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The Antimicrobial Lipopeptide Tridecaptin A_1 Selectively Binds to Gram-Negative Lipid II

Authors: Stephen A. Cochrane,[1†] Brandon Findlay,[1†] Alireza Bakhtiary,[1] Jeella Z. Acedo,[1] Eva M. Rodriguez-Lopez,[1] Pascal Mercier[2] and John C. Vederas.[1*]

Affiliations:

[1] Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2G2

[2] NANUC, University of Alberta, Edmonton, Alberta, Canada, T6G 2E1

*Correspondence to: john.vederas@ualberta.ca

†Authors contributed equally to this work

Abstract

Tridecaptin A_1 ($TriA_1$) is a non-ribosomal lipopeptide with selective antimicrobial activity against Gram-negative bacteria. Herein we show that $TriA_1$ exerts its bactericidal effect by binding to the bacterial cell wall precursor lipid II on the inner-membrane, disrupting the proton motive force. Biochemical and biophysical assays show that binding to the Gram-negative variant of lipid II is required for membrane disruption, and that only the proton gradient is dispersed. The NMR solution structure of $TriA_1$ in dodecylphosphocholine micelles with lipid II has been determined and molecular modeling was used to provide a structural model of the $TriA_1$-lipid II complex. These results suggest that $TriA_1$ kills Gram-negative bacteria by a novel mechanism of action using a new lipid II binding motif.

Significance

The increasing development of antimicrobial resistance is a major global concern and there is an urgent need for the development of new antibiotics. In this article we show that the antimicrobial lipopeptide tridecaptin A_1 selectively binds to the Gram-negative analogue of peptidoglycan precursor lipid II, disrupting the proton motive force and killing Gram-negative bacteria. To the best of our knowledge this is the first example of the selective targeting of Gram-negative lipid II and a completely new binding mode to this peptidoglycan precursor. No persistent resistance develops against tridecaptin A_1 in *E. coli* cells exposed to sub-inhibitory concentrations of this peptide during a one-month period. We believe this study showcases the excellent antibiotic properties of the tridecaptins in an age where new antibiotics that target Gram-negative bacteria are desperately needed.

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Introduction

Recently, a lot of media coverage has been focused on the problem of antimicrobial resistance. A report commissioned by the UK government predicts that by 2050, antimicrobial resistance will have caused 300 million premature deaths and cost the global economy over $100 trillion.(1) Even more worrying is the lack of new classes of antibiotics active against Gram-negative bacteria. In the last 50 years, only a few structurally and mechanistically distinct classes of antibiotics have been clinically approved to treat systemic infections (including

fidaxomicin, bedaquiline, linezolid and daptomycin), yet none of these are active against Gram-negative bacteria.(2),(3) Two new classes of Gram-negative targeting antibiotics in the clinical pipeline are POL7080 and brilacidin.(4),(5) Both of these compounds are modeled on antimicrobial peptides, which are becoming increasing important in the fight against antibiotic resistance.(6) Bacteria produce a wealth of antimicrobial peptides, both ribosomally, including the lantibiotics,(7),(8) and non-ribosomally, including lipopeptides.(9) In particular, lipopeptides are a rich source of antimicrobial compounds and several examples with activity against Gram-positive(10),(11) and/or Gram-negative bacteria(12) have been recently characterized.

Tridecaptin A_1 (TriA_1) is a member of the tridecaptin family, a group of non-ribosomal lipopeptides produced by *Bacillus* and *Paenibacillus* species (Fig. 1).(13),(14),(15) This acylated tridecapeptide displays strong and selective antimicrobial activity against Gram-negative bacteria, including multidrug resistant strains of *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Escherichia coli*.(16) TriA_1 analogues have low cytotoxicity and have been shown to treat *Klebsiella pneumoniae* infections in mice.(16),(17) Therefore, we believe that tridecaptin A_1 could be an excellent antibiotic candidate. However, prior to our investigations little was known about how TriA_1 exerts its selective bactericidal effect against Gram-negative bacteria. A previous structure activity relationship study by our group suggested that TriA_1, akin to many other lipopeptides, is a membrane-targeting agent. We found that removal of the N-terminal lipid tail abolishes antimicrobial activity, however the chiral lipid tail could

be replaced with an octanoyl chain to give Oct-TriA_1 (Fig. 1), which retains full activity.(16) We therefore sought to identify the precise mode and mechanism of action by which TriA_1 kills Gram-negative bacteria.

Results

Tridecaptin A_1 targets the cell membrane

Our initial efforts focused on identifying the mode of action of TriA_1. Measuring the time taken for an antibiotic to exert its antimicrobial effect can provide valuable information on the cellular process targeted by that compound. Bacteriostatic agents halt cell division but do not reduce the number of viable cells and antibiotics that target protein synthesis and nucleic acid synthesis typically fall within this category. Bactericidal agents reduce the population of viable bacterial cells and the time taken for a bactericidal agent to kill bacteria provides further information on its target. Therefore, we monitored the growth kinetics of *Escherichia coli* cells exposed to TriA_1 and a number of other antibiotics (Fig. 2). Lipopeptides like polymyxin B are generally bactericidal within minutes of exposure, due to the formation of large, non-specific pores in the bacterial membrane, while many other antibiotics (like ampicillin) exert their killing effect over several hours. Bacteriostatic agents (like chloramphenicol) halt cell growth, but do not kill cells. Optical density measurements showed that cells exposed to TriA_1 grew at a reduced rate for 20 minutes, at which point a steady decrease in cell count was observed (Fig. 2A). Polymyxin B reduced the cell count immediately, while ampicillin displayed an expected three-hour lag and

chloramphenicol did not reduce optical density. Complementary results were obtained from a time-kill assay (Fig. 2B), which revealed that cells exposed to $TriA_1$ showed slightly reduced cell viability after 15 minutes of exposure, a significant reduction after 30 minutes and complete killing after 60 minutes. Polymyxin B acted faster, significantly reducing the viable cell population after 5 minutes and killing all cells by 30 minutes. These results suggested that $TriA_1$ is a membrane targeting peptide, but does not act by a generic membrane lysis mechanism like polymyxin B. This is further supported by the selectivity of $TriA_1$ against Gram-negative bacteria, as a peptide that operates through a generic lysis mechanism would also target Gram-positive organisms.

$TriA_1$ binds to lipopolysaccharide on the outer-membrane

Gram-negative organisms are protected from their environment by an outer-membrane, which reduces diffusion of many antibiotics into the cell or periplasmic space. In order for $TriA_1$ to attack the bacterial membrane of Gram-negative bacteria, it must first cross this barrier. Polymyxin B traverses the outer-membrane by binding to the lipid A portion of lipopolysaccharide (LPS), followed by insertion into the membrane.(18) We postulated that tridecaptin A_1 may also cross the outer-membrane through an interaction with LPS, given that it possess several cationic 2,4-diaminobutyric acid residues (Dab) like polymyxin B. Isothermal titration calorimetry (ITC) was therefore used to determine if $TriA_1$ binds to LPS. Our studies revealed that $TriA_1$, Oct-$TriA_1$, the enantiomeric form of $TriA_1$ (Ent-$TriA_1$) and unacylated $TriA_1$ (H-$TriA_1$) bind to LPS with similar affinities

(SI Appendix, Fig. S1). We have previously shown that H-TriA$_1$ has weak antimicrobial activity, but at sub-MIC concentrations it can enhance the activity of hydrophobic antibiotics like rifampicin and vancomycin over 100-fold.(17) Therefore, it is likely that LPS binding is the mechanistic basis for this disruption of the outer-membrane. We have also found that the enantiomer of TriA$_1$ (Ent-TriA$_1$) is four-fold less active than the natural peptide, suggesting that TriA$_1$ interacts with a chiral target.(19) Ent-TriA$_1$ contains the same amino acids and lipid tail as TriA$_1$, and therefore retains the same amphiphilic properties. It is likely that the observed activity at higher concentrations is due to membrane lysis by a non-specific detergent-like effect, akin to the mode of action of the polymyxins. This observation that both TriA$_1$ and its enantiomer bind to LPS with similar affinities suggested that another chiral receptor is involved in the mode of action.

TriA$_1$ disrupts the proton motive force

With a rationale for how TriA$_1$ crosses the outer-membrane, we next sought to uncover the mechanism by which it acts on the inner-membrane. To this end, a series of experiments were performed using fluorescent dyes (Fig. 3). DiBAC$_4$ is a common dye used to monitor membrane depolarization.(20) It enters depolarized cells and binds to proteins or membrane components, exhibiting enhanced fluorescence. Addition of TriA$_1$ to DiBAC$_4$ treated *E. coli* cells resulted in no membrane depolarization, whilst application of polymyxin B lead to a rapid increase in fluorescence (Fig. 3A). To further assess the effect of TriA$_1$ on the inner-membrane, its ability to form large pores was assessed using the dye

SYTOX Green (Fig. 3B). Upon lysis of the inner-membrane, this dye binds to nucleic acids in the cytoplasm, causing an increase in fluorescence.(21) Immediate pore formation was not observed when *E. coli* cells pretreated with SYTOX Green were exposed to $TriA_1$, whereas addition of the surfactant Triton X-100 caused an instant fluorescence increase. Instead, $TriA_1$ caused a very gradual increase in fluorescence at a rate consistent with the death of cells based on the time-kill assays, suggesting that this increase is due to cells that are already dead and not an immediate effect of $TriA_1$. To further probe the action of $TriA_1$ on the inner membrane, an *ortho*-nitrophenyl-β-galactosidase (ONPG) assay was performed on *E. coli* ML-35 cells, which lack the *lac* permease. The chromophore *ortho*-nitrophenol is released by the action of cytoplasmic galactosidase on *ortho*-nitrophenol galactoside only if large pores are formed on the inner-membrane. ONPG assays (SI Appendix, Fig. S2) did not show immediate membrane lysis upon $TriA_1$ addition to *E. coli* cells.

The rapid killing of *E. coli* by $TriA_1$ stood in stark contrast to the lack of membrane lysis or depolarization observed. The essential nature of the hydrophobic tail for antimicrobial activity and the overall amphiphilic structure of $TriA_1$ support an interaction with the bacterial membrane, therefore we examined the function of the inner-membrane in detail. Bacteria use both protons and potassium ions to generate adenosine triphosphate (ATP) via the proton motive force, a process that is essential for cell growth.(22),(23) Other antibiotics are known to affect this vital cellular process. For example, nigericin and valinomycin shuttle a combination of K^+ and H^+ across the bacterial membrane,(23) while the

bacteriocin subtilosin forms proton-specific pores in the Gram-positive bacterium *Gardnerella vaginalis*.(24),(25) To determine if TriA$_1$ affects the proton motive force, we adapted the pro-dye BCECF-AM for use in Gram-negative bacteria. In the presence of ethylenediaminetetraacetic acid (EDTA), this dye readily crosses the bacterial membrane where it is hydrolyzed by non-specific esterases to give a pH sensitive dye, whose fluorescence decreases as cytoplasmic pH decreases.(25) Addition of glucose to BCECF-AM treated *E. coli* cells resulted in a fluorescence increase, indicating they were actively respiring and that BCECF was correctly showing the increase in cytoplasmic pH (Fig. 3C). To assess the effect on the proton gradient, the potassium gradient was first dispersed by addition of valinomycin, which exchanges K^+ with H^+, causing a small increase in fluorescence. Subsequent addition of TriA$_1$ resulted in a significant fluorescence decrease, signifying pore formation and disruption of the proton gradient. Addition of the proton shuttle peptide nigericin also had a similar but more rapid effect, strongly suggesting that TriA$_1$ disrupts the proton motive force.

TriA$_1$ selectively binds to Gram-negative lipid II

Having identified a probable mode of action for tridecaptin A$_1$, we next sought to identify the chiral receptor involved in its mechanism of action. Given the lower antimicrobial activity of the Ent-TriA$_1$, and the fact that TriA$_1$ acts on the bacterial membrane, we postulated that binding of TriA$_1$ to a chiral receptor on the surface of the inner-membrane is important. Lipid II is the final monomeric intermediate in peptidoglycan biosynthesis and a target of several antimicrobial

peptides, including nisin,(26),(27) plectasin(28) and teixobactin.(10) It is synthesized in the cytoplasm and anchored to the interior of the inner-membrane by an undecaprenyl chain. Lipid II is subsequently flipped to the exterior of the inner-membrane, where it is then elongated into peptidoglycan. The structure of lipid II differs between Gram-positive and Gram-negative bacteria, namely on residue three of the pentapeptide portion. In most Gram-negative bacteria, this amino acid is *meso*-diaminopimelic acid (DAP), whereas in most Gram-positive bacteria it is lysine (Fig. 4A). Using ITC, we evaluated the binding affinity of $TriA_1$ to Gram-negative (Fig. 4B) and Gram-positive lipid II (SI Appendix, Fig. S3). $TriA_1$ binds to Gram-negative lipid II in a 1:1 stoichiometry and with a K_d of 4 μM, which is close to the MIC of $TriA_1$ against *E. coli* (~3 μM). We were surprised to find that although $TriA_1$ binds strongly to Gram-negative lipid II, it has a much weaker binding affinity to the Gram-positive variant. This observation would explain why $TriA_1$ is approximately 50-fold less active against Gram-positive bacteria, therefore we sought to further investigate this phenomenon. Gratifyingly, Ent-$TriA_1$ does not bind to Gram-negative lipid II (SI Appendix, Fig. S4), confirming that this is an important chiral receptor for $TriA_1$. When $TriA_1$ was premixed with one equivalent of Gram-negative lipid II, its activity was completely abolished in a spot-on-lawn assay against *E. coli* (Fig. 4C). In contrast, premixing $TriA_1$ with Gram-positive lipid II had very little effect on the antimicrobial activity of the peptide, further evidencing this selective interaction. The same experiment with Ent-$TriA_1$ and Gram-negative lipid II had not effect on activity (SI Appendix, Fig. S4). To the best of our knowledge, this is the first reported instance of an

antimicrobial compound binding selectively to lipid II from Gram-negative bacteria.

In vitro assays link lipid II binding to membrane disruption

To corroborate our theory that $TriA_1$ binds to lipid II on the inner-membrane and disrupts the proton motive force, we developed an *in vitro* assay using large unilamellar vesicles (LUV) and BCECF acid to measure intravesicle pH changes. BCECF was encapsulated in 50 nm LUVs, such that the internal pH is set to pH 8.0 (Fig. 5A). Transferring these vesicles into a pH 6 buffer did not lead to an observable change in fluorescence over 30 minutes of incubation, whilst an immediate decrease was observed on the addition of triton X-100, confirming the integrity of the vesicles. Oct-$TriA_1$, which has identical antimicrobial activity to $TriA_1$ and also binds to Gram-negative lipid II, was used in these studies. Addition of Oct-$TriA_1$ to LUVs containing 1 mol% Gram-negative lipid II at concentrations mimicking the bacterial MIC resulted in a rapid decrease in fluorescence, signifying pore formation (Fig. 5B). A much weaker decrease in fluorescence occurred with LUVs containing 1 mol% Gram-positive lipid II, which was comparable to the effect observed against vesicles lacking any lipid II. These studies provide further evidence that $TriA_1$ selectively recognizes Gram-negative lipid II and link this recognition to the observed bactericidal activity.

Synthesis of Gram-negative lipid II analogue for NMR studies

In an effort to understand the interaction between $TriA_1$ and Gram-negative lipid II on a molecular level, we sought to elucidate the NMR solution structure of $TriA_1$ in dodecylphosphocholine (DPC) micelles containing Gram-negative lipid II. Natural lipid II, which contains a fatty undecaprenyl chain, is not very amenable to NMR studies. It is prone to micelle formation in aqueous solvent and the multiple methyl and methylene signals from the C_{55} chain can drown out important signals in its binding partner needed for complete structural characterization. Breukink and coworkers have previously shown that a Gram-positive lipid II analogue with an (*E,E*)-farnesyl (C_{15}) chain retains full binding affinity to nisin,(29) and this analogue was used in the elucidation of the NMR solution structure of the nisin-lipid II complex.(27) Although they found that the isoprene units in the chain were not necessary for nisin binding, it was unclear if this region of lipid II was required for interactions with $TriA_1$. Therefore we embarked on the synthesis of (*Z*,*Z*)-farnesyl Gram-negative lipid II (**1**) (Fig. 6). The total synthesis of Gram-positive lipid II was previously reported by Van Nieuwenhze and coworkers,(30),(31) whereas a semi-synthesis of Gram-negative lipid II has been reported by Walker and Kahne.(32) By modification of these literature procedures, we performed the first total synthesis of Gram-negative lipid II analogue (**1**) (Fig. 6, SI Appendix). The disaccharide core was constructed by a glycosylation between acetimidate **2** (SI Appendix, Scheme S1) and glycol **3** (SI Appendix, Scheme S2-S3). After protecting group manipulation and phosphorylation of the anomeric position of alanyl disaccharide **4**, the appropriate

tetrapeptide (**5**, SI Appendix, Scheme S4-S5) was coupled to the alanine carboxylate to yield pentapeptidyl disaccharide **6**. Deprotection of the benzyl phosphate, followed by coupling to CDI activated (*Z*,*Z*)-farnesyl phosphate and global deprotection gave the desired Gram-negative lipid II analogue **1**.

Characterization of TriA$_1$-lipid II complex by NMR and molecular modeling

With Gram-negative lipid II analogue **1** in hand, we then proceeded to elucidate the NMR solution structure of Oct-TriA$_1$ in DPC micelles doped with Gram-negative lipid II. First, the chemical shifts (SI Appendix, Table S1) and Nuclear Overhauser Effect (NOE) correlations of Oct-TriA$_1$ in DPC micelles were assigned and its solution structure calculated using CYANA (SI Appendix, Fig. S5).(33) Without lipid II, Oct-TriA$_1$ adopts a looped structure with all hydrophobic residues on one face. Upon addition of one equivalent of lipid II analogue **1** (SI Appendix, Table S2) to Oct-TriA$_1$ in DPC micelles, significant amide chemical shift changes occurred on D-Val1, D-Dab2, and D-Dab8 in TriA$_1$ (SI Appendix, Fig. S6); and on D-γ-Glu2, Ala4 and Ala5 in lipid II (SI Appendix, Fig. S7). This is indicative of a conformational change in both molecules and confirms they interact (SI Appendix, Tables S3-S4). With lipid II present, Oct-TriA$_1$ adopts a more open and amphiphilic structure with an apparent π-stacking interaction between D-Trp5 and Phe9 (Fig. 7A). Interestingly, D-Dab8, which we have previously shown is the critical residue for Oct-TriA$_1$ activity,(19) is located at the base of a possible binding pocket.

To obtain an experimentally derived model of the Oct-TriA$_1$-Lipid II complex, the structure of lipid II analogue **1** in the presence of Oct-TriA$_1$ was also calculated using CYANA and then docked into the NMR solution structure of Oct-TriA$_1$ (Fig. 6A) using AutoDock Vina (Fig. 7B).(34) The model suggests that the N-terminal lipid tail and D-Trp5 are in close proximity to the lipid II terpene chain. This would be expected as the terpene tail of lipid II anchors it to the exterior of the inner-membrane and would associate with the more hydrophobic residues of TriA$_1$. The model also suggests there is a H-bonding interaction between the γ-amino group of D-Dab8 and the ε-carboxylate on DAP3 in lipid II (Fig. 7C). D-Dab8 is essential for the antimicrobial activity of the tridecaptins and DAP3 in lipid II is essential for TriA$_1$ binding. Notably, the model does not show interactions between Oct-TriA$_1$ and the pyrophosphate moiety of lipid II. No shift in the ^{31}P-NMR of lipid II is observed on addition of Oct-TriA$_1$, confirming that the pyrophosphate is not involved in Oct-TriA$_1$ binding (SI Appendix, Fig. S8). To the best of our knowledge, this is the first reported instance of an antimicrobial peptide that does not make use of a pyrophosphate cage.

TriA$_1$ displays low levels of resistance development

The results from the mode of action and mechanism of actions studies suggested that tridecaptin A$_1$ exerts its bactericidal effect by binding to lipid II on the inner-membrane and disrupting the proton motive force. Lipid II is a late stage intermediate in peptidoglycan biosynthesis, and the proposed binding site for TriA$_1$ would be difficult for bacteria to modify without affecting its subsequent

processing by enzymes in the peptidoglycan biosynthesis pathway. One would therefore expect that resistance development against $TriA_1$ would be limited. An *in vitro* evolution study was performed, in which *E. coli* cells were continuously exposed to sub-MIC concentrations of Oct-$TriA_1$ or ciprofloxacin for one month. During this experiment, the activity of the nucleic acid synthesis inhibitor ciprofloxacin decreased eight-fold, whereas no persistent resistance developed against Oct-$TriA_1$ (Fig. 8).

Discussion

We have shown that tridecaptin A_1 exerts its bactericidal effect on Gram-negative bacteria by binding to lipid II on the surface of the inner-membrane and disrupting the proton motive force. Monitoring the time taken for $TriA_1$ to kill *E. coli* cells by growth kinetic measurements and time-kill assays suggested that it targets the cell membrane (Fig. 2). However, it does not act as quickly as polymyxin B, which kills bacteria by forming large non-specific pores on the inner-membrane. A study of the effect that $TriA_1$ has on the bacterial membrane using fluorescent dyes (Fig. 3) revealed that $TriA_1$ does not depolarize the membrane or kill Gram-negative bacteria by the formation of large pores. Initially this result was quite surprising as several pieces of evidence suggested that it interacts with the cell membrane. Firstly, the lipid tail of $TriA_1$ is essential to its antimicrobial activity. This is similar for many other lipopeptides, which must insert their lipid tail into the cell membrane for membrane disruption. Also, the time taken by $TriA_1$ to kill Gram-negative bacteria is consistent with membrane

acting antibiotics and its overall amphiphilic structure further suggested a membrane interaction. This lead us to consider that $TriA_1$ targets the bacterial membrane by a mode of action that is not membrane lysis. The proton motive force is a vital process that occurs across the bacterial membrane because it is the primary method by which bacteria produce ATP. The observation that other peptide antibiotics like nigericin, valinomycin and subtilosin can disrupt the proton motive force lead us to develop an *in vivo* assay using BCECF-AM to assess the impact that $TriA_1$ has on this important cellular function. This experiment showed that addition of $TriA_1$ rapidly decreases the cytoplasmic pH of *E. coli* cells, which was visualized as a rapid decrease in fluorescence (Fig. 3C). This signifies the formation of pores, allowing the transport of protons from the more acidic extracellular buffer into the cytoplasm. Combining this result with the observation that $TriA_1$ does not depolarize the bacterial membrane or form large pores led us to conclude that $TriA_1$ forms proton specific pores. This will ultimately block the synthesis of ATP and kill bacteria.

Having identified a probable mode of action, we next became interested in the mechanism of action. ITC revealed that like polymyxin B, $TriA_1$ binds to LPS on the outer-membrane of Gram-negative bacteria. Removal of the lipid tail from $TriA_1$ does not affect LPS binding but the unacylated derivative of $TriA_1$ has substantially lower activity than the natural peptide. Therefore, the lipid tail is important for either outer- or inner-membrane penetration. The observation that the enantiomer of $TriA_1$, which is less active than $TriA_1$, binds to LPS with a similar binding affinity shows that another chiral receptor is involved in the

mechanism of action. As several other antimicrobial peptides bind to the peptidoglycan precursor lipid II, which is presented on the surface of the inner-membrane, we investigated if this was also a target for $TriA_1$. A combination of ITC (Fig. 4B, SI Appendix, Fig. S1), inhibition assays (Fig. 4C) and *in vitro* assays (Fig. 5) provided compelling evidence that $TriA_1$ selectively binds to the Gram-negative variant of lipid II, which contains DAP rather than lysine on its pentapeptide chain. $TriA_1$ has strong activity against Gram-negative bacteria and strongly binds to lipid II from these organisms. Its activity is sequestered through complex formation with this analogue and the presence of Gram-negative lipid II in model membranes substantially increases the pore forming ability of $TriA_1$. With $TriA_1$ having much lower activity against Gram-positive bacteria, one would expect it to not bind very strongly to lipid II from these organisms, not lose activity when mixed with Gram-positive lipid II and not show increased activity against model membranes doped with Gram-positive lipid II. This is exactly what our experiments show.

We synthesized an analogue of Gram-negative lipid II and used this to calculate the NMR solution structure of tridecaptin A_1 in DPC micelles containing one equivalent of Gram-negative lipid II (Fig. 7A). Molecular docking studies were then used to produce a model of the $TriA_1$-lipid II complex (Fig. 7B-7C), which is supported by several lines of experimental evidence. Firstly, the amide chemical shifts of lipid II that underwent the largest change upon addition of Oct-$TriA_1$ are on the pentapeptide portion. Secondly, no interaction is predicted with the pyrophosphate of lipid II and no change in the ^{31}P-NMR upon addition of Oct-

TriA$_1$ to lipid II analogue **1** was observed. Thirdly and most importantly, a key hydrogen bond is predicted between the γ-amino group of D-Dab8 and the ε-carboxylate on DAP3 in lipid II. A previous SAR study showed that D-Dab8 is absolutely essential for activity, and its substitution to D-Ala decreases its activity against Gram-negative bacteria to the level observed against Gram-positive organisms. If residue three on the pentapeptide portion of lipid II is lysine rather than DAP, the binding affinity of TriA$_1$ is significantly reduced. Therefore, this predicted interaction perfectly describes these experimental observations and led us to conclude that it is the presence of DAP on lipid II that gives rise to the remarkable selectivity of tridecaptin A$_1$ against Gram-negative bacteria. The observation that resistance development against Oct-TriA$_1$ is limited (Fig. 8) is also important, given the lack of new antibiotics that target Gram-negative bacteria, and we believe that the tridecaptins are an attractive class of future antibiotic candidates.

Materials and Methods

Whole cell studies: See supplementary information.

BCECF containing LUV preparation: 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (16 mg) and 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) (4 mg) were dissolved in chloroform (2 mL). If necessary G+LII or G-LII was then added as a solution in 2:3:1 $CHCl_3$:MeOH:H_2O to 1 mol%. The solution was thoroughly mixed, the solvent was removed under reduced pressure and the film dried in the dark under high

vacuum overnight. The desiccated lipids were rehydrated with potassium phosphate buffer (50 mM, pH 8, 2 mL) and BCECF acid (2 mM, 10 µL). In dim light the solution was shaken thoroughly, vortexed and transferred to a 5 mL cryovial. The vial was frozen in liquid nitrogen, and thawed at 37 ^{o}C. The lipids were shaken thoroughly until finely suspended and re-frozen. This process was repeated five times in total. The now finely dispersed vesicles were extruded 21 times (back and forth 10.5 times) through a lipid extruder (Avanti Polar Lipids, AL) containing a 100 nm pore. Non-encapsulated dye was removed by passing the pale yellow solution through a Sephadex G-50 size exclusion column (50 mM potassium phosphate buffer as eluent, pH 8). The vesicles were then stored in the dark on ice at 4 ^{o}C for further use. Phosphate concentration was determined using the Stewart method.(35)

***In vitro* assay using BCECF LUVs:** Excitation and emission wavelengths were set to 500 nm and 522 nm respectively. Freshly prepared lipid vesicles (10 µL) were added to potassium phosphate buffer (50 mM, pH 6, 2 mL). The fluorescence was monitored, with stirring, for approximately 100 seconds to establish a baseline. Oct-TriA_1 was added, and fluorescence was monitored until stable. Triton X-100 (1%, 50 µL) was added to quench fluorescence. Experiments shown are representative of results from three technical replicates.

NMR spectroscopy: Oct-TriA_1 and lipid II were dissolved separately in 600 µl of a 180 mM dodecylphosphocholine-d_{38} (DPC) solution (10% D_2O/90% H_2O in 10 mM sodium phosphate buffer at pH 6) to a final concentration of 4 mM. All spectra were referenced to the methylene protons in DPC at 1.52 ppm. One-

dimensional (1D) ^{1}H-NMR and two-dimensional homonuclear (2D) ^{1}H^{1}-H total correlation spectroscopy (TOCSY) and Nuclear Overhauser Effect spectroscopy (NOESY) experiments were acquired at 27 °C on a four channel 600 MHz Varian VNMRS spectrometer with a HCN z-axis pulsed-field gradient probe. The acquisition software used was VNMRJ 4.2A. TOCSY and NOESY experiments used a 8,000 Hz spectral window in both the directly and indirectly detected dimensions. 512 experiments were used to define the indirectly detected dimension, with 32 and 64 scans for each experiment for the TOCSY and NOESY spectra, respectively, with a total of 4,882 real and imaginary points acquired in the directly detected dimension. A spin lock mix time of 250 ms was used for TOCSY spectra and a mix time of 150 ms was used for NOESY spectra. Water suppression was achieved by presaturation during the relaxation delay. For NOESY, saturation of the water peak was also applied during the mix time. NMRPipe and NMRView were used for data processing. Chemical shift assignments were performed manually. After complete spectral assignment of Oct-TriA_1 and the Gram-negative lipid II analogue, the solutions were mixed and the TOCSY (70 ms mix time) and NOESY (125 ms mix time) data were acquired for the TriA_1-Lipid II complex at 20 °C on a triple resonance HCN cryoprobe-equipped Varian VNMRS 700 MHz spectrometer with z-axis pulsed-field gradients and VNMRJ 4.2A as host control. Spectral width for both experiments was 10,000 Hz in both the directly and indirectly detected dimensions. 256 acquisitions were used to define the indirectly detected dimension, with 96 and 128 scans for each experiment for the TOCSY and NOESY spectra, respectively,

with a total of 8,192 real and imaginary points acquired in the directly detected dimension.

Structure calculations: CYANA 2.1(33) was used to calculate the structure of Oct-TriA_1 in DPC micelles with and without lipid II using automatically assigned NOE cross peaks. Custom library files for non-canonical amino acids were created based on energy minimized structures drawn with Avogadro. For Oct-TriA_1 in DPC micelles without lipid II, 211 crosspeak NOEs were selected and 134 of these automatically assigned by CYANA (101 short-range, 13 medium-range and 20 long-range) were used in the structure calculation. For Oct-TriA_1 with lipid II, 188 crosspeak NOEs (168 short-range, 14 medium-range and 6-long-range) were used in the structure calculation. Seven cycles were done with 10,000 steps per cycle giving 20 structures. Coordinates for the structure of Oct-TriA_1 in DPC micelles without lipid II have been deposited in the PDB (2n5w) and the chemical shifts deposited to BMRB (accession number 25737). Coordinates for the structure of Oct-TriA_1 in DPC micelles with lipid II have been deposited in the PDB (2n5y) and the chemical shifts deposited to BMRB (accession number 25741). Structures were generated from the CYANA output .pdb files using MacPyMOL. The structure with the lowest target function was used in subsequent docking studies.

Docking studies with lipid II: CYANA was used to calculate the structure of lipid II in the presence of Oct-TriA_1 using 67 NOEs restraints (64 short-range and 3 medium-range). Out of the 20 calculated structures, the first structure had the lowest target function value and was used as the input ligand structure for

docking studies. AutoDockTools (v. 1.5.6) was used to convert the lipid II and Oct-TriA$_1$ .pdb files to .pdbqt files. The exhaustiveness was set to 8. *Z,Z*-Farnesyl Gram-negative lipid II was then docked into Oct-TriA$_1$ using AutoDock Vina.(34)

Lipid II analogue synthesis: See supplementary information.

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Author Contributions

SAC performed ITC, lipid II inhibition studies, peptide synthesis and lipid II synthesis. BF performed whole cell experiments and *in vitro* vesicle assays. AB synthesized some lipid II precursors. AB and PM consulted on NMR experiments. SAC and JA performed NMR experiments and docking studies. ERL synthesized DAP. PM and BF created custom residues for CYANA. SAC prepared the manuscript, with input from the other authors. SAC, BF, JA and JV conceptualized the experiments.

Competing Financial Interests

The authors declare no competing financial interests.

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Figure legends

Figure 1. Structures of the tridecaptin analogues $TriA_1$ and Oct-$TriA_1$

Figure 2. (A) Bacterial growth kinetics. Optical densities of *E. coli* cells exposed to 2x minimum inhibitory concentration (MIC) of ampicillin (8 μg/mL), chloramphenicol (32 μg/mL), tridecaptin A_1 (6.25 μg/mL) and polymyxin B (4 μg/mL). Tridecaptin A_1 reduces cell density after twenty minutes of exposure. (B) Time-kill assays. *E. coli* cells were treated with 10xMIC of each antibiotic (50 μM) and the number of viable cells determined at different time points. Tridecaptin A_1 kills cells more slowly than polymyxin B.

Figure 3. Inner-membrane assays. (A) Membrane depolarization assay. Addition of $TriA_1$ to $DiBAC_4$ treated *E. coli* cells decreases fluorescence by dilution but no membrane depolarization is observed. Polymyxin B leads to expected fluorescence increase as inner-membrane is depolarized. (B) Membrane disruption assay. Addition of $TriA_1$ to SYTOX Green treated *E. coli* cells does not cause immediate pore formation. Triton X-100 causes rapid inner-membrane lysis. (C) Disruption of proton motive force detected as fluorescence decrease from BCECF-AM treated *E. coli* cells. Glucose increases the proton motive force, increasing the cytoplasmic pH and increasing fluorescence. Valinomycin disperses the electrochemical gradient and addition of $TriA_1$ rapidly decreases

fluorescence. Subsequent addition of nigericin further decreases fluorescence at a faster rate than $TriA_1$.

Figure 4. (A) Structure of Gram-negative and Gram-positive lipid II. (B) ITC of $TriA_1$ + Gram-negative lipid II. (C) Spot-on-lawn assay with *E. coli* cells. Left = $TriA_1$ (50 μM), middle = 1:1 $TriA_1$ (100 μM):G^+LII (100 μM) and right = 1:1 $TriA_1$ (100 μM):G^-LII (100 μM). $TriA_1$ is active and premixing with Gram-positive lipid II (G+LII) slightly reduces the zone of inhibition. Premixing with Gram-negative lipid II (G-LII) abolishes activity.

Figure 5. (A) *In vitro* assay measures formation of small proton pores. BCECF is encapsulated in LUVs with an internal pH of 8, whilst the external buffer is pH 6. Pore formation results in a proton gradient and decrease in fluorescence. (B) BCECF LUVs with no lipid II, 1 mol% Gram-negative lipid II or 1 mol% Gram-positive lipid II are treated with 1.8 μM Oct-$TriA_1$. Gram-negative lipid II significantly accelerates pore formation.

Figure 6. Total synthesis of (*Z*,*Z*)-farnesyl Gram-negative lipid II (**1**). (a) TMSOTf, 4Å MS, CH_2Cl_2, rt, 18h, 61%; (b) (i) $ZnCl_2$, AcOH/Ac_2O, rt, 24h (i) Zn, THF/AcOH/Ac_2O, rt, 24h, 63% (2 steps); (c) (i) H_2, Pd/C, MeOH, rt, 3h, (ii) $(^iPr)_2NP(OBn)_2$, tetrazole, CH_2Cl_2, rt, 2h, (iii) 30% H_2O_2/THF, -78 ^oC, 2h, 84% (3 steps); (d) DBU, CH_2Cl_2, rt, 0.5h, quant.; (e) Tetrapeptide **5**, TFA/CH_2Cl_2, 2h; HATU, DIPEA, DMF, rt, 24h, 78%; (f) (i) H_2, Pd/C, MeOH, rt, 2.5h, (ii) CDI activated (*Z*,*Z*)-farnesyl phosphate, DMF, rt, 4d, (iii) NaOH, H_2O/dioxane, 37 ^oC, 2 h, 25 % (3 steps).

Figure 7. (A) NMR solution structure of $TriA_1$ in DPC micelles containing Gram-negative lipid II. Orange = hydrophobic residues, purple = D-Dab8 and cyan = other residues. (B) Lipid II analogue **1** docked into $TriA_1$. Hydrophobic residues interact with the lipid II terpene tail and the pentapeptide occupies the binding pocket. (C) Modeled interaction shows H-bonding between D-Dab8 and DAP3.

Figure 8. Resistance study with *E. coli* cells.