Synthesis and Characterization of Retro-Inverso Peptides (VVRRVV and vvrrvv) and Their Derivatives towards Antimicrobial Applications

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Abstract

This study investigates the antibacterial activities of hexapeptides and derivatives. The use of solid phase and liquid phase synthesis produces a pair of enantiomeric hexapeptides (VVRRVV and vvrrvv) and the relevant derivatives (Ac-vvrrvv and Ac-vvrrvv-C12) in moderate yields. VVRRVV and vvrrvv form heterogeneous aggregates in PBS buffer at pH 7.4 and show little toxicity on the mammalian cells tested. Both VVRRVV and vvrrvv inhibit 55% of E. coli growth at 16 h and 35% of growth at 24 h at the concentrations ranging from 20 µM to 100 µM. N-terminal acylation (e.g., Ac-VVRRVV and Ac-vvrrvv) results in decreased inhibition activities. C-terminal modification via addition of a long hydrocarbon chain leads to slight increase of the inhibition activity. The co-incubation with Ac-vvrrvv-C12 and vancomycin decreases the activity of vancomycin against E. coli. These results suggest that Ac-vvrrvv-C12 likely leads to cell death by embedding itself in the lipid bilayer, altering the composition and properties of the bacterial membrane, and disrupting homeostasis. It is also possible that Ac-vvrrvv-C12 forms aggregates around the outer membrane and prohibits ion exchange, an assumption that agrees with declined inhibition by vancomycin in the presence of Ac-vvrrvv-C12.
Introduction

Emergence of Antimicrobial Drug Resistance

Antimicrobial resistance is the ability of microbes to counter the effects of antibacterial drugs. Microbes are small organisms that are invisible to the naked eyes. Microbes are classified as bacteria, viruses, parasites, and fungi. Many bacteria are capable of causing severe diseases in humans. For example, tuberculosis is caused by *Mycobacterium tuberculosis*, whooping cough by *Bordatella Pertussis*, and Bubonic plague by *Yersinia pestis*. As a result, human develop antibiotic drugs as a way to treat bacterial infectious diseases.

Although antibiotic drugs are exceptionally effective at treating bacterial infections, they also give rise to the development of drug resistance among bacteria via selectivity. The selectivity induced by antibiotics weeds out susceptible bacteria and selects the ones that are resistant. Some resistant bacteria could become even more resistant under the selective pressure by initiating single genetic mutations or acquiring exogenous resistant genetic material from the environment.¹

Intensive and frequent use of antibiotic drugs escalates bacterial resistance in both hospitals and communities. The relationship between frequent antibiotic use and the development of resistance has been recognized in hospitals. Evidence of such is the increasing resistance seen in *Enterococci* against vancomycin. *Enterococci* is the second most commonly found bacterium in hospitals that results urinary tract infections and wound infections. Vancomycin is heavily used to treat infectious disease caused by *Enterococci*. Due to the excessive use of vancomycin, vancomycin resistance among *Enterococci* has surged from 0.5% in 1989 to more than 18% in 1997 within hospitals in the United States.² Furthermore, the resistance problem is also encountered persistently in communities. High antibiotic resistance is associated with the overuse and misuse of antibiotics in many
communities, as noted in several communities in the United States and several countries in Asia.\(^3\) Resistant strains can be traced from hospitals to communities and vice versa, indicating that drug resistance is delocalized.\(^4\)

It is not surprising that some bacteria are showing multidrug resistance when considering their high level of resilience. The inevitability of bacteria to find ways to resist various types of drugs poses obstacles in treating bacterial infectious diseases. For example, *Pneumococci*, a bacterium that causes ear infections and pneumonias, particularly in children, first showed penicillin resistance, followed by macrolide and tetracycline resistance.\(^5\) Researchers have predicted that multidrug resistant bacteria will outgrow the single drug resistant bacteria in ten years.\(^6\) Strains of *N. gonorrhoeae* even exhibit triple resistance to penicillin, fluoroquinolones, and tetracycline.\(^7\)

Antibiotic drug resistance that emerged from overuse and misuse of drugs continues to be a global issue. Resistance in some strains of *Escherichia coli* (*E. coli*) to the commonly used drug fluoroquinolones poses obstacles in treating urinary tract infection. In certain parts of southeast Asia and China, about 70% of *E. coli* adopt resistance to fluoroquinolones\(^8,9\) In the United States, 10% of *E. coli* show resistance to fluoroquinolones.\(^10\) About 24% of *E. coli* showed resistance to multiple drugs when treating urinary tract infection in the Netherlands and about 39% in Belgium.\(^11\)

The rise of antibiotic resistance not only poses a great challenge for public health, but also compromises our ability to treat bacterial infectious disease. Development of new antibiotic drugs is a myopic act in terms of long-term inhibition, as bacteria will continue to evolve to resist new drugs. Therefore, a novel approach to control drug resistant bacteria is urgent and necessary.
**Application of Peptide Based Hydrogel**

Antimicrobial peptide (AMP) emerges as a new approach to kill microbes by disrupting cell membrane without displaying toxicity. These peptides are derived from precursor proteins in animals and plants that play an important role in innate defense mechanisms.\(^1\) AMPs are amphipathic oligopeptides that contain three to over a hundred amino acid residues.\(^2\) They constitute cationic portions due to multiple lysine and arginine residues, as well as hydrophobic portions.\(^3\) They are able to form amphipathic secondary structures such as alpha-helices and beta-sheets. The positive charges on the peptide allow them to interact with anionic bacterial membranes via electrostatic forces. Hydrophobicity in the secondary structures helps them integrate into the bacterial membranes.\(^4\) Incorporation of foreign molecules causes stress in the bacterial phospholipid bilayer, leading to membrane perturbation.

**Figure 1.** Mechanisms of membrane permeabilization by antimicrobial peptides.\(^5\)

Peptides imposing positive curvature strain on membranes by expanding the polar head group region form the ‘toroidal’ pore, unless strongly inhibited by the bilayer. The peptides can translocate into the inner leaflet upon disintegration of the pore. (B) In the presence of negative curvature-inducing lipids, large amounts of peptides (P/L≈1/10) are accumulated on the bilayer surface, eventually leading to irreversible membrane disruption.
René Dubos discovered the first AMP in 1939. Dubos extracted an antimicrobial agent of a \textit{Bacillus} soil strain that was able to protect mice from pneumococci infection.\textsuperscript{15-16} In the following year, the extract was named gramicidin, which was used to treat wounds and ulcers.\textsuperscript{17} While most naturally occurring AMPs are activated through post-translational modification, synthetic AMPs require modification to either achieve desired antimicrobial activities or to improve antimicrobial abilities.\textsuperscript{18} For instance, Hu and colleagues found that N-terminal acylation of AMP chitosan enhanced the inhibition of \textit{E. coli} and \textit{P. aeruginosa} by 200\% to 600\%.\textsuperscript{19} Other chemical modifications include \textit{N}-methylation, glycosylation, and heterocyclic ring formation.\textsuperscript{20} Acylation of cationic AMPs also results in a wider microbial inhibition spectrum and less susceptibility to proteolytic degradation.\textsuperscript{21}

As nanotechnology continues to advance at a staggering rate, researchers are starting to design peptides that can self-assemble into well-ordered nanostructures. Many synthetic peptides have biocompatible applications such as 3D cell culture, drug delivery, tissue engineering, and antimicrobial ability.\textsuperscript{22} It is noteworthy is that self-assembly of bio-macromolecules is a common prevalent process in nature. Phospholipid bilayers and polymeric nucleic acid housing genetic information are manifestations of bio-macromolecule self-assembly.\textsuperscript{23}

Among the materials that formed by peptide, hydrogels are the most promising ones due to their advantages. Hydrogels have gelling like structure, which consist of more than 99\% weight of water and only 1\% weight of hydrogelators. Self-assembly of hydrogelators is driven by noncovalent interactions such as aromatic stacking, ionic interactions, and London dispersion force between the hydrogelators or with the aqueous solvent. Hydrogelators then form nanofibrous structures that entangle with each other to form hydrogel.\textsuperscript{24}
**Objective**

With increasing understanding of protein functions, researchers are starting to incorporate peptide epitopes as the functional moiety of hydrogelators for a wide range of biomedical applications, such as promoting stem cell differentiation.

My project is to design and synthesize a retro-inverso supramolecular peptidic hydrogel that mimics the function of apoptosomes, which could activates a caspase cascade, inhibiting bacterial growth. Retro-inverso peptides constitute D-amino acids in a reversed sequence, and therefore could enhance the stability of the peptide. Research has shown a 5-fold increase in potency among retro-inverso peptides compared to D-amino acid peptides. Furthermore, they are more resistant to proteolytic degradation.\textsuperscript{25}

Epitope of human gasdermin D (GSDMD) was examined because it is an important protein that participates in inflammatory caspase cascade that triggers pyroptosis, which is inflammatory cell death.\textsuperscript{26} Under microbial infection, inflammatory caspases are activated and cleave human GSDMD to generate N-terminus cleavage product.\textsuperscript{27} The cleavage product oligomerizes within the cell, binds to cell membranes, and induce cell death.

A short peptide sequence vvrrvv was identified in the N-terminus of human GSDMD. Retro-inverso modification was performed on the sequence to enhance both cytotoxicity and resistance to proteolytic degradation. The arginine residues in the sequence bear positive charges at physiological pH. Therefore, vvrrvv and its relevant derivatives are promising AMPs capable of inhibit microbial growth.
Materials and Methods

General Methods

The following procedures were performed in air unless specified. Reactions were carried out in oven-dried glassware and stirred magnetically under room temperature. All reagents and solvents were purchased from commercial vendors and were used without further purification unless specified. Peptides were synthesized manually by the Fmoc method via solid phase synthesis on chloride resins. All peptides were dissolved in MeOH and further purified via reverse-phase High-Performance Liquid Chromatography with a Water Delta6000 HPLC system equipped with an XTerra C18 RP column and in-line diode UV detector. 0.1% TFA in HPLC grade distilled water and 0.1% TFA in HPLC grade acetonitrile were the HPLC eluents. Purification method was set at a flow rate of 8 mL/min and an absorbance wavelength of 220 nm. All compounds were analyzed by Liquid Chromatography-Mass Spectrometry that has a Water Acquity Ultra Performed LC with Waters MICRO-MASS detector. A Morgagni 268 transmission electron microscope was used to characterize peptides. A DTX880 Multimode Detector was used in cellular toxicity assessment. Chemical names were abbreviated as the following: DIEA= N, N-diisopropylethylamine; HBTU= N, N, N’, N’- Tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate; DMF= dimethylformaide; DCM=dichloromethane, TFA= trifluoroacetic acid, TIS= triisopropylsilane. CFU=colony forming units

Solid Phase Peptide Synthesis

Synthesis of VVRRVV and vvrvv

Resin (0.4 g, 0.5 mmol) was swelled in anhydrous DCM (20 mL) for 5 min. To the swelled resin, Fmoc-Val-OH (0.51 g, 1.5 mmol), DCM (20 mL), and DIEA (1.3 mL) were combined and allowed to react for 1h. The resin was washed with DCM (5x 20 mL). A mixture of
DCM: DIEA: MeOH 80:5:15 (20 mL) was added to block the unreacted resin for 15 min. The resin was washed with DCM (5x 20 mL) followed by DMF (5x 20 mL). Piperidine: DMF 20:80 by volume (6 mL) was added to remove the Fmoc group for 30 min. The resin was washed with DMF (5x 20 mL). A solution of Fmoc-Val-OH (0.51 g, 1.5 mmol), HBTU (0.57 g, 1.5 mmol) and DIEA (1.3 mL) in DCM (20 mL) was added to the resin and the reaction was allowed to react for 1h. The resin was washed with DMF (5x 20 mL). Piperidine: DMF 20:80 by volume (6 mL) was added to remove the Fmoc group for 30 min. After all amino acids were added to the resin and the Fmoc group of the last amino acid was removed, the resin was washed with DMF (5x 20 mL) followed by DCM (5x 20 mL). TFA: TIS: dH$_2$O 19 mL: 0.5 mL: 0.5 mL was added to the resin to cleave off the peptide for 2h. The solution was dried in air to give a clear oil, to which diethyl ether (20 mL) was added to afford crude peptide as white precipitates (0.81 g, 70% yield). 150 mg of the crude peptide was dissolved in MeOH (20 mL). 1M HCl was used to adjust the pH until solution became clear and homogenous. The resulting solution was filtered with a microfilter and purified by HPLC to give 24 mg (16% yield).
Scheme 1. Solid Phase Synthesis of vvrrvv and VVRRVV

Synthesis of AcVVRRVV and Acvvrrvv

Resin (0.4 g, 0.5 mmol) was swelled in anhydrous DCM (20 mL) for 5 min. To the swelled resin, Fmoc-Val-OH (0.51 g, 1.5 mmol), DCM (20 mL), and DIEA (1.3 mL) were combined and allowed to react for 1h. The resin was washed with DCM (5x 20 mL). A mixture of DCM: DIEA: MeOH 80:5:15 (20 mL) was added to block the unreacted resin for 15 min. The resin was washed with DCM (5x 20 mL) followed by DMF (5x 20 mL). Piperidine: DMF 20:80 by volume (6 mL) was added to remove the Fmoc group for 30 min. The resin was washed with DMF (5x 20 mL). A solution of Fmoc-Val-OH (0.51 g, 1.5 mmol), HBTU
(0.57g, 1.5 mmol) and DIEA (1.3 mL) in DCM (20 mL) was added to the resin and the reaction was allowed to react for 1 h. The resin was washed with DMF (5x 20 mL).

Piperidine: DMF 20:80 by volume (6 mL) was added to remove the Fmoc group for 30 min. After all amino acids were added to the resin and the Fmoc group of the last amino acid was removed, DMF: Acetic Anhydride: DIEA 14 mL:1 mL: 3.3 mL was added to the resin. The reaction was allowed to react for 1 h. The resin was washed with DMF (5x 20 mL) followed by DCM (5x 20 mL). TFA: TIS: dH₂O 19 mL: 0.5 mL: 0.5 mL was added to the resin to cleave off the peptide for 2 h. The solution was dried in air to give a clear oil, to which diethyl ether (20 mL) was added to afford crude peptide as a white precipitate (0.93g, 81% yield). 600 mg of the crude peptide was dissolved in MeOH (20 mL). 1M HCl was used to adjust the pH until solution became clear and homogenous. The resulting solution was filtered with a microfilter and purified by HPLC to give 180 mg (30% yield).
Scheme 2. Solid Phase Synthesis of Acvvrrvv and AcVVRRVV

**Synthesis of AcVVRRVVC12 and AcvvrrvvC12**

To a 100 mL round bottom flask, C$_{12}$H$_{28}$ClN (0.05 g, 1.1 equiv.), Acvvrrvv (0.14 g, 0.19 mmol, 1 equiv.) HBTU (0.071 g, 1 equiv.) and DMF (40 mL) were combined. The pH of the solution was adjusted to 8 with DIEA. The solution was stirred for 48 h and dried in air to give opaque oil (163 mg, 94% crude yield). The compound was precipitated with diethyl
ether and dissolved in MeOH (20 mL) for HPLC. 1M HCl was used to adjust the pH until the solution became clear and homogenous. AcVVRRVVC12 was lost during HPLC. Purified AcvvrrvvC12 had a weight of 0.012 g.

![Chemical Structure](image)

**Scheme 3.** C-terminal Modification of Acvvrrvv and AcVVRRVV

**TEM Sample Preparation of VVRRVV, vvrrvv, and mixture of VVRRVV and vvrrvv**

Each compound (1 mg) was dissolved in PBS buffer (100 µL) to give a 1% w/v mixture. For the mixed gel, 1 mg of each compound was dissolved in PBS buffer (200 µL) to give a 1% w/vv mixture. 1M NaOH and 1M HCl was used adjust the pH of all mixture to 7.4. The mixture was left for 24h. 4 µL of each gel was loaded onto a separate carbon film coated with 400 mesh copper grids that were previously glowed and discharged. All gels were washed with ddH2O three times, stained with uranyl acetate (2% w/v) three times, and dried in air. A TEM image of each mixture was taken right away.
HeLa Cell Culture

HeLa Cell line was purchased from American-type Culture Collection (ATCC, USA). HeLa Cells were cultured in MEM Medium supplemented with 10% v/v fetal bovine serum, 100 µMure Collection (ATCC, USA). HeLa Cells were in an humidified environment of 37 °C and 5% CO₂.

MTT Assay

MTT Assay was used to test the cytotoxicity of the compounds. HeLa cell lines were seeded in 96-well plates at 1×10⁵ cells/ well for 24 h followed by culture medium removal. Afterwards, various concentrations (31.25µM/ 62.5 µM/ 125µM/ 250µM/ 500µM) of vvrvvv, VVRRVV and the mixture of the two were added to each well along with culture medium. At designated time (24 h/ 48 h/ 72 h), 5 mg/mL MTT solution (10 µL) was added to each well and incubated at 37 °C for additional 4 h, followed by the addition of SDS-HCl solution (100 µL) to quench by the reduction reaction and dissolve the purple formazan. Cytotoxicity was evaluated by direct visualization with phase-contrast microscopy and cell counting.

Bacterial Culture

E. coli (Wild-type K12, ATCC 25922) was obtained from the American Type Culture Collection (ATCC). Bacteria were cultured at initial concentration of 10⁵ CFU ml⁻¹ in LB medium (Sigma-Aldrich) in a shaker-incubator at 37°C and 250 r.p.m. Bacteria were pre-inoculated on a shaker-incubator and aliquots placed into 96-well plates at optical density at 600 nm of 0.005. Bacterial growth kinetics was measured in 96-well plates in LB sterile medium at 37°C without shaking for 16 h and 24 h.
Bacterial Growth Inhibition

*E. coli* was cultured in LB medium overnight to stationary growth phase. After dilution to optical density at 600 nm of 0.005, bacterial was sub-cultured in the same medium until they reached an optical density of 1. Then the bacteria that were in exponential growth phase were diluted to optical density at 600 nm of 0.005. Each well was placed with 200 µl of bacteria. All compounds were added at a final concentration of 20, 40, 60, 80, 100 µM. In all plates, the optical density at 600 nm was measured after 16 h and 24 h of incubation at 37°C. Negative controls (1% DMSO) and positive controls (0.8 µg/mL, 1.65 mM kanamycin), which inhibit *E. Coli* growth were run in each plate. The percentage bacterial growth inhibition was computed as: percentage inhibition=100×(ODnegative control−ODtest compound)/(ODnegative control−ODpositive control).
Results

TEM characterization

Figure 2: The TEM images of 1% W/V (A) vrrvv, (B) VVRRVV, and (C) a 1:1 mixture of vrrvv and VVRRVV in PBS buffer at pH 7.4.

1% W/V of vrrvv, VVRRVV, and a mixture of D and L were characterized by transmission electronic microscopy (Figure 2). The TEM images showed that all three samples did not formed uniform aggregates.

MTT Assay

Figure 3. Cytotoxicity of HeLa cells measured at 24 h, 48 h, 72 h upon the addition of VVRRVV, vrrvv, and a mixture of the two at a concentration of 31.25 µM, 62.5 µM, 125
µM, 250 µM, and 500 µM at pH 7.4.

HeLa cell viability in the presence of each compound at various concentrations was shown in Figure 3. For vrrrvv, cell viability did not change much with time for a given concentration. Among all concentrations, 500 µM of vrrrvv resulted in the lowest bacteria viability of 85% at all time points. For VVRRVV, cell viability was between 90% to 100% for all concentrations at every time point. For the mixture of D and L, cell viability changed neither with concentration nor with incubation time. All three compounds hardly caused cell death at all concentrations, thus they are compatible with cells.

**Bacterial Growth Inhibition**

![Bacterial Growth Inhibition](image)

**Figure 4.** Percent of inhibition against *E. coli* growth after incubating with VVRRVV and vrrrvv at pH 7.4.

Both D and L stereoisomers showed similar inhibition activities for a given concentration and
time. Inhibition activity did not depend on concentration nor was specific to each stereoisomer. Decreasing inhibition activities with respect to time suggested that both compounds did not reduce the growth of \textit{E. coli}.

![Figure 5](image-url)

**Figure 5.** Percent of inhibition against \textit{E. coli} growth in the presence of Ac-VVRRVV at pH 7.4.

The inhibition activities of Ac-VVRRVV did not change with increasing concentration. Compared to the inhibition activities of VVRRVV, Ac-VVRRVV showed some antibacterial activities with respect to time. At all concentrations, VVRRVV reduced its percent of inhibition from 55% to 35% with time. However, Ac-VVRRVV showed similar antibacterial activity of 35% with time. Although acylation of the N terminal of VVRRVV lowered the inhibition activities at 16h, it did prevent further bacterial growth at some extent.
The antibacterial ability of Ac-vrrrvv did not change much with increasing concentration. The percent inhibition declined from 50% at 16 h to 35% at 24 h; therefore Ac-vrrrvv did not inhibit further bacterial growth. Compared to the L stereoisomer, which had an inhibition against *E. coli* of 35% at 16 h and 24 h, the D stereoisomer showed greater inhibition activity at 16 h but a similar inhibition activity at 24 h. Prior to Ac modification, vrrrvv possessed an inhibition of 55% at 16 h and 35% at 24 h. Ac-vrrrvv showed similar inhibition ability as vrrrvv. Acylation of the N-terminal did not alter the antibacterial effects of the original sequence.
Figure 7. Percent of inhibition against *E. coli* growth upon the addition of Ac-vvrrvv-C12 at various concentrations.

The inhibition activity of Ac-vvrrvv-C12 was positively correlated with concentration. Although the antibacterial activity was lower at 24 h than 16 h at a concentration of 20 µM and 40 µM, the antibacterial activity was very similar between the two time points at a concentration of 60 µM and beyond. It was reasonable to conclude that the compound was able to inhibited further bacterial growth at a higher concentration. Modification of the C-terminal with long hydrocarbon chain enhanced the antibacterial ability.
Figure 8. Percent of inhibition against *E. coli* growth in the presence of vancomycin, Ac-vvrrvv-C12, and vancomycin and Ac-vvrrvvC-12 mixture at various concentrations. Negative controls (1% DMSO) and positive controls (0.8 mg/mL, 1.65 mM kanamycin) were run in each plate. Percentage inhibition = $100 \times \frac{(\text{OD}_{\text{negative control}} - \text{OD}_{\text{test compound}})}{(\text{OD}_{\text{negative control}} - \text{OD}_{\text{positive control}})}$. 
Discussions

Vancomycin is an effective antibacterial drug that has a unique means of action against gram-positive bacteria. It inhibits the second stage of cell wall synthesis of susceptible bacteria, specifically the peptidoglycan layer synthesis. Peptidoglycan is an essential component of the cell wall as it protects the cell from osmotic pressure and cell lysis. Unlike gram-positive bacteria, gram-negative bacteria such as *E. coli* have additional outer membrane outside the peptidoglycan layer (Figure 9).

![Figure 9. Cell wall of gram-negative bacteria.](image)

During cell wall synthesis, transpeptidase enzymes bind to short peptide monomers and facilitate cross-link formation. Vancomycin recognizes and binds to a highly specific region, D-alanyl-D-alanine (D-Ala-D-Ala) at the free carboxyl end, of the peptidoglycan precursors that are only present in the cell wall. It is postulated that the vancomycin-peptide conjugate sterically inhibits the transpeptidase from approaching the active site of the peptide, thus disabling peptidoglycan layer synthesis and making cells more susceptible to osmotic pressure.
Gram-negative bacteria have multiple ion channels integrated in the outer membrane that are capable of facilitating diffusion of small molecules of less than 700 Dalton. However, vancomycin is too large (with a molecular weight of 1440 Dalton) to fit into the ion channels and diffuse to the peptidoglycan layer during stationary phase of the *E. Coli*. On the other hand, vancomycin is able to get in contact with the peptidoglycan layer during cell division due to the nature of crosslinked peptidoglycan network. Covalent bonds must be broken to allow cell division. One of the consequences of bond breakage is that the peptidoglycan layer is exposed to the environment. As a result, vancomycin is able to bind to D-ala-D-ala sequence, inhibit transpeptidase from approaching the active site, and prevent crosslinking between peptidoglycan precursors during cell division (Figure 10).

![Figure 10](image)

**Figure 10.** Inhibition of transpeptidase’s active site by vancomycin during cell division.

Previous data showed that increasing concentration of Ac-vvrrvv-C12 led to greater inhibition. Furthermore, at a concentration greater and equal to 60 µM, Ac-vvrrvv-C12 was able to inhibit further bacterial growth between 16 h and 24 h (Figure 6, 7). Compared to
inhibition activity of Ac-vvrrvv at 24 h, modification of the C-terminal with long hydrocarbon chain enhanced the antibacterial ability from 40% to 60% at 80 µM and 40% to 70% at 100 µM.

Ac-vvrrvv-C12 is an amphiphilic molecule that could potentially interact with the hydrophobic phospholipid layers within the outer membrane through its hydrocarbon tail. It is possible that Ac-vvrrvv-C12 partially embeds itself in the lipid raft, changes the integrity of the phospholipid bilayer, and results in cell lysis. It is also likely that Ac-vvrrvv-C12 forms aggregates outside the cell, prohibits ion exchange, and alter cellular homeostasis.

Figure 11. Possible interactions between Ac-vvrrvv-C12 and E. coli cell wall and steric hindrance posed by Ac-vvrrvv-C12 on vancomycin approaching peptidoglycan.

Since Ac-vvrrvv-C12 was capable of inhibiting bacterial growth, a greater inhibition activity was expected when E.coli was co-incubated with Ac-vvrrvv-C12 and vancomycin. However, the mixture of vancomycin and Ac-vvrrvv-C12 exhibited lower inhibition effects as opposed to vancomycin alone at any concentration. This result suggested that Ac-vvrrvv-C12 jeopardizes vancomycin’s ability to disrupt peptidoglycan synthesis, resulting in
declining inhibition. It is might pose steric hindrance for vancomycin to approach the cell. Therefore its inhibition ability was undermined (Figure 11).

Considering its amphiphilic nature, Ac-vvrrvv-C12 might embed itself in the lipid raft and sterically prevent vancomycin from approaching the outer membrane during cell division. The side chain of arginine has a pKa of 12.5. The cellular environment has a pH of 7.4. Therefore Ac-vvrrvv-C12 bears positive charge, which allows electrostatic interaction with the negatively charged cell membrane. Ac-vvrrvv-C12 that are proximal to the cell surface could insert its long hydrocarbon tail into the membrane and cause steric hindrance against incoming vancomycin. It is also likely that Ac-vvrrvv-C12 forms aggregates around the outer membrane. When vancomycin is introduced, these aggregates block vancomycin from approaching the cell. More experiments are needed to investigate the action and mechanism of Ac-vvrrvv-C12 inhibition.
Conclusion

We have successfully synthesized two stereoisomers of vvrqvq and their relevant derivatives Ac-vvrqvq and Ac-vvrqvq-C12 via solid phase synthesis and liquid phase synthesis. We showed that VVRVV, vvrqvq, and a mixture of the two isomers did not form uniform aggregates in PBS buffer at pH 7.4. All three compounds did not have a significant effect on HeLa cell viability so that they are compatible with mammalian cells. Both VVRVV and vvrqvq inhibited 55% of *E. coli* growth at 16 h and 35% of growth at 24 h across concentrations from 20 µM to 100 µM. Ac-VVRVV and Ac-vvrqvq showed decreasing inhibition activities compared to their pre-modification analogs at the same concentration and time. However, inhibition by Ac-vvrqvq-C12 was positively correlated with its concentration. Ac-vvrqvq-C12 slightly increased the inhibition at a concentration beyond 60 µM. It was reasonable to speculate that C-terminal alkylation improved antibacterial ability. Upon co-incubation with Ac-vvrqvq-C12 and vancomycin, less *E. coli* was inhibited as opposed to incubation with vancomycin alone.

It is speculated that Ac-vvrqvq-C12 embeds itself in the lipid raft and disrupts cellular homeostasis. It is also likely that Ac-vvrqvq-C12 forms aggregates around the outer membrane and prohibits essential ion exchange. This was consistent with the observation that vancomycin showed attenuated inhibition in the presence of Ac-vvrqvq-C12. More experiments are needed to investigate the action and mechanism of Ac-vvrqvq-C12 inhibition.
Future Direction

Although bacterial inhibition by Ac-vvrrv-C12 showed dependency on concentration, the result was not yet proved to be statistically significant. The experiment will be replicated on multiple *E.coli* samples to perform t-test.

Additional experiments will be performed to investigate the mechanism of Ac-vvrrvv-C12 inhibition. Gellation tests and subsequent TEM imaging will be performed to see if Ac-vvrrvv-C12 forms aggregates under cellular environment. Ac-vvrrvv-C12 will be modified with fluorescent NBD compound and visualized with confocal imaging to determine the location of the compound upon incubation. In the same time, moiety of the phospholipid membrane in the presence of Ac-vvrrvv-C12 would be studied to examine Ac-vvrrvv-C12 and phospholipid membrane interaction.

Ac-VVRRVV-C12 will also be synthesized and used to test its antibacterial activity against *E.coli*. Ac-vvrrvv showed greater inhibition than Ac-VVRRVV at any given concentration and time. It is possible that D-stereoisomers have better inhibition ability.

N-terminal alkylation of antimicrobial peptides has been shown to improve antibacterial activities. N-terminal alkylated analogs of vvrrvv would be synthesized and used to study its inhibition ability to investigate whether or not acylation at different terminal would cause variance in antibacterial ability.
Supporting Figures

S1. LC-MS of Ac-vvrrvv

S2. LC-MS of Ac-VVRRVV
S3. LC-MS of Ac-VVRRVV-C12 and Ac-vrrvvv-C12
Reference


