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Effect of Ester to Amide or *N*-methyl Amide Substitution on Bacterial Membrane Depolarization and Antibacterial Activity of Novel Cyclic Lipopeptides

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Abstract

Cyclic lipopeptides derived from the fusaricidin/LI-F family of naturally occurring antibiotics represent particularly attractive candidates for the development of new antibacterial agents. In comparison to natural products, these derivatives may offer better stability under physiologically relevant conditions and lower nonspecific toxicity, while preserving their antibacterial activity. In this study we have assessed the ability of cyclic lipodepsipeptide **1**, and its amide **2**, *N*-methyl amide **3** and linear peptide **4** analogues to interact with the cytoplasmic membranes of selected Gram-positive bacteria, as well as their bacteriostatic/bactericidal modes of action and in vivo potency using a *Galleria mellonella* model of MRSA infection. Cyclic lipopeptides **1** and **2** depolarize the cytoplasmic membranes of Gram-positive bacteria in a concentration-dependent manner. The degree of membrane depolarization was influenced by the structural and physical properties of **1** and **2**, with more flexible and hydrophobic peptide **1** being most efficient. However, membrane depolarization does not correlate with bacterial cell lethality, suggesting that membrane-targeting activity is not the main mode of action for this class of antibacterial peptides. Conversely, substitution of the depsipeptide bond in **1** with an *N*-methyl amide bond **3**, or its hydrolysis **4**, lead to a complete loss of antibacterial activity, and indicate that the conformation of cyclic lipopeptides plays a role in their antibacterial activities. Cyclic lipopeptides **1** and **2** are also capable of improving the survival of *G. mellonella* larvae infected with MRSA with different efficiencies reflecting their in vitro activities. Gaining more insights into the structure-activity-relationship and mode of action of these cyclic lipopeptides may enable the development of new antibiotics of this class with improved antibacterial activity.

Keywords

antibiotics; depsipeptide; biological activity; membranes; isosteric analogues

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Supporting information for this article is available on the WWW under <http://www.chemmedchem.org> or from the author

Introduction

Fusaricidins or LI-Fs are a family of cyclic lipodepsipeptide antibiotics isolated from *Paenibacillus* sp.^[1-4] These natural products are structurally unrelated to existing drugs and are active against Gram-positive bacteria with MICs ranging from 0.78-3.12 $\mu\text{g mL}^{-1}$.^[1-2] Analysis of the *Bacillus subtilis* transcriptome upon treatment with a mixture of fusaricidin natural products showed induction of sets of genes similar to those induced by membrane active antibiotics.^[5] Structural modifications of these natural products that include incorporation of a somewhat simpler lipidic tail, and substitution of an ester bond with an amide bond, resulted in comparably potent analogues with improved stability and greatly decreased nonspecific cytotoxicity.^[6] However, the precise mode of action of fusaricidins/LI-Fs natural products and their analogs is yet to be determined.

Cationic antimicrobial peptides (CAMPs) initiate their antibacterial activity through electrostatic interaction with negatively charged phospholipids in bacterial membranes, leading to formation of channels or pores that cause leakage of intracellular components and, ultimately, cell death.^[7-10] However, for some CAMPs, their interaction with bacterial membranes is an intermediate step, leading to cytoplasmic uptake where peptides reach their true antimicrobial target(s).^[8, 11] The extent of CAMP interaction with bacterial membranes depends on multiple factors, including conformational flexibility, overall charge, hydrophobicity and amphiphilicity of the peptides.^[9, 12] Interestingly, anionic molecules, such as the cyclic lipodepsipeptide antibiotic daptomycin, can also interact with negatively charged bacterial cytoplasmic membranes. Daptomycin interacts with bacterial membranes in a calcium dependent manner to initiate its antibacterial activity.^[13-17] Daptomycin is one of our last-line of defense antibiotics for the treatment of severe infections caused by resistant Gram-positive bacteria.^[18] However, the utility of this drug has been somewhat limited due to the emergence of bacterial strains displaying reduced susceptibility.^[19-21] Changes in phospholipid composition, leading to a reduction in the negative charge of membranes, and, consequently, to reduced affinity for daptomycin, have been associated with resistance to daptomycin.^[21-23] Antibacterial agents with different modes of action from daptomycin could be promising candidates against daptomycin-nonsusceptible strains.

In contrast to typical CAMPs or daptomycin, fusaricidin/LI-F natural products^[1-4] and their synthetic derivatives^[6] possess a neutral amino acid sequence and a single positive charge located at the termini of their lipidic tails. These structural differences also suggest a different mode of interaction with bacterial cytoplasmic membranes as an initial step of antibacterial action. A better understanding of the mode of antibacterial action of this class of cyclic lipopeptides may provide additional rationale for their structural modification and optimization for further development.

Herein, we report our studies on the interaction of cyclic lipopeptides **1-3** and linear peptide **4**, derived from fusaricidin/LIF natural products, with the cytoplasmic membranes of selected Gram-positive bacteria, as well as the assessment of their bacteriostatic/bactericidal modes of action, and *in vivo* potency using a *Galleria mellonella* model of *S. aureus* (MRSA) infection.

Results

Synthesis of cyclic lipopeptides

The amino acid sequences and head-to-tail bond of synthesized cyclic lipopeptides **1-3** are shown in Figure 1. An efficient Fmoc solid-phase synthesis was developed that involved resin attachment of the first amino acid *via* a side chain, successful use of combination of four quasi-orthogonal removable protecting groups, stepwise solid-phase synthesis of linear peptide analogues, lipidic tail attachment followed by terminal amino acid coupling *via* ester **1** or amide **2** bond and on-resin head-to-tail macrolactamization.^[6] In comparison to depsipeptide **1**, amide **2** exhibits comparable antibacterial activity, with improved stability and decreased human cell cytotoxicity.

To further probe the effect of substituting the ester bond for an isosteric equivalent, we synthesized an *N*-methylated cyclic lipopeptide **3** possessing *N*^β-methyl-Dap¹ instead of Thr¹. The linear precursor of **3** was synthesized using the same solid-phase strategy as described for **1** and **2**. The Fukuyama–Mitsunobu alkylation procedure was used to introduce methyl substituent to β-amino group of Dap¹, Scheme 1.^[24-25] In the case of **3**, however, the amino group at the end of the lipidic tail was converted into the corresponding guanidine before methylation of *N*^β-Dap¹. This was necessary because Fmoc protection of the lipidic tail's amino group is cleaved under the basic conditions required for removal of nosyl group (2-nitrobenzenesulfonyl, *o*-NBS), which served as a temporary protection to ensure monomethylation of the Dap¹ β-amino group.^[24-25] Following the *N*-methylation reaction, the *o*-NBS group was removed using DBU and 2-mercaptoethanol.^[26] Although *N*-methylation changes the stereoelectronic properties of the amino group, subsequent coupling of Alloc-D-Ala-OH was successfully accomplished using the HOAt/HATU/NMP coupling procedure.^[27] Protocols for the removal of Allyl/Alloc protecting groups, head-to-tail cyclization and *N*-methylated cyclic lipopeptide **3** cleavage from the resin were identical to those used for synthesis of cyclic lipopeptide **2**.^[6]

All peptides were purified using preparative RP HPLC, with purity (>98%) confirmed by analytical RP HPLC and MALDI-TOF MS.

Conformational analysis of cyclic lipopeptides

Our previous conformational studies revealed that depsipeptide **1** can adopt different conformations in polar and nonpolar environments, whereas conformational differences for amide analogue **2** under the same conditions are less pronounced, suggesting a more rigid peptide backbone structure.^[6] Since *N*-methylation facilitates formation of the *cis*-peptide bond, and reduces the number of intramolecular hydrogen bonds,^[28] we expected that substitution of an ester or amide bond in cyclic lipopeptides **1** and **2** with an *N*-methyl amide bond would induce significant conformational changes. The structural features of cyclic lipopeptides **1-3** were monitored by circular dichroism (CD) spectroscopy, Figure 2. CD spectra were recorded in aqueous medium, and in less polar tri-fluoroethanol (TFE); a membrane-mimicking solvent.^[29] Although complete interpretation of the cyclic lipopeptides **1-3** CD spectra is difficult due to the presence of multiple conformers, the obtained spectra clearly show that substitution of the depsipeptide bond in **1** with an amide

bond, analogue **2**, or with *N*-methyl amide, analogue **3**, induces relatively large conformational changes in the cyclic lipopeptide backbone. In addition, *N*-methyl amide analogue **3** exhibits significant conformational rigidity, as indicated by small differences in its CD spectra recorded in water and TFE.

Assessment of cyclic lipopeptides interaction with the cytoplasmic membrane of Gram-positive bacteria

The ability of fusaricidin/LI-F type cyclic lipopeptides to destabilize the membrane of Gram-positive bacteria was assessed by evaluating their effect on membrane potential using DiSC₃(5) (a membrane potential-sensitive fluorescent dye), Figure 3. The dye loses fluorescence intensity in polarized membranes; however, it becomes highly fluorescent once released into the media.^[30] The membrane depolarization experiments were performed with three different species of staphylococci *Staphylococcus aureus* Wichita (ATCC 29213), *Staphylococcus aureus* Mu50 (ATCC 700699), and *Staphylococcus epidermidis* GA (ATCC 27626) using cyclic lipopeptides **1-3**, linear lipopeptide **4** and a 1% Triton X-100 control (maximal fluorescence). The *S. aureus* Wichita and *S. epidermidis* GA strains differ in bacterial membrane lipid composition, whereas *S. aureus* Mu50 has an identical membrane composition to *S. aureus* Wichita strain but a significantly thicker peptidoglycan layer^[31-32] Both, depsipeptide **1** and amide **2** were active against all bacterial strains used in this study, with MIC values ranging from 8-16 µg mL⁻¹.^[6]

The antibacterial activity of these peptides appears to be salt and serum independent. The presence of physiological concentrations of CaCl₂ (2 mM), MgCl₂ (1 mM) and NaCl (150 mM),^[33] and the presence of 25% human serum in MIC assays did not affect the antibacterial activities of **1** and **2** (Supporting information). Under the same experimental conditions, at concentration up to 128 µg mL⁻¹, neither linear lipopeptide **4** nor *N*-methyl amide **3** showed antibacterial activity. In addition, the antibacterial activities of **1** and **2** remain unchanged in the presence of 100 mM KCl; a concentration of KCl required for DiSC₃(5) assays to balance the chemical potential of K⁺ ions outside and inside the cells (Supporting information).^[30] The DiSC₃(5) assays were carried out at two different temperatures, 25 °C and 37 °C, to assess the effect of bacterial growth and metabolic activity on the ability of cyclic lipopeptides **1-3** to depolarize Gram-positive bacterial membranes. As shown in Figure 3, depsipeptide **1** and amide **2** depolarized the cytoplasmic membrane of all three Gram-positive bacteria tested in a concentration-dependent manner.

Overall, the more flexible and hydrophobic depsipeptide **1** showed faster membrane depolarization in comparison to its amide analogue **2**. At 48 µg mL⁻¹ maximum depolarization for depsipeptide **1** was observed within 10 min of addition, whereas amide **2** required >30 min to yield the same effect. Interestingly, low or no depolarization was observed at MIC for both cyclic lipopeptides, Figure 3.

No significant differences were observed in depolarization of *S. aureus* Wichita and *S. aureus* Mu50 membranes by depsipeptide **1** and amide **2**, Figures 3A and 3B. On the other hand, the higher content of negatively charged phospholipids in bacterial cytoplasmic membranes, as with *S. epidermidis* GA, has a much greater effect on the rate and level of

depolarization by **1** or **2**, Figure 3. In the case of *S. epidermidis*, membrane depolarization induced by depsipeptide **1** at $48 \mu\text{g mL}^{-1}$ reached maximum levels within 5 min. At the same concentration, a much longer time (>20 min) was required for the amide **2** to induce maximum depolarization. Similar to our previous observations, at concentrations corresponding to MIC, cyclic lipopeptides **1** and **2** induced low or no depolarization of *S. epidermidis* cytoplasmic membranes, Figure 3. Temperature was not observed to have an effect on the ability of **1** and **2** to depolarize bacterial membranes, as evidenced by almost identical depolarization results obtained at 25 °C and 37 °C (see Figure 3 and Supporting information). *N*-methyl amide **3** and linear peptide **4**, which were not active in broth-microdilution antibacterial assays, did not depolarize bacterial membranes under the same experimental conditions, even at the highest tested concentration of $48 \mu\text{g mL}^{-1}$.

To evaluate whether the depolarization of Gram-positive cytoplasmic membranes is directly correlated to the ability of cyclic lipodepsipeptide **1** and its amide analogue **2** to kill bacteria,^[17] we monitored bacterial survival using conditions required for the DiSC₃(5) assay. In these experiments bacteria were treated with $48 \mu\text{g mL}^{-1}$ of either depsipeptide **1** or amide **2**, with untreated bacteria serving as controls. At 10 min intervals over the course of 1 h, aliquots of each bacterial suspension were plated onto nutrient agar. Agar plates were incubated for 24 h at 37 °C, and the resulting viable colonies were counted, and calculated as $\log(\text{CFU mL}^{-1})$. Different results were obtained depending on the peptide and the temperature of the DiSC₃(5) assay. When the assay was carried out at 25 °C, neither the depsipeptide **1** nor the amide **2** caused a reduction in the number of *S. aureus* Wichita colonies over the course of the experiment, Figures 4A. Approximately 2-logs decrease in the number of *S. aureus* Mu50 viable colonies was observed for depsipeptide **1**, and no reduction in bacterial colonies was observed for **2**, Figure 4B. In the case of *S. epidermidis* GA, significantly different activities of **1** and **2** were observed, Figure 4C. Depsipeptide **1** reduced the number of viable colonies by approximately 3-logs, whereas amide **2** had no effect on bacterial survival. If the membrane depolarization assays were performed at 37 °C, Figure 4, the depsipeptide **1** caused a decrease of 4 logs in viable colonies for all three tested bacterial strains. In contrast, increasing the temperature of the DiSC₃(5) assay to 37°C did not affect the activity of amide **2**, Figure 4.

Characterization of the antibacterial activity of cyclic lipopeptides **1** and **2** using time kill assays

The time-kill studies were performed to evaluate the *in vitro* activities of cyclic lipopeptides **1** and **2** at $48 \mu\text{g mL}^{-1}$ against *S. aureus* Wichita, *S. aureus* Mu50 and *S. epidermidis* GA. Time-kill curves were plotted graphically as $\log(\text{CFU mL}^{-1})$ versus time, Figure 5.

The depsipeptide **1** showed bactericidal activity against all three tested Gram-positive bacteria, with the kinetics of killing proving similar for all strains; very fast killing within the first hour with a 3-log drop in the number of viable colonies.^[34] In contrast, under the same experimental conditions, amide **2** exhibited bacteriostatic activity. No significant change in viable colony counts was observed for all three tested Gram-positive bacteria upon incubation with **2**, Figure 5. The time-kill studies were performed at medium- and high

density of bacterial colonies, and both cyclic lipopeptides **1** and **2** were able to tolerate high inoculum, up to 1×10^9 CFU mL⁻¹.

Assessment of cyclic lipopeptides **1** and **2** *in vivo* efficacy in a *Galleria mellonella* model of systemic MRSA infection

The *in vivo* efficacy of depsipeptide **1** and amide **2** was assessed in a wax worm (*Galleria mellonella*) model of systemic *S. aureus* infection, Figure 6. The wax worm model has been widely used as a preliminary model for assessing the *in vivo* efficacy of antibacterial agents before proceeding to mammalian models.^[35] Depsipeptide **1** and amide **2** exhibit different efficacies when administrated to larvae one day after *S. aureus* USA100 (MRSA) inoculation, Figure 6. A single dose of depsipeptide **1** at 5×MIC greatly improves survival of larvae infected with MRSA. In contrast, amide **2** under the same experimental conditions had a modest effect on larvae survival.

Discussion

The structures of cyclic lipopeptides **1-3** and the control linear peptide **4** used in this study are shown in Figure 1. The main distinguishing structural characteristic of cyclic lipopeptides **1-3** is the absence of the depsipeptide (ester) bond in analogs **2** and **3**. Linear peptide **4** represents an analogue with a hydrolyzed depsipeptide bond. As demonstrated previously, substitution of an ester bond in depsipeptides with an amide bond results in significant alteration of the chemical and biological properties of this class of cyclic lipopeptides.^[6] In the case of amide **2**, this includes improved stability under physiologically relevant conditions, lower overall hydrophobicity/amphiphilicity, reduced conformational flexibility and reduced nonspecific toxicity towards human cells. Similarly, the presence of *N*-methylated amino acids in cyclic peptide sequences has a significant impact on their proteolytic stability, membrane permeability, and conformation.^[28, 36-37] Therefore, *N*-methyl amide analog **3** was/is expected to exhibit different antibacterial activity from **1** and **2**.

Positive charge, conformational flexibility and hydrophobicity have previously been reported as the major factors affecting interaction of CAMPs with anionic lipids^[9, 12, 38-40]. In addition, the phospholipid composition of bacterial cytoplasmic membranes is not uniform, and these differences may also determine the response of bacteria to antibacterial agents,^[38-39] including cyclic lipopeptides **1-3**. Considering this, we found it of particular interest to examine the interaction of cyclic lipopeptides **1-3** with Gram-positive bacterial cells.

Both, depsipeptide **1** and amide **2** in a DiSC₃(5) assay show concentration-dependent depolarization of *S. aureus* and *S. epidermidis* cytoplasmic membranes, Figure 3. For both cyclic lipopeptides **1** and **2**, a low level of depolarization was observed at MIC, whereas maximum depolarization was reached at much higher concentrations. In comparison to amide **2**, the observed faster membrane depolarization by **1** may be explained by its greater hydrophobicity, facilitating spontaneous insertion into membranes, and its conformational flexibility allowing for better adaptability to the membrane.^[6, 38] However, substitution of depsipeptide bond in **1** with an isosteric *N*-methylamide bond, analogue **3**, resulted in a

complete loss of the peptide's ability to interact with bacterial membranes, and loss of its antibacterial activity (see Supporting information). Conformational restraints induced by the incorporation of *N*^β-methyl-Dap¹ into the cyclic peptide sequence may enhance the population of inactive conformers resulting in a loss of biological activity for cyclic peptide **3**. Opening the peptide's ring by hydrolysis of the ester bond, as illustrated with linear peptide **4**, and allowing for more structural flexibility, also leads to a complete loss of antibacterial activity.

Although thick bacterial cell walls may serve as a physical barrier against the penetration of antibacterial agents,^[31] this is not the case for cyclic lipopeptides **1** and **2**. As shown in Figure 3B, both peptides were able to reach cytoplasmic membrane in *S. aureus* Mu 50 strain and cause efficient depolarization. The composition of bacterial membranes had a much greater effect on the interaction of cyclic lipopeptides **1** and **2** with Gram-positive cells. The cytoplasmic membrane of *S. epidermidis* GA possesses a higher percentage of anionic phosphatidylglycerol in comparison to that of *S. aureus*. Consequently, more efficient membrane depolarization of *S. epidermidis* GA by both **1** and **2** was observed, Figure 3. On the other hand, temperature did not have an effect on depolarization by **1** or **2**, as almost identical depolarization results, within the range of experimental error, were obtained at 25 °C and 37 °C (Figure 3 and Supplemental material). Quite interestingly, neither depsipeptide **1** nor its amide analogue **2** require the presence of divalent cations for activity in our DiSC₃(5) and MIC assays. Since Ca²⁺ is necessary for the activity of daptomycin, these data suggest that cyclic lipopeptides **1** and **2** exhibit different modes of action from that of daptomycin.

It has been reported previously that protein binding may affect the activity of antibacterial agents.^[41-44] Therefore, the assessment of *in vitro* antibacterial activity in the presence of human serum may be a better indicator of *in vivo* potential for antibacterial agents.^[45] To assess such an effect on the antibacterial activity of **1** and **2** we performed MIC assays in the presence of 25% human serum and determined that *in vitro* antibacterial activity was not altered by the presence human serum.

Despite the almost identical sequences between depsipeptide **1** and amide **2**, these two cyclic lipopeptides exhibit different modes of antibacterial action. Time-kill assays, following the membrane depolarization studies, showed a lack of correlation between depolarization and the ability of depsipeptide **1** and amide **2** to kill bacteria. In these assays amide **2**, at concentrations of 48 μgmL⁻¹, exhibited bacteriostatic activity against all three tested organisms, regardless of their membrane composition and metabolic activity. This was not the case, however, for depsipeptide **1**, Figure 4. At the same concentration, depsipeptide **1** is bacteriostatic against *S. aureus* strains but bactericidal against *S. epidermidis* GA at 25°C, whereas at 37°C depsipeptide **1** exhibits bactericidal activity against all tested bacteria. The bactericidal activity of **1** and bacteriostatic activity of **2** were confirmed by time-kill assays, in which bacterial survival was monitored over a period of 18 h, Figure 5. Taken together, all of these data indicate that the *in vitro* antibacterial activity of **1** and **2** requires bacterial cell division or active metabolism, and suggest that the mechanism of action for these cyclic lipopeptides involves some event other than disruption of the cytoplasmic membrane.

Both, depsipeptide **1** and amide **2** were shown to be protective in a wax worm larvae (*Galleria mellonella*) model of systemic methicillin-resistant *S. aureus* (MRSA) infection, Figure 6. However, MRSA-infected larvae treated with depsipeptide **1** had a significantly greater percentage survival compared with the larvae treated with amide **2**. This observation is consistent with the data from *in vitro* antibacterial assays indicating better efficiency of **1**.

Conclusion

In summary, we have demonstrated that cyclic lipopeptides that belong to the fusaricidin/LI-F family of antimicrobial peptides are capable of depolarizing the cytoplasmic membrane of Gram-positive bacteria. Bacterial membrane composition as well as the structural and physical properties of **1-4**, such as conformation and hydrophobicity, have a major effect on their ability to depolarize bacterial membranes. Among the synthesized fusaricidin/LI-F analogs, depsipeptide **1** and amide **2** depolarize cytoplasmic membranes of Gram-positive bacteria in a concentration-dependent manner. More flexible and hydrophobic cyclic lipodepsipeptide **1** more efficiently depolarizes bacterial membranes. However, our data suggests that the interaction with bacterial membranes is likely an intermediate step in the uptake of these peptides into the cytoplasm where they exert their antibacterial activity. Conversely, substitution of the depsipeptide bond with an *N*-methyl bond drives cyclic lipopeptides into inactive conformations, as suggested by the inability of analog **3** to depolarize bacterial cytoplasmic membranes, and a lack of activity in MIC assays. Hydrolysis of the depsipeptide bond also leads to a loss of antibacterial activity.

Although **1** and **2** belong to the same class of cyclic lipopeptide antibacterial agents, they exhibit significantly different modes of action. Under optimal conditions for bacterial growth, depsipeptide **1** showed bactericidal activity against all tested strains, whereas its amide counterpart **2** was bacteriostatic. Both cyclic lipopeptides **1** and **2** improved survival of wax worm larvae infected with MRSA, with cyclic lipodepsipeptide **1** being most efficient.

The unique structure and reported potent antibacterial activities of the fusaricidin/LI-F class of cyclic lipopeptides such as **1** and **2** make them an attractive source of lead compounds for the development of new antibacterial agents; capable of treating infections caused by multidrug-resistant Gram-positive pathogens. Studies are currently underway to determine the exact mechanisms of antibacterial action for the depsipeptide and amide analogues.

Experimental Section

Chemicals and instrumentation

TentaGel S RAM resin was obtained from Advanced ChemTech (Louisville, KY, USA). 2-Chlorotriethyl chloride was obtained from Novabiochem (Gibbstown, NJ, USA). Fmoc-protected amino acids and coupling reagents (HOBt, HBTU, PyBOP) were purchased from Chem-Impex (Wood Dale, IL, USA) or Novabiochem. DIC was purchased from Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA). DMAP and DiSC₃(5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals, solvents, and buffers were purchased from Sigma-Aldrich or Fisher Scientific (Atlanta, GA) and were

HPLC grade or better. Linear peptidyl-resin precursors were synthesized on a PS3 automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA). Mass spectrometry was performed on MALDITOF Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) in reflector mode using α -cyano-4-hydroxycinnamic acid as a matrix and positive mode. Analytical RP HPLC analyses and peptide purifications were performed on 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) liquid chromatography systems equipped with a UV/Vis detector. For analytical RP HPLC analysis, a C18 monomeric column (Grace Vydac, 250×4.6 mm, 5 mm, 120 Å), 1 mLmin⁻¹ flow rate, and elution method with a linear gradient of 2→98% B over 30 min, where A is 0.1% TFA in H₂O, and B is 0.08% TFA in CH₃CN was used. For peptide purification, a preparative C18 monomeric column (Grace Vydac, 250×22 mm, 10 mm, 120 Å) was used. The elution method was identical to the analytical method except for the flow rate, which was 19 mLmin⁻¹. CD spectra were recorded on a JASCO 810 spectropolarimeter (Easton, MD, USA) using a quartz cell of 0.1 mm optical path length. Spectra were measured over a wavelength range of 180–250 nm with an instrument scanning speed 200 nmmin⁻¹ and a response time of 1 s. The concentrations of peptides were 0.1–0.2 mM. Bacterial strains used in this study; *Staphylococcus aureus* Wichita ATCC 29213, *S. aureus* Mu50 ATCC 700699, and *Staphylococcus epidermidis* ATCC 27626, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). For the wax worm tests, a clinical isolate, MRSA CBD-635 (USA100) was used.^[46] Media and agar were purchased from BD (Franklin Lakes, NJ). Concentrations of peptides in all experiments were determined using analytical RP HPLC and calibration curves based on the depsipeptide **1**. The peptide content of **1** was determined by quantitative amino acid analysis to be 62.32 %.

Peptide synthesis

Cyclic lipodepsipeptide **1**, its amide analogue **2** and control linear peptide **4**, Figure 1, were synthesized using standard Fmoc-SPPS methodology as reported previously.^[6, 47–48] *N*-methylamide analogue **3** was synthesized on amide TentaGel S RAM resin (substitution 0.26 mmol g⁻¹, 0.25 mmol scale) using the same strategy as described for **2**, with the exception that the amino group at the end of the lipidic tail was converted into a guanidine moiety before *N*-methylation reaction, Scheme 1. *N*-methylation of Dap¹ was completed as described previously.^[24, 49] In brief, after *N*-terminal Mtt protecting group removal under mild acidic conditions (1% TFA in DCM, 30 min), the resulting deprotected peptidyl-resin was washed with DCM (3×1 min) and NMP (3×1 min). A solution of *o*-NBS-Cl (4 eq) and collidine (10 equiv) in NMP (2 mL) was added to the peptidyl-resin, and the reaction mixture was shaken for 15 min at room temperature. The peptidyl-resin was washed with NMP (3×1 min) and the previous step was repeated. Next, the *o*-NBS protected peptidyl-resin was washed with NMP (3×1 min) and anhydrous THF (3×1 min), and a solution of PPh₃ (5 equiv), MeOH (10 equiv) and DIAD (5 equiv) in THF (1 mL) was added portion-wise over 50 min (200 μ L aliquots were added every 10 min). The peptidyl-resin was washed with THF (5×1 min) and the *o*-NBS protection was removed by adding a solution of 2-mercaptoethanol (10 equiv) and DBU (5 equiv) in NMP, and shaking the peptidyl-resin for 5 min at room temperature. The peptidyl-resin was repeatedly treated with the deprotection mixture until the release of yellow color was no longer observed (cca 30 min).^[25] Total time required for the complete removal of *o*-NBS was 30 min. The Alloc-D-

Ala-OH was coupled using 3 equiv excess of this amino acid and HOAt/HATU/DIEA coupling protocol. The removal of Allyl/Alloc protecting groups and cyclization were performed as described previously.^[6] Efficiency of *N*-methylation and Alloc-D-Ala-OH coupling was assessed by RP HPLC and by MALDI-TOF MS (Supporting information). In all cases peptides were removed from the resin using TFA:thioanisole:H₂O (95:2.5:2.5 v/v/v) for 3 h. Crude peptides were precipitated with cold methyl *tert*-butyl ether, and purified by preparative RP HPLC. HPLC fractions were analyzed for purity, combined and lyophilized to give a white powder. The final purity of synthesized peptides was confirmed by analytical RP HPLC and MALDI-TOF MS, and was 98% in all cases (ref. [6] and Supporting information). The yields for all peptides were 40-60% after RP HPLC purification.

Minimal inhibitory concentration determination

Minimal inhibitory concentrations (MICs) were determined by 2-fold serial dilution of peptides **1-4** in sterile 96-well plates containing Mueller-Hinton broth (MHB) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.^[50] To determine the effect of physiological salt concentration and the presence of human serum on the antibacterial activity of **1** and **2**, MICs were also determined in MHB containing 2 mM Ca²⁺, 1 mM Mg²⁺, 150 mM Na⁺ and 25% human serum (Supporting information).^[33]

Membrane depolarization assay

Cytoplasmic membrane depolarization was assessed using membrane potential sensitive cyanine dye DiSC₃(5)^[51] as described previously.^[30, 51-52] Stocks of microorganisms maintained at -80 °C in 15% glycerol were thawed and grown at 37 °C and 220 rpm in medium recommended by ATCC. The following day, an aliquot of the bacterial suspension was transferred into fresh medium and incubated for 4-5 h until OD₆₀₀ of the suspension reached 0.3-0.4. Following centrifugation at 2800 rpm for 10 min, cells were washed one time with HEPES (5 mM) buffer supplemented with 5 mM glucose (pH 7.2), and resuspended in HEPES buffer supplemented with 5mM glucose and 100 mM KCl (pH 7.2) to a final OD₆₀₀=0.5 (approximately CFU mL⁻¹).^[30] The DiSC₃(5) dye was then added to bacterial suspensions at a final concentration of 0.4 μM. Aliquots (100 μL) of the bacterial suspension were placed in a 96-well non-culture treated white flat-bottomed plate and incubated until the intensity of the fluorescence signal stabilized (up to 30 min). The fluorescence signal was monitored using a Synergy H4 microplate reader (BioTek, Winooski, VT) with an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Once the fluorescence intensity stabilized, 100 μL of solutions containing peptides, at concentrations of 8, 12, 16, 24, 32 and 48 μg mL⁻¹, were added to the wells and the fluorescence was monitored at 2 min intervals over 60 min. Untreated bacteria were used as a control. The level of depolarization was expressed as a percentage of the maximum fluorescence caused by 1% Triton X-100. To evaluate the potential influence of temperature on the outcome of experiments, membrane depolarization assays were performed at 25 °C and at 37 °C. All measurements were performed in triplicate and repeated twice.

In order to correlate bacterial survival and membrane depolarization, viable bacteria counts were determined in parallel with the DiSC₃(5) assay. In brief, aliquots of bacterial

suspensions treated with peptides **1** and **2** ($48 \mu\text{g mL}^{-1}$) were taken out of the microtiter plate wells at 10 min intervals and, following proper dilutions, plated on nutrient agar. After 24 h incubation at 37°C , colonies were counted to determine the CFU mL⁻¹. Untreated bacteria were used as a control.

CD spectra

All CD spectra were recorded on a JASCO 810 spectropolarimeter at 25°C using a 0.1 cm path length cell. Spectra were acquired in the range 180–250 nm, 1 nm bandwidth, four accumulations and 200 nm min^{-1} scanning speed. All spectra were obtained using 0.1–0.2 mM concentrations in 0.5% AcOH in H₂O, and 100% TFE solution. Each experiment was repeated at least once and at various concentrations. No concentration-dependent CD spectral changes were observed.

Time-kill assays

The time-kill assays were performed according to CLSI guidelines.^[34] Bacteria (10^6 CFU mL⁻¹) were incubated for 18 h in the presence of $48 \mu\text{g mL}^{-1}$ of cyclic lipopeptides **1** and **2**. At various time points (0, 0.5, 1, 2, 4, 8 and 18 h) aliquots of the bacterial suspension were taken and, following proper dilutions, plated on nutrient agar, and incubated for 24 h at 37°C . Untreated bacteria were used as the control.

Treatment of *S. aureus*-infected wax worm larvae with cyclic lipopeptides **1** and **2**

To determine the *in vivo* efficacy of peptides a *G. mellonella* of *S. aureus* infection was used as previously described.^[53] Briefly, 1 mL aliquots of overnight cultures of *S. aureus* USA100 were pelleted by centrifugation and washed in sterile PBS, before being resuspended in 100 μL of PBS. *G. mellonella* larvae (N=10) that weighed 200–300 mg were then inoculated with 5 μL of *S. aureus* (1×10^9 CFU) into the last left proleg. Larvae were then treated with **1** or **2** at 1 hour post-inoculation. Treatments were performed in the same manner as infection, except that injections were into the next left pro-leg moving towards the head of the larvae. Larvae were then incubated at 37°C and mortality rates were monitored for 78 h; larvae were considered dead if they did not respond to physical stimuli. Data was analyzed for statistical significance using a Log Rank and Chi Squared Test with 1-degree of freedom.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AcOH	acetic acid
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Alloc	allyloxycarbonyl
12-ADA	12-aminododecanoic acid
CD	circular dichroism
CFU	colony-forming unit
Dap	diaminopropionic acid
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DIC	diisopropylcarbodiimide
DIEA	diisopropylethyl amine
DiSC₃(5)	3-propyl-2-[5-[3-propyl-2(3 <i>H</i>)-benzothiazolylidene]-1,3-pentadienyl]benzothiazolium iodide
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DMSO	dimethyl sulfoxide
Fmoc	fluorenylmethyloxycarbonyl
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HOBt	N-hydroxybenzotriazole
HBUTU	2-(1 <i>H</i> -benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MeOH	methanol
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant <i>S. aureus</i>
Mtt	4-methyltrityl chloride
<i>o</i>-NBS-Cl	2-nitrobenzenesulfonyl chloride
NMM	N-methylmorpholine
NMP	1-methyl-2-pyrrolidinone
PPh₃	triphenylphosphine
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RP HPLC	reversed-phase high-performance liquid chromatography

SPPS	solid-phase peptide synthesis
TIA	thioanisole
TFA	trifluoroacetic acid
TFE	trifluoroethanol

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Figure 2.
CD spectra of **1** (...), **2** (---) and **3** (—) in a) 0.5% AcOH and b) 100% TFE.

Figure 3. Time- and concentration-dependent membrane depolarization of a) *S. aureus* Wichita; b) *S. aureus* Mu50; c) *S. epidermidis* GA with cyclic lipopeptides **1** and **2** performed at 25°C. Concentrations of peptides were 48 (•), 32 (■), 24 (x), 16 (▲) and 8 (+) $\mu\text{g mL}^{-1}$.

Figure 4. Bacterial survival following membrane depolarization with depsipeptide 1 (•) and amide 2 (▲) at 48 µg mL⁻¹. a) *S. aureus* Wichita; b) *S. aureus* Mu50; c) *S. epidermidis* GA. Untreated bacteria (◆).

Figure 5. Antibacterial activities of depsipeptide **1** (•) and amide **2** (▲) at $48 \mu\text{gmL}^{-1}$ against selected Gram-positive strains: a) *S. aureus* Wichita; b) *S. aureus* Mu50; c) *S. epidermidis* GA. Untreated bacteria (◆).

Figure 6.

In vivo efficacy testing using a *G. mellonella* model of infection. *G. mellonella* larvae (n=10 for each group) were injected with a lethal dose (1.0×10^9 CFU) of MRSA USA100. After 1h, groups were injected with either vehicle (DMSO), or single doses ($5 \times \text{MIC}$) of depsipeptide 1 ($P = 0.0005$) and amide 2 ($P=0.155$). Data was analyzed for statistical significance using a Log Rank and Chi Squared Test with 1-degree of freedom. Mock = untreated *G. mellonella* larvae infected with MRSA USA100.

Scheme 1.

Synthesis of cyclic lipopeptides **1–3**. Reagents and conditions: a) Fmoc-D-Asp-Oallyl and Fmoc-AA-OH, standard Fmoc SPPS deprotection and coupling protocols; b) Fmoc-Thr-OH, standard Fmoc SPPS deprotection and coupling protocols; c) Fmoc-Dap(Mtt), standard Fmoc SPPS deprotection and coupling protocols; d) Fmoc-ADA-OH, , i), standard Fmoc SPPS deprotection and coupling protocols; e) Alloc-D-Ala-OH (4 equiv), DIC (4 equiv), DMAP (0.2 equiv), DCM, RT, 18 h; f) Pd(Ph₃P)₄ (0.1 equiv), HN(CH₃)₂-BH₃ (4 equiv), DCM, RT, 2×10 min; g) TFA/CH₂Cl₂ (1% v/v), RT, 30 min; h) PyBOP/HOBt/DIEA (2:2:6 equiv), DMF, 18 h; i) piperidine/DMF (20% v/v), RT, 25 min, *N,N*-bis(*tert*-butoxycarbonyl)thiourea (3 equiv), 2-chloro-1-methylpyridinium iodide (3 equiv), TEA (4 equiv), DMF, RT, 18 h; j) *o*-NBS-Cl (4 equiv), collidine (10 equiv), NMP, RT, 2×10 min; k) PPh₃ (5 equiv), MeOH (10 equiv), DIAD (5 equiv), THF, RT, 1 h; l) HSCH₂CH₂OH (10 equiv), DBU (5 equiv), NMP, RT, 30 min; m) Alloc-D-Ala-OH (3 equiv), HOAt (3 equiv), HATU (3 equiv), DIEA (6 equiv), NMP, RT, 1 h; n) TFA/TIA/H₂O (95:2.5:2.5 v/v/v), RT, 3 h.