# Synthesis and Structure–Activity Relationship Studies of N-Terminal Analogues of the Antimicrobial Peptide Tridecaptin A(1)

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# Synthesis and Structure–Activity Relationship Studies of N-Terminal Analogues of the Antimicrobial Peptide Tridecaptin $A_1$

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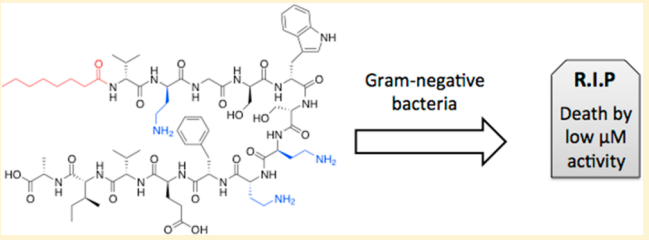
Supporting Information

**ABSTRACT:** Chemical synthesis was used to increase the potency of the antimicrobial lipopeptide tridecaptin $A_1$. Lipid tail modification proved to be an ideal platform for synthesizing structurally simpler analogues that are not readily accessible by isolation. The stereochemical elements of the tridecaptin $A_1$ lipid tail are not essential for antimicrobial activity and could be replaced with hydrophobic aliphatic or aromatic groups. Some simpler analogues displayed potent antimicrobial activity against Gram-negative bacteria, including *Campylobacter jejuni*, *Escherichia coli* O157:H7, and multidrug resistant *Klebsiella pneumoniae*.




## INTRODUCTION

The increasing emergence of multidrug resistant bacteria has highlighted the need for the development of new classes of antibiotics.[1] Antimicrobial peptides are already being used for food preservation and present an attractive alternative for treatment of infections by such resistant bacteria.[2,3] In particular, bacterial lipopeptides often exhibit potent antimicrobial activity. Some bacterial lipopeptides have been approved for clinical use, including colistin (polymyxin E), which is used as a topical antibiotic and a last line of defense treatment against multidrug resistant Gram-negative infections.[4]

The tridecaptins are a group of linear cationic lipopeptides analogous to the polymyxins. These acyl tridecapeptides were first isolated in 1978 and display antimicrobial activity against Gram-positive and Gram-negative bacteria.[5] Until recently, very little work has been published on this interesting group of antimicrobial lipopeptides. Tridecaptin $A_1$ (Figure 1) has been isolated by our group from *Paenibacillus terrae* NRRL B-30644 and shown to display activity against the common foodborne pathogen *Campylobacter jejuni*.[6,7] *C. jejuni* is responsible for between 2 and 4 million cases of food poisoning in the U.S. each year, with approximately 0.1% resulting in death.[8] The use of antimicrobial peptides as food preservatives is a possible method to prevent *C. jejuni* related illnesses, and several bacteriocins have been reported to inhibit its growth.[9] Our group recently performed the first chemical synthesis of tridecaptin $A_1$ to determine the stereochemistry of the lipid tail. Lipid tail peptide stereoisomers **1**–**4** were synthesized for comparison with the natural peptide (Figure 1).[7] Surprisingly, analogue **2**, in which the stereochemistry of the 6-methyl group is inverted, and analogue **3**, in which the 3-hydroxyl group is inverted, retained their antimicrobial activity. However, the stereoisomer in which both positions were inverted showed diminished activity.

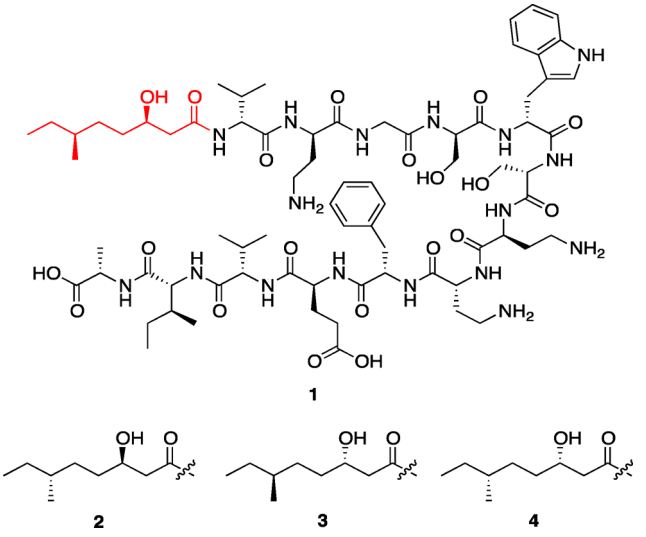



**Figure 1.** Structures of natural tridecaptin $A_1$ (**1**) and the synthetic lipid tail peptide stereoisomers.

The chemical synthesis of tridecaptin $A_1$ is an attractive alternative to isolation (~2 mg/L), as it allows much larger quantities of the antimicrobial peptide to be obtained. However, the synthesis of the activated lipid tail used in the SPPS of tridecaptin $A_1$ requires several linear steps from expensive chiral starting materials. The observation that the

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**Scheme 1. Synthesis of Tridecaptin $A_1$ Lipid Tail Analogues**

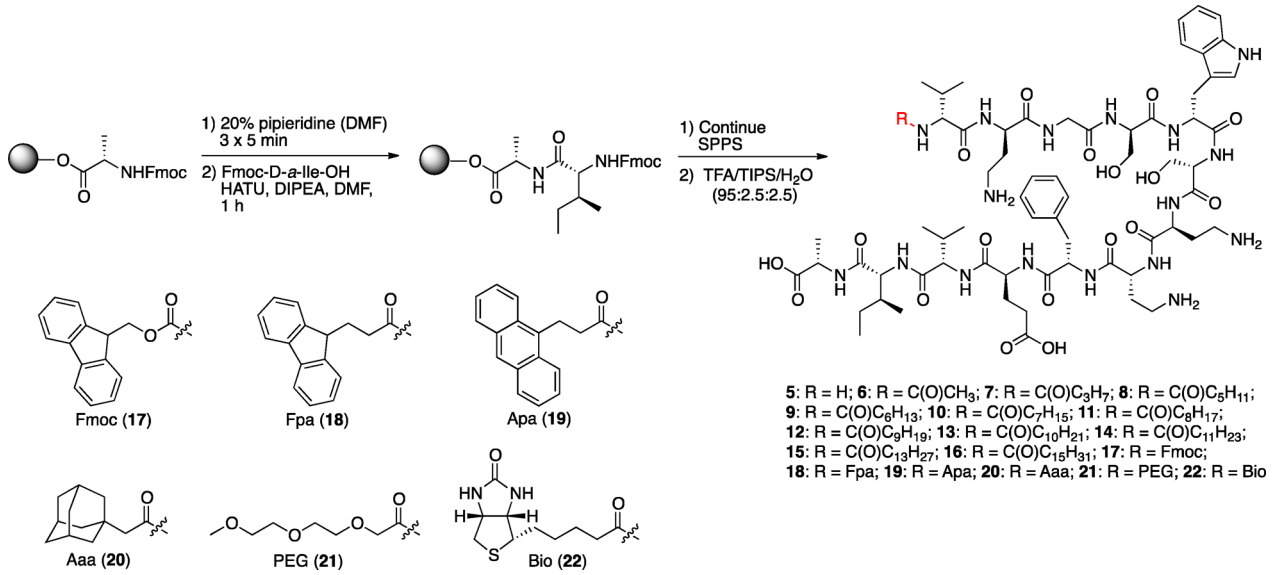


**Table 1. Comparison of the Antimicrobial Activity of Synthetic Analogues with Tridecaptin $A_1$ [a]**

| analogue | *E. coli* ATCC 25922 | *S. enterica* ATCC 13311 | *P. aeruginosa* ATCC 27853 | *C. jejuni* NCTC 11168 | *K. pneumoniae* ATCC 13883 | *A. baumannii* ATCC 19606 | *E. faecalis* ATCC 29212 | *S. aureus* ATCC 29213 | *L. monocytogenes* ATCC 19434 | *E. faecium* ATCC 19434 |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 3.13 | 6.25 | 50 | 1.56 | 3.13 | 12.5 | >100 | >100 | 50 | >100 |
| **2** | 3.13 | 6.25 | 50 | 3.13 | 6.25 | 12.5 | >100 | >100 | 100 | >100 |
| **3** | 6.25 | 6.25 | 50 | 1.56 | 6.25 | 25 | >100 | >100 | 100 | >100 |
| **4** | 12.5 | 25 | 100 | 3.13 | 12.5 | 50 | >100 | >100 | >100 | >100 |
| **5** | 100 | 100 | 100 | >100 | 50 | >100 | >100 | >100 | >100 | >100 |
| **6** | 50 | 100 | >100 | 100 | 50 | >100 | >100 | >100 | >100 | >100 |
| **7** | 12.5 | 25 | >100 | 12.5 | 25 | 100 | >100 | >100 | >100 | >100 |
| **8** | 3.13 | 6.25 | 50 | 3.13 | 3.13 | 25 | >100 | >100 | 100 | >100 |
| **9** | 6.25 | 12.5 | 50 | 1.56 | 6.25 | 12.5 | >100 | >100 | 50 | >100 |
| **10** | 3.13 | 6.25 | 25 | 0.78 | 3.13 | 12.5 | 100 | 100 | 25 | 50 |
| **11** | 12.5 | 6.25 | 100 | 0.78 | 12.5 | 50 | 100 | 100 | 25 | 100 |
| **12** | 6.25 | 12.5 | >100 | 0.4 | 6.25 | 12.5 | >100 | >100 | 25 | 100 |
| **13** | 6.25 | 12.5 | >100 | 0.4 | 6.25 | 12.5 | >100 | >100 | 25 | 100 |
| **14** | 12.5 | 50 | >100 | 0.4 | 12.5 | 50 | >100 | >100 | 50 | 100 |
| **15** | >100 | >100 | >100 | 12.5 | >100 | >100 | >100 | >100 | >100 | >100 |
| **16** | >100 | >100 | >100 | 50 | >100 | >100 | >100 | >100 | >100 | >100 |
| **17** | 6.25 | 6.25 | >100 | 0.78 | 6.25 | 12.5 | 50 | 100 | 12.5 | 25 |
| **18** | 6.25 | >100 | >100 | 1.56 | 100 | >100 | >100 | >100 | 100 | >100 |
| **19** | 6.25 | 12.5 | >100 | 1.56 | 12.5 | 25 | >100 | >100 | 50 | >100 |
| **20** | 6.25 | 6.25 | 50 | 1.56 | 12.5 | 12.5 | 50 | 50 | 25 | 50 |
| **21** | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| **22** | 50 | 50 | >100 | 50 | 50 | >100 | >100 | >100 | >100 | >100 |

[a]Activities are reported in $\mu$g/mL.

stereochemical elements on the lipid tail could be varied while retaining activity led us to synthesize a library of lipid tail analogues. Our goal was to make structurally simpler analogues and determine if the antimicrobial activity could be increased through lipid tail modification (Scheme 1).

## RESULTS AND DISCUSSION

The MIC values for tridecaptin $A_1$ against some common pathogenic Gram-positive and Gram-negative bacteria were determined using broth-dilution assays (Table 1). Tridecaptin $A_1$ displays potent activity against Gram-negative organisms (excluding *Pseudomonas aeruginosa*) but is less active against Gram-positive bacteria. Synthetic analogues were compared to natural tridecaptin $A_1$ using the same assay.

The tridecaptin $A_1$ lipid tail analogues were synthesized using standard Fmoc-SPPS (Scheme 1). The N-terminus was acylated on-resin using either the acyl chloride or the activated acid. To determine if the hydroxyl and methyl moieties were required for the antimicrobial activity of tridecaptin $A_1$, the octanamide analogue Oct-Tri$A_1$ (**10**) was synthesized. Oct-Tri$A_1$ (**10**) fully retained its activity against all organisms. Oct-Tri$A_1$ (**10**) is synthetically more accessible than natural tridecaptin $A_1$ and enables large quantities of peptide to be obtained for biological studies (~100 mg from 0.1 mmol of


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resin). Encouraged by this result, we then proceeded to synthesize lipid tail analogues **6**–**16** with varying alkyl chain lengths, as well as the unacylated tridecapeptide $H_2N$-Tri$A_1$ (**5**), to test how the length of the lipid tail affects the antimicrobial activity.

The unacylated peptide **5** was substantially less active than the natural peptide, typically requiring concentrations of 100 μg/mL or more to kill most organisms. The general mode-of-action of antibacterial lipopeptides involves permeabilizing the bacterial membrane, resulting in cell death. For the analogous polymyxins, this requires insertion of the lipid tail into the phospholipid bilayer, with unacylated polymyxin analogues showing no activity.[10] The absence of a lipid tail on tridecaptin $A_1$ likely perturbs antimicrobial activity by a similar effect. Analogues in which the lipid tail was shorter than $C_6$ or longer than $C_{12}$ were also significantly less active than the natural peptide. The optimal length of linear lipid tails ranged between $C_8$ and $C_{12}$, with Oct-Tri$A_1$ (**10**) displaying the strongest activity against most organisms. The trend observed for the linear lipid tail analogues shows that chain length is important for the antimicrobial activity of tridecaptin $A_1$. Shorter chains are likely less effective at disruption of the cell membrane. Long alkyl chains can self-associate through hydrophobic interactions, which could prevent membrane disruption and explain their decreased activity.

Our previous SAR studies on the two-component lantibiotic lacticin 3147 found that a bis(desmethyl) analogue of lacticin 3147 A2, in which the N-terminal pentapeptide (13 linear step synthesis) was replaced with the Fmoc protecting group, retained its synergistic activity with lacticin 3147 A1.[11] It was postulated that the Fmoc protecting group mimics the pentapeptide because of its planarity and hydrophobicity. Fmoc-Tri$A_1$ (**17**) was therefore synthesized and tested. The Fmoc analogue **17** retained activity against all the tested Gram-positive and Gram-negative organisms. An alternative explanation for the activity of Fmoc-Tri$A_1$ (**17**) is that cleavage of the Fmoc-carbamate occurs in vivo, releasing the toxic benzofulvene moiety. Benzofulvene is a potent Michael acceptor and could potentially react with a variety of cellular nucleophiles, causing cell death. To rule out this mode of action, 3-fluorenylpropanoic acid was prepared and used in the synthesis of Fpa-Tri$A_1$ (**18**).[12] This analogue cannot undergo cleavage like Fmoc-Tri$A_1$ (**17**). Fpa-Tri$A_1$ (**18**) only retained activity against *E. coli* and *C. jejuni*. The activity against the remaining organisms was significantly lower than the natural peptide. The possibility of carbamate deprotection being responsible for bactericidal effects could therefore not be ruled out. To further probe this hypothesis, 3-anthracenylpropanoic acid was prepared and used in the synthesis of Apa-Tri$A_1$ (**19**).[13,14] In contrast to the Fpa analogue **18**, anthracene analogue **19** retained comparable activity to the natural peptide. This suggests that carbamate deprotection is not responsible for the observed activity but does not explain the reduced activity of **18**. A possible explanation is that the planarity of the lipid tail is decreased when the carbamate is replaced with an ester. This substitution could make it less effective at membrane disruption, lowering activity.

To test the importance of flexibility in the lipid tail, Aaa-Tri$A_1$ (**20**) was synthesized by acylating the tridecapeptide with adamantane acetic acid. The activity of Aaa-Tri$A_1$ (**20**) against Gram-negative bacteria was comparable to that of the natural peptide against most organisms. PEGylation has been shown to be an effective strategy for increasing the solubility of drug molecules while retaining their activity.[15] PEG tails have the advantage of being soluble in water and organic solvents and could therefore be an ideal lipid tail modification to improve the solubility of tridecaptin $A_1$. PEG-Tri$A_1$ (**21**), in which the N-terminus is acylated with a triethylene glycol analogue, was synthesized. This length of PEG chain was chosen, as it lies within the $C_8$–$C_{12}$ range for optimal activity found during the screening of the linear alkyl analogues. Analogue **21** was inactive against all organisms at the concentrations tested. The presence of heteroatoms in the lipid tail possibly prevents effective interaction with the cell membrane or alters the conformation of the peptide, abolishing the antimicrobial activity. This effect is further highlighted by the lower activity of the biotin analogue **22**. These experiments show that provided the hydrophobicity of the tail is maintained and the carbon chain length is not too long, the antimicrobial activity of the tridecaptin $A_1$ analogues is conserved.

To further probe the utility of tridecaptin $A_1$ analogues as therapeutic agents, the hemolytic activities of six analogues were tested. To be clinically useful, the peptide should not be hemolytic at its MIC. The extent of rabbit erythrocyte hemolysis was determined using a peptide concentration of 83 μg/mL, over 20-fold greater than the MIC against most Gram-negative bacteria (Table 2). Replacement of the natural lipid tail with octanoic acid slightly increased the hemolytic activity of the peptide from 3.2% to 4.7%. The $C_{12}$ and Fmoc analogues caused substantial hemolysis, whereas the hemolytic activities of the $C_4$ and $NH_2$ analogues were negligible. Oct-Tri$A_1$ was therefore chosen as our lead compound, as it was the most active analogue and had an MIC significantly lower than the concentrations required for hemolysis.

**Table 2. Hemolytic Activities**

| analogue | % hemolysis |
|---|---|
| natural Tri$A_1$ (**1**) | 3.2 |
| H-Tri$A_1$ (**5**) | 0.5 |
| But-Tri$A_1$ (**7**) | 0.8 |
| Oct-Tri$A_1$ (**10**) | 4.7 |
| Laur-Tri$A_1$ (**14**) | 82.2 |
| Fmoc-Tri$A_1$ (**17**) | 100.0 |

Oct-Tri$A_1$ (**10**) was tested against some more clinically relevant bacterial strains (Table 3). *E. coli* O157:H7, one of the most dangerous strains of this species, causes severe food poisoning. The *Klebsiella* and *Enterobacter* strains are clinically isolated strains that are resistant to numerous antibiotics including ertapenem, meropenem, gentamicin, cefoxitin, and norfloxacin (see Supporting Information). *Clostridium difficile* 630 is a multidrug resistant and highly transmissible strain. Oct-Tri$A_1$ displayed excellent antimicrobial activity against the Gram-negative strains but was predictably less active against the Gram-positive *C. difficile* strain. To further assess the potential clinical applications of Oct-Tri$A_1$, cytotoxicity data were

**Table 3. Antimicrobial Activity of Oct-Tri$A_1$**

| organism | MIC (μg/mL) |
|---|---|
| *E. coli* O157:H7 | 0.43 |
| *K. pneumoniae* KpCG | 2.33 |
| *Enterobacter cloacae* 849 | 0.43 |
| *C. difficile* 630 | 12.7 |


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obtained using an MTT assay. The synthetic analogue was 2-fold more toxic (MIC = 100 $\mu$g/mL) against HEK 293 cells than tridecaptin $A_1$ (MIC = 200 $\mu$g/mL). This could be due to the increased hydrophobicity of the lipid tail. Regardless of this higher toxicity, the MIC of Oct-Tri$A_1$ against the Gram-negative bacteria listed in Table 3 is significantly lower, making it an interesting candidate for further development.

## CONCLUSION

In summary, the lipid tail of the antimicrobial peptide tridecaptin $A_1$ proved to be an ideal site for modification. In particular, acylation of the tridecapeptide with octanoic acid yielded an analogue that retained strong activity against foodborne pathogens *C. jejuni* and *S. enterica* and showed good activity against clinically relevant strains of *E. coli* and *K. pneumoniae*. The ability to replace the chiral lipid tail with simple linear hydrocarbons allows rapid synthesis of tridecaptin $A_1$ analogues by solid-phase peptide synthesis. The synthetic simplicity and potent activity of Oct-Tri$A_1$ could make it an ideal candidate for further medicinal development.

## EXPERIMENTAL SECTION

**General.** NMR spectra were recorded on Varian Inova 500 and 600 spectrometers. For $^1$H NMR spectra, $\delta$ values were referenced to $D_2O$ (4.79 ppm). Mass spectra were recorded on a Bruker 9.4 T Apex-Qe FT-ICR. 4-Hydroxy-$\alpha$-cyanocinnamic acid was used as matrix. HPLC was performed on a Gilson preparative HPLC system equipped with a model 322 HPLC pump, GX-271 liquid handler, 156 UV−vis detector, and a 10 mL sample loop or a Varian Prostar analytical system equipped with a Rheodyne 7225i injector fitted with a 100−1000 $\mu$L sample loop. The columns used were a Vydac $C_{18}$ column (5 $\mu$m, 4.6 mm × 250 mm) for analytical scale and Phenomenex $C_{18}$ column (5 $\mu$m, 21.2 mm × 250 mm) for preparative scale. HPLC solvents were filtered with a Millipore filtration system under vacuum before use.

**Peptide Synthesis.** Solid-phase peptide synthesis was carried out on a 10 or 25 $\mu$mol scale using Fmoc chemistry on preloaded L-alanine-2-chlorotrityl resin (ChemImpex, 0.824 mmol/g loading). Reactions were performed in a custom-built 20 mL glass fritted column fitted with a T-joint and three-way T-bore PTFE stopcock. The resin was preswollen by bubbling in DMF (5 mL, 10 min) with argon. Between deprotections and couplings the vessel was drained under argon pressure and washed with DMF (3 × 5 mL). The Fmoc group was removed by bubbling with 20% piperidine in DMF (3 × 5 mL × 5 min). The deprotection steps were monitored by spotting each filtrate on a silica gel TLC plate and visualizing the benzofulvene piperidine adduct with a 254 nm UV lamp. Fmoc-D-*allo*-isoleucine (5 equiv) was preactivated by shaking with HATU (5 equiv) and DIPEA (10 equiv) in DMF (5 mL) for 5 min. The resin was bubbled in the coupling solution for 1 h, drained, and washed with DMF (3 × 5 mL). The deprotection and coupling steps were continued to complete the peptide synthesis. The N-terminus was acylated by one of two methods: (1) bubbling the resin in a 20% solution of the desired acyl chloride in DMF (5 mL, 15 min); (2) activation and coupling of the appropriate carboxylic acid using the previously described coupling procedure. The resin-bound peptide was washed with $CH_2Cl_2$ (3 × 5 mL) and dried under argon for 20 min. The resin was transferred to a screw top vial containing TFA/TIPS/$H_2O$ (95:2.5:2.5, 5 mL) and gently shaken for 2 h. The cleavage solution was filtered and concentrated in vacuo, and the crude peptide was precipitated with cold diethyl ether. The crude peptide was dissolved in $H_2O$/MeCN (1:1, 5 mL) and purified using preparative-scale $C_{18}$-RP-HPLC: Phenomenex $C_{18}$ column, flow rate 10 mL/min, detection at 220 nm, gradient starting from 20% MeCN (0.1% TFA) and 80% water (0.1% TFA) for 5 min, ramping up to 55% MeCN over 30 min, then ramping up to 95% MeCN over 3 min, staying at 95% MeCN for 3 min, ramping down to 20% MeCN over 2 min, then staying at 20% MeCN for 5 min. The product containing fractions were pooled, concentrated, frozen, and lyophilized to yield the product as a white powder. A small amount of product was reinjected on an analytical scale. All peptides showed >99% purity.

**Bacterial Growth Conditions.** All cultures were grown from glycerol stocks. All organisms in Table 1 (excluding *C. jejuni*) were grown in Mueller−Hinton (MH) broth at 37 °C, 225 rpm. *C. jejuni* was grown on MH agar plates at 37 °C under 10% $CO_2$, 5% $O_2$, and 85% $N_2$. Gram-negative organisms in Table 2 were grown in tryptic soy broth at 37 °C, 225 rpm. *Clostridium difficile* 630 was grown in brain−heart infusion broth anaerobically.

**Broth-Dilution Assay.** All MIC values were determined using CLSI guidelines.[16] Polymyxin B was used as a control, and MIC values determined against CLSI QC strains were within the range of published values.

**Hemolytic Assay.** The hemolytic activity of tridecaptin analogues was tested following a previously described method.[17] Defibrinated rabbit blood (1 mL; Hemostat Laboratories; Dixon, CA, USA), within 7 days of receipt, was diluted with PBS (19 mL) and mixed gently. This mixture was centrifuged (1000*g*, 5 min, 4 °C) and the supernatant carefully removed. This was repeated three more times to remove free hemoglobin. The resulting pellet was gently resuspended in 19 mL of PBS. Aliquots of the blood solution (100 $\mu$L) were added to the wells of a 96-well plate. To these wells, 50 $\mu$L of a 250 $\mu$g/mL solution of tridecaptin analogue (in PBS) was added, and the samples were mixed gently by pipetting. Triton X-100 (50 $\mu$L of a 0.1% solution) was used as a positive control, while PBS (50 $\mu$L) was used as a blank. Each sample and control were tested in triplicate. The 96-well plate was then incubated at 37 °C for 30 min. The samples were gently mixed by pipetting, and 20 $\mu$L of each was added to 200 $\mu$L of PBS. Once again, the samples were gently mixed. The diluted samples were then centrifuged (13 000 rpm, 5 min) using a tabletop centrifuge. Supernatant (120 $\mu$L) from each sample was added to a new 96-well plate, and the absorbance was measured at 415 nm. Percent hemolysis of the peptides was calculated relative to Triton X-100, while the PBS negative control was used as a blank.

## ASSOCIATED CONTENT

### Supporting Information

Characterization of peptide analogues, information of multidrug resistant strains, and MTT assay protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

S.A.C. synthesized and characterized the peptide analogues and conducted biological testing. C.T.L., J.R.B., and G.M. conducted biological testing.

### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

Aaa, adamantane acetic acid; Apa, 3-anthracenylpropanoic acid; *C. difficile*, *Clostridium difficile*; *C. jejuni*, *Campylobacter jejuni*; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; *E. faecium*, *Enterococcus faecium*; *E. coli*, *Escherichia* coli; Fmoc, fluorenylmethyloxycarbonyl; Fpa, 3-fluorenylpropanoic acid; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HEK 293, human embryonic kidney cell line 293; *L. monocytogenes*, *Listeria monocytogenes*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PEG, polyethylene glycol; *P. aeruginosa*, *Pseudomonas aeruginosa*; RP-HPLC, reverse-phase high pressure liquid chromatography; *S. enterica*, *Salmonella enterica*; SAR, structure−activity relationship; SPPS, solid-phase peptide synthesis; *S. aureus*, *Staphylococcus aureus*; TFA, trifluoroacetic acid; TIPS, triisopropylsilane

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