactone ring by a lanthionine ring. Along the peptide chain, we replaced positively charged ornithines
by lysines, which are the closest resembling proteinogenic residues. Moreover, a fatty acid tail was mimicked by introducing two or three hydrophobic amino acid residues at the N terminus. Following this strategy, we discovered ribosomally produced compounds with antibacterial activity against Gram-negative pathogenic bacteria.

Figure 3. MALDI-TOF MS Data of HPLC-Purified Peptides (A) RiPep2, (B) RiPep3, (C) RiPep4, and (D) RiPep5.
C-terminal ring is essential for the activities of brevicidine and laterocidine, consistent with previous studies demonstrating that the C-terminal ring is essential for antibacterial activity of RiPeps. These results are consistent with previous studies demonstrating that the methyllanthionine ring was correctly formed in the peptide at the C terminus. In contrast, Thr9 in RiPep2 was fully dehydrated, and the LC-MS/MS analysis indicated that the C-terminal ring of RiPep1 had escaped dehydration and, therefore, lanthionine-ring formation was precluded. This provided a suitable control, since this peptide also lacked any antimicrobial activity, stressing the importance of a ring structure in the peptide at the C terminus. In contrast, Thr9 in RiPep2 was fully dehydrated, and the LC-MS/MS analysis indicated that the C-terminal methyllanthionine ring was correctly formed in RiPep2. Collectively, the results demonstrated that RiPep2 showed antimicrobial activity against Gram-negative bacteria. However, RiPep1 did not show antibacterial activity against the indicator strains used in our study. MALDI-TOF MS (Figure S3A) demonstrated that Thr9 of RiPep1 had escaped dehydration and, therefore, lanthionine-ring formation was precluded. This provided a suitable control, since this peptide also lacked any antimicrobial activity, stressing the importance of a ring structure in the peptide at the C terminus. In contrast, Thr9 in RiPep2 was fully dehydrated, and the LC-MS/MS analysis indicated that the C-terminal methyllanthionine ring was correctly formed in RiPep2. Collectively, the results demonstrate that the methyllanthionine ring mimicked the brevicidine crosslink and that the C-terminal ring is essential for the antibacterial activity of RiPeps. The importance of the C-terminal ring of RiPep2 was further evidenced by a linear Thr9-dehydrated RiPep2. These results are consistent with previous studies demonstrating that the C-terminal ring is essential for the activities of brevicidine and laterocidine (Li et al., 2018b).

The N-terminal acyl chain plays a critical role in the antibacterial activity of NRPs (Winn et al., 2016), which depends, within limits, on the length of the acyl chain (Baltz et al., 2005; Malina and Shai, 2005). In the engineering of the second-generation mimetics, the hydrophobic and aliphatic amino acid Ile was selected to mimic the branched fatty acid chain of brevicidine (Eisenberg, 1984). Because brevicidine only has a 7-carbon acyl chain, tails were designed with up to three hydrophobic residues. Increasing hydrophobicity from RiPep2 to RiPep5 resulted in longer retention times in HPLC traces (Figure S7). Likewise, the antibacterial activity against Gram-negative pathogenic bacteria of RiPep2 increased 4- to 8-fold by the N-terminal hydrophobic residues, which is consistent with previous studies (Baltz et al., 2005; Malina and Shai, 2005; Winn et al., 2016). In addition, the N-terminal hydrophobic moiety was proved to be important for the capacity of pore formation of RiPep3, RiPep4, and RiPep5, which explains the increased antimicrobial activity of RiPep3, RiPep4, and RiPep5. Together, the results clearly demonstrate that mimicking the acyl chain with hydrophobic amino acids contributes to the antibacterial properties of the peptides studied here. In future studies, an N-terminal acyl chain could be chemically introduced to explore whether it will further enhance the antibacterial activity. Moreover, the biosynthetic machinery of a recently discovered RiPP, microvionin, could potentially be used to mimic non-ribosomally produced lipopeptides, because its gene cluster contains genes for the biosynthesis of an N-terminal acyl chain and to connect it to the N terminus of the RiPP (Wiebach et al., 2018).

In this study, L-amino acids were used instead of D-amino acids of the studied NRPs, and Lys residues instead of Orn. Following these designs, the mimicked peptides (Table 1) showed antibacterial activity against Gram-negative pathogenic bacteria. However, D-amino acids have been shown to play an important role on the antibacterial activity and the stability of some NRPs (Guo et al., 2018; Monaim et al., 2018). Peptide epimerases form a class of enzymes that can change L-amino acid residues to D-amino acid residues, including YydG, OspD, and PoyD (Fuchs et al., 2016; Nakano et al., 2019; Parent et al., 2018). Recently, substantial progress has been made in the study of peptide epimerases (Benjdia et al., 2017; Morinaka et al., 2017; Ogasawara, 2019; Vagstad et al., 2019). These increasing achievements will likely make it possible that in the near future D-amino acids can be introduced into various types of RiPPs. Alternatively, some D-amino acids can be introduced into RiPPs by LtnJ-catalyzed hydrogenation of LanB-introduced dehydro-amino acids (Mu et al., 2015).

Recently, a ribosomally synthesized small peptide was found in an NRP synthetic pathway, which serves as a scaffold for NRP extension and chemical modification (Ting et al., 2019). These results suggest that Nature can partially recruit the ribosomal pathway for producing NRPs. In the present study, our results demonstrate an alternative strategy for ribosomal biosynthesis and post-translational modifications of NRP-antimicrobial mimics, which uses an RiPP strategy for the synthesis of NRPs. This may enable the future generation of large genetically encoded peptide libraries of NRP-mimicking structures to screen for antimicrobial activity against relevant pathogens.

In conclusion, our results show that peptides with antibacterial activity against Gram-negative pathogenic bacteria can be successfully obtained by mimicking NRPs by employing RiPP machineries and rational replacement of non-canonical residues. The peptides with activity against Gram-negative bacteria obtained in this study demonstrate that the methyllanthionine ring can be used to functionally mimic the lactone ring in brevicidine. L-amino acid residues can replace D-amino acid residues, at least in some cases, although this might come at a cost of reduced proteolytic stability. Moreover, we demonstrate that...
the acyl chains can be mimicked by adding a series of hydrophobic amino acid residues. Hence, these results justify the conclusion that structural and functional conversion of NRPs to RiPPs is possible and offers great opportunities for engineering a wide range of effective antibiotics.

**SIGNIFICANCE**

Many non-ribosomally produced peptides show potent therapeutic effects on human and animal diseases, particularly in the treatment of bacterial infections. However, the development of variants of such peptides is currently hampered by the difficulty to functionally express and engineer the corresponding large biosynthetic complexes. In this study, we show a strategy that circumvents the difficulty to engineer non-ribosomally produced peptide (NRP) variants. As an example, a recently found NRP, brevicidine, was used as the template for engineering its mimics by RiPP synthetic machinery. The engineered mimics show antimicrobial activities against Gram-negative pathogenic bacteria susceptible to brevicidine, which demonstrates that structural and functional conversion of NRPs to RiPPs is possible. Since the class of NRPs harbors many potent antibiotics, already in therapeutic use, this approach will greatly speed up the development of NRP-based libraries of structural and functional analogs to be screened for antimicrobial activity against human and veterinary bacterial pathogens. High-throughput screening of such libraries
has recently been published, which shows the feasibility of combining the two approaches for antibiotic discovery (Schmitt et al., 2019).

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.chembiol.2020.07.005.

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AUTHOR CONTRIBUTIONS

O.P.K. conceived the project and strategy, supervised the work, and corrected the manuscript. Z.L. conducted part of the antibacterial activity test. 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.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


## STAR METHODS

### KEY RESOURCES TABLE

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<td>oligonucleotide primers</td>
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<td><strong>Experimental Models: Strains</strong></td>
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<td><em>Lactococcus lactis</em> NZ9000 MG1363 derivative; NisRK⁺</td>
<td>(Kuipers et al., 1997)</td>
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<td><strong>Oligonucleotides</strong></td>
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<td>See Table S2</td>
<td>Lab collection or this paper</td>
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<tr>
<td>See Table S3</td>
<td>Biologio B.V.</td>
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</table>

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RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Oscar P. Kuipers (o.p.kuipers@rug.nl)

Materials Availability
All strains and plasmids generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability
This study did not generate any unique datasets or code not available in the manuscript files.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

General Materials and Methods
*L. lactis* NZ9000 was used as host cell for plasmid maintenance and protein expression. Plasmid and strains used in this study is shown in Table S2. Constructed plasmids were sequenced at Macrogen Inc. (Amsterdam, NL). All peptide concentrations were measured by nanodrop under the mode of Mol. Ext. Coeff. with the molecular weights and Ext. coefficient values of peptides (Walker, 2005).

Microbial Strains Used and Growth Conditions
*L. lactis* NZ9000 was used for plasmid construction, plasmid maintenance and protein expression. *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 plasmid selection. For protein expression, stationary-phase cultures were inoculated (20-fold diluted) on minimal expression medium and induced with nisin (5 ng/ml) at an optical density at 600 nm (OD600) of about 0.4.

*A. baumannii*, *E. coli* and *P. aeruginosa* were used as indicator strains for MIC assays. *A. baumannii*, *E. coli* and *P. aeruginosa* were grown in LB medium at 37 °C under shaking (220 rpm).

*X. campestris* was used as indicator strain for agar diffusion assays, MIC assay and propidium iodide assay. *X. campestris* was grown in LB medium at 28 °C under shaking (220 rpm) or cation-adjusted Mueller-Hinton broth containing 1% (w/v) agar at 28 °C.

*Brevibacillus laterosporus* DSM 25 was used to produce brevicidine. *Brevibacillus laterosporus* DSM 25 was grown in LB medium at 37 °C under shaking (220 rpm).

Molecular Biology Techniques
Oligonucleotide primers used for PCR, cloning, and sequencing in this study are provided in Table S3; all of the oligonucleotide primers were purchased from Biologio B.V. (Nijmegen, The Netherlands). Plasmids encoding the peptides were constructed by amplifying template plasmid using a phosphorylated downstream sense (or upstream antisense) primer and an upstream antisense (or downstream sense) primer. Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA) was used to amplify the DNA. Self-ligation of the PCR product 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) (Holo and Nes, 1995).
mutations were verified by sequencing using the PrXZ12 reverse primer. By sequencing, the correct sequence of all plasmids was verified.

METHOD DETAILS

Expression, and Purification of Designed Peptides

*Streptococcus pyogenes* NZ9000, containing the plasmids with the genes of the nisin biosynthetic system and of the designed peptide, was used to inoculate 50 mL of GM17 broth containing erythromycin (5 µg/mL) and chloramphenicol (5 µg/mL). The cultures were grown overnight at 30˚C, and then used to inoculate 1 L (20-fold dilution) of minimal expression medium. After that, cultures were grown at 30˚C to an OD600 of 0.4. Peptide expression was induced by 5 ng/mL nisin, and cultures were grown at 30˚C for overnight. After centrifugation of the overnight expressed cultures, the supernatants were collected and adjusted the pH to 7.2. After that, the cultures were applied to CM Sephadex™ C-25 column (GE Healthcare) equilibrated with MQ. The flow-through was discarded, and the column was subsequently washed with 12 column volumes (CV) of MQ. The peptide was eluted with 6 CV 2 M NaCl. The eluted peptide was then applied to a SIGMA-ALDRICH C18 Silica gel spherically equilibrated with 10 CV of 5% aq. MeCN containing 0.1% trifluoroacetic acid. After washing with a 10 CV of 5% aq. MeCN containing 0.1% trifluoroacetic acid, peptide was eluted from the column using up to 10 CV of 40% aq. 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) containing an appropriate amount of NisP leaderprotease and incubated at 37˚C for 2 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 × 4.6 mm, 3.6 µm particle size, 100 Å pore size). Acetonitrile was used as the mobile phase, and a gradient of 22-35% aq. MeCN over 18 min at 1 mL per min was used for separation. Peptide was eluted at 28-32% MeCN. After lyophilization, peptides were dissolved in sterilized water and stored in -80˚C.

Mass Spectrometry Analysis

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 at University of Groningen. Briefly, a 1 µL sample was spotted on the target, and dried at room temperature. Subsequently, 0.6 µL of matrix solution (5 mg/mL of a-cyano-4-hydroxycinnamic acid) was spotted on each sample. After the samples had dried, MALDI-TOF MS was performed.

LC-MS/MS Analysis

To gain insight into the lanthionine bridging pattern we performed MS/MS. LC-MS was performed using a Q-Exactive mass spectrometer fitted with an Ultimate 3000 UPLC, an ACQUITY BEH C18 column (2.1 × 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 flowrate 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 ion of the compound of interest.

Antibacterial Activity Measurement by Agar Diffusion Assay

To preliminary assess the antibacterial activity of peptides on an agar plate, an overnight cultured of *X. campestris* was added to the cation-adjusted Mueller-Hinton broth containing 1% (w/v) agar (at 42˚C), and then the mixture was poured to the plates, 10 mL per plate. After that, 50 µL of the RiPPs (400 µM) was added into the well of agar plates, and brevicidine at a concentration of 100 µg/mL and sterilized water were added (at the same volume) used as positive and negative control, respectively. The plates were then transferred to the 28˚C incubator for incubation overnight. The relative antimicrobial activity of designed peptides was determined by measuring the zone of inhibition.

Minimum Inhibitory Concentration (MIC) Assay

MIC values were determined by broth micro-dilution according to the standard guidelines (Wiegand et al., 2008). Briefly, the test medium was cation-adjusted Mueller-Hinton broth. Cell concentration was adjusted to approximately 5 × 10⁸ 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. Experiments were performed with biological replicates.

Propidium Iodide Assay

*X. campestris* Culture was pelleted at 4000 x g for 5 min and washed three times in 10 mM HEPES with 10 mM glucose (pH 7.2]). After that, cell density was normalized to an OD600 of 0.2, loaded with propidium iodide at a finial concentration of 2.5 µg per mL, and incubated at 37˚C for 2 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 × 4.6 mm, 3.6 µm particle size, 100 Å pore size). Acetonitrile was used as the mobile phase, and a gradient of 22-35% aq. MeCN over 18 min at 1 mL per min was used for separation. Peptide was eluted at 28-32% MeCN. After lyophilization, peptides were dissolved in sterilized water and stored in -80˚C. The expression levels for brevicidine was 6 mg/L.

**Cell Chemical Biology**

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incubated for 5 min in the dark. After incubation, peptides were added to a final concentration of 10 μM. Fluorescence was monitored for 10 min, with the antibiotics added after ~60 s. The excitation and emission wavelengths on the fluorescence spectrometer (Varioskan™ LUX multimode microplate reader, Thermo Fisher) were 533 nm and 617 nm, respectively. Experiments were performed in triplicate, and data are represented as mean values.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

GraphPad Prism 8.0 was used to fit the data of propidium iodide assay, in Figure 6, experiments were conducted in triplicate, and data are represented as the mean value of triplicate experiments.