Conversion of a Short Lipopeptoid into Longer Non-lipidated Repeat Peptoids with Improved Activity

By

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Abstract

The need for new antibiotics to treat antibiotic resistant bacteria has been a growing problem worldwide over the past several years. Bacteria are quickly developing modifications to render antibiotics useless due to overuse and misuse of medications. With the need for new classes of antibiotics, developing peptoids as potential antibiotics to treat nosocomial pathogens is the overarching goal of this research project. The ESKAPE bacteria are the focus of this research because of their well characterized global health risk. A series of monomer sequence repeat peptoids, termed the MNT series, were designed from a previously discovered lipopeptoid, termed ALA1. This was done in an effort to determine the peptoid length requirement when converting a short lipopeptoid into a longer non-lipidated peptoid. It was hypothesized that removing the lipid tail would reduce toxicity while lengthening the peptoid would allow for retention of antibacterial activity. A total of four MNT series peptoids (MNT1, MNT2, MNT3, and MNT4) ranging from 3 to 12 monomers in length without a lipid tail were synthesized and characterized for antibacterial efficacy and mammalian cytotoxicity. This research determined that MNT3, which is 9 monomers in length, was the most promising ALA1 derivative. This compound was more potent towards gram-positive pathogens, such as *E. faecium, E. faecalis,* and *S. aureus* than ALA1 or the longer MNT4 peptoid. In general, all non-lipidated MNT series peptoids were less effective against gram-negative bacteria than ALA1. Cytotoxicity testing of the MNT series peptoids and ALA1 against HepG2 liver cells and red blood cells indicated that MNT3 had lower cytotoxicity than ALA1 or MNT4. The shortest derivatives, MNT1 and MNT2 displayed no antibacterial activity or cytotoxicity, demonstrating the dependence of peptoid length or lipidation for both of these parameters.

Table of Contents

List of Figures

List of Tables

Chapter One: Introduction

History of Antibiotics

Infections can originate from different sources and are transferred through either direct or indirect contact between humans, animals, or various environmental sources.[1] Until the last century, people did not know that bacteria were the cause of infections. Discovered by some of the earliest civilizations, such as Ancient Egypt, various molds and plant extracts can be used to treat infections. This led to the discovery of antibiotics which greatly impacted the world both medically and historically. Over the past eighty years, antibiotics have enabled tremendous advances in modern medicine and revolutionized agricultural and industrial practice.[2] Today, antibiotics are prescribed to treat or prevent different types of bacterial and fungal infections.

In 1928, the first naturally occurring antibiotic, penicillin, was discovered by Alexander Fleming. Penicillin was found by extracts from mold and had an antibacterial effect on staphylococci and other gram-positive pathogens.[3] It was first developed to treat *streptococcal septicemia* and successfully treated a patient for the first time in the United States in 1942. People began using penicillin to treat various infections in the early 1940s, which led to Alexander Fleming's Nobel Prize in Medicine and Physiology in 1945 for penicillin's beneficial effects on society. However, as early as 1945, Fleming was concerned about antibiotic overuse which did ultimately lead to resistance.

According to The Centers for Disease Control and Prevention (CDC), antimicrobial resistance (AMR) happens when bacteria or fungi begin to develop the ability to inactivate the drugs designed to kill them. The overuse or misuse of antibiotics drives the evolution of resistance.[4] Antibiotics can be misused through unnecessary prescriptions by physicians,

patients not adhering to correct dosing or course duration, large scale agricultural use for disease treatment, or growth promotion in animal husbandry and food production.[2]

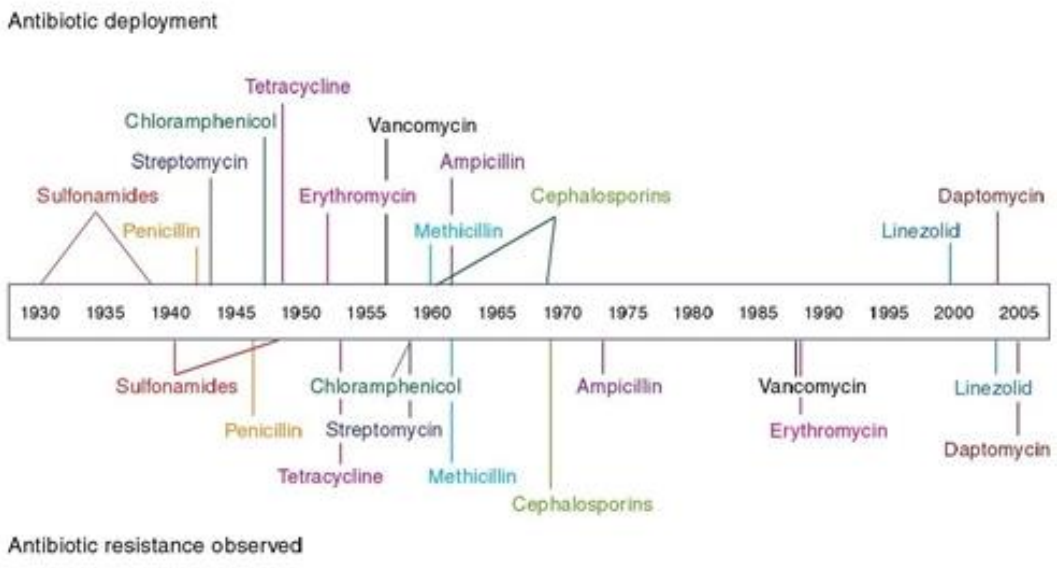


Figure 1 : Timeline of when a major antibiotic was developed and when resistance was first observed.[5]

According to the CDC, each year in the United States, at least 2.8 million people are infected with antibiotic-resistance bacteria and more than 35,000 people die as a result. **Figure 1** above shows the year that major antibiotics were developed, and the year bacteria were discovered that were resistant to the antibiotic. The growing numbers of antimicrobial resistant pathogens is alarming for the healthcare system and have important global cost,

such as high mortality and morbidity rates, increased treatment cost, diagnostic uncertainties, and lack of trust in orthodox medicine.[1,6] As antibiotic development declines and resistance rises, health-care-associated infections (HAIs) remain a constant threat to patient welfare. Therefore, it is very important to explore classes of compounds that can be used to treat the antibiotic-resistant bacteria.

ESKAPE Pathogens

It is critical to identify new potential antibiotics for the ESKAPE pathogens because they are a top threat to global public health.[7] The ESKAPE microbes represent deadly bacterial pathogens with rapidly growing multi-drug resistant properties. The acronym ESKAPE consists of the following pathogens: *Enterococcus faecium* & *Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter species.* These pathogens are known to "escape" the biocidal action of antimicrobial agents due to their ability to modify antibiotic target sites.[8,9] They are also able to reduce accumulation of drug by increasing efflux of the drug or reducing permeability, at times using a complex, protective matrix known as a biofilm.[8]

Many antibiotics, such as penicillin and cephalosporins, are β-lactam antibiotics which inhibit cell wall biosynthesis.[10] β-Lactam antibiotics have been one of the most commonly prescribed drug to treat bacterial infections.[11] β-Lactam antibiotics have a β-lactam ring that allows it to be highly reactive, inhibiting cell wall synthesis by the pathogens.[11] However, many of the ESKAPE pathogens have enzymes, such as β-lactamase, that irreversibly modify and inactivate the antibiotics by breaking open the β-lactam ring.[1]

Enterococcus species

Enterococcus faecalis and *Enterococcus faecium* are gram-positive, facultative anaerobes that live as commensals in the GI tract in living organisms.[12] Enterococci show multidrug resistances and are found in mostly healthcare-associated infections (HAIs). Vancomycin-resistant enterococci (VRE) arose during the late 1980s and are responsible

for approximately 12% of all nosocomial infections in the United States.[13,14] Many patients with VRE infections require prolonged in-hospital stays and unfortunately display high mortality rates.[15]

Staphylococcus aureus

Beating enterococci for the leading cause of gram-positive nosocomial infections are staphylococci. *Staphylococcus aureus* is a gram-positive pathogen with cells arranged in characteristic grape-like cluster. The mortality associated with *S. aureus* bacteremia is approximately 20-40%.[16] This bacterium is normally found as part of the normal skin flora, especially the nose, axillae, and perineum of humans and animals.[17] Its primary mode of transmission is through direct contact, commonly skin-to-skin contact with an infected individual or contaminated objects.[18] S. *aureus* is notorious for being resistant to antibiotics because it was the first microorganism recognized to rapidly form biofilms.[2] Methicillin-resistant S. *aureus* (MRSA) and community-associated MRSA (CA-MRSA) are strains of S. *aureus* that are common in hospitals and are a leading cause of nosocomial deaths.[17]

Klebsiella pneumoniae

In the *Enterobacteriaceae* group, K. *pneumoniae* is a gram-negative bacterium that is nonfastidious, nonmotile, and usually encapsulated. It is generally found on the surface of mucosa in the human and animal oropharynx and gastrointestinal (GI) tract; it is also ubiquitously found in the environment, such as water and soil.[19] This bacterium is responsible for infectious diseases, such as urinary tract infections (UTI), pneumonia, liver abscesses, and septicemia.[19,20] Like many other multi-drug resistant pathogens, *K. pneumoniae* is characterized by a high mortality rate, prolonged hospitalization, and high cost of infection treatment.[20] *K. pneumoniae* is well known for its ability to form biofilms

and its virulence factor, capsule polysaccaride.[21] This gives the bacteria the ability to survive by producing a thick layer of extracellular biofilm that supports bacterial attachment and prevents antibiotics from penetrating.[21]

Acinetobacter baumannii

From the Moraxellacae family, *Acinetobacter baumannii* is an aerobic, non-fermenting, nonfastidious, and non-motile gram-negative pathogen.[22] This pathogen is also responsible for the high rates of HAIs worldwide and has a mortality rate ranging from 7.8-43%.[23] Infections are commonly found in the respiratory tract, skin, bloodstream, urinary tract, and soft tissues.[24] *A. baumannii* virulence factors are porins, capsular polysaccharides, lipopolysaccharides, phospholipases, outer membrane vesicle, and protein secretion systems.[24] They are able to produce β-lactamase enzymes, regulate multidrug efflux pumps, decrease antibiotic membrane permeability, and alter antibiotic target sites, demonstrating multiple, complex mechanisms of resistance.[22,24]

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic gram-negative pathogen. This pathogen is the leading cause of morbidity and mortality in cystic fibrosis patients and immunocompromised individuals.[25] It has the ability to tolerate a variety of conditions that allow it to persist in both community and hospital settings.[26] According to the CDC, in 2017, *P. aeruginosa* caused an estimated 32,600 infections among hospitalized patients and 2,700 estimated deaths in the United States. It was identified as the fifth most frequently isolated nosocomial pathogen, second leading cause of nosocomial pneumonia, and third most common cause of UTIs.[26] *P. aeruginosa* can acquire a high level of intrinsic resistance to most antibiotics through restricted outer membrane permeability, efflux

systems that pump antibiotics in and out of the cell, and production of β-lactamase enzymes.[25]

Enterobacter species

Another gram-negative bacterium in the Enterobacteriaceae family is *Escherichia coli*. Similar to *Klebsiella pneumoniae*, this bacterium is also the cause of UTIs, bloodstream infections, and health-care-associated pneumonias.[27] According to World Health Organization (WHO), this bacterium can be easily spread through a population by fecal contamination of water and food, especially in raw meat, raw milk, and raw vegetables. *E. coli* shows great resistance to antimicrobial agents due to its ability to accumulate resistance genes, mostly through horizontal gene transfer.[28] In the enterobacterial gene pool, *E. coli* is able to act as a donor and as a recipient of resistance genes to acquire resistance genes from other bacteria and not pass it on.[28] Another virulence factor of *E.coli* is its ability to produce extended-spectrum β-lactamases (ESBLs).

Gram-Negative versus Gram-Positive pathogens

The ESKAPE pathogens are divided into two different groups, gram-positive and gram-negative bacteria, based on their ability to take up the Gram stain. The structure of gram-positive versus gram-negative pathogens correlates with resistance to antibiotic drugs.

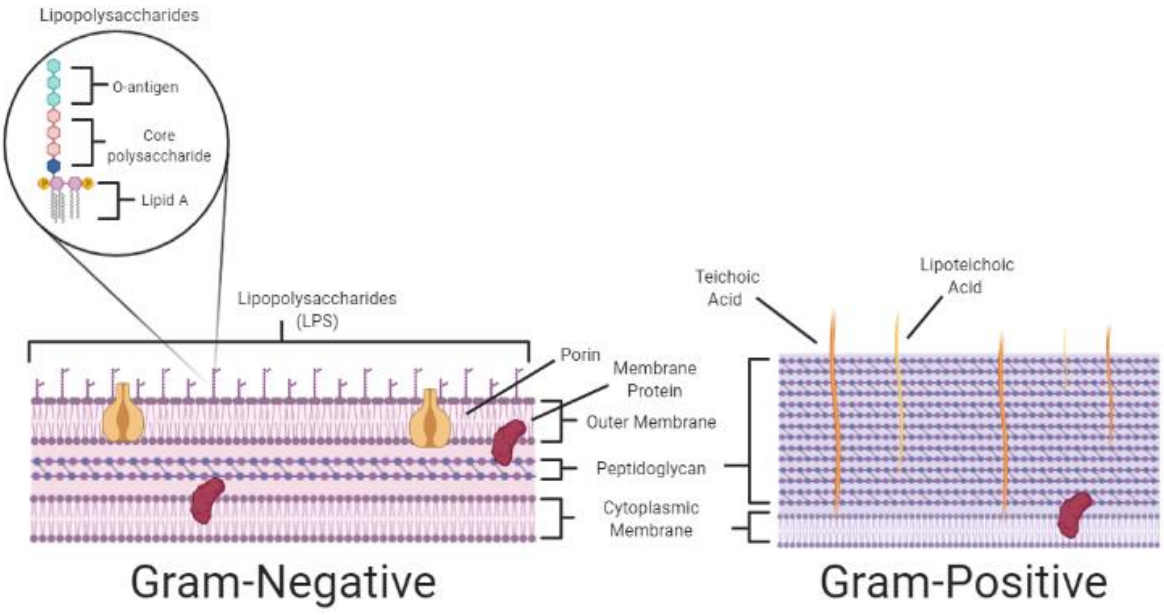


Figure 2: Gram-negative and gram-positive bacterial cell walls

In gram-positive bacteria, the cell walls are composed of thick layers of peptidoglycan. In contrast, gram-negative bacterial cell walls have a thin layer of

peptidoglycan but have a second outer phospholipid cell membrane. Gram-negative bacteria have an envelope that consists of three principal layers: the outer membrane containing lipopolysaccharide, the peptidoglycan cell wall with peptide chains, and the cytoplasmic membrane.[29,30] Gram-negative bacteria are more resistance towards antimicrobial agents due to its structural differences in the penetration and retention of chemical agents.[29] Gram-positive bacteria have a thick peptidoglycan layer that is still permeable to small molecules and ultimately develop resistance slower. Gram-positive bacteria lack the outer membrane that is the key feature serving as a permeability barrier in gram-negative bacteria. Understanding each of the bacterial resistance mechanisms is crucial for the development of an antimicrobial agent.

Example mechanisms of drug resistance are as follows: drug inactivation/alteration, modification of drug binding sites/targets, changes in cell permeability, and biofilm formation.[1,31] As shown in **Figure 2**, gram-negative bacteria contain proteins called porins which allows the passage of many hydrophilic substances. Membrane proteins also have the ability to export antimicrobial agents from the cell and maintain their low-intracellular concentrations through efflux pumps.[32] A common mechanism of resistance is through natural variations or acquired changes to prevent a drug from binding to its target sites.[32] Target sites can typically be changed by spontaneous mutation of a bacterial gene on the chromosome.[32] Most antimicrobial agents must pass the outer membrane of gram-negative bacteria to access their target sites. In order to inactivate antibiotics, some bacteria have enzymes, such as β-lactamase, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases.[32]

Alternative Antibiotics

There have been many attempts to find new structural or mechanistic classes of antibiotics. Many alternative agents work well *in vitro* but not *in vivo.* Antimicrobial peptides (AMPs) have been developed and successfully been used to treat several pathologies such as acne vulgaris, periodontal disease, and cancer.[33] AMPs are biologically active compounds that are produced and found within all living organisms. Most AMPs show therapeutic characters because of their high positive charge and amphiphilic α-helical peptide structure.[34] AMPs are relatively short, ranging from 12-50 amino acids.[35] AMPs are able to damage the bacterial cell through general membrane permeabilization, making it difficult for certain pathogens to develop resistance towards them.[8] AMPs are effective against a broad-spectrum of microorganism such as bacteria, fungi, and viruses.[33]

There have been many antimicrobial peptides that have been studied and developed in research groups. Jiwon Seo's research group in Korea has developed and analyzed AMPs that are undergoing clinical and preclinical trails against various infectious diseases.[36] Most AMPs are classified into two structural categories: linear cationic amphipathic peptides and macrocycle peptides. Seo discovered that prominent class of AMPs comprises the calcium-dependent antibiotics (CDAs); they are anionic macrocyclic peptides that bind with a calcium ion to exert antimicrobial activity.[36]

Even though AMPs show potential as antibiotic candidates *in vitro* they fail to show the same results *in vivo*. Overall, AMPs are generally toxic towards mammalian cells, especially their toxic side effects in haemolytic activity.[36,37,38] They are unable to achieve high antimicrobial activities under physiological salt, pH, and serum conditions due to the loss of electrostatic interactions between peptides and the cell membrane.[36,39] There are

also other drawbacks to AMPs, such as sensitivity to enzymatic degradation, low bioavailability, loss of activity at low salt concentrations, and the high cost of synthesis.[35] Therefore, in our research lab, we are looking at peptide mimics known as peptoids, an N-alkylation class of peptidomimetics.

Peptoid vs. Peptide

As stated before, peptides have potential to act as alternatives to conventional antibiotics; however, they are unstable and have drawbacks. Peptoids, oligomers of N-substituted glycine, are mimics of antimicrobial peptides with a slight modification in the structure.[40]

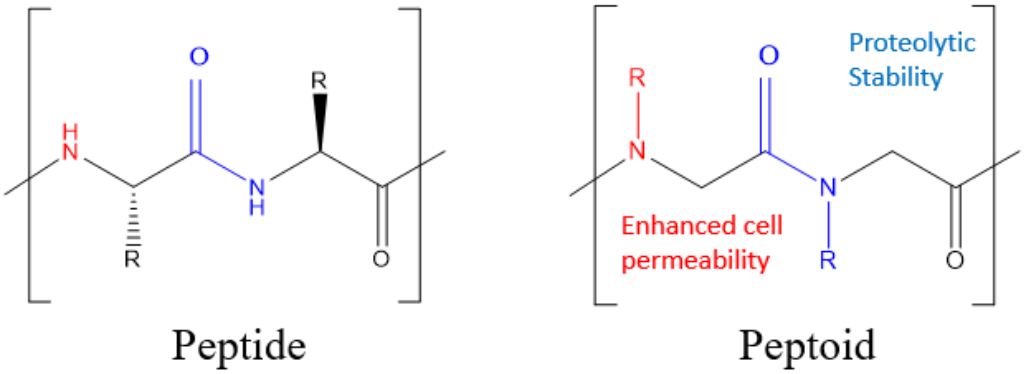


Figure 3: Structure differences between peptide and peptoid. R (side chains) are attached to the nitrogen atom in peptoid rather than to the alpha carbons in peptide.

In peptoids, the side chains are attached to the nitrogen atom of the peptide backbone rather than to the alpha-carbons. Research has shown that peptoids are a promising class of antibiotics because of demonstrated antimicrobial activity against a broad spectrum of pathogens, a non-specific mode of action, decreased susceptibility to enzymatic

degradation, stability to heat, and their relative ease of synthesis.[41] Peptoids have been shown to act via disruption of bacterial membranes and have increased membrane permeability when compared to peptides.[41] Peptoids also have an increase *in vivo* half-life compare to peptides due to their structural difference, with *in vivo* half-lives on the order of 24 hours or more.[42] A result of increase in half-lives can be due to the human body not being able to recognize the peptoid as quickly compared to peptides or proteins.[43] Antimicrobial peptoids are created to mimic the structural characteristics of AMPs with varying compound length, various degrees of cationic charge, and a general amphiphilic nature.[34] In our research group, we have focused on the discovery of antimicrobial peptoids and explored how to modify peptoid structure to improve biological activity.

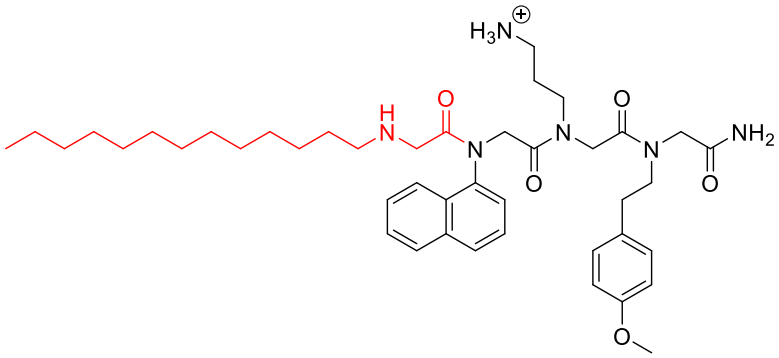
A research group in Santa Clara University studies how length, charge, and side chain of water-soluble peptoids impact binding to phospholipid membrane.[44] They have monitored three to fifteen residue long peptoids with at least one aromatic residue and its adsorption to supported lipids bilayers (SLBs). They discovered that the best drug design are small organic molecules with hydrophobic substituents and overall cationic charge will likely absorb to and accumulate in phospholipids.[44] However, they still were not able to clarified the contributions of sequence length to the mechanisms of peptoid-lipid interactions.[44]

Thesis Statement: MNT Peptoid Series

Characterizing and identifying a therapeutic compound to treat infections caused by drug-resistant bacteria has been one of the biggest challenges in modern research. Our lab has mainly focused on the development of short peptoids containing a long aliphatic tail (lipopeptoids). While lipopeptoids are easy to identify from our high-throughput Peptoid Library Agar Diffusion (PLAD) assay,[45] the aliphatic tail contributes significantly to mammalian cytotoxicity. The overall goal of this research is to explore the conversion of a short lipopeptoid into a longer, non-lipidated peptoid to determine the peptoid length that could maintain potency towards ESKAPE pathogens and have reduced mammalian toxicity. ALA1 was discovered in our research group by Angelica Aguila from a combinatorial peptoid library using the high-throughput PLAD assay screening against *E. coli*. Developed by the Bicker Lab, the PLAD assay uses solid-phase combinatorial libraries of peptoids that enable multiple beads to be screened at once for possible "hits" that exhibit biological activity against microbes of interest.[45] As shown in **Figure 4,** ALA1 is made of three peptoid monomers with a N-terminal 13-carbon lipid tail.

Researchers have shown that converting a long, repeated-sequence peptoid into a short, lipidated peptoid generally produces a lipopeptoid with similar efficacy to the longer, repeated-sequence peptoid.[40] However, previous results from our lab have shown that the lipid tail is the key source of toxicity in lipopeptoids.[46] Therefore, we synthesized a series of ALA1 derivatives that are repeats of the ALA1 trimer (Nnap-Nap-Nmpe) but without the lipid tail, known as the MNT series of peptoids (**Figure 5**). ALA1 overall has promising results against the ESKAPE pathogens but the cytotoxicity of the compound was higher than desired (data shown and discussed below).

Peptoid MNT1 is the ALA1 trimer with no lipid tail. Peptoids MNT2, MNT3, and MNT4 are 2x, 3x, 4x repeats of MNT1, respectively. The purpose of the repeats is to test the effect of non-lipidated peptoid length on efficacy and toxicity. We hypothesized that as we add repeats, the biological activity of the compound will increase. As stated before, since MNT series peptoids do not have a lipid tail, we hypothesized that all the MNT series peptoids will be less toxic than ALA1 and that longer repeats should maintain activity against the ESKAPE pathogens. ALA1 and the MNT series of peptoids were synthesized and purified prior to biological testing. Minimum inhibitory concentrations against the ESKAPE bacteria were determined to compare the efficacy of each compound against several bacteria. Lastly, the cytotoxicity of these peptoids was tested through multiple assays, evaluating hemolytic activity and mammalian cell toxicity against HepG2 liver cells.

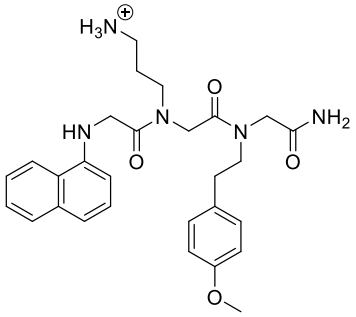


(Ntri-Nnap-Nap-Nmpe) :: ALA1

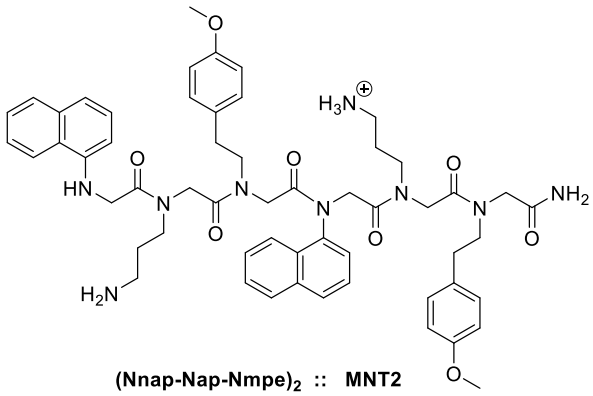
Chemical Formula: $C_{43}H_{65}N_6O_5^+$

Molecular Weight: 746.0295

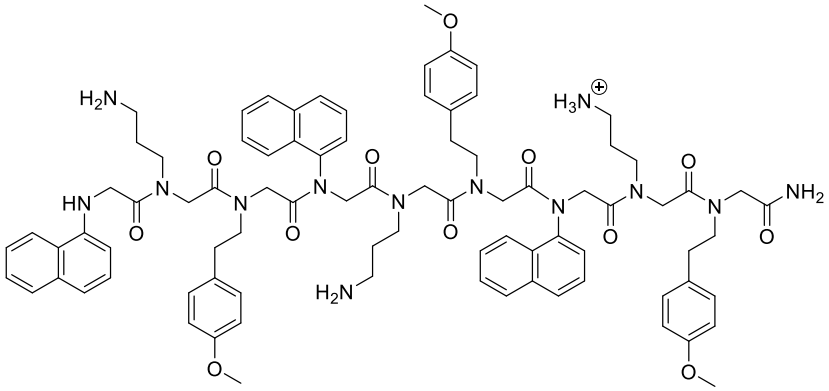
Figure 4 : ALA1 Structure. ALA1 consists of three monomers with a tridecylamine tail in the N-terminus. ALA1 has a molecular weight of 746.03 g/mol with a cationic charge. Each monomer is labeled with their code Ntri-Nnap-Nap-Nmpe from N-terminus to C-terminus.



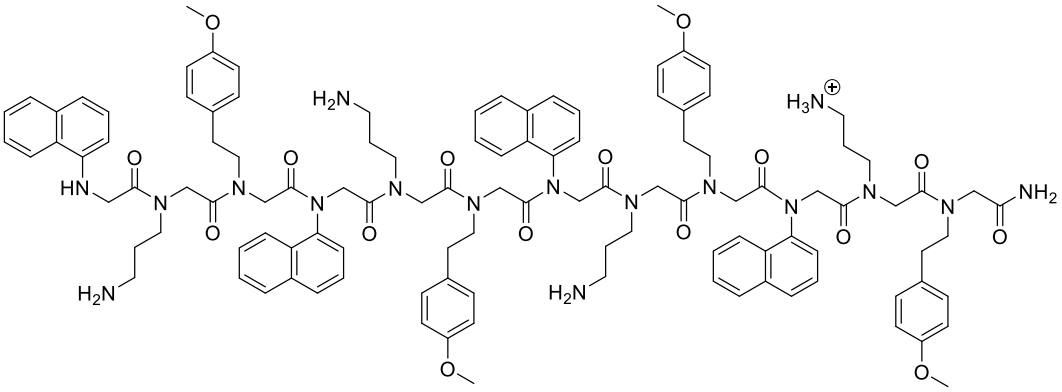
(Nnap-Nap-Nmpe) :: MNT1
Chemical Formula: $C_{28}H_{36}N_5O_4^+$
Molecular Weight: 506.6265



(Nnap-Nap-Nmpe)$_2$ :: MNT2
Chemical Formula: $C_{56}H_{68}N_9O_8^+$
Molecular Weight: 995.2145



(Nnap-Nap-Nmpe)$_3$ :: MNT3
Chemical Formula: $C_{84}H_{100}N_{13}O_{12}^+$
Molecular Weight: 1483.8025



(Nnap-Nap-Nmpe)$_4$ :: MNT4
Chemical Formula: $C_{112}H_{132}N_{17}O_{16}^+$
Molecular Weight: 1972.3905

Figure 5: Structures of MNT series peptoids, which are repeats of the ALA1 structure (Nnap-Nap-Nmpe) without the lipid tail.

Chapter Two: Experimental Methods

Peptoid Synthesis

Peptoids are relatively easy and inexpensive to synthesize. Synthesis of antimicrobial peptoids has been explored in many ways but the most common technique is the submonomer method using solid phase synthesis.[47]

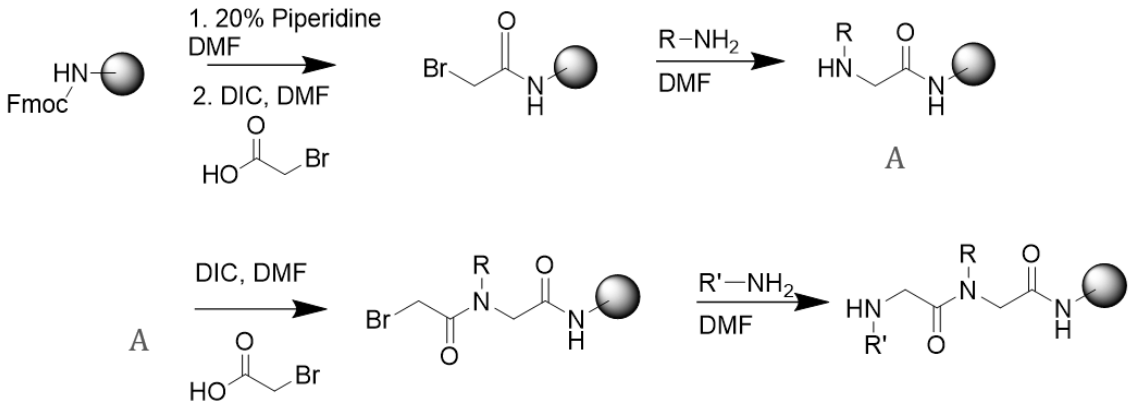


Figure 6 : Scheme of Peptoid Synthesis. Rink amide resin is first deprotected using 20% piperidine with DMF. Bromoacylation is performed by adding bromoacetic acid in anhydrous DMF and activating with diisopropylcarbodiimide (DIC). Amination is done by adding the desired amine to displace bromine in an S_N2 reaction. This process is repeated until the desired peptoid is synthesized.

MNT1

As shown in **Figure 6,** this process was used to synthesize all MNT series peptoids and ALA1. MNT1 was synthesized in a synthesis vial using 1 g of Rink Amide resin (0.75 mmol/g loading level). The resin was swollen with dimethylformamide (DMF) and rocked for 20 minutes. After 20 minutes, the solvent was drained and 20% piperidine in DMF was added 2x for 10 minutes to deprotect the Fmoc groups on the Rink Amide resin. The resin was washed with DMF three times before bromoacylation. Bromoacylation was performed by adding 2 M bromoacetic acid and 3.2 M diisopropylcarbodiimide (DIC) in anhydrous DMF to the resin, microwaving at 10% power for 15 seconds twice, and rocking for 15 minutes. After each bromoacylation, resin was washed 3x with DMF and a Kaiser test was run to test for a successful reaction. A 2 M solution of the first desired amine, 2-(4-methoxyphenyl) ethylamine, also abbreviated as Nmpe in the peptoid sequence, was added to the resin, microwaved for 15 seconds twice, and rocked for 15 minutes. Resin was washed 3x with DMF and a Kaiser test was run to test for positive coupling. After positive coupling is confirmed, bromoacylation is performed again and the next desired amine is added. The second amine added was Boc-diaminopropane, abbreviated as Nap. Finally, bromoacylation was run again before adding the last amine which was 1-naphthylamine. After synthesis of MNT1 was done, the peptoid structure was confirmed by mass spectrometry (Waters ESI-TOF MS) before cleaving with TFA:water:triisopropylsilane (95:2.5:2.5) for 1 hour. Purification was done by RP-HPLC as described below.

MNT2-MNT4

After MNT1 was synthesized and before cleaving, half of the resin was transfer to a new synthesis vial to synthesize MNT2. The same three monomers were added to MNT1

following the methods described above to synthesize MNT2. MNT3 and MNT4 were synthesized in the same way by adding monomers to obtain the desired peptoid. Once each peptoid was synthesized, it was confirmed, cleaved, and purified as described.

ALA1

Just like MNT1, ALA1 was synthesized using the submonomer method described above. In addition, after amination with 1-naphthylamine, a 13-carbon lipid tail was added to the N-terminus to produce ALA1. This was done by bromoacylating and then aminating using tridecylamine, as described above. Once synthesis was complete, the structure was confirmed by mass spectrometry, cleaved, and purified as described.

Purification

After each peptoid was synthesized, purification was done by reverse-phase high performance liquid chromatograph (RP-HPLC). HPLC is an analytical technique that is used for separation, detection, and quantification of a sample.[48] It is a column chromatography technique that pumps the mobile phase at high pressure through a column with an immobilized chromatographic packing material.[48] The solvents used in HPLC for the purification of the MNT peptoids were water and acetonitrile, ranging from 0% to 100% with 0.05% TFA. The column used was a Supelco Ascentis C18 (25 cm x 21.2 mm, 5µm). Each peak was collected, and compound identity determined using mass spectrometry. Once all samples of a compound were purified and collected, they were dried down *in vacuo* using a rotary evaporator and lyophilized. Each compound was resuspended in water to make a stock concentration of 20 $\frac{mg}{mL}$.

Minimum Inhibitory Concentration

Once each compound was purified, the broth microdilution method was used to determine the minimum inhibitory concentration (MIC; **Figure 7**). MIC_{90} is the lowest concentration (in μg/mL) of a compound that inhibits the growth of a strain of bacteria greater than 90%. Performing this assay will help us determine which compound has better efficacy towards the ESKAPE pathogens.

Each strain of the ESKAPE bacteria (*Enterococcus faecium* ATCC 6569; *Enterococcus faecalis* ATCC 29212; *Staphylococcus aureus* ATCC 29213; *Klebsiella pneumoniae* ATCC 13883; *Acinetobacter baumannii* ATCC 19606; *Pseudomonas aeruginosa* ATCC 25619; and *Escherichia coli* ATCC 25922) were tested against each MNT series compound. Briefly, Lysogeny Broth (LB) agar plates were streaked from frozen bacterial cultures of the ESKAPE pathogens and incubated between 18-24 hours at 37 °C. After incubation, colonies of bacteria were picked using a sterile loop and resuspended in Tryptic Soy Broth (TSB) to an OD_{600} between 0.08-0.15. This solution was then diluted 1:200 in Cation Adjust Muller-Hinton Broth (CAMHB) for a final concentration of ~$5x10^5$ CFU/mL. Peptoid stock solutions (10x concentration) were prepared in water in two-fold serial dilutions ranging from 100 to 1.56 $\frac{\mu g}{mL}$. In a 96-well plate, 90 μL of 1:200 cell inoculum was added to each well. Then, 10 μL of the 10x compound stocks were added to the appropriate wells, diluting to 1x in the well with a final well volume of 100 μL. Tetracycline (20 $\frac{\mu g}{mL}$) was used as a positive control and water (vehicle) was used as a negative control. The plates were incubated overnight at 37 ºC. After the plates had incubated overnight, 10 μL of PrestoBlue was added to each well and

incubated for additional 1-2 hours at 37 ºC. The plate was then read for fluorescence on a SpectraMax M5 Plate reader with an excitation of 555 nm and emission of 585nm. Assays were tested in triplicate on at least three different days.

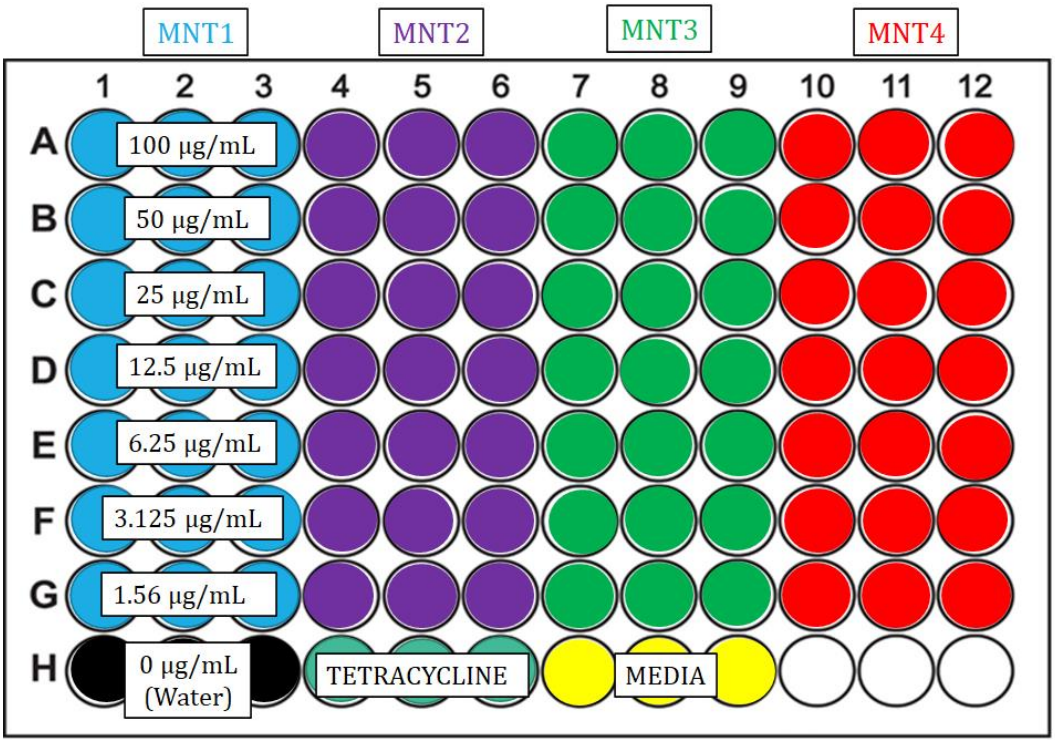


Figure 7: An example of a 96-well plate testing MIC against a bacterium with all MNT series compounds. The highest concentration of compound is 100 $\frac{\mu g}{mL}$ with the lowest concentration of 1.56 $\frac{\mu g}{mL}$. Tetracycline and water are used as positive and negative controls, respectively. Media was also used as a media control to ensure no contamination.

Mammalian Toxicity Assay/MTT Assay

Mammalian cytotoxicity was determined against HepG2 hepatocellular carcinoma cells using the MTT assay as previously described (**Figure 8**).[49] Briefly, cells were grown in a T-75 flask in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin, streptomycin, glutamine (PSG) and incubated at 37 ºC in a humidified 5% CO_2 incubator. Once the cells reached above 70% confluence, an MTT assay was prepared. Briefly, the media from the T-75 flask of cells was removed. The cells were washed with 10 mL of phosphate buffered saline (PBS). Trypsin (2 mL) was added to the flask and incubated at 37ºC for 5-10 minutes to separate cells from the culture flask. After 5-10 minutes, 8 mL of phenol-red free media containing 10% FBS and 1% PSG was added to the flask and the entire cell solution transferred to a 15 mL centrifuge tube. Cells were pelleted by centrifugation at 1000 rpm for 5 minutes in a swing bucket centrifuge. After centrifugation, the supernatant is removed, and the cells are resuspended gently in 7.5 mL of phenol-red free media. The cell concentration is determined using a hemacytometer and diluted with more media if necessary, to $1x10^5$-$4x10^5 \frac{cells}{mL}$. In a tissue-culture treated 96-well plate, 100 µL of cell solution is pipetted into each well and incubated at 37ºC for 2-3 hours to allow the cells to adhere to the plate. After 2-3 hours, 11.1 µL of 2-fold serially diluted 10x compound stocks were added to each well in triplicate with the highest concentration being 200 $\frac{\mu g}{mL}$ and the lowest concentration being 6.25 $\frac{\mu g}{mL}$. The vehicle used was water for the positive control. The 96-well plate was then incubated at 37 ºC for ~72 hours (3 days). After three days, 20 µL of 5 $\frac{mg}{mL}$ (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in water was added to each well and

incubated for an addition three hours at 37 ºC. Three hours later, the MTT was removed from the well using a glass Pasteur pipette. 100 µL of dimethyl sulfoxide (DMSO) was then added to each well and incubated for 15 minutes at 37 ºC to lyse cells and solubilize metabolized MTT dye. After 15 minutes, the 96-well plate was read for absorbance at 570 nm on a SpectraMax M5 plate reader. The compound concentration resulting in a 50% reduction in viable cells (toxicity dose 50%; TD_{50}) was then calculated using Excel and GratFit.

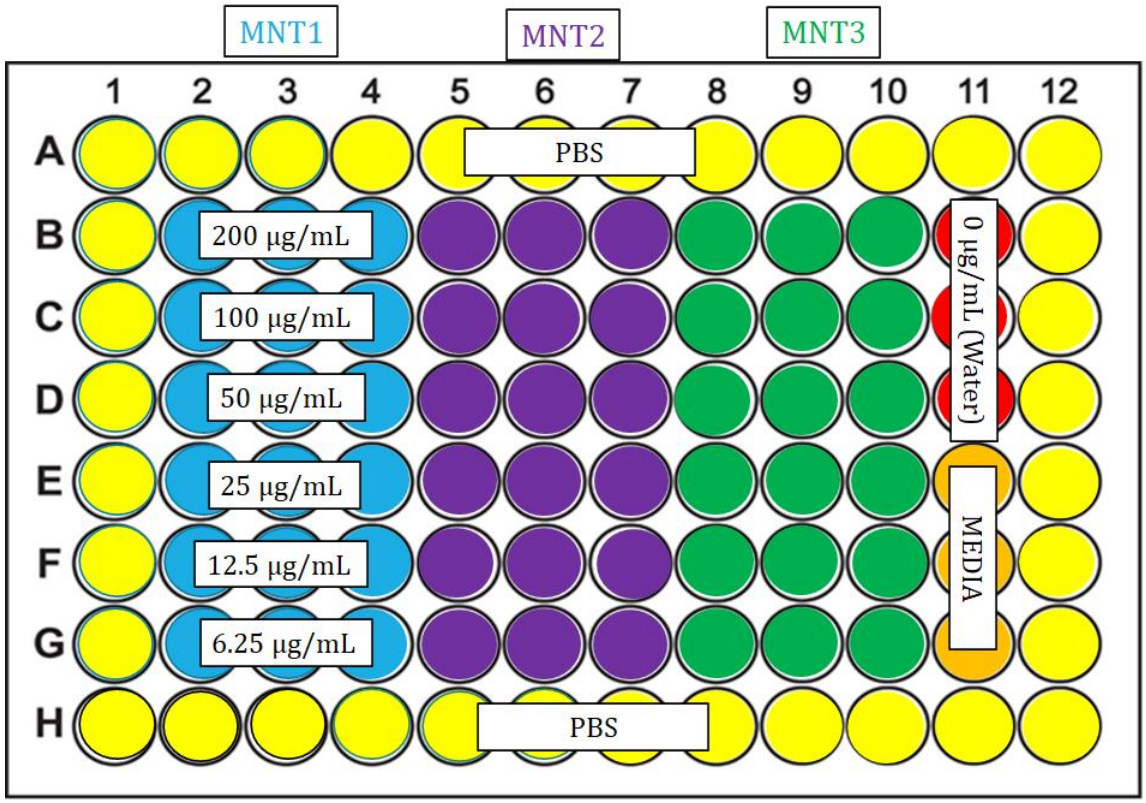


Figure 8: An example of a MTT assay setup to test peptoids against HepG2 cells. The 96-well plate is surround with PBS to minimize evaporation. The 96-well plate example above is used to test against three different compounds with concentrations from 200 µg/mL to 6.25 µg/mL. Water is used as the vehicle.

Hemolytic Toxicity Assay

The hemolytic activity of peptoids was determined against human red blood cells as previously reported.[49] This assay is donor blood specific and human red blood cells were acquired from a single donor source from Innovative Research, Inc (DONOR-INB3ALS40ML-33449). First, two-fold serial dilutions of 10x peptoid were prepared in PBS at concentrations ranging from 400 $\frac{\mu g}{mL}$ to 3.13 $\frac{\mu g}{mL}$. Human red blood cells (hRBCs) were aliquoted out into a 15 mL centrifuge tube and pelleted by centrifugation at 1000 rpm for ten minutes in a swing bucket centrifuge. The supernatant was removed, and cells were washed again with 10 mL of PBS a total of three times. Lastly, the hRBCs were resuspended in PBS and 100 µL aliquoted into individual wells of a 96-well plate. In triplicate, 11.1 µL of 10x peptoid solution was pipetted into the appropriate wells. The positive control for this assay was 1% Triton X-100 and negative control was PBS. The plate was then incubated for one hour at 37 ºC. After incubation, the plate was centrifuged at 1000 rpm for 10 minutes to pellet intact cells. In a new 96- well plate, 5 µL of sample supernatant was transferred into 95 µL of PBS. The plate was then analyzed for absorbance at 405 nm on a SpectraMax M5 Plate reader. The percent hemolysis was then calculated in Excel and GratFit using the following equation.

$$\%\ hemolysis = \frac{(OD_{405nm}sample - OD_{405nm}negative\ control)}{(OD_{405nm}positive\ control - OD_{405nm}sample)}$$

Chapter Three: Results and Discussion

Peptoid Synthesis

The MNT series peptoids and ALA1 were synthesized through solid-phase synthesis with a polystyrene Rink Amide resin using traditional Fmoc-based and sub-monomer approaches. Peptoid synthesis yields ranged from 4-56% (**Table 1)**. All compounds were identified and purified using Waters EI-MS and HPLC, respectively. The percentage of acetonitrile was recorded at the time of elution from HPLC as a measure of compound hydrophobicity. This value was then compared with a calculated distribution coefficient ($cLogD_{7.4}$) that was determined using ChemAxon's Marvin Sketch Calculator Plugin.[50] This parameter is a prediction of the partitioning of a molecule between aqueous and hydrophobic environments, taking into account the compound's ionization at pH 7.4.[44] A high $cLogD_{7.4}$ is an indicator of increased compound hydrophobicity. Determining the hydrophobicity of a compound aids in predicting the potential efficacy and cytotoxicity of a peptoid.

Compound	Yield	Expected m/z	Observed m/z	$cLogD_{7.4}$	% Acetonitrile
ALA1	4.8%	745.50	745.36	1.57	60%
MNT1	52%	506.63	506.20	-1.82	52%
MNT2	20%	995.21	994.38	-2.99	54%
MNT3	56%	1483.80	1482.60	-4.15	56%
MNT4	16%	1972.39	1971.94	-5.31	56%

Table 1: Structure characteristics table of all compounds of its percent yield, expected mass over charge, observed mass over charge from mass spectrum, $cLogD_{7.4}$, and percentage of acetonitrile. $cLogD_{7.4}$ = calculated distribution coefficient at pH 7.4

Previous research has emphasized the importance of hydrophobic residues for peptoids' selective interactions with bacterial versus mammalian membranes.[44] The positive value of calculated hydrophobicity for ALA1 (1.57) is due to the aliphatic tail in this peptoid, and the acetonitrile percentage analysis also confirms ALA1 to be the most hydrophobic compound tested here. Removal of the tail to give MNT1 results in a large drop in hydrophobicity as measured by both $cLogD_{7.4}$ and percent acetonitrile. However, as we increase the number of repeats in the MNT series, the $cLogD_{7.4}$ value decreased, indicating a decrease in hydrophobicity as peptoid length increases. Contrary to these results, the acetonitrile percentage analysis shows that an increase in peptoid monomer repeats causes an increase in hydrophobicity. The discrepancies in this analysis could be for several reasons. First, $cLogD_{7.4}$ is a calculated metric and should therefore be

considered secondary to experimental measurements of hydrophobicity. Second, the calculated value was determined at a pH of 7.4, while the HPLC solvent is quite acidic, with a low pH due to the inclusion of trifluoroacetic acid in the mobile phase.

Mass Spectroscopy

Each compound was purified by RP-HPLC and the mass confirmed using a Waters electrospray ionization mass spectrometer. The expected molecular weight for each peptoids was verified against the mass spectrometry parent peak (ALA1, **Figure 9**; MNT1, **Figure 10**; MNT2, **Figure 11**; MNT3; **Figure 12**; MNT4; **Figure 13)**. The expected mass and the observed mass are also stated in **Table** 1. With larger peptoids, multiply charged ions were observed in the mass spectra, allowing for confirmation of peptoid structure using m/1, m/2, and m/3 signals.

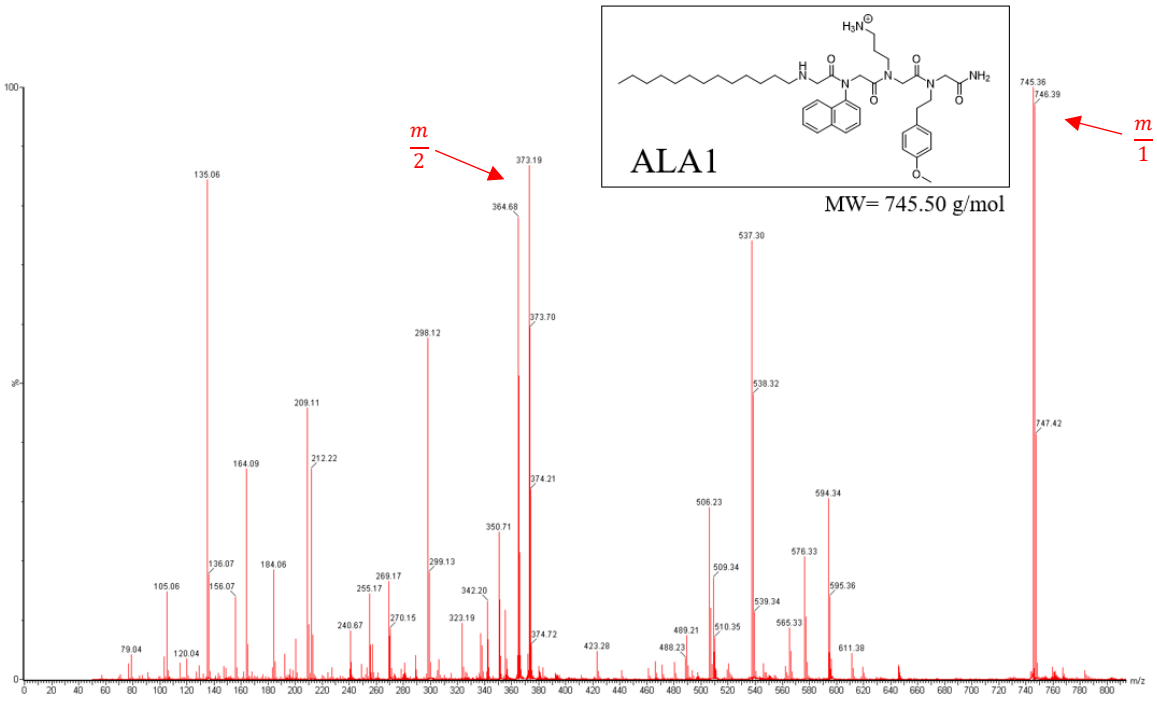


Figure 9: Mass spectra for ALA1 confirming the expected mass of 745 g/mol.

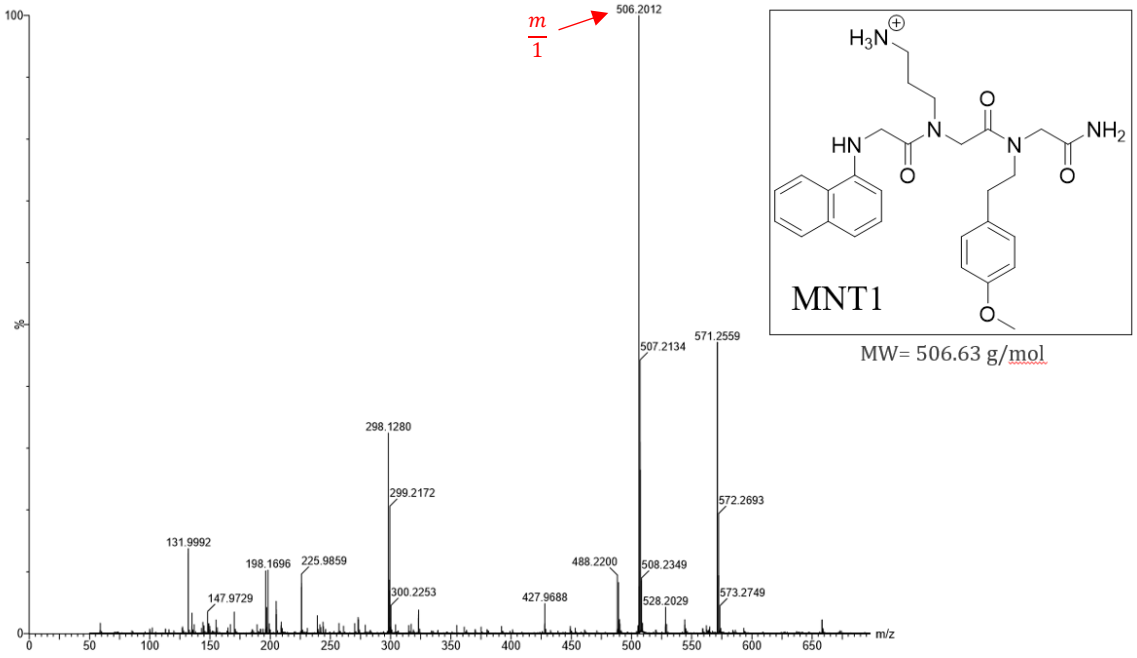


Figure 10: Mass spectra for MNT1 confirming the expected mass of 506 g/mol.

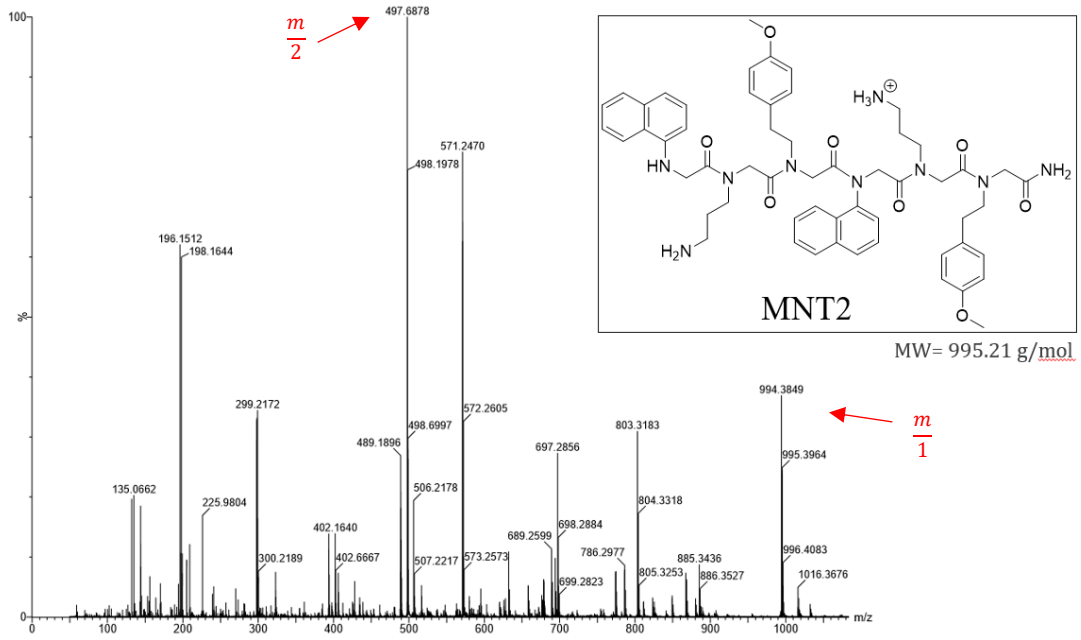


Figure 11: Mass spectra for MNT2 confirming the expected mass of 995 g/mol.

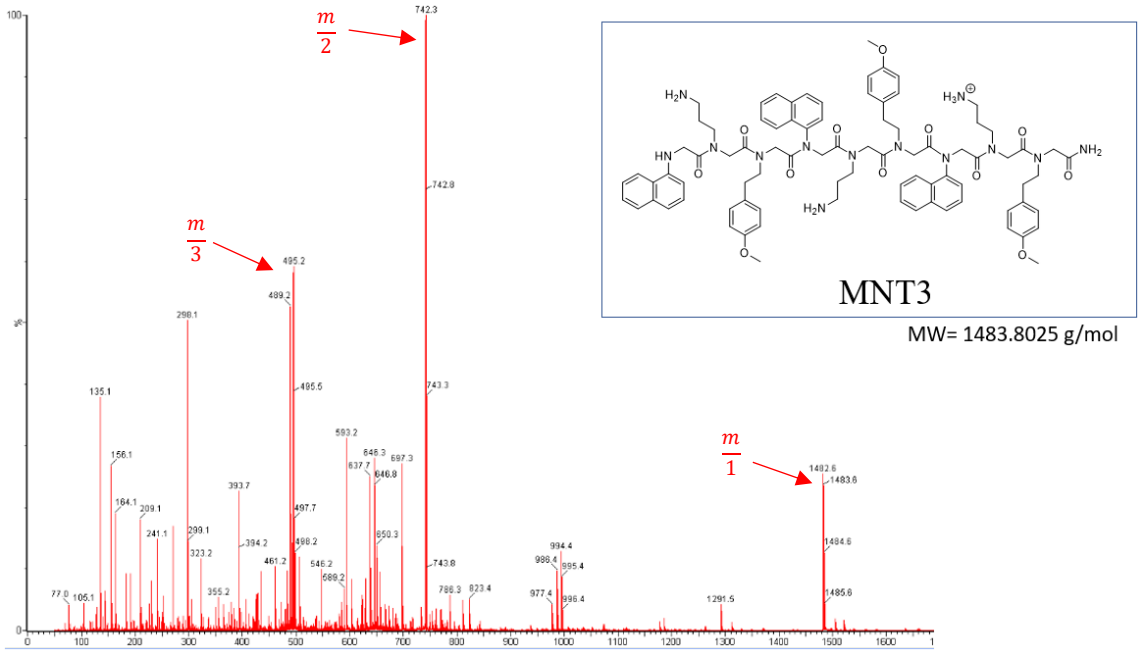


Figure 12: Mass spectra for MNT3 confirming the expected mass of 1483 g/mol.

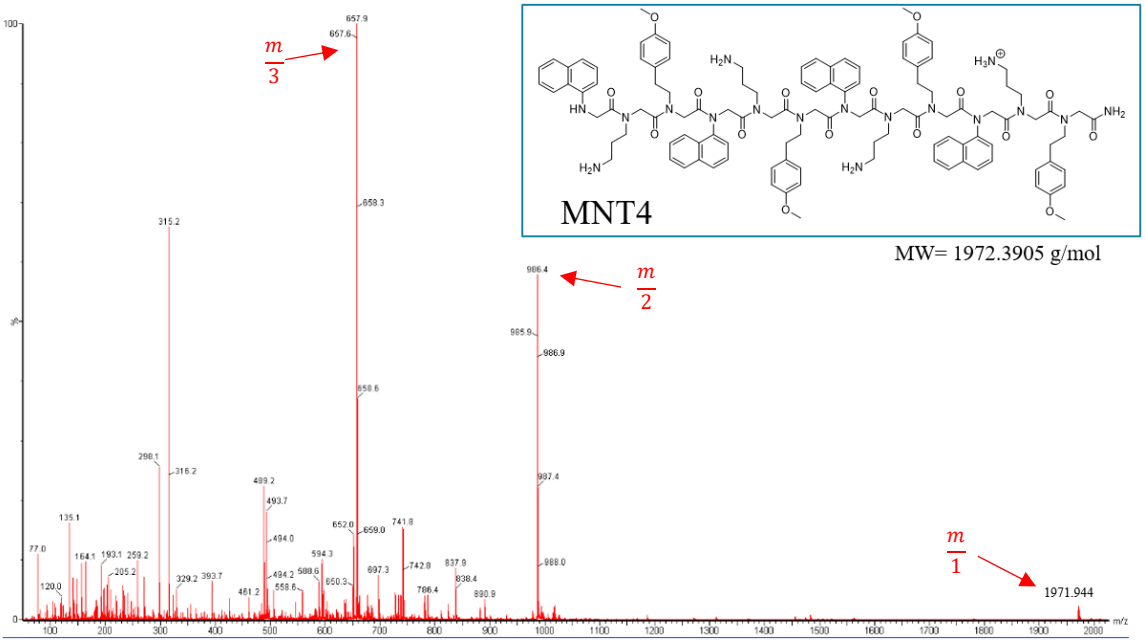


Figure 13: Mass spectra for MNT4 confirming the expected mass of 1972 g/mol.

MIC Results

MIC Values (µg/mL)					
PATHOGENS	**MNT1**	**MNT2**	**MNT3**	**MNT4**	**ALA1**
***E**nterococcus faecium*	>100	25	6.25	12.5	12.5
***E**nterococcus faecalis*	>100	25	12.5	25	25
***S**taphylococcus aureus*	>100	25	6.25-12.5	25	6.25
***K**lebsiella pneumoniae*	>100	100	50	>100	50
***A**cinetobacter baumannii*	>100	50	12.5-25	25	25
***P**seudomonas aeruginosa*	>100	100	>100-100	>100-100	50
***E**scherichia coli*	>100	50	100	100	12.5

Table 2: MIC values for MNT series and ALA1 against the ESKAPE bacteria. MIC is defined as the compound concentration resulting in greater than 90% inhibition of bacterial growth. Data were run in triplicate and the assay was repeated on three different days.

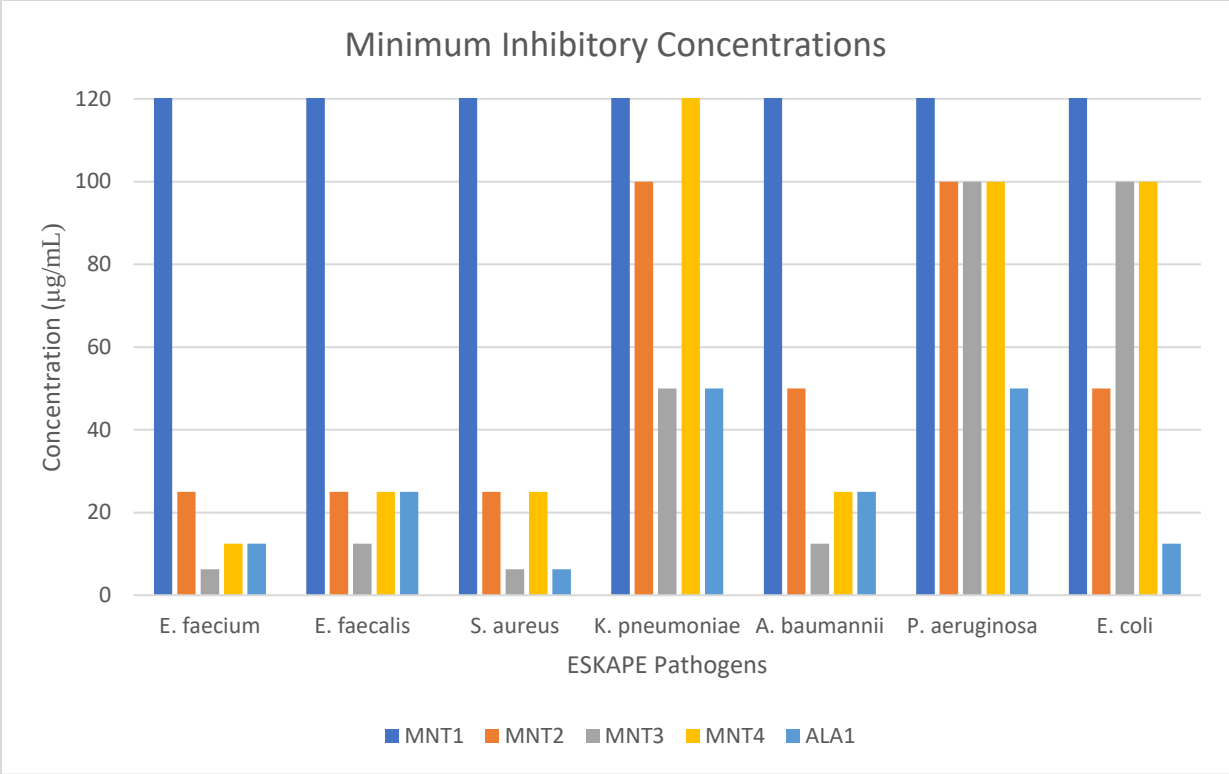


Figure 14: Graphic visualization of minimum inhibitory concentrations from **Table 1**. Concentrations of 120 µg/mL indicate that the concentration is greater than 100. Gram-positive pathogens (*E. faecium, E. faecalis, S. aureus).* Gram-negative pathogens (*K. pneumoniae, A. baumannii, P. aeruginosa, E. coli).*

Minimum inhibitory concentration (MIC) was determined using the broth microdilution method for each MNT series peptoid and compared with the MIC for ALA1 (**Table 2**; **Figure 13**). MIC is defined as the compound concentration resulting in greater than 90% inhibition of bacterial growth. As expected, MNT1 had the highest MIC values, greater than 100 µg/mL against all ESKAPE bacteria, due to being a short, non-lipidated

peptoid. Other studies have shown that changes to peptoid structure, such as altering the length of peptoids, does affect antimicrobial activity.[51,52] The MIC values decreased as peptoid monomer repeats were added (MNT2 and MNT3) for all pathogens tested. However, with the fourth repeat, MNT4, the MIC values increased rather than continued to decrease as expected, indicating a loss in antibacterial activity when progressing from 9 to 12 monomers. Similar research by Steven Cobb's group has explored peptoids with varying lengths and side chains to test the effect of these changes on antibacterial activity and toxicity.[52] Cobb's studies have reviewed many peptoid structures and observed different MIC value trends as the number of repeats are increased. With certain bacteria, the MIC values decreased as the number of repeats increased from 9 to 12 monomers. While with other peptoids, there was an increase in MIC values as repeats increased from 9 to 12 monomers, similar to the results seen here with MNT3 and MNT4. Lastly, extending peptoid length beyond 9 monomers had little effect on some microbes, indicating that the relationship between peptoid length and antimicrobial activity can be very dependent on monomer composition.

MNT3 proved to be more effective towards both *Enterococcus species* (*E. faecium* = 6.25 µg/mL, *E. faecalis* = 12.5 µg/mL) than any other peptoids tested, including ALA1. MNT3 retains similar efficacy compared to ALA1 towards *S. aureus* (6.25-12.5 µg/mL), *K. pneumonia* (50 µg/mL), and *A. baumannii* (12.5-25 µg/mL). However, ALA1 was more effective than MNT3 towards *P. aeruginosa* and *E. coli,* unsurprising given that ALA1 was originally discovered from a PLAD assay against *E.coli.* Overall, MNT3 appears to be a more effective compound towards gram-positive pathogens than ALA1.

Mammalian Cell Toxicity Results:

Cytotoxicity testing against mammalian cells is a very important part of developing a potential antibiotic because it can predict the effect a compound might having within the body.[53] To study *in vitro* the toxic effects of each peptoid in the MNT series and ALA1, two different cytotoxicity assays were used; mammalian cytotoxicity against HepG2 hepatocellular carcinoma cells and hemolytic activity against red blood cells, following previously published methods.[49,54] As stated before, we hypothesized that the unwanted cytotoxicity of the MNT series will be lower compared to ALA1 due to the absence of the lipid tail, a confirmed major contributor of toxicity in previous studies.[46] We tested the compounds against mammalian cells, specifically HepG2 liver cells, because the first pass of any molecule that enters the blood stream is through the liver.[55] Human red blood cells (hRBCs) were also tested since high hemolytic activity is disqualifying for any compound being developed for circulation in the blood stream. HepG2 cytotoxicity is calculated as TD_{50} (toxicity dose 50%), which is the compound concentration that results in 50% cell growth inhibition compared to the control.[56] Hemolytic toxicity is reported as HC_{10} (hemolytic concentration 10%), which is the compound concentration that results in 10% lysis of hRBCs compared to the control. Both of these metrics were calculated from curves constructed with the graphing program, Gratfit, that plots the cell survival percentage or percent hemolysis versus compound concentration.

HepG2 Liver Cell Toxicity

HepG2 Cytotoxicity Assay		
Compound	**TC_{50} (µg/mL)**	**Standard Deviation**
MNT1	>200	ND
MNT2	>200	ND
MNT3	84.7	±6.2
MNT4	38.9	±4.4
ALA1	40.4	±2.6

Table 3: HepG2cell toxicity for MNT series and ALA1. TC_{50} values obtained by MTT assay for all compounds after 72 hours of incubation. Data were run in triplicate and the assay was repeated on three different days. ND = not determined.

All compounds were evaluated in two-fold serial dilution against HepG2 hepatocellular carcinoma cells. Cytotoxicity against HepG2 cells was determined by using the MTT assay. Cells were incubated with varying concentration of MNT series peptoid, ALA1, or appropriate controls for three days, followed by the addition of MTT. MTT is a yellow dye that is metabolized to a purple dye by active mitochondria, therefore serving as a reporter of cell viability. Toxicity against HepG2 cells was defined as the compound concentration resulting in 50% cell death (TD_{50}). Data shown in **Table 3** represent the TD_{50} of each compound against HepG2 cells. MNT1 and MNT2 have such low toxicity that a

TD_{50} value could not be calculated and was reported to be above 200 μg/mL, the highest concentration tested. MNT3 have a TD_{50} concentration against HepG2 cells of 84.7 ± 6.2 μg/mL. With an additional repeat of the trimeric sequence, the cytotoxicity of MNT4 increased roughly 2-fold to 38.9 ± 4.4 μg/mL. The toxicity of MNT4 is very similar to the toxicity for ALA1 (TD_{50} = 40.4 ± 2.6 μg/mL), indicating that a 4-mer of the trimeric sequence displays similar toxicity to the lipidated monomer of the trimeric sequence. Of the compounds that maintain good efficacy against the ESKAPE bacteria, MNT3 is the least toxic compound against HepG2 cells.

Hemolytic Results

Hemolytic Assay		
Compound	**HC_{10} (μg/mL)**	**Standard Deviation**
MNT1	>400	ND
MNT2	>400	ND
MNT3	25.0	±13.1
MNT4	23.3	±5.5
ALA1	11.7	±10.2

Table 4: Hemolytic activity for MNT series peptoids and ALA1 incubated with hRBCs for 1 hour. Hemolysis reported as HC_{10} (peptoid concentration resulting in 10% hemolysis). Data were run in triplicate and the assay was repeated on three different days. ND = not determined.

Human red blood cells (hRBCs) from a single donor were incubated with MNT series peptoids, ALA1, or appropriate controls for 1 hour. Hemoglobin released by hemolysis was measured by UV-vis spectroscopy. The peptoid concentration resulting in 10% hemolysis compared to control (HC_{10}) was calculated using GraFit and Excel. Similar to the result of the HepG2 MTT assay, MNT1 and MNT2 hemolytic activity was so low that an HC_{10} value could not be determined even though the concentration tested here was increased to 400 μg/mL (**Table 4**; **Figure 14**). Interestingly, MNT3 (HC_{10} = 25 ± 13.1

µg/mL) had very similar hemolytic activity to MNT4 (HC_{10} = 23.3 ± 5.5 µg/mL), however, both MNT3 and MNT4 had lower hemolytic activity than ALA1 (HC_{10} = 11.7 ± 10.2 µg/mL). While liver cell toxicity and hemolytic activity generally follow similar trends from compound to compound, this is not always the case as reported previously.[54]

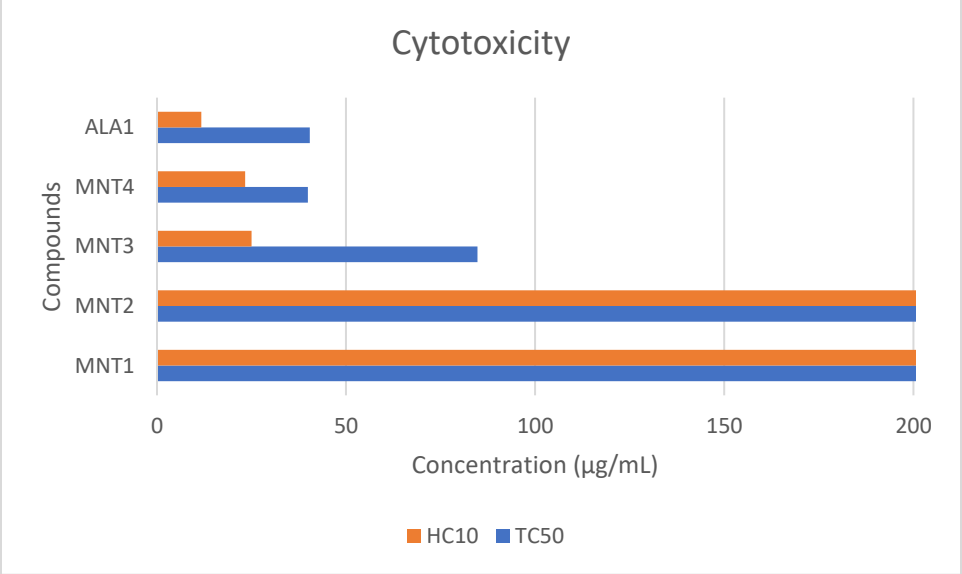


Figure 15: Graphic visualization of cytotoxicity results from **Table 2** and **Table 3.** Compounds with bars reaching 200 µg/mL represent toxicity values greater than 200 µg/mL.

Chapter Four: Conclusions and Future Outlook

Conclusion

The goal of this research was to determine if converting a short lipopeptoid into longer non-lipidated repeat peptoids would improve ESKAPE antibacterial efficacy and lower mammalian toxicity. We hypothesize that since the hydrophobic tail was the key contributor to toxicity, removing this tail would decrease overall cytotoxicity. We also hypothesize that as we increased the length of the peptoid, it would also increase the antibacterial efficacy of the peptoid. The MNT series of peptoids derived from lipopeptoid ALA1 was synthesized, characterized, and compared to ALA1. Out of the four peptoids in the MNT series, MNT3 a 9-mer with three repeats of the ALA1 trimer appears to be the most promising compound. Our results show that as expected, removal of the lipid tail did decrease the toxicity of the compound, comparing ALA1 (TD_{50} = 40.4 μg/mL; HC_{10} = 11.7 μg/mL) to the non-lipidated trimer MNT1 (TD_{50} = >200 μg/mL; HC_{10} = >400 μg/mL). However, a total loss of antibacterial activity also accompanied removal of the lipid tail. Again, as expected, adding further repeats of the ALA1 trimer eventually recovered antibacterial activity, however 9-mer MNT3 ultimately displayed better overall efficacy against the ESKAPE bacteria than 12-mer MNT4. Additionally, MIC results indicate that MNT3 was actually more potent against gram-positive pathogens compared to ALA1. Unfortunately, MNT3 potency against gram-negative bacteria was maintained or diminished compared to ALA1. Though MNT3 was more cytotoxic than MNT1 or MNT2, it was less cytotoxic than ALA1 or MNT4, indicating that the overall selectivity of a lipopeptoid can be improved by generating sequence repeats without the lipid tail.

Future Outlook

Given the unexpected decrease in antibacterial efficacy progressing from MNT3 to MNT4, one future plan is to synthesize a 15-mer of the ALA1 trimer to create MNT5 and test its antibacterial efficacy and cytotoxicity. Based on the original hypothesis, we assume that MNT5 efficacy should increase compared to other MNT series peptoids; however, our results with MNT4 suggest that there is an upper limit to peptoid length, after which antibacterial efficacy can begin to decrease. It would be interesting to see if that trend continues and if cytotoxicity also continues to increase as the peptoid is lengthened.

An additional avenue of future research would be to add the 13-carbon lipid tail back onto MNT2 or MNT3. MNT2 displayed low cytotoxicity but also had diminished antibacterial activity. Adding the lipid tail to MNT2 or MNT3 may improve antibacterial activity while maintaining lower cytotoxicity compared to AlA1.

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