“Design and Identification of Antimicrobial Peptoids Against the ESKAPE Pathogens”

By

Angelica L. Aguila

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Middle Tennessee State University

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Dissertation Committee:

Dr. Kevin Bicker, Chair

Dr. Scott Handy

Dr. Justin Miller

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April 10, 2020

Approved:

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Dr. Kevin Bicker, Committee Chair

_____
Dr. Scott Handy, Committee Member

_____
Dr. Justin Miller, Committee Member

_____
Dr. Greg Van Patten, Department Chair

_____
Dr. David L. Butler, Dean, College of Graduate Studies

ABSTRACT

A globalized health threat rises each year as pathogens quickly continue to develop modifications rendering previously prosperous antibiotics ineffective. Discovery or synthetically constructed antibacterial compounds are a necessary emphasis for new therapeutic options as there is currently a decline in drug discovery. While antimicrobial peptides are an intriguing option for antibiotic design; peptoids, which structurally contain the same backbone as peptides but with a slight modification, are better adapted to surviving degradation within the body. Combinatorial libraries were generated and optimization of peptoid design was further investigated to determine antibacterial efficiency against each pathogen in the ESKAPE panel. This study highlighted 6 combinatorial libraries with varied distribution of hydrophobic residues, overall charge ranging from a potential +1 to +7, and length varying from 3 to 7 residues long. Information gathered will guide future structure optimization for drug design aimed at Gram-negative pathogens. Antibacterial potency and mammalian toxicity were additionally determined for antibacterial peptoid K9 and compared with thiazole and oxazole K9 derivatives.

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CHAPTER I: INTRODUCTION

GLOBAL HEALTH CONCERNS:

The discovery of antibiotics along with their significant capabilities towards human survival is arguably one of the greatest medical breakthroughs to date. Becoming known as the "modern antibiotic era", infections that plagued the world years ago were halted with discoveries of naturally occurring antibiotics such as sulfa drugs and penicillin. Overzealously however, assumptions circulated amongst all individuals of that time period. The expectation lay in belief that any disease or illness that arose, then and in the future, would be eradicated by antibiotics of the time. Unfortunately, this led to massive production and utilization at any hint of sickness or for unnecessary means such as feeding livestock to accelerate animal growth. Alexander Fleming, who discovered penicillin in 1928[20], also expressed excitement for this newfound discovery that went on to increase survival rates from bacterial infections tremendously, but also skepticism towards life expectancy of antibiotics due to resistance development. In fact, he encouraged all to use caution when he presented his Nobel prize speech in 1945 stating that, "There is the danger that the ignorant man may easily under dose himself and by exposing his microbes to non-lethal quantities of the drug making them resistant." [33] Caution fell on deaf ears however, leading to the beginning of a global wide threat when bacteria resistant to penicillin were identified shortly after discovery in 1940.[14] Evolving bacteria have charted a dangerous path with destruction of any possibility for eradication on the horizon. Bacteria that once were killed off in quick succession are now flourishing with modifications. These changes are rendering previously valuable antibiotics to become useless by disrupting binding

affinity or by pumping it right back out of the cell membrane. This in turn is forcing the discovery or synthesis of new therapeutic options for bacteria that have developed resistance to almost all antibiotics on the market today. These bacteria are known as multi-drug resistant bacteria (MDRB).

Multi-drug resistant bacteria have become a global concern that requires quick attention as mortality rates skyrocket, healthcare cost increases, and numbers of antibiotics that still work efficiently are becoming few and far between. The CDC first published an antibiotic resistant (AR) threats report in 2013 that stated 23,000+ people die each year in the United States alone out of 2 million people with antibiotic-resistant infections.[10,21] Currently, mortality rates are at an all-time high associated with antibiotic resistant bacteria with 35,000 + deaths per year in the United States alone[3] and around 700,000 a year globally.[3,10] The AR threats report published in 2019, six years later, shows a 40% increase in the number of infected individuals which sits at 2.8 million[3] and a 52% increase in the number of deaths reported each year. Alarmingly this statistic is expected to escalate to an estimated 10 million deaths per year globally by the year 2050.[36,10] This upsurge is directly related to healthcare costs, as well, as more people are requiring medical attention and further hospital stays after surgery or major treatments. Necessary emphasis on drug development is critical to combat this public health threat yet there is a decline in antimicrobial agent discovery. Problematically, bacteria are mutating and developing resistance at a quicker rate than antimicrobial agents are being developed. The search for new drugs can be daunting, time consuming, and costly often ending in failure due to limitations of toxicity and failure to demonstrate efficiency on the intended target. Whether

it is lack of knowledge concerning the disease target or stricter regulations in clinical trials, drug discovery or rather synthesis is becoming quite complex and unsuccessful. Since 2000 only four new classes of antibiotics have been discovered out of 15 new antibiotics[7,9] which is not reassuring when antibiotic life expectancy is at only 50 to 60 years. (Figure 1.1)

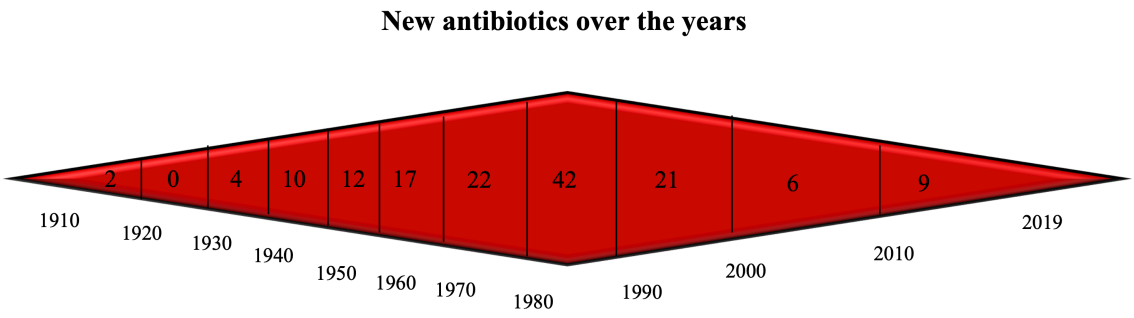


Figure 1.1:Timeline of antibiotics. The 1970s and 1980s demonstrated an upsurge of antibiotic production which has now declined tremendously.

Linezolid, for example, is an antibiotic used for last-resort infections against Gram-positive pathogens and was introduced to the market in 2000. In 2002, isolation of bacteria with developed resistance were already noted. [36] With such a short life expectancy it is crucial, if not life dependent, to discover or synthetically introduce a wide range of prospective antibiotics rendering the post antibiotic era a myth.

ESKAPE PATHOGENS:

The ESKAPE pathogens are an accumulation of highly resistant bacterial species, exhibiting quick adaptability, and are responsible for a high degree of antibiotic-resistant deaths globally. This acronym is a formulation of seven bacterial strains with high levels of resistance development and prevailing mortality rates which are *Enterococcus faecium, Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species.* Although this is a clever acronym that indicates each pathogen by name, the acronym most effectively points out the ability of these microbes to "escape" modern antibiotic mechanisms.[24] These pathogens are a top priority not only due to their ability to resist almost all antibiotics on the market, but it is of clinical significance in the medical field because they thrive in a compromised immune system. Without proper antibiotics, medical procedures such as cancer treatments and organ transplants will be considered more harmful than good. The World Health Organization, which is largely responsible for the initiative of MDR control, reported a pathogens list of priority of critical, high, and medium. *A. baumannii, P. aeruginosa,* and *Enterobacteriaceae* (includes *E. coli* and *K. pneumoniae*) are listed as critical priority with *E. faecalis*, *E. faecium,* and *S. aureus* listed as a high priority.[24] The Centers for Disease Control and Prevention categorize bacterial threats into Urgent, Serious, and Concerning with *Enterobacteriaceae* and *A. baumannii* listed under Urgent threats while *E. faecalis, E. faecium, S. aureus,* and *P. aeruginosa* are considered serious.[3]

Methicillin-resistant *Staphylococcus aureus* (MRSA), a gram-positive pathogen, is of high concern due to cultivation of serious infections and even death. In fact, out of US reported deaths per year due to antibiotic-resistant infections, MRSA alone is responsible for 36% of those cases. Therefore, it comes as no surprise that more emphasis is devoted to Gram-positive studies allowing more options therapeutically than are available for Gram-negative bacteria. In fact, there have not been any new advances for Gram-negative bacteria in 50 years.[7] However, Gram-negative bacteria are arguably just as dangerous as their Gram-positive counterparts. Out of US reported deaths per year, 46% of those casualties are due to *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and E. coli,* all of which are Gram-negative pathogens.[3] While 46% does not sound significant, it in fact equals 13,600 deaths per year in the United States alone.[3.] (Figure 1.2).

Antibiotics on the market are currently more adapt at maintaining control of Gram-positive based infections while Gram-negative infections are harder to treat as they are developing resistance to essentially all antibiotics. Gram-negatives of the ESKAPE panel, *K. pneumoniae, A. baumannii, P. aeruginosa,* and. *E. coli,* are all listed as critical threats globally not only due to extreme resistance towards antibiotics but also selectivity of antibiotics towards Gram-negative pathogens prove difficult due to membrane structure. Their membranes make it more difficult to develop new drugs for treatment as compared to Gram-positive pathogens due to another layer of security. This indicates that research on Gram-negative structure and potential antimicrobial drugs against them is a top priority.

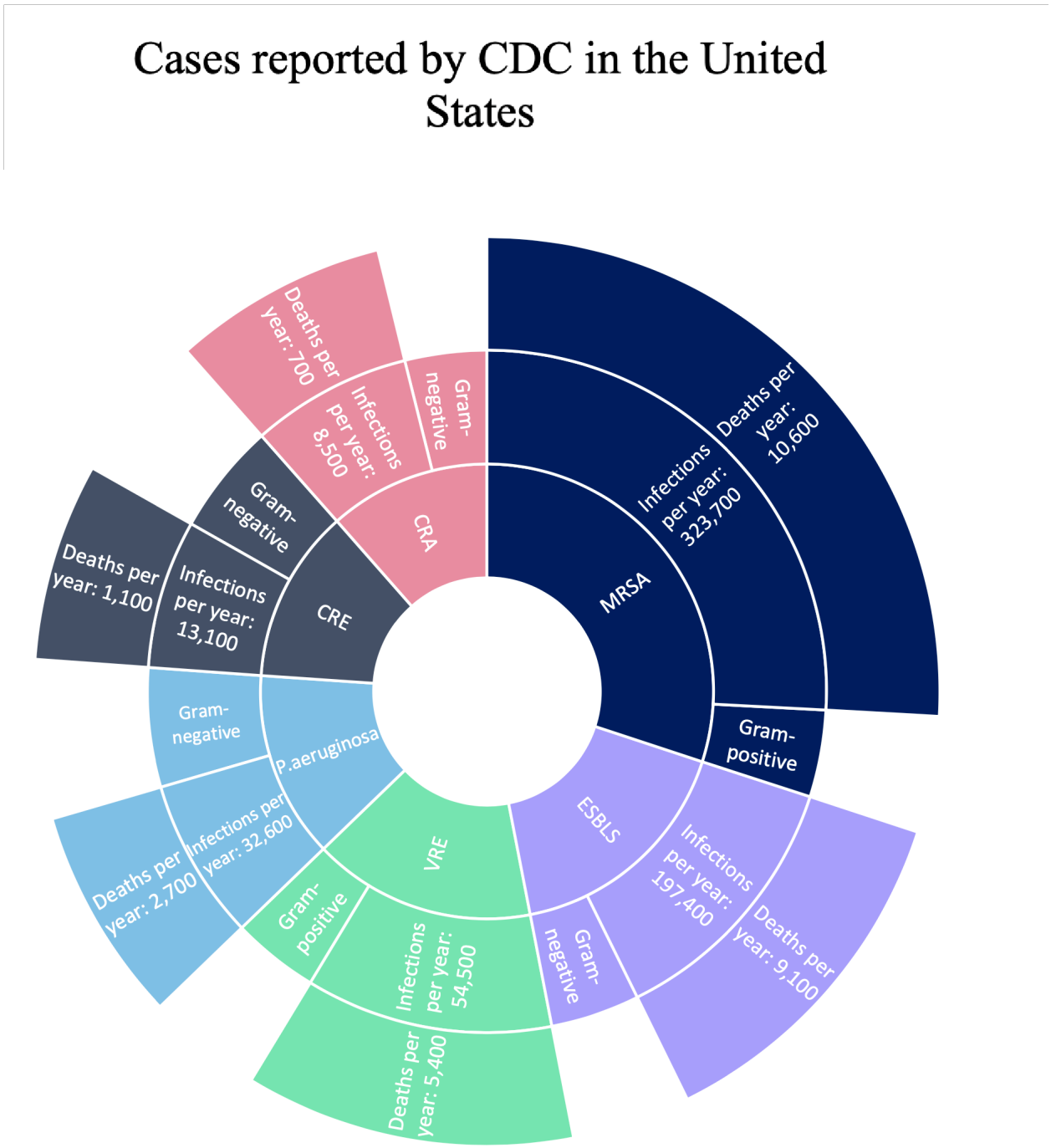


Figure 1.2 Representation of reported deaths per year for each ESKAPE pathogen.

CRA = Carbapenem-resistant *A. baumannii;* **CRE** = Carbapenem-resistant *Enterobacteriaceae* (most commonly *E. coli* and *K. pneumoniae*); **ESBLS** = Extended spectrum Beta-lactamase producing Enterobacteriaceae (most commonly *E. coli* and *K. pneumoniae*); **MRSA** = Methicillin-resistant *Staphylococcus aureus*; **VRE** = Vancomycin-resistant Enterococci

ENTEROCOCCUS:

Enterococcus bacterial pathogens are not normally considered dangerous species as they colonize in human gastrointestinal tracts and uterine walls as common flora. Infections do occur, however, in individuals with a compromised immune system most commonly in hospital environments. *Enterococcus* consists of numerous species but only two have been associated with serious infections such as urinary tract infections (UTIs), intra-abdominal infections, pelvic infections, endocarditis, and rarely meningitis. *E. faecalis* is responsible for 90% of *enterococcus* based human infections, but *E. faecium* has shown more resistance towards vancomycin.[16] Vancomycin is a last resort drug for Gram-positive pathogens approved for use in 1958 [12] against infections that proved resistant against common antibiotics such as penicillin. The rise of penicillin resistance led to increased use of vancomycin which resulted in the first isolation of vancomycin-resistant *Enterococcus* (VRE) in 1986 reported in Europe.[15]

STAPHYLOCOCCUS AUREUS:

Future use of vancomycin is of concern and might prove ineffective in the near future as serious infections caused by vancomycin-resistant *Enterococcus* and now *Staphylococcus aureus* are rising. *Staphylococcus aureus* first developed resistance towards benzylpenicillin in the 1950s but was quickly replaced in 1959[34] with methicillin which was an adaptation of benzylpenicillin with methoxy groups substituted into the compound. Unfortunately, two years later methicillin-resistant *Staphylococcus aureus* (MRSA) was isolated[34] and physicians were forced to turn to vancomycin as nosocomial MRSA rates were significantly rising every year eventually doubling to 57% by the year

2000. Serious infections can result leading to severe skin infections, bloodstream infections, or infectious wounds and are now commonly spread through the community rather than just the hospital. Vancomycin-resistant *Staphylococcus aureus* was discovered in 2002[12] causing grave concern for the future.[19]

ENTEROBACTERIACEAE:

A significant portion of hospital-acquired infections emergence as a result of *Enterobacteriaceae* leading to complicated urinary tract infections, causes of major sepsis, and cases of hospital-acquired pneumonia. Difficulties arise as bacterial infections, largely influenced by *K. pneumoniae* and *E. coli,* are acquired in healthcare settings with developed resistance towards penicillin and cephalosporins. Gene modifications have developed enzymes termed Extended-spectrum beta-lactamases[19] responsible for inactivation of beta-lactam rings that characteristically guides these antibiotics. In response, implementation of the last resort drug for Gram-negative pathogens, carbapenems, was utilized consistently by physicians leading to a bigger problem. Carbapenem-resistant *Enterobacteriaceae* (CRE) and Extended-spectrum β lactamase (ESBL) *Enterobacteriaceae* were listed as part of the critical priority list proposed by WHO, as mentioned previously, in addition to the urgent and serious threats revealed by the CDC. In 2013, the CDC reported a 6.6% mortality rate due to CRE infections[3] that are have colonized further since then. To combat CRE, polymyxins were reintroduced despite concerns of nephrotoxicity due to anxiety of transference of carbapenemases to other bacterial species.

ACINETOBACTER:

Acinetobacter began demonstrating major hospital-acquired infections with resistance around 25 years ago.[18] Poor clinical outcomes have been reported in relation to nosocomial outbreaks with hospital-acquired pneumonia, wound infections, and cases of sepsis. Similar to *K. pneumonia* and *E. coli,* therapeutic options have evolved to necessary emphasis on polymyxins as resistance to carbapenems is considered more dangerous than the toxicity polymyxins provide. According to CDC, 60% of *Acinetobacter* infections are resistant to 3 or more antibiotics making treatment difficult as represented by a statistic from 2017 stating that out of 8,500 hospital-acquired infections 700 deaths occurred in the US.[39]

PSEUDOMONAS AERUGINOSA:

Pseudomonas aeruginosa infections strongly impact individuals with chronic respiratory diseases. Patients with cystic fibrosis, for example are commonly victims of hospital-acquired *P. aeruginosa* infections. However, multi-drug resistant *p. aeruginosa* only constitute 10% of hospital-acquired infections.[1] Development in resistance for this species still contributes to a significant need for new antibiotics as quick adaptiveness is limiting treatment options.

EARLY AND MODERN ANTIBIOTICS:

The discovery of antibiotics impacted the direction of the world both medically and historically. Not only were previously life-threatening bacterial infections essentially wiped out but there was also significant impact for the soldiers in World War II. Sulfa

drugs were utilized often in the field, but they are not considered the first true antibiotics. Though they made tremendous difference in lives saved, their significance lay in bacteriostatic capabilities meaning it only inhibits further growth of bacteria. Penicillin, however, is the first true antibiotic because it is bactericidal meaning it kills bacteria. Antimicrobial properties were accidently discovered in the fungus *penicillium notatum* by Alexander Fleming leading to penicillin and penicillin derivative antibiotics for multiple infectious diseases.[20] However, Fleming warned of a post antibiotic era where bacteria were resistant to treatment by over and unnecessary use of antibiotics. The effectiveness of antimicrobial drugs stem from their ability to disrupt bacterial cell membrane integrity or by inhibiting metabolic pathways intracellularly. Exposure, however, transforms remnants of bacterium inadvertently into "super bugs" with acquired resistance. Developed resistance occurs through modifications when pathogens are exposed to antibiotics such as genetic mutations or resistance transferred from another bacterium. With an incredibly rapid reproduction rate, microbes are able to develop defense mechanisms efficiently and quickly. Utilization of efflux pumps are instrumental in pumping antibiotics out of the membrane, targeted sites are altered preventing attachment of antimicrobial drugs, and genes are transferred among both progeny and different species. [2]

ALTERNATIVE ANTIBIOTICS:

Antimicrobial peptides (AMPs) are biologically active compounds found within the domains of the human body, on the skin of frogs, in the leaves of plants around us, and various other organisms. They serve as "host defense peptides" that respond immediately to threats within their environment. The ability to participate as a first line of defense is

significant as is their broad-spectrum antimicrobial properties that combat various species of bacteria, fungi, parasites, and viruses. Identification of AMPs were originally recognized in prokaryotes and proved to be natural endogenous antibiotics with activity towards various bacterial species. Utilization of AMPs by eukaryotes remained unknown for a short period as the adaptive immune system was widely understood to be responsible for protection against exposure to threats. Speculation arose, however, due to infection free plants and insects which are known to be lacking an adaptive immune system. The first discovery of an AMP was reported by Alexander Fleming[5] when he observed lysing capabilities of nasal mucus. These properties were briefly studied after Fleming noticed inhibited bacterial growth on a culture plate where his own mucus had made contact. He termed this antimicrobial enzyme a "lysozyme"[38] based on its ability to break apart bacterial cells and further explored its properties determining that it was found additionally in saliva, tears, breastmilk, and cervical mucous among others.[38] However, its classification as an AMP proved inadequate when years later its mechanism of action was determined to be enzymatic destruction instead of non-enzymatic mechanisms utilized by AMPs. [27] Identification of lysozymes were significantly overshadowed when it was determined to be limiting towards disease causing pathogens but sparked an interest in Fleming in the study of antimicrobial agents. This lasted from the initial discovery of lysozyme in 1921 until his groundbreaking uncovering of the antibiotic penicillin in 1928.[27] The year 1944 yielded an AMP, Gramicidin S,[13] that would two years later actively be utilized as an antibiotic. Isolated from the bacteria *Bacillus brevis,* Russian microbiologist Georgyi Frantsevitch Gause[28] discovered its ability to target both Gram-positive and some Gram-negative bacteria. Based on these therapeutic properties,

Gramicidin S was widely used in Soviet military hospitals during World War II. Polymyxins were isolated in 1947[28] and used briefly in Japan, Europe, and the United States to treat infections but were halted in the early 1970s[27] due to extreme toxicity outcomes. Polymyxin B and Polymyxin E, also known as Colistin, were isolated from a bacterial species *Paenibacillus polymyxia* found in a soil environment. [27]

Interest in further physiochemical characterizations and study of structural components of AMPs was diminished as the "golden age of antibiotics" provided a false sense of security. Engagement in AMP research resurfaced only with the revelation of antibiotic resistant bacteria in the late 1960s. Penicillin which was the trademark for this era and hope for the future was now showing instances of resistance by *Streptococcus pneumoniae* in 1967 and *Neisseria gonorrhoeae* in 1976. [20] Polymyxins were reintroduced as a last resort antibiotic as its narrow spectrum towards Gram-negative pathogens proved necessary as multi-drug resistant bacteria began to accumulate and difficulty targeting Gram-negatives was presented. Colistin is considered a valuable therapeutic option by physicians despite nephrotoxicity levels as it is the only effective antibiotic towards multi-drug resistant bacteria specifically *K. pneumoniae, A. baumannii, P. aeruginosa, and E. coli.* [8] In 1967, an AMP with broad-spectrum activity was found to be a key component in the venom of *Apis mellifera.* [41] The AMP isolated from the venom is known as melittin and while is antibacterial it is most known for its ability to produce anti-inflammatory effects leading to better outcomes in diseases such as rheumatoid arthritis as well as its antitumor activities in various cancers. [41] In 1970, the first AMP found within the skin secretions of an amphibian[27] were isolated from the frog *Bombina variegate* and respectively named

bombinin. The first insect antimicrobial peptides, cecropins, were observed in 1974 and were not inducible until 1981.[42] First isolated from the hemolymph of pupae in the giant silk moth *Hyalophora cecropi,*[42]*,* cecropins have since been discovered in other insects and demonstrate broad-spectrum antimicrobial activity.[10] The year 1985 expressed human alpha defensin (HNPs) of which two additional types would be discovered later in 1991 and 1999.[25] Alpha-defensins were indicated in the neutrophils within rabbits and now are understood to be abundant in the neutrophils and macrophages in humans[27]. Beta-defensins, characterized in 1991, and gamma-defensins, characterized in 1999[27], are related AMPs to alpha-defensins that differ in their cysteine motif pairings[17] allowing stabilization by disulfide bonds.

Continuation of AMP isolation and characterization studies have determined the successful components of peptide design that are attributed to their capacity to disrupt or transverse into the membrane of pathogens. Thousands of AMPs to date have been recorded with a wide array of diversity concerning the structural elemental design of each peptide. Categorically, the most abundant feature of AMPs is their cationic nature in conjunction with relatively short peptide lengths of 12 to 50 amino acids.[35] The ability of AMPs to orientate themselves and penetrate the membrane surface is based on their abundance of basic amino acids granting them a net positive charge of +2 or higher in addition to implementation of >50% of hydrophobic residues. [35] It is assumed that AMPs work by electrostatic interactions with anionic bacterial membranes which lead to interference of membrane integrity due to amphipathic characteristics. Loss of viability is due to generated pores in which cytoplasmic leakage and cell death occurs or may be a

result of disruption of important intracellular components. Pore formation is theorized to occur in one of three different mechanisms based on the structural components of the AMP involved. However, the key feature between them all is the ability to form pores through interactions between the positively charged AMP and the anionic environment of phospholipids, lipopolysaccharides present in Gram-negative bacteria, or lipoteichoic acid present in Gram-positive bacteria. Mammalian cell membranes exhibit weak interactions as their surface is zwitterionic with dispersion of groups such as cholesterol enabling AMPs to demonstrate cell selectivity. [35] Also known as host defense peptides, AMPs have already proven affective as part of our innate immune system making them therapeutically intriguing options for antibiotic design. However, synthetic AMPs have continuously failed in pre-clinical trials and have not produced any reputable antibiotics to combat the multi-drug resistant pandemic. Challenges of high magnitude concerning levels of toxicity and quick recognition by proteases lead to insufficient half-life's that hinder AMP clinical success. These concerns, in addition to costly production, have led to alternate synthetic sequences that will address the fundamental drawbacks of original designs.

PEPTOIDS AS ANTIMICROBIAL AGENTS:

Peptoids are a class of peptidomimetics that are synthesized to mimic the components of an antimicrobial peptide leading to the same characteristical behaviors but with enhanced capabilities.[40] Synthetic representations of antimicrobial peptides constitute the foundations for improved antibiotic design. Physiochemical components of AMPs that attribute to their characteristic behaviors which are deemed successful in antimicrobial activity should be maintained. Charge and amphiphilicity, specifically, are key features

that enable bacterial membrane interaction and will dictate much of future design. Structural changes would lower the occurrence of protease recognition leading to half-lives within an acceptable range. Structurally, peptoids contain the same backbone as peptides but, with a slight modification that increases proteolytic stabilities. (Figure 1.3) Relocation of the side chain from the alpha carbon to the nitrogen atom of the backbone enhances these abilities and allows new prospects for novel treatments against multi-drug resistant bacteria.

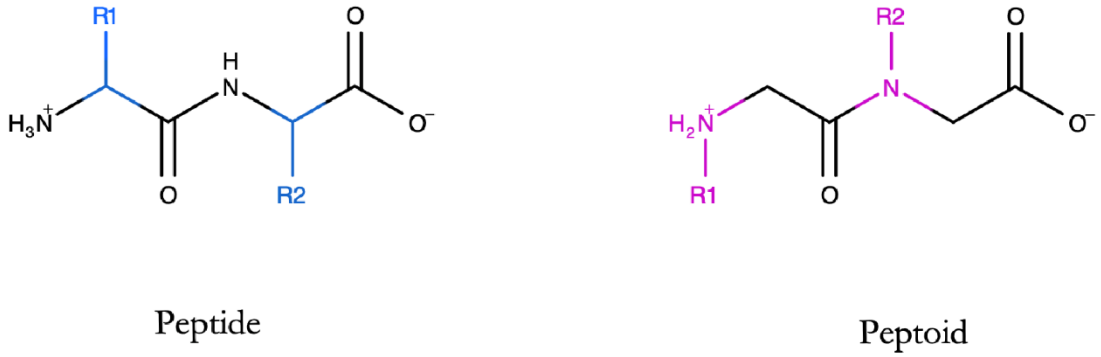


Figure 1.3: Peptides vs. Peptoids. [40] Structural differences between peptides and peptoids.

This relocation increases the half-life, time it takes for 50% to be eliminated from the body, from around twenty minutes to as much as twenty-four hours. Utilizing solid phase synthesis, huge diverse libraries of peptoids can be designed and later screened for potential antibacterial properties.

Bacteria quickly evolving resistance modifications have left current antibiotics ineffective and has created the need for new therapeutic options. In this work combinatorial libraries have been constructed to synthesize large numbers of compounds with potential antimicrobial activity specifically to target species of the ESKAPE panel. Focus has been on the optimization of peptoid design with regards to compound length, difference in tail design, and degree of hydrophobicity and charge. Comparison of what characteristics prove most effective in each library will help identify features specifically targeting Gram-negative pathogens over Gram-positive pathogens. Utilizing our lab's high-throughput PLAD assay, antimicrobial peptoids can be rapidly identified, and efficacy can then be measured through minimum inhibitory concentration (MIC). While peptoids will be tested for potency and cytotoxicity against Gram-positive bacteria, emphasis will focus more on Gram-negative bacteria.

CHAPTER II: PEPTOID COMBINATORIAL LIBRARY DESIGN AND OPTIMIZATION

2.1 INTRODUCTION:

Synthesis of antimicrobial peptoids are widely being explored as a new therapeutic option to overcome conventional antibiotic downfalls of bacterial resistance and the challenges presented by synthetic peptides of natural AMP design. In 1992, Reyna J. Simon designed an isomer of peptides with important structural differences to enhance the diversity possible and effectively target the biggest setback of peptide drug design, protease degradation. [32] In fact, most research obtained today concerning the advancements of peptoid design and their capabilities as possible drug candidates for antibiotic resistant bacteria stem from Ronald Zuckermann whom was a coauthor on Reyna's paper from 1992. Zukerman provided an alternate method, later in 1992, for synthesis that provided ease and introduced further submonomer options to introduce more opportunities for peptoids with significant biological activity. [43] This method is still widely adopted today utilizing a procedure of alternating acylation steps followed by a nucleophilic displacement step. Synthesis of peptoids involves a two-step process which starts with acylation of a receptive amine by a haloacetic acid, bromoacetic acid in this case, that is activated by the addition of diisopropylcarbodiimide (DIC). A primary amine of choosing is then introduced which displaces the bromine in a S_N2 reaction generating a secondary amine ready for acylation. This process is repeated until the desired length is achieved. (Figure 2.1)

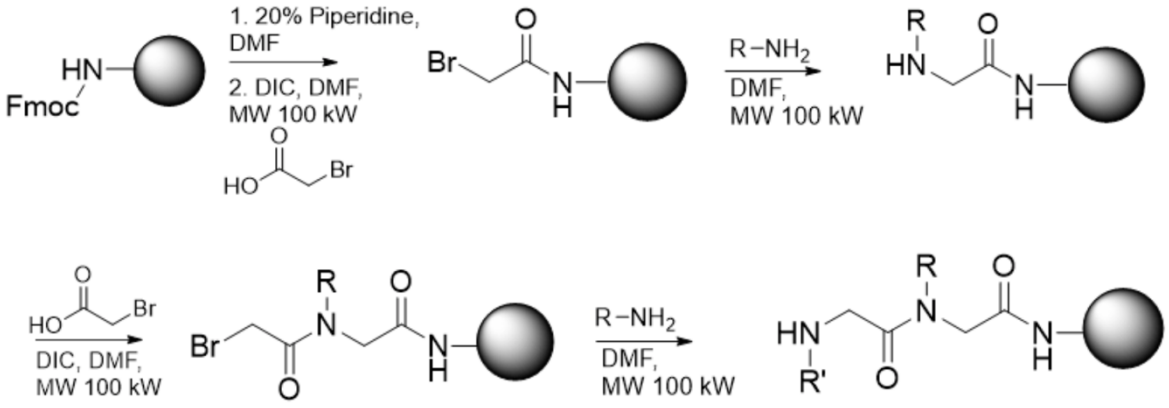


Figure 2.1: [40] **Peptoid Synthesis.** Two-step process of adding a submonomer by acylation and amine coupling.

Peptoid synthesis involves solid-phase chemistry and is adaptable to a spilt-and-pool method for the synthesis of combinatorial libraries. By adopting this technology, the benefits of combinatorial libraries extraordinarily accelerate synthesis capabilities leading to a tremendous diversity of compounds in massive quantities. The split-and-pool method essentially involves four sequential steps for each coupling "cycle" (Figure 2.2). Acylation with bromoacetic acid, that is activated by the addition of DIC, constitutes the first step which makes it possible for a primary amine to be coupled in step three. Before coupling can take place, resin beads are separated into various vessels, depending on how many monomers will be tested. An amine of choosing is then introduced displacing the bromine in a S_N2 reaction generating a secondary amine ready for acylation. Beads are then pooled together before repeating the process of acylation, separation, and coupling until a desired length is reached.

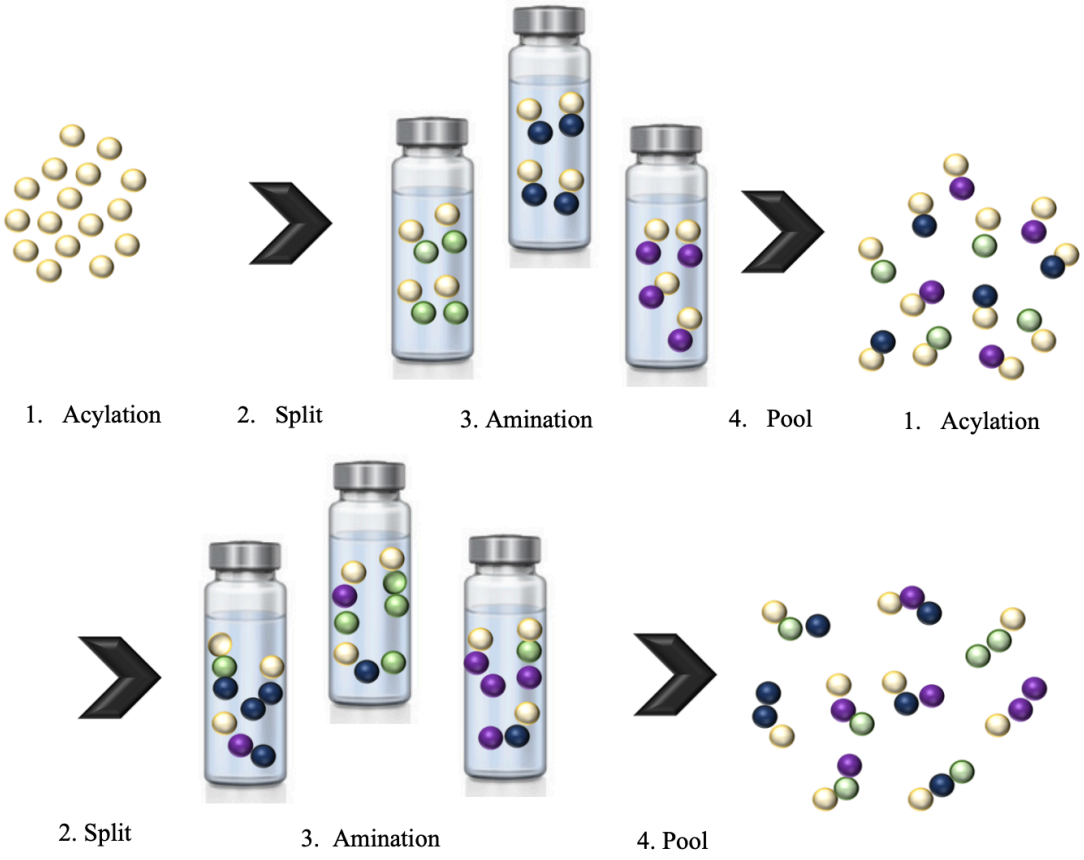


Figure 2.2: Split-and-Pool Synthesis. A schematic representing a split-and-pool method to generate a large diverse combinatorial library utilizing three submonomers undergoing two couplings. The yellow circles depict resin beads that molecules build upon. The green, blue, and purple circles represent different submonomers implemented. The last step of the schematic shows a variety of unique compounds produced from this method.

A high-throughput assay, termed the Peptoid Library Agar Diffusion (PLAD) assay, was developed by the Bicker lab to enable multiple beads in a combinatorial library to be screened at once for possible "hits" to determine and sequence which peptoids within a combinatorial library exhibit biological activity against targets of interest. Its true effectiveness lies with the PLAD linker section implemented into the growing peptoid sequences synthesized. This section utilizes a chemically cleavable linker system in which two identical sequences of peptoid can be synthesized (Figure 2.3). These two identical peptoids, labeled α and β-strand, can individually cleave under different chemical conditions allowing identification of antimicrobial beads and also sequencing of the unknown peptoid. These unknown molecules are exposed and incubated with the target(s) of interest to determine if any interaction between the two proceeds. This is achieved by inoculating a pathogenic bacterium of interest in soft agar and then incorporating library compound beads and a reducing agent into this mixture. While incubating overnight, the reducing agent will reduce the disulfide bond of the branched linker releasing the β-strand into a uniform lawn of bacteria. If the compound released exhibits antimicrobial behavior, indicated by a zone of inhibition, then that bead can be removed and prepared for sequencing via tandem mass spectrometry (Figure 2.4). The α-strand is then cleaved from the PLAD linker with cyanogen bromide (CNBr) to determine the peptoid sequence of that bead. Studies can then be performed to further understand the properties of the peptoid of interest.

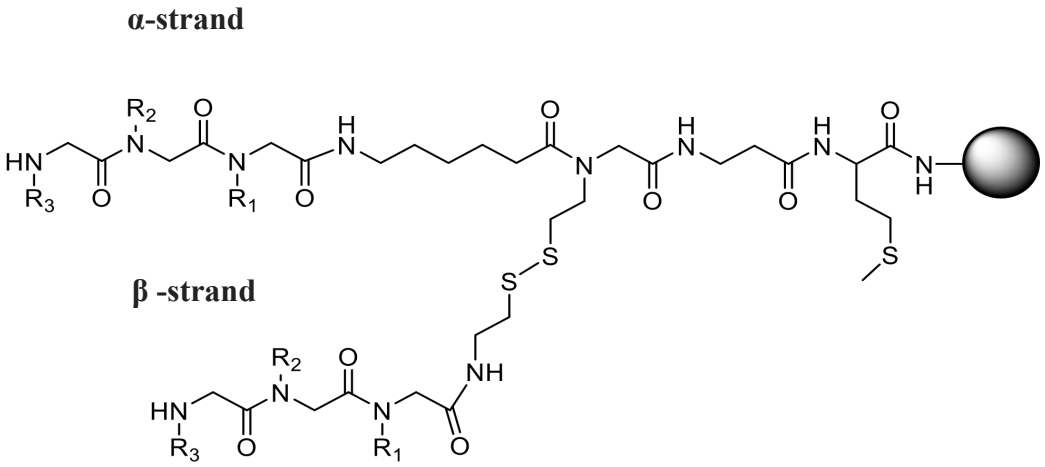


Figure 2.3: Linker, α-strand, and β-strand.[40] Representation of a cleavable linker utilized to produce two identical strands of a peptoid that can be released for both antibacterial property identification and for sequencing.

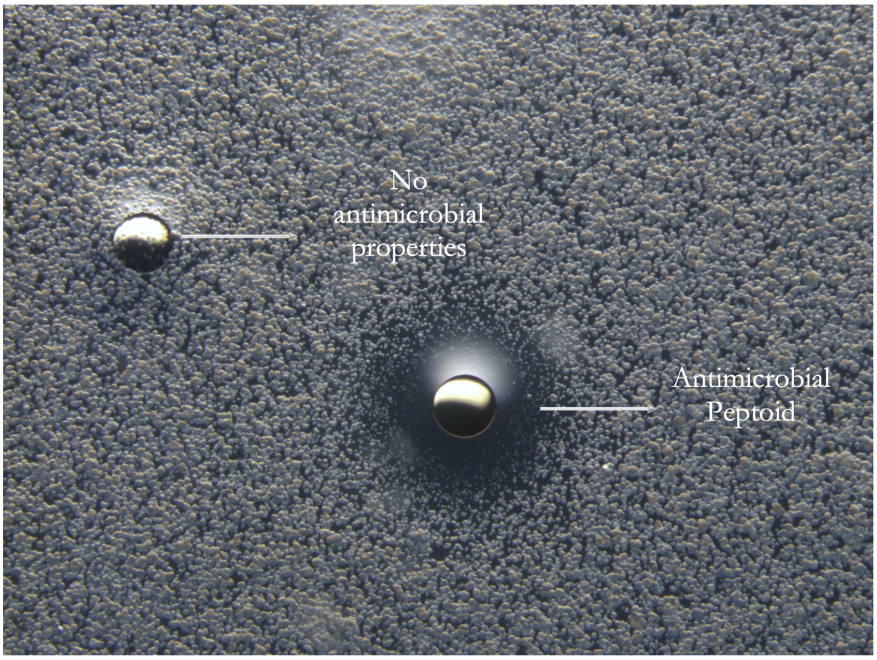


Figure 2.4: Peptoids with or without antimicrobial properties. Antimicrobial properties indicated by zone of inhibition or no antimicrobial properties indicated by no zone of inhibition.

Peptoid design was tailored towards features that would selectively target Gram-negative membranes. Screening for antibacterial properties, however, still included Gram-positive pathogens as discovered antimicrobial agents against *E. faecalis, E. faecium,* and *S. aureus* still prove necessary therapeutically. Additionally, antimicrobial compound "hits" obtained for Gram-negative bacteria vs Gram-positive bacteria can be used in comparison to determine structure characteristics that could potentially favor one membrane type over the other. The composition of Gram-negative membranes adds extra security that makes cell permeability quite difficult as compared to the membranes of Gram-positive pathogens which are comparatively easier to target. Challenges presented for potential antibiotics to effectively target Gram-negative bacteria are due to the outer membrane (OM) it possesses in addition to its inner cytoplasmic membrane (ICM) (Figure 2.5). The OM is responsible for inhibiting antibiotic connection and implementation through the membrane or porin portals present. This is due largely to a plethora of lipopolysaccharides (LPS) dispersed without the OM.

The LPS structure as a whole provides benefits of tremendous diversity that further stabilizes the OM, compresses fluidity, allows recognition of potential danger, and are even toxic themselves. [31] Stability is offered through negatively charged phosphate groups, found on the ends of glucosamines, within the lipid A structure of an LPS. These phosphate groups are connected through a magnesium divalent cation bridge that diminishes the fluidity of the membrane (Figure 2.6). While the hydrophobicity of the Lipid A portion allows implementation into the membrane, the other two domains are hydrophilic in nature and extend into the extracellular environment for recognition potential and restrictive

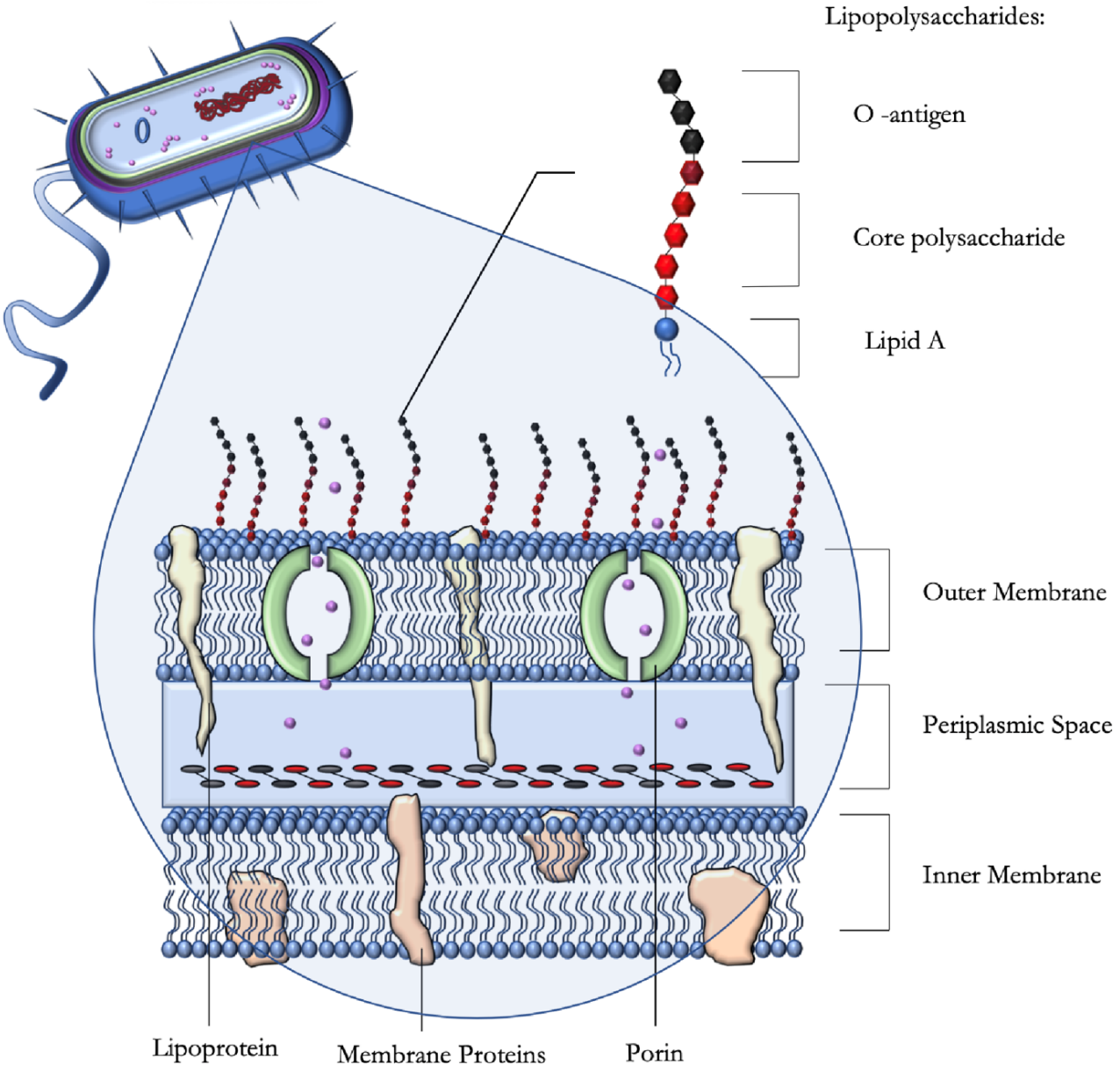


Figure 2.5: Membrane of Gram-Negative Bacteria. The membrane consists of two components, an outer membrane and a cytoplasmic inner membrane. A thin layer of peptidoglycan is present in the middle constituted as the periplasmic space which is a gel-like matrix composed of various proteins. The outer membrane, largely responsible for difficulties experienced in targeting, is composed of phospholipids and lipopolysaccharides (LPS). An LPS has a highly hydrophobic region knows as Lipid A embedded into the outer leaflet of the outer membrane, and two hydrophilic regions which are the core polysaccharides indicated by the red octagons as well as the O-antigen region indicated by the black octagons.

capabilities. Lack of successful hydrophobic antibiotics could arguably be due to the presence of both the hydrophilic domains of LPS and also the lowered OM fluidity due to adjacent lipid A connections of LPS.

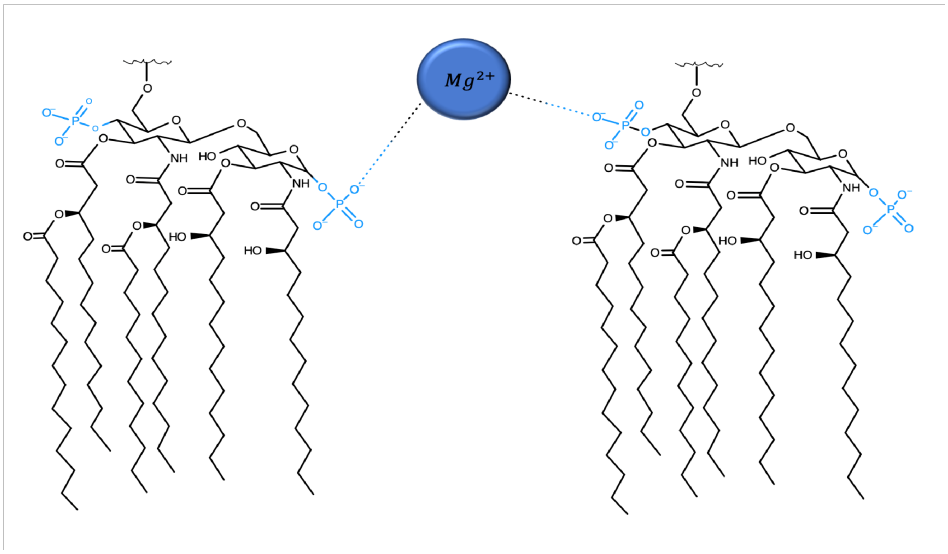


Figure 2.6: Structure of Lipid A. Mg^{2} bridge between two adjacent negatively charged phosphate groups found within the lipid A portion of lipopolysaccharides in the outer membrane of *E. coli.*

The membrane of Gram-positive bacteria (Figure 2.7) exhibits structural characteristics that differentiate them from Gram-negatives, specifically their lack of an OM and a relatively massive cell wall in comparison. The peptidoglycan section, present in all bacterium, consists of multiple layers dispersed with teichoic and lipoteichoic acids making it much thicker than the peptidoglycan layer found in Gram-negative membranes. Teichoic acids, adhered to peptidoglycan, and lipoteichoic acids, attached to membrane lipids, make up >50% of the cell wall.[4] Without an OM present, diffusion more readily occurs through the porous peptidoglycan wall despite the thickness contribution.

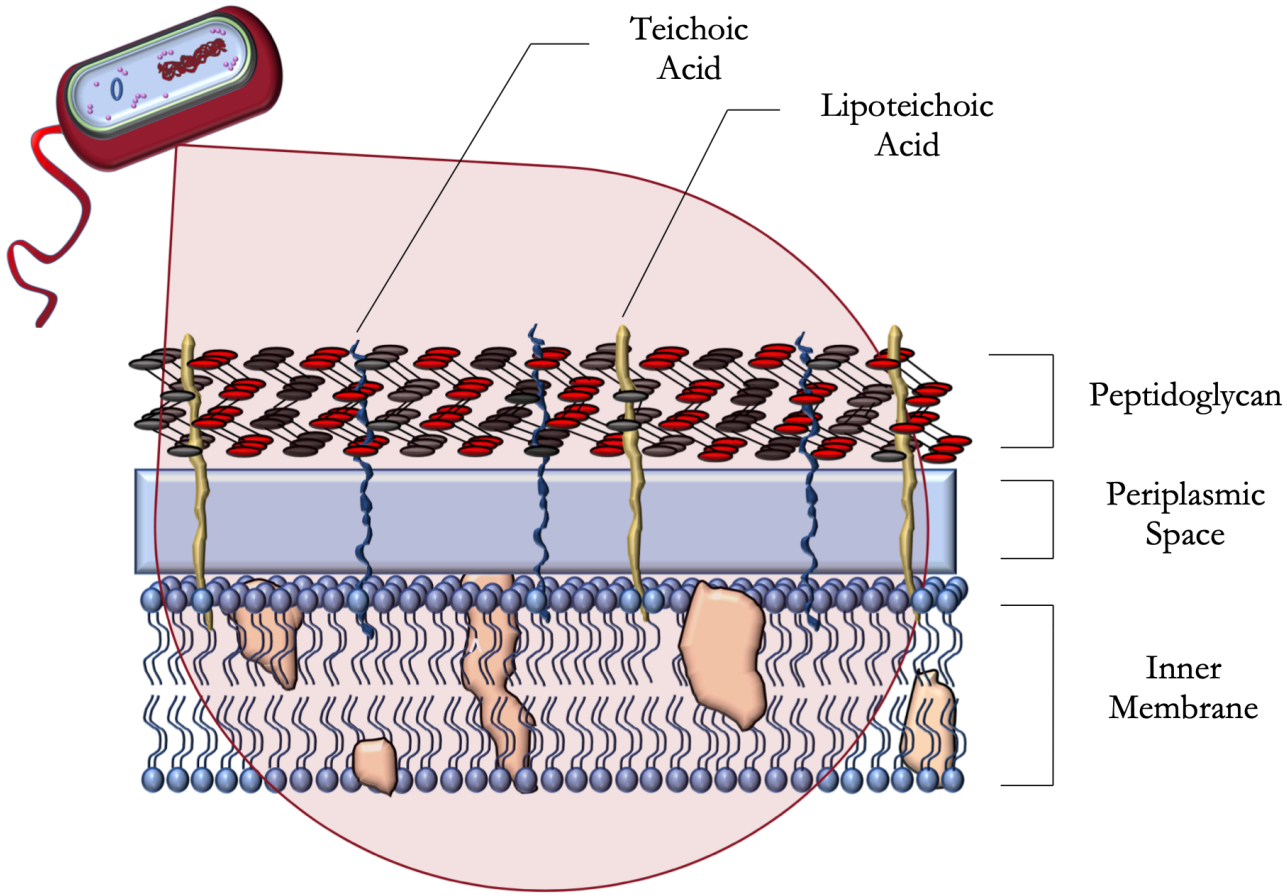


Figure 2.7: Membrane of Gram-Positive Bacteria. A thick peptidoglycan layer persists in gram-positive bacteria, but they lack the outer membrane that gram-negative bacteria have. This actively leads to better antibiotic reception. Teichoic and lipoteichoic acid are distributed within the peptidoglycan layer and the periplasmic space is present but thinner compared to a large volume found in Gram-negatives.

2.2. PEPTOID DESIGN AND OPTIMIZATION OF LIBRARY:

Advancements in generating effective antimicrobial peptoids were reported previously by this lab describing key features necessary for successful outcomes. While a long alkyl carbon tail proved to increase cytotoxicity, it also significantly demonstrated heightened potency towards the bacterium tested. Further investigation of characteristics that benefit potency are of importance while still maintaining the efficacy of a 13-carbon tail. Emphasis is focused primarily on development of antibacterial peptoids against the ESKAPE pathogens as they are a top priority concern due to their overwhelming and constantly evolving resistance mechanisms. Combinatorial libraries focused on designs that resemble the characteristics of natural antimicrobial peptides with ranging levels of compound lengths, various degrees of cationic charges, and hydrophobicity are key for strong biological activity. Implementing these components ensures that developed compounds will exhibit mechanisms similar to already successful antimicrobial peptides found within the body but with the additional benefits peptoid structures provide. Gram-positive bacteria have more readily available treatment options still available in comparison to Gram-negatives who often are treated with last resort drugs. Furthermore, designs for drug development are more easily attributed to the bacterial membranes of Gram-positive pathogens as they are more accessible to compounds than Gram-negatives. This makes generating effective antibacterial peptoids for these species optimal, however, priority is tailored more towards the permeability of Gram-negative bacteria which proves difficult. Success may be observed with moderately-sized compounds generated in succession with multiple cationic groups providing a charge preferably higher than +1 for specific targeting

of Gram-negative pathogens. Molecules with extended length might be attracted further to bacterial membranes due to enhanced capacity for charge buildup potentially increasing the probability of developing antibacterial peptoids effective towards the ESKAPE panel. Smaller molecules, on the other hand, would have more success navigating through the porins found implemented in the outer membrane of Gram-negative pathogens which allow passage of small, hydrophilic, and/or cationic molecules. Hydrophobicity is an essential component utilized in the design of peptoids. A proper balance of hydrophobic residues implemented into the backbone of peptoids can prove to be difficult to achieve as they have been determined, in previous studies, to enhance the antimicrobial activity achieved but additionally are responsible for higher evaluated toxicity against mammalian cells. [22] Hydrophobic submonomers implemented might benefit from staying at a ratio of 50% as is often observed when taking antimicrobial peptides into consideration. Increased hydrophobicity may not be the most effective strategy pertaining to membrane disruption in regard to Gram-negative species though and rather might benefit from high net positive charge.

Seven submonomers of various levels of hydrophobicity and cationic charge were randomly incorporated into libraries 001 and 002. Diversity of unique compounds produced will contain a variety of aromatic and heterocyclic residues, specifically 3, 4-methylenedioxybenzylamine (1), cyclohexylamine (2), tetrahydrofurfurylamine (3), 2-thiophenemethylamine (4), 4-methoxyphenethylamine (5), N-Boc-1,4-diaminobutane (6), and N-Boc-1,3-propanediamine (7). Similarity between the two libraries remains with the amines of choice utilized along with the tail, tridecylamine (X), implemented in the N-

terminal position. The significant difference is associated with the length outcome of the peptoids built. Library 001 has a theoretical diversity of 823,543 unique compounds as seven-residue sequences (Figure 2.8) are produced while four-residue sequences are the result of Library 002 (Figure 2.9) giving a theoretical diversity of 2,401. Previous studies have obtained lower MIC values as repeated motifs were added to a sequence in structure-activity determinations. Compound length was fluctuated between libraries to increase biological hits obtained. MIC values, from previous studies, have demonstrated lowered MIC values as residue length extended from 6 to 12 positions. *E. coli* decreased from 27 μM for $(N_{lys}N_{spe}N_{spe})_2$ to 9.1 μM for $(N_{lys}N_{spe}N_{spe})_3$, and further down to 3.5 μM for $(N_{lys}N_{spe}N_{spe})_4$. However, continuous efforts showed to have a reverse effect with an MIC of 5.5 μM for $(N_{lys}N_{spe}N_{spe})_5$. A peptoid sequence of 6 residues (*N*ah*N*phe*N*phe)2 in length, for example, exhibited a MIC value of >100 μM for *E. coli.*[6] This decreased to 50 μM as the sequence expanded to 9 residues (*N*ah*N*phe*N*phe)3 and further dropped to 13 μM at 12 residues (*N*ah*N*phe*N*phe)4.[6]

Library 001

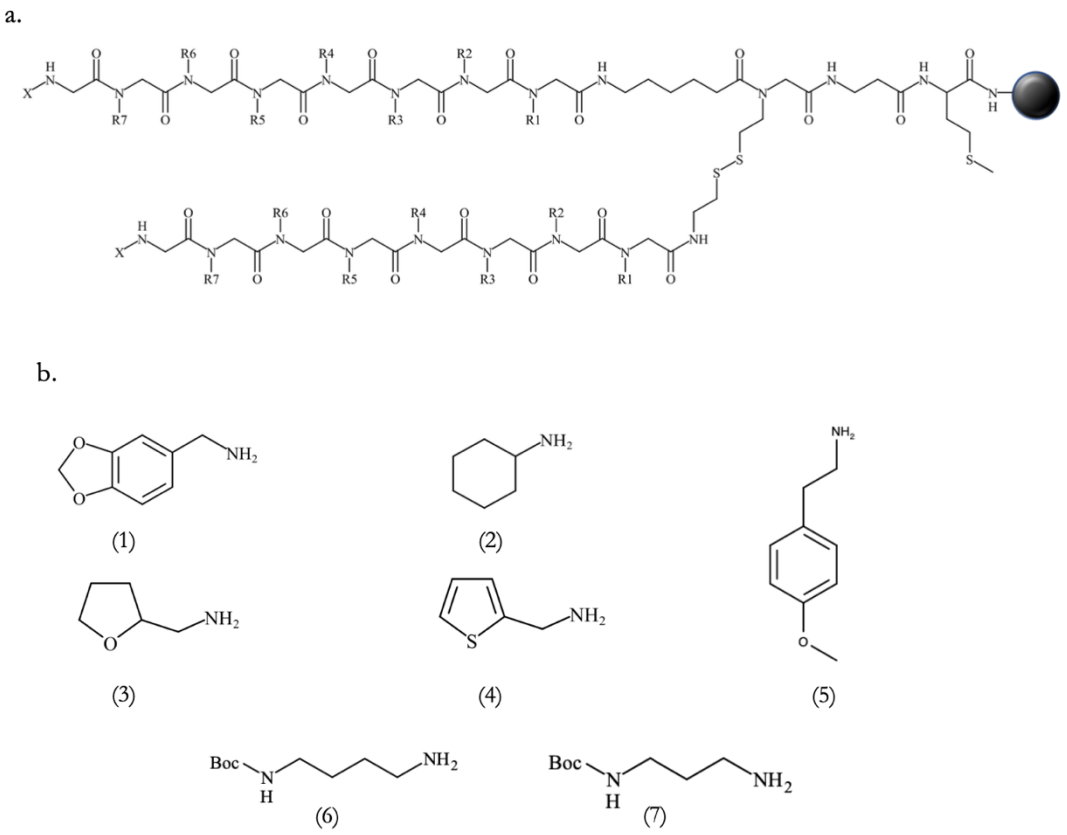


Figure 2.8: Library 001. A completely random combinatorial library with seven different submonomers utilized within seven positions. Theoretical diversity equals 823,543 possible compounds containing aromatic and heterocyclic residues. (1) 3, 4-methylenedioxybenzylamine (2) cyclohexylamine (3) tetrafurfurylamine (4) 2-thiophenemethylamine (5) 4-methoxyphenethylamine (6)N-Boc-1,4-diaminobutane (7) N-Boc-1,3-propanediamine

Library 002

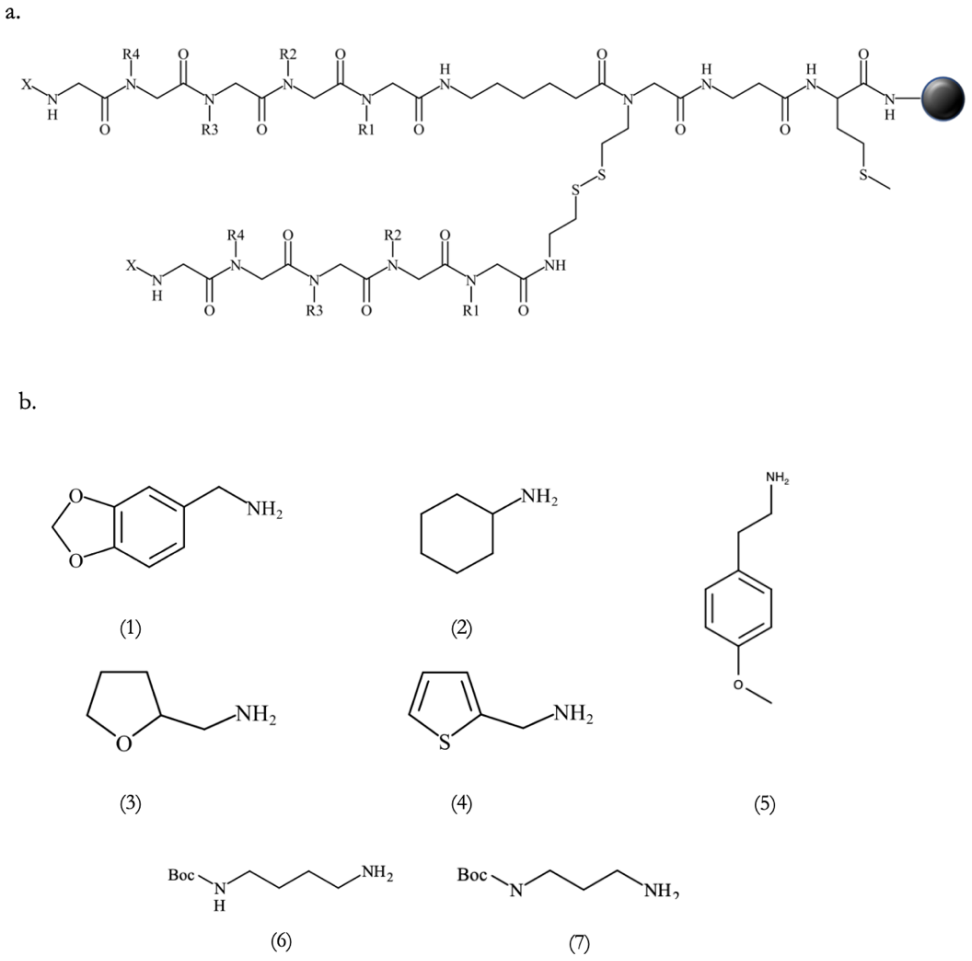


Figure 2.9: Library 002. Theoretical Diversity equals 2,401 possible unique compounds containing aromatic, heterocyclic, and cationic groups. Seven submonomers were incorporated randomly into four different positions (1) 3, 4-methylenedioxybenzylamine (2) cyclohexylamine (3) tetrafurfurylamine (4) 2-thiophenemethylamine (5) 4-methoxyphenethylamine (6) N-Boc-1,4-diaminobutane (7) N-Boc-1,3-propanediamine

Library 003 (Figure 2.10) was constructed based on the design of library JTL_{13}[40] developed by Jeremy A. Turkett in our lab in previous years. Multiple PLAD screenings for JTL_{13} determined a hit rate of 16.7% for *E. faecalis* and 16.6% for *A. baumannii. S. aureus* expressed minimal zone of inhibitions in comparison to the pathogens mentioned previously with a hit rate of 4.2%. [40] Biological activity was not tested for *E. faecium, K. pneumoniae, P. aeruginosa,* or *E. coli* possibly due to challenges presented at structure characterization. Therefore, library 003 was similarly constructed due to the potential outcomes associated with JTL_{13} and the rest of the ESKAPE panel in hopes of more success concerning sequencing difficulties. Submonomers utilized were of similar design by implementing five of the groups found in JTL_{13}, altering one, and eliminating two. The five groups carried over were (1) isopropylamine, (2) isobutylamine, (3) hexylamine, (4) N-Boc-1,4-diaminobutane, and (5) β-phenylethylamine. Furfurylamine was incorporated into JTL_{13}, but was switched out for (6) tetrahydrofurfurylamine while tryptamine and N-Boc-1,2-diaminoethane were left out of the library. As porins allow small, hydrophilic, and/or cationic groups to pass, smaller submonomers were chosen to facilitate this navigation. Theoretical diversity calculates to 1,296 compounds with residue positions extending to four possible submonomers making this one of the shortest lengths tested.

Library 003

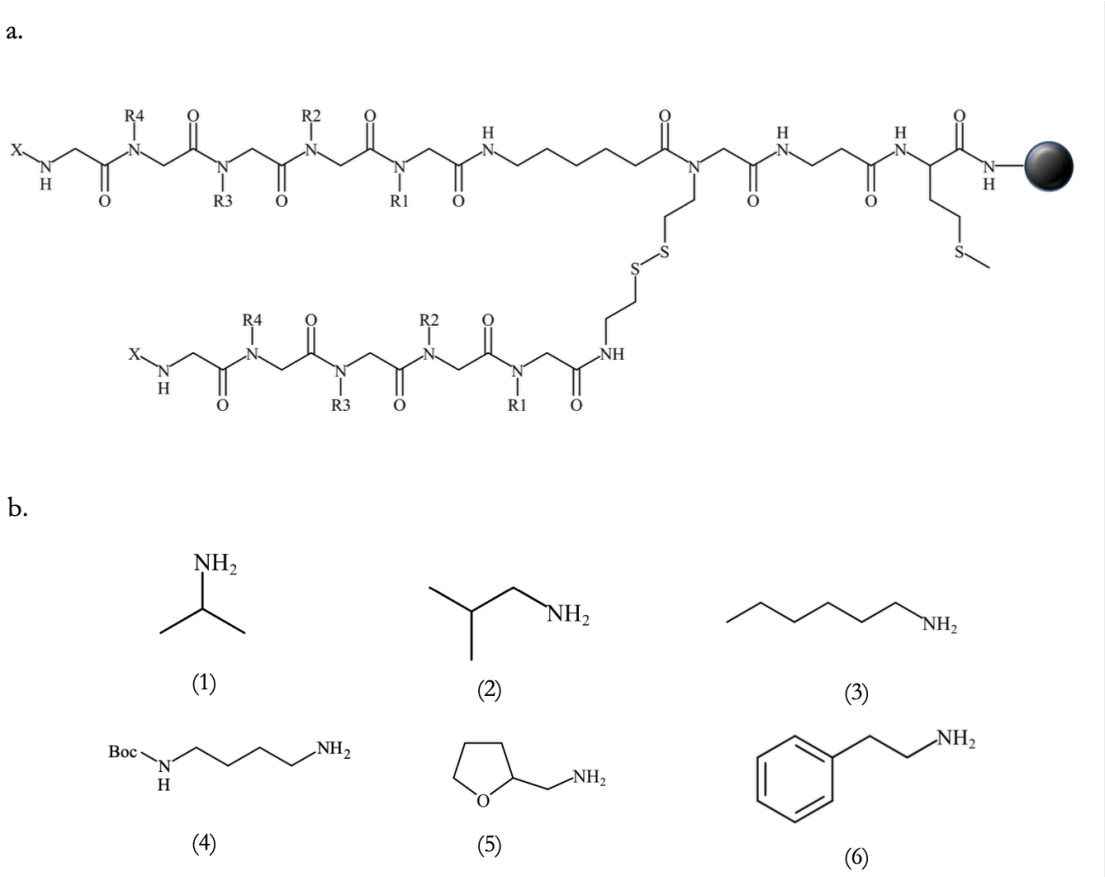


Figure 2.10: Library 003. Combinatorial library randomly incorporated with six different submonomers at four positions equaling a theoretical diversity of 1,296 compounds. (1) isopropylamine (2) isobutylamine (3) hexylamine (4) N-Boc-1,4-diaminobutane (5) tetrahydrofurfurylamine (6) β-phenylethylamine

Bulky aromatic residues were considered to promote selective antibacterial activity. Structure-activity relationship studies have previously demonstrated enhanced activity against *E. coli* and *P. aeruginosa* within a 9-residue sequence. [22] Few implementations, ~25-50% instead of ~70-80%, of bulky residues could balance out the toxicity that is additionally correlated with increased hydrophobicity. This balance could be maintained by an overall increase in charge in relation to bulky aromatic residues. Library 004, containing randomized inclusions of cationic, aromatic, and heterocyclic submonomers, is shorter than other libraries tested with a total of three residues and a tridecylamine tail (Figure 2.11). This completely random combinatorial library focuses on implementing both cationic and hydrophobic groups with a smaller overall size to potentially be more effective towards the Gram-negative bacteria *K. pneumoniae, A. baumannii, P. aeruginosa, and. E. coli.* Theoretical diversity is lower than other libraries prepared at 343 possible compounds but shorter sequences with hydrophobic character might exhibit ease targeting the outer membrane through the lipopolysaccharides extended outward. Another possible benefit from a shorter sequence would be passage through porins in the outer membrane due to small size and cationic charges into the periplasmic space of the pathogen. In fact, a lower percentage of hydrophobic residues in relation to various cationic groups utilized in a peptoid sequence might lead toward targeting the Gram-negative membrane. A specific nonpolar residue, dehydroabietylamine, was selected as a suitable contribution and was expected to provide antibacterial effects. This was the outcome in a study where dehydroabietylamine was determined to be a key component for antibacterial activity. In this specific study, every peptoid that has at least one Dehydroabietylamine group exhibits

some sort of activity towards MRSA strains.[30] Ultimately, this article provided further insight and optimal direction to follow concerning side chain optimization for peptoid combinatorial libraries. Dehydroabietylamine, based on this consistent data, is a promising addition for future antibacterial peptoids. Unfortunately, this data collected is based solely on one strain of bacteria, MRSA. Further analysis with additional strains, specifically Gram-negative, would be beneficial and might offer conclusions concerning dehydroabietylamines antibacterial effects on Gram-positive vs. Gram-negative bacteria.

Library 005 (Figure 2.12) was designed similarly to Library 004 by implementation of the same submonomers 4-methoxyphenethylamine, naphthylamine, dehydroabietylamine, N-Boc-1,3-propanediamine, N-Boc-1,4-diaminobutane, and tetrafurfurylamine. Submonomer 3,4-methylenedioxybenzylamine was not used as a residue option like it was in Library 004, however. Success from library 004 encouraged further testing to see if additional length enhanced or decreased antimicrobial properties as more residue options allows further opportunities for charge expansion. The additional charge could encourage diffusion through the porin portals found within the OM of Gram-negative pathogens. Whereas the highest possible charge distribution is +3 in library 004, it extends to a possible +5 in library 005. Additionally, this allows more opportunity for expansion regarding possible unique compounds where in Library 004 the theoretical diversity was quite small at 343 with 3 submonomer positions. In library 005, the theoretical diversity increases to 7,776 with 5 submonomer positions utilized while still maintaining the amines implemented in library 004 that proved successful.

Library 004

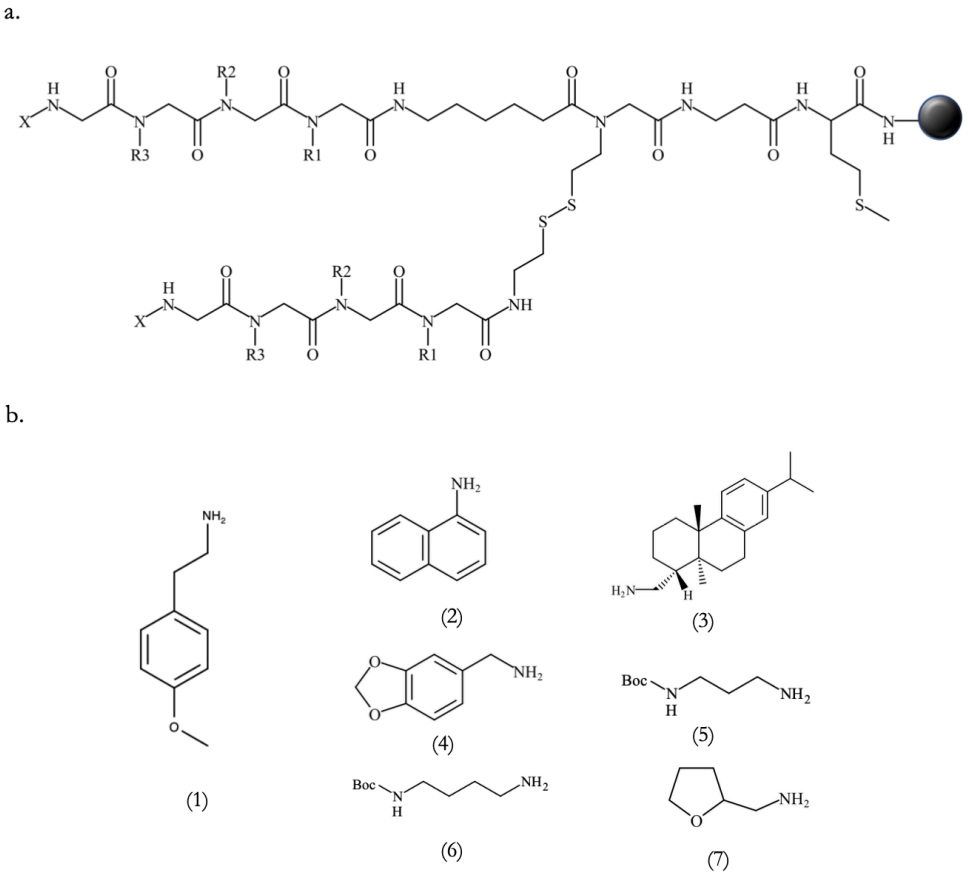


Figure 2.11: Library 004. Random combinatorial library on a smaller scale. Residues extend to three positions with seven possible submonomer incorporations. This brings theoretical diversity to 343 unique possible compounds. (1) 4-methoxyphenethylamine (2) naphthylamine (3) dehydroabietylamine (4) 3,4-methylenedioxybenzylamine (5) N-Boc-1,3-propanediamine (6) N-Boc-1,4-diaminobutane (7) tetrafurfurylamine

Library 005

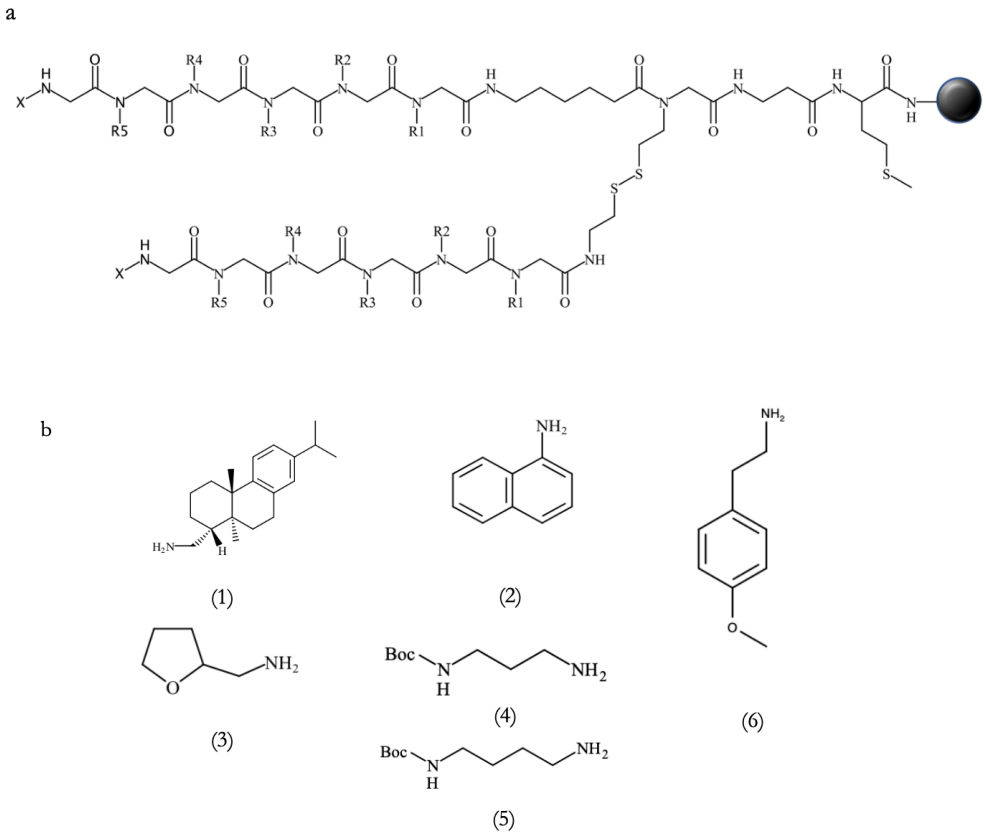
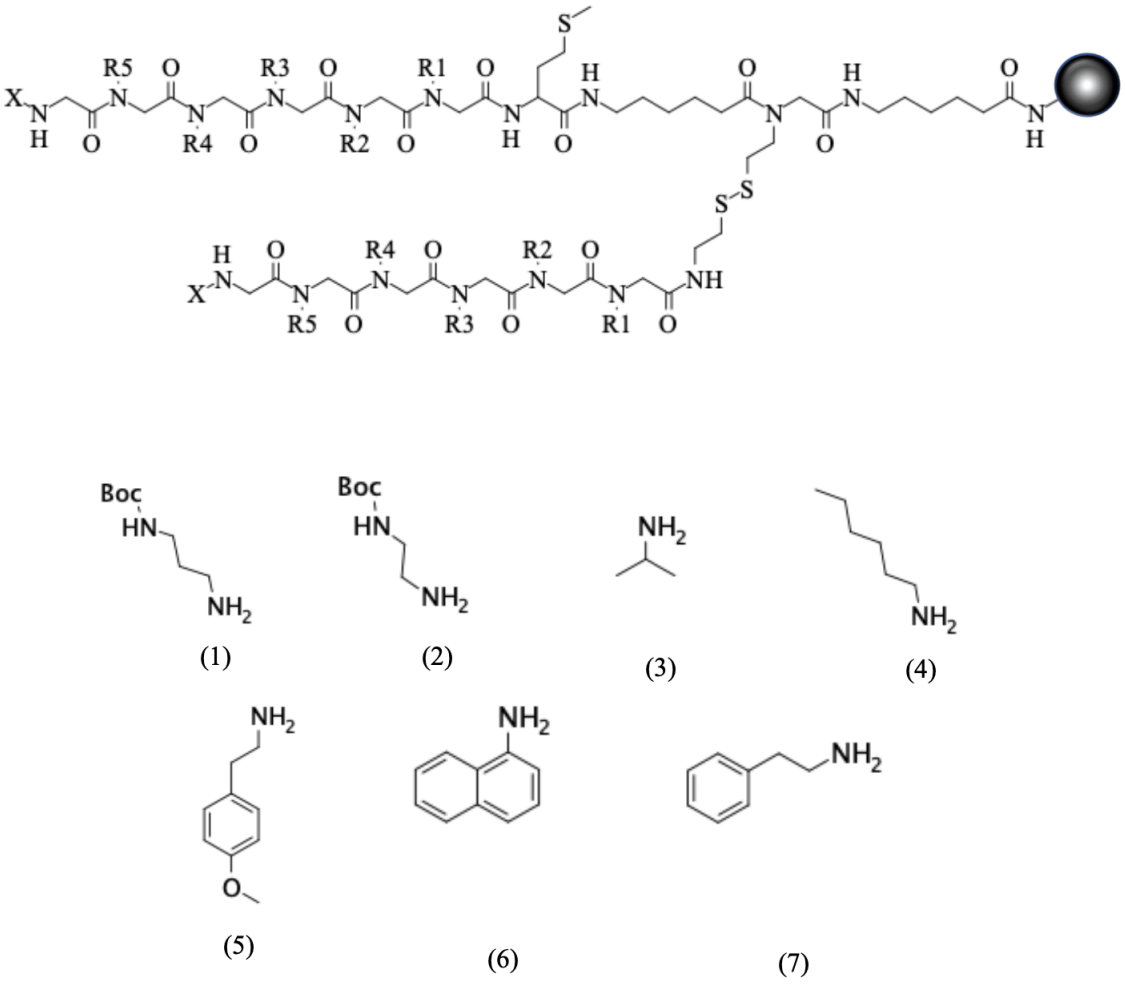


Figure 2.12: Library 005. Random combinatorial library with similar design to library 004. Differences include length extension by two more residue positions and one less submonomer implementation. (1) dehydroabietylamine (2) naphthylamine (3) tetrafurfurylamine (4) N-Boc-1,3-propanediamine (5) N-Boc-1,4-diaminobutane (6) 4-methoxyphenethylamine

Combining the characteristics found in Library 003 and Library 005 led to the development of a new library, labeled Library 006. Three submonomers presumed accountable to promote activity were selected from both libraries and utilized to synthesize five-residue compounds. (1) isopropylamine, (2) hexylamine, and (3) β-phenylethylamine from Library 003 remained as potential side chains while (1) isobutylamine, (2) N-Boc-1,4-diaminobutane, and (3) tetrahydrofurfurylamine were not used. Additionally, (1) naphthylamine, (2) 4-methoxyphenethylamine, and (3) N-Boc-1,3-propanediamine were implemented as inspiration from Library 005 while (1) dehydroabietylamine was removed. A shorter catatonic residue, N-Boc-1,2-diaminoethane, not present in either libraries, was utilized to test efficiency of cationic side chain length. Compounds were synthesized on a modified linker, discussed in section 2.4, in hopes of generating more successful sequencing outcomes.

Library 006



Theoretical Diversity $= 7^5 = 16{,}807$

Figure 2.13: Library 006. Combinatorial library with a theoretical diversity of 16,807. Peptoids are built on a modified linker to determine if sequencing is benefited and reveals more outcomes. (1) = N-Boc-1,3-propanediamine (2) N-Boc-1,2-diaminoethane (3) Isopropylamine (4) hexylamine (5) 4-methoxyphenethylamine (6) Naphthylamine (7) β-phenylethylamine

2.3 PEPTOID LIBRARY AGAR DIFFUSION (PLAD) ASSAY SCREENING OF ESKAPE PATHOGENS:

Libraries 001-006 were synthesized to investigate the structural characteristics that represent a successful design of compounds with antimicrobial activity. Peptoids were constructed based on a variety of features that contribute to interaction with bacterial membranes for permeabilization. Screening each library against the ESKAPE pathogens will give insight into which components derive strong activity aimed at Gram-negative bacteria vs Gram-positive. By utilizing combinatorial libraries in conjunction with the PLAD assay described previously, each library was able to undergo screening against all seven pathogens in a relatively short period of time. Zone of inhibitions are a quick and effective method at determining if a species is susceptible to interactions from certain compounds. The extent of bactericidal efficacy is hard to speculate however, based solely on the size distributions of the zone of inhibitions presented. Zone of inhibition could be indicative of antimicrobial strength or potentially is correlated with diffusion from the bead.

Screening of library 001 and library 002 yielded no results, however, screening of Library 003 indicated minimal biological activity associated with these compounds. Peptoids did display cell selectivity towards certain bacterial species, *E. faecalis, E. faecium,* and *E. coli,* but demonstrated no activity in the other ESKAPE pathogens screened. The activity was theorized to be greater amongst the Gram-negative pathogens due to smaller produced compounds but only produced zone of inhibitions for one of the

four tested. *A. baumannii* had negligible activity (Figure 2.13) in comparison to results obtained for JTL_{13} which might illustrate that tryptamine or furfurylamine are possible candidates that increased biological activity for JTL_{13}. This led to the design of library 004 which consisted highly of bulky aromatic residues.

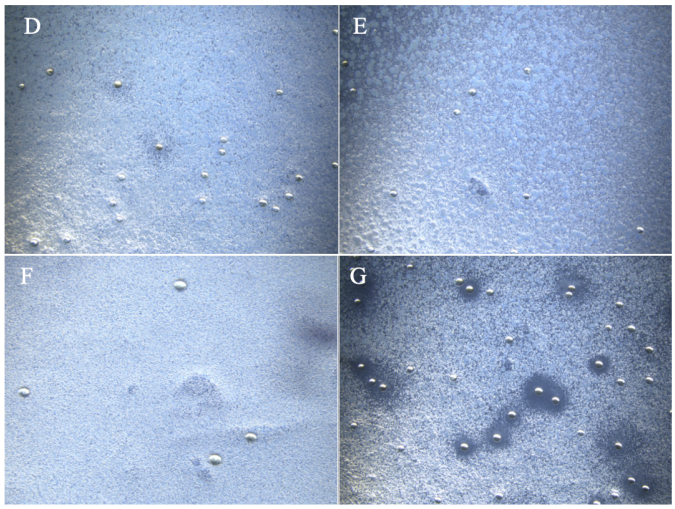


Figure 2.14: "Hits" obtained from Library 003 against the Gram-negative ESKAPE pathogens. *(D)= K. pneumoniae (E) = A. baumannii. (F) = P. aeruginosa (G)= E. coli*

S. aureus continued to express no indication of antimicrobial activity along with *K. pneumoniae,* and *P. aeruginosa. E. coli, E. faecium,* and *E. faecalis* presented multiple zones of inhibition that were selected and carried out for sequencing.

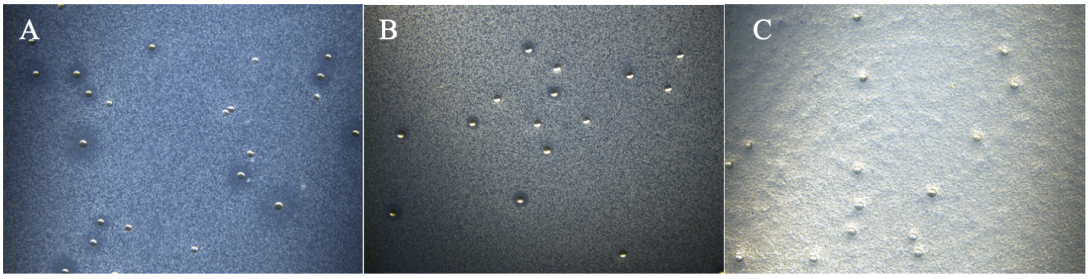


Figure 2.15: "Hits" obtained from Library 003 against the Gram-positive ESKAPE pathogens. (A)= *E. faecalis* (B) =*E. faecium* (C)= *S. aureus*

Library 004 showed significant antibacterial properties in regard to *E. coli*. This combinatorial library identified peptoids with a strong emphasis on Gram-negative pathogens. (Figure 2.16) Quantities ranging from 4.5-6.0 mg of library beads containing unique compounds were incubated separately with one of the ESKAPE pathogens. No visible zone of inhibitions was observed amongst the lawn of *E. faecalis, E. faecium,* nor *S. aureus* (Figure 2.17) which are the top priority Gram-positive pathogens of the ESKAPE family. Forty-one beads with zones of inhibition presented in *E.coli* were extracted and taken to mass spectrometry for sequencing.

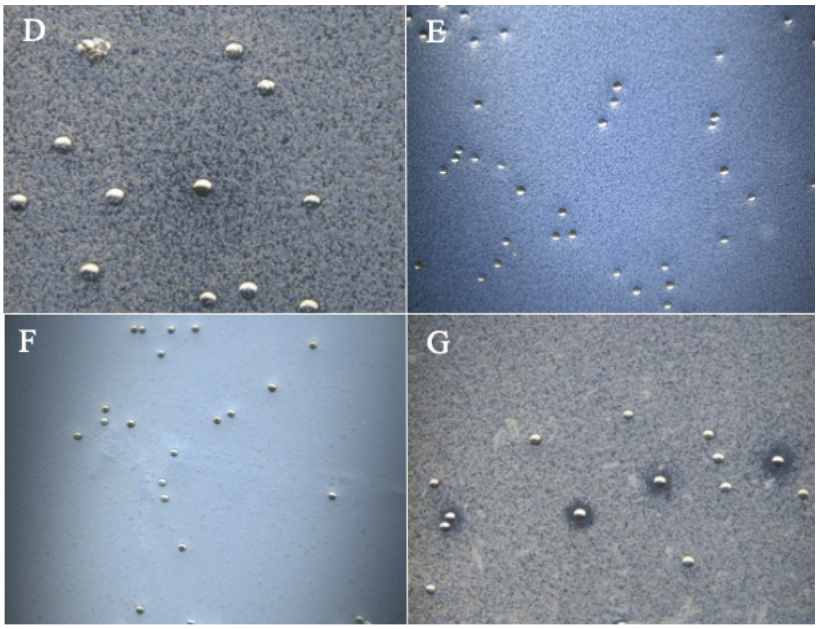


Figure 2.16: "Hits" obtained from Library 004 against the Gram-negative ESKAPE pathogens. *(D)= K. pneumoniae (E) = A. baumannii (F) = P. aeruginosa (G)= E. coli*

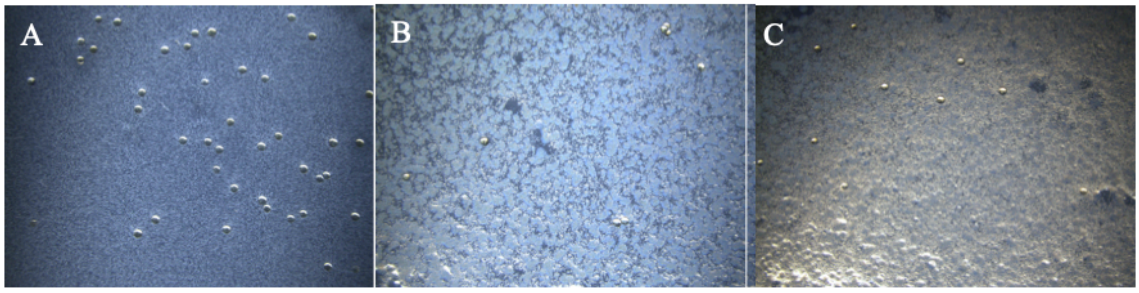


Figure 2.17: "Hits" obtained from Library 004 against the Gram-positive ESKAPE pathogens. (A)= *E. faecalis* (B) =*E. faecium* (C)= *S. aureus*

Escherichia coli *Klebsiella pneumoniae*

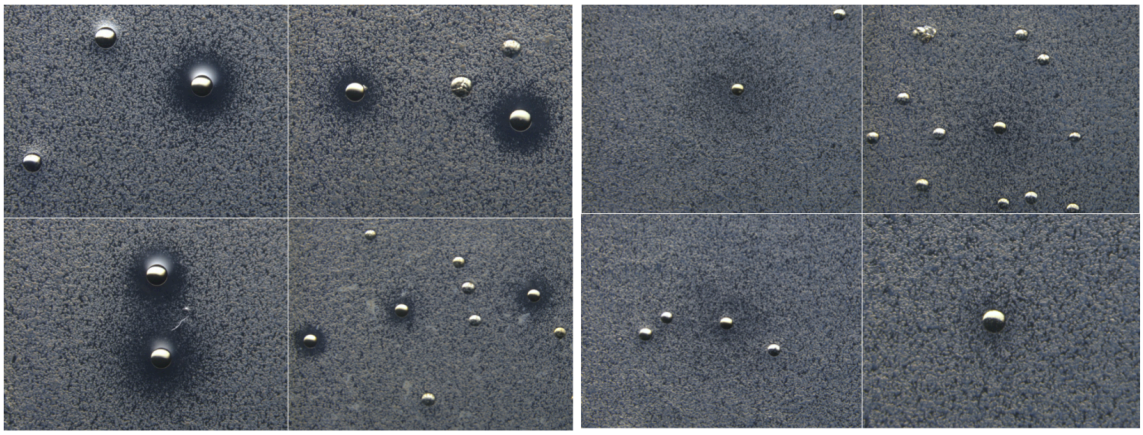


Figure 2.18: Zoomed in *E. coli* and *K. pneumoniae* "hits". Multiple zones of inhibition discovered from Library 004 after overnight incubation. Beads with no zone of inhibition indicate that there are no antibacterial properties present in that compound. Zones of inhibition show that there are antibacterial properties found within that compound and were extracted for sequencing.

Library 005 produced zones of inhibitions for ESKAPE Gram-positive pathogen *E. faecium* (Figure 2.19) but was most effective towards all four of the Gram-negative pathogens *K. pneumoniae, A. baumannii, P. aeruginosa,* and *E. coli* tested. Screening library 005 showed enhanced potential for antibacterial peptoids as more bacterial pathogens were seen to be susceptible towards compounds maintained on the beads. Gram-negatives were especially affected by this library design as every species tested was susceptible to a certain degree whether mildly, moderately, or extremely.

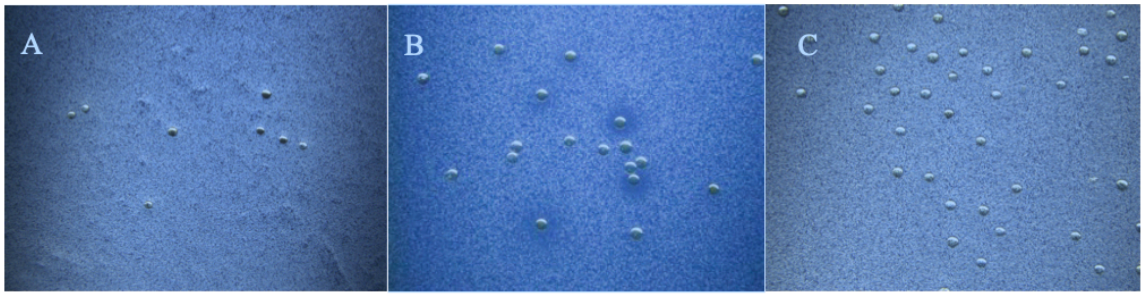


Figure 2.19: "Hits" obtained from Library 005 against the Gram-positive ESKAPE pathogens. (A)= *E. faecalis* (B) =*E. faecium* (C)= *S. aureus*

A. baumannii and *E. coli* exhibited extreme disarray displaying anywhere between 40 – 70% produced zones of inhibition after overnight incubation. *K. pneumoniae* and *P. aeruginosa* expressed mild susceptibility towards library 005 compared to the degree the other Gram-negatives were affected. This is an improvement from library 004 where no zones of inhibition were visible for *A. baumannii* nor for *P. aeruginosa*. Furthermore, while *K. pneumoniae* is still only mildly affected by Library 005, there is noticeable enhanced

susceptibility from what is expressed in Library 004. (Figure 2.20) Factors that differentiate between library 004 and 005 are specifically in relation to the length associated with each combinatorial library where library 005 is extended further by two additional residues. Maintaining a relatively low mass while allowing the overall charge to theoretically increase from a potential (+1 - +3) to a potential (+1 - +5) increases targeting capabilities. This direction was an attempt to selectively target Gram-negative bacteria and surprisingly this seems to be the case as the Gram-positive bacteria tested are scarcely affected by the activity of the peptoids.

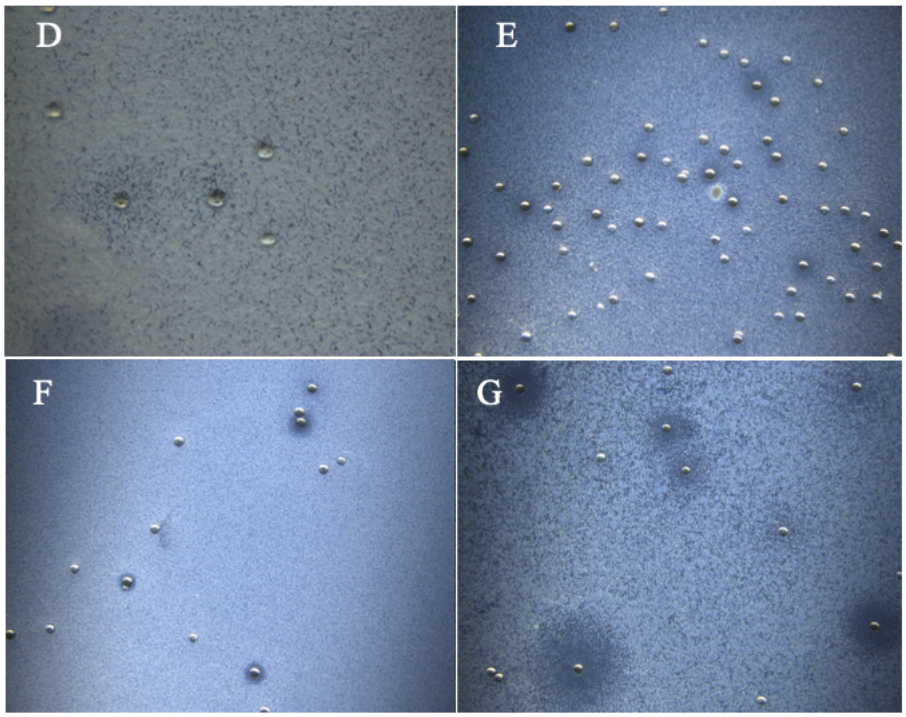


Figure 2.20: "Hits" obtained from Library 005 against the Gram-negative ESKAPE pathogens. *(D)= K. pneumoniae (E) = A. baumannii (F) = P. aeruginosa (G)= E. coli*

Library 006 displayed strong inhibition for various bacterial pathogens, both Gram-positives and Gram-negatives. High quality zones of inhibition were dispersed within the uniform lawn of *E. coli,* numerous hits obtained for *K. pneumoniae,* and mild interactions observed for *P. aeruginosa.* More effectively, library 006 expressed significant biological activity targeted at *E. faecalis* and *E. faecium* with an 85-90% hit rate. This determines that library 006 is more effective towards Gram-positive bacteria.

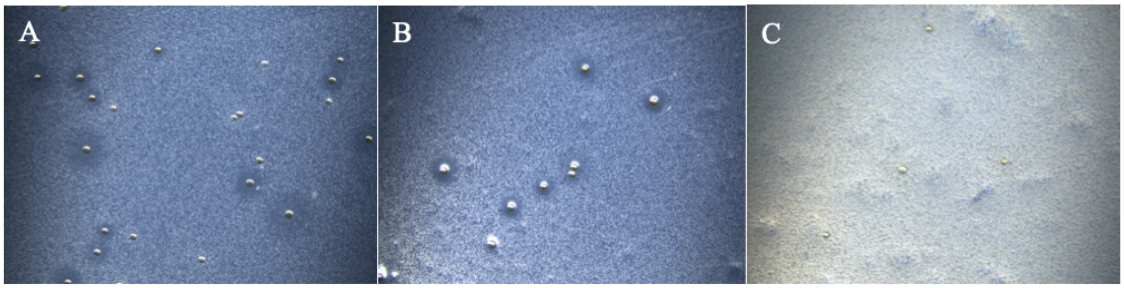


Figure 2.21: "Hits" obtained from Library 006 against the Gram-positive ESKAPE pathogens. (A)= *E. faecalis* (B) =*E. faecium* (C)= *S. aureus*

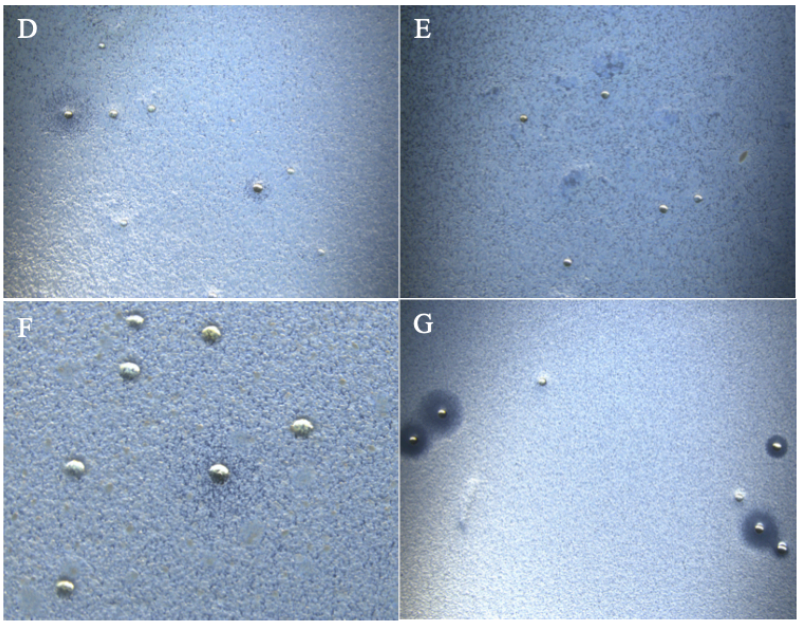


Figure 2.22: "Hits" obtained from Library 006 against the Gram-negative ESKAPE pathogens. *(D)= K. pneumoniae (E) = A. baumannii (F) = P. aeruginosa (G)= E. coli*

Peptoids produced from library 005 demonstrated significant biological activity selective for the Gram-negative pathogens. Enhancement of Gram-negative selectivity is noticeable between library 004 and library 005 who share the same submonomer composition but differ in the length of randomized positions. Difference in length reflects the ability of longer peptoids to exhibit enhanced antimicrobial activity as library 005 was extended by two additional residues synthesizing five-residue compounds. Length alone has the potential to be responsible for increased hit rates directed at Gram-negatives or additional charge could be an important factor in enhancement. Future design will be directed on these key characteristics as increased hydrophobicity has shown to not be effective at targeting the membranes of Gram-negatives. [22] Biological activity was improved when residues were not confined to shorter sequences. The shortest residue peptoids synthesized from Library 004 expressed mild activity against *K. pneumoniae* and displayed large zones of inhibition for only one pathogen tested, *E. coli.* The second shortest library, with four residues, had mild interactions as well, but displayed biological activity against *E. faecalis, E. faecium,* and, *E. coli.* Additionally, bulky aromatic residues show potential contribution towards permeability against Gram-negatives demonstrated by screening outcomes of individual libraries. Library 004 and 005 were designed with bulky aromatics and exhibited zones of inhibition selectively towards these bacterium. Library 006 was theorized to exhibit broad-spectrum biological activity with hit rates decreasing for Gram-negative pathogens and increasing for Gram-positives based on moderately included bulky aromatics. In response the degree of Gram-negative selectivity minimized from hits against all four Gram-negatives to two significantly impacted and one mildly effective in addition to Gram-positive efficacy slightly enhanced. While library 004 had

no interactions and library 005 had activity towards *E. faecium*, library 006 had strong activity against both *E. faecalis* and *E. faecium.* Furthermore, library 003 had a moderate amount of bulky aromatic submonomers and was consistent with the zone of inhibition pattern described above. Interactions with Gram-negatives decreased against *K. pneumoniae* and *E.coli* with mild interactions towards *P. aeruginosa* in library 006 to mild interactions with *K. pneumonia* and strong activity against *E. coli* in library 003. Gram-positive interactions remained consistent between library 003 and 006.

2.4 MASS SPECTROMETRY CLEAVAGE OPTIMIZATION:

Design, synthesis, and screening of combinatorial libraries has achieved exploration of large diverse collections of unknown potential lead compounds. However, this proves to not be enough. While synthesis of small molecules is an essential aspect associated with drug discovery, it has little value if hit identification is unachievable. Sequencing hits for structure characterization proved to be the most difficult aspect regarding further study of antibacterial properties. Determining the structure of the α-strand mass proved to be challenging when sequenced through tandem mass spectrometry often presenting limitations on peptoid library design. This frequently inhibits further progress as multiple promising "hits" can be discovered but are lost when attempts are made to sequence the structure of the peptoid. The unique compound that showed antibacterial activity is attached to a single bead, therefore making it difficult to determine the structure due to low concentration and small quantities of resuspension solvent. If no structure is determined, then that potential antimicrobial peptoid cannot be resynthesized and retested. Running mass spectrometry with minuscule volumes is difficult, however adding solvent would further dilute the concentration, which is already minimal due to single-bead analysis. Therefore, all aspects of the cleavage process were studied for optimization by testing various conditions and solvent changes.

Cyanogen bromide (CNBr) is an inorganic compound capable of cleaving peptides at methionine residues making fragmented sections possible or in this case cleavage of an entire peptoid from the resin it was designed on. A nucleophilic substitution takes place

with the addition of CNBr (Figure 2.23). The nucleophilic sulfur in methionine attacks the carbon leading to bromine displacement by sulfur. Subsequently, a five-membered ring is formed leading to a homoserine lactone as a result of cleavage with CNBr.

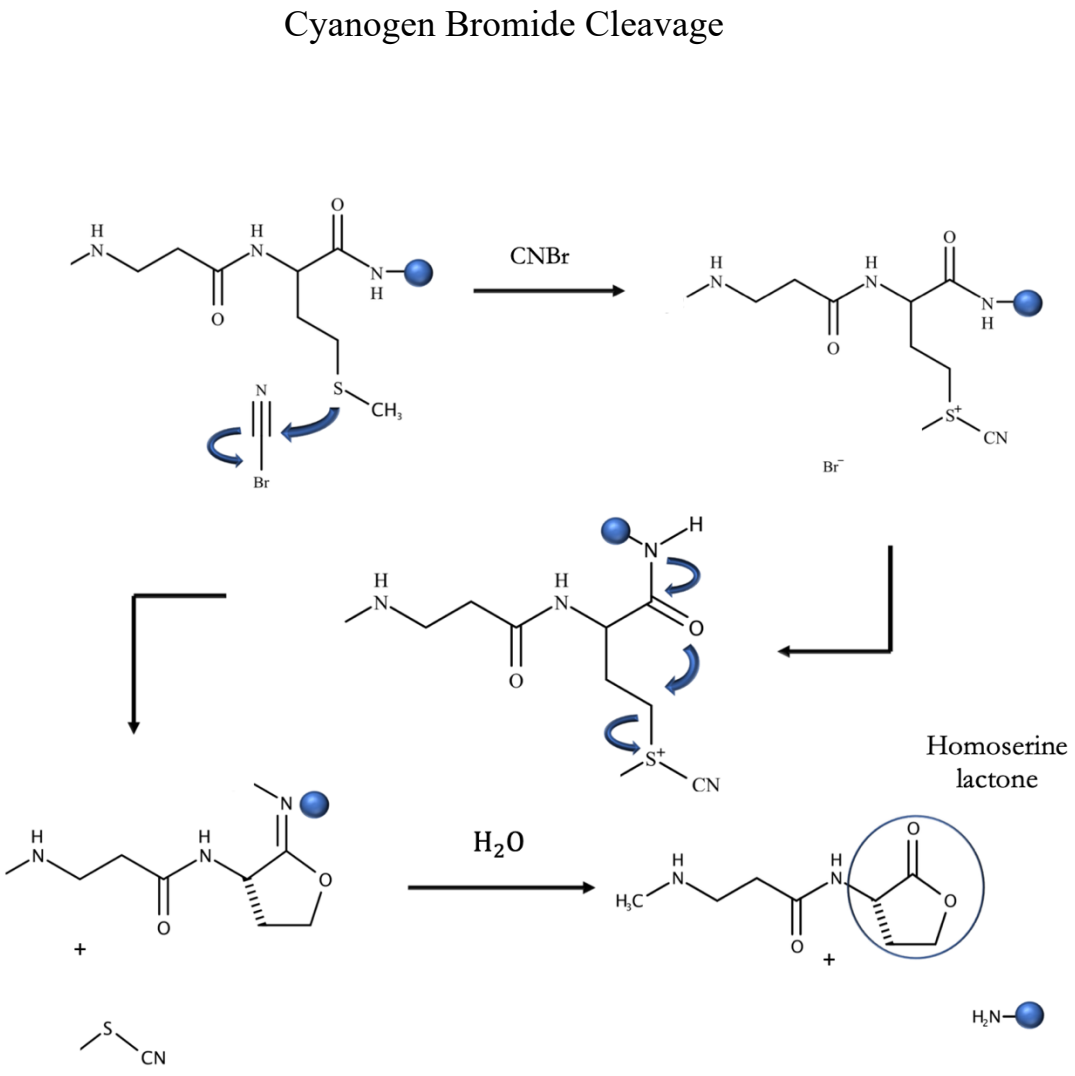


Figure 2.23: Cyanogen Bromide cleavage from methionine residue. CNBr cleaves methionine from the resin to release the α-strand in preparation for mass spectrometry. The nucleophilic sulfur attacks the electrophilic carbon eventually leading to a homoserine lactone, seen above.

A possibility leading to lack of success and consistency during CNBr cleavage might be due to the inability of the peptoid to diffuse from the resin and instead remaining inside the bead. Additionally, the cleavage cocktail used to cleave the compound from the bead might produce unwanted degradation if left in solution for an extended period of time. Alternate CNBr solutions, resuspension solvents, and incubation times to improve CNBr cleavage and resuspension of peptoids were tested. A test peptoid (Figure 2.24) was synthesized and cleaved off the resin under multiple CNBr conditions to determine the optimal conditions to achieve high success rates for sequencing single beads. The α-strand has a relatively low mass at 915.12 g/mol as the four-residue extension consists of smaller amines furfurylamine, methoxyethylamine, benzylamine, and isobutylamine.

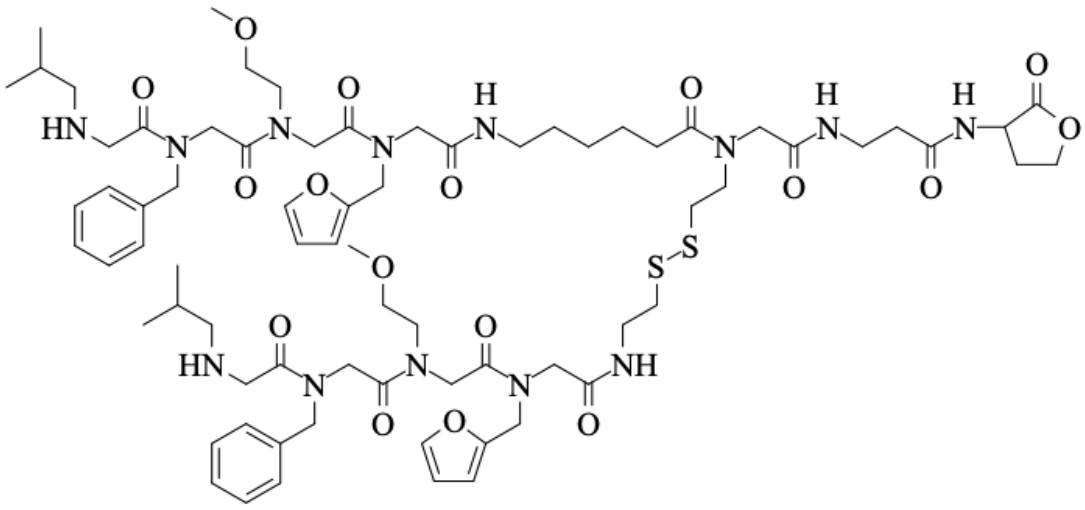


Figure 2.24: Peptoid with four Submonomers. Test peptoid used for CNBr cleavage condition testing. Molecular weight whole sequence = 1502.85 g/mol α-strand = 915.12 g/mol β-strand = 589.75 g/mol

MS analysis has shown to be more successful when a large quantity of beads are prepared for analysis, compared to success rates for single bead analysis, due to heightened concentration. However, this only shows that the resuspension solvent ratio is sufficient and gives little insight into single bead analysis. A ratio of 80:20 acetonitrile/water in 0.05% trifluoroacetic acid (TFA) produces accurate results when analyzing groups of beads and occasionally when single bead analysis is done. However, single bead determination is not always successful. Successful single bead analysis, however, has been more consistent with a more hydrophobic 80:20 acetonitrile/water solution than was observed with a previously used 50:50 with 0.05% TFA ratio.

Conditions were varied to encourage diffusion of potential lead compounds from beads. Temperature adjustments were evaluated to determine if room temperature or an elevated temperature of 47°C would assist in diffusion. Additional time for diffusion of compounds into resuspension solvent was tested. Furthermore, the addition of agitation to assist in diffusion was analyzed. Single bead analysis was conducted with CNBr cleavage in 5:4:1 acetonitrile/acetic acid/water (ACN/AA/H_2O) instead of 0.1 M HCl in H_2O, previously used, with A1-A5 and resuspended in 80:20 acetonitrile/water in 0.05% TFA succeeding in 4 out of 5 attempts (Table 2.1) . This further emphasized the importance of the acetonitrile/water ratio and indicated that composition of the resuspension solvent was more important in obtaining sequencing results than agitation or elevated temperature. A1'-A4' (Table 2.1) assessed if extended time spent in solvent would increase efficiency.

Twenty minutes proved successful while surprisingly 1.5 hours and >23 hours did not produce any sufficient sequencing data.

	Conditions	Success Determination
A1	Rocked 20 mins. in 50:50	Yes
A2	Rocked 20 mins. in 80:20	Yes
A3	In 80:20	Yes
A4	47° C in 50:50	No
A5	47° C in 80:20	Yes
A1’	Rocked 1 ½ hrs. in 80:20	No
A2’	Rocked 1 ½ hrs. in 80:20	No
A3’	Rocked 20 mins. in 80:20	Yes
A4’	Rocked 23 hrs. in 80:20	No

Table 2.1: CNBr cleavage conditions. Conditions demonstrating that cleavage with CNBr in 5:4:1 acetonitrile/acetic acid/water resuspended in 80:20 acetonitrile/water in 0.05% TFA prove most optimal.

Reproducibility was tested to confirm cleavage conditions determined optimal for single bead analysis. A1-A10 consisted of single beads cleaved in 5:4:1 ACN:AA: H_2O and Q1-7 were cleaved in 0.1 M HCl in H_2O. Both were resuspended in 80:20 acetonitrile/water in 0.05% TFA for 20 minutes after the solution was evaporated. Results proved not to be reproducible as only 2 out of the 7 single beads tested for group A were analyzable matching the success rate of group Q. (Table 2.2).

Often times, sequencing proves to be the most challenging step of identifying antibacterial peptoids using the PLAD assay. Modifying the linker could attribute to more successful outcomes in terms of sequencing hits by tandem mass spectrometry. If a

structural adjustment to the linker can overcome previous limitations in peptoid sequencing, then higher rates of hit identification might be an outcome. A redesigned linker must exhibit (1) stability towards both synthesis and screening conditions, (2) facilitate synthesis

	Success Determination		Success Determination
A1	No	Q1	No
A2	No	Q2	No
A3	Yes	Q3	Yes
A4	No	Q4	No
A5	No	Q5	No
A6	Yes	Q6	No
A7	No	Q7	Yes

Table 2.2: CNBr Cleavage Reproducibility. A1-A7 cleaved in 5:4:1 acetonitrile/water/water produced 2 successes out of 7. Q1-Q7 cleaved in .1 M HCl in water produced 2 successes out of 7.

of two identical strands, (3) allow side chain deprotection of protecting groups without cleaving from the resin, and (4) be cleaved from the bead after screening and before sequencing. The current design constitutes implementation of Fmoc-Met-OH first to the resin as a cleavage point to remove the peptoid from the resin before undergoing sequencing. β-alanine is then introduced to ensure proper space is provided between the linker and the resin before coupling Boc-cystamine. Finally, Fmoc-6-aminohexanoic acid is coupled to provide space between the two identical strands built during synthesis. This

leaves an additional mass of 402 that must be considered when performing mass spectrometry sequencing of hits. As overall mass increases, sequencing efficiency usually decreases. Therefore, any unnecessary mass can be excluded to encourage sequencing success. A modified linker (Figure 2.25) would maintain current chemical cleavage conditions but would result in cleavage at a different position. Fmoc-6 aminohexanoic acid would be coupled first, followed by Boc cystamine, and then another Fmoc-6 aminohexanoic acid before attaching Fmoc-methionine. This allows cleavage to occur directly ahead of the randomized compound sequence rather than cleavage directly from the bead, significantly minimizing hit mass. (Figure 2.26).

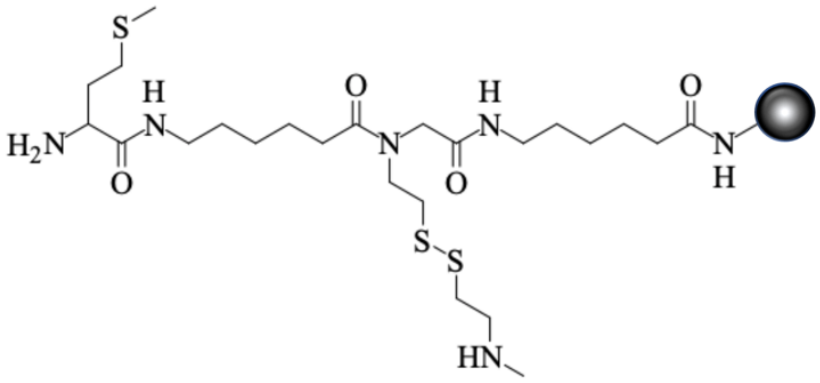


Figure 2.25: Modified Linker. Fmoc- 6 aminohexanoic acid coupled first, followed by Boc cystamine, an additional Fmoc-6 aminohexanoic acid, and lastly Fmoc-methionine.

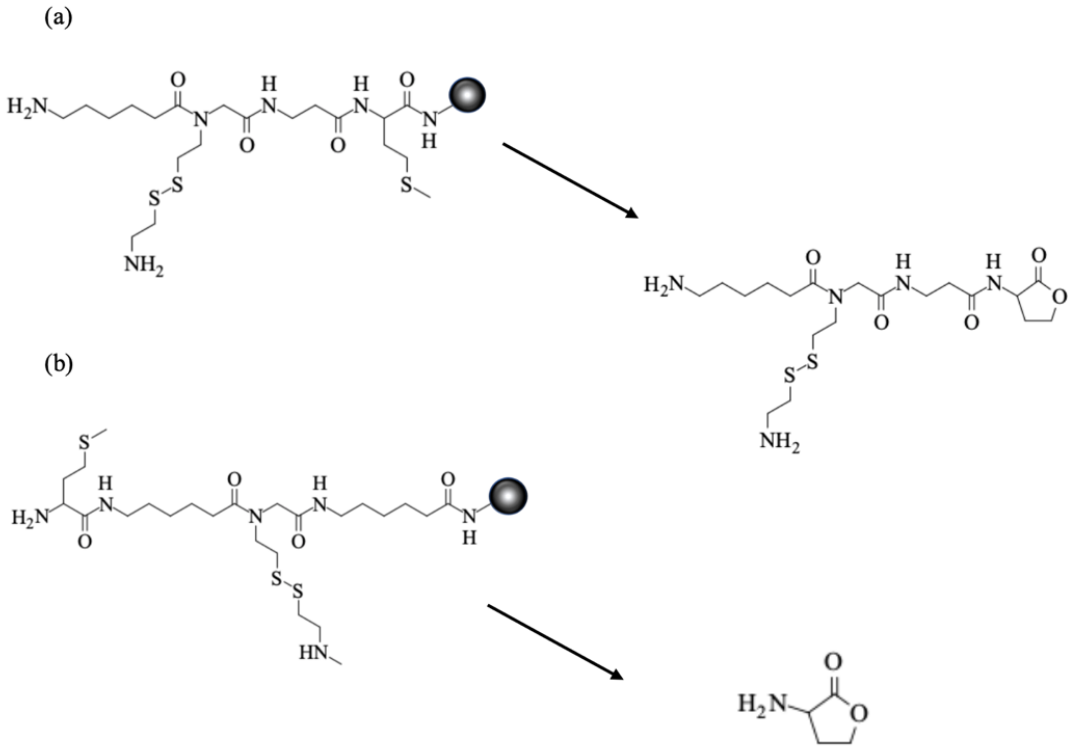


Figure 2.26: Methionine Cleavage. (a) The original linker cleaved directly from the bead. (b) Modified linker cleaved directly ahead of the sequence to be determined.

2.5 SEQUENCING OF LIBRARY "HITS"

Qualitative data of each hit collected must be determined following PLAD screening as the compound with antimicrobial activity has been identified but the sequence is unknown. Tandem mass spectrometry allows structural study to occur through ionization and fragmentation of peptoids. Compounds are ionized, in the ionization source, with either positive or negative charges before traveling further through the analyzer and fragmented at different intervals[5] .Fragmentation occurs at the amide bond providing predictable b-ions and y-ions allowing structure determination as indicated below (Figure 2.27).

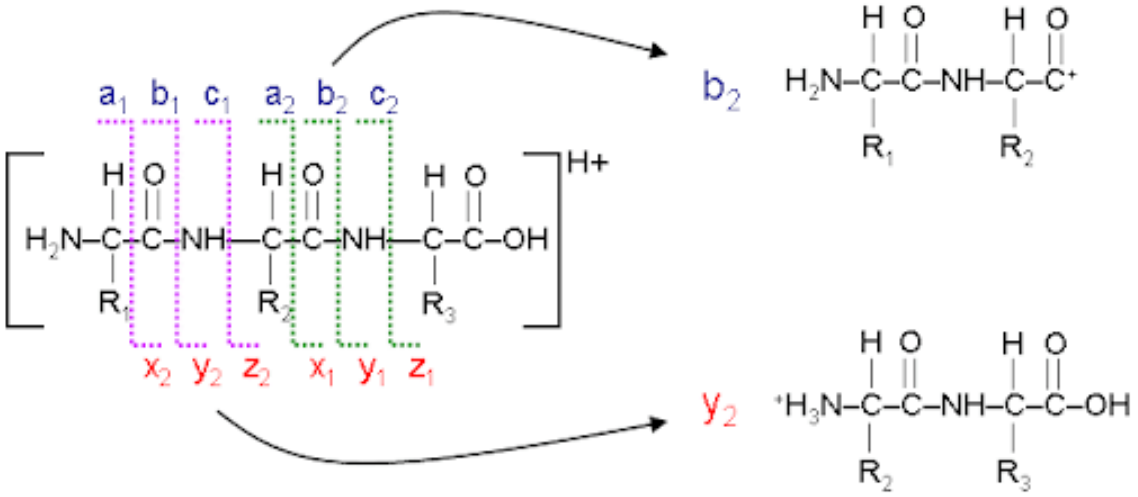


Figure 2.27 Sequencing.[23] Example of peptide sequencing that occurs in the same nature for peptoids. Residues along the backbone are cleaved at intervals revealing a y-ion (C to N) and a b-ion (N to C). Mass differences between peaks will allow structural determination.

Figure 2.28. Tandem Mass Spectrometry of ALA1.

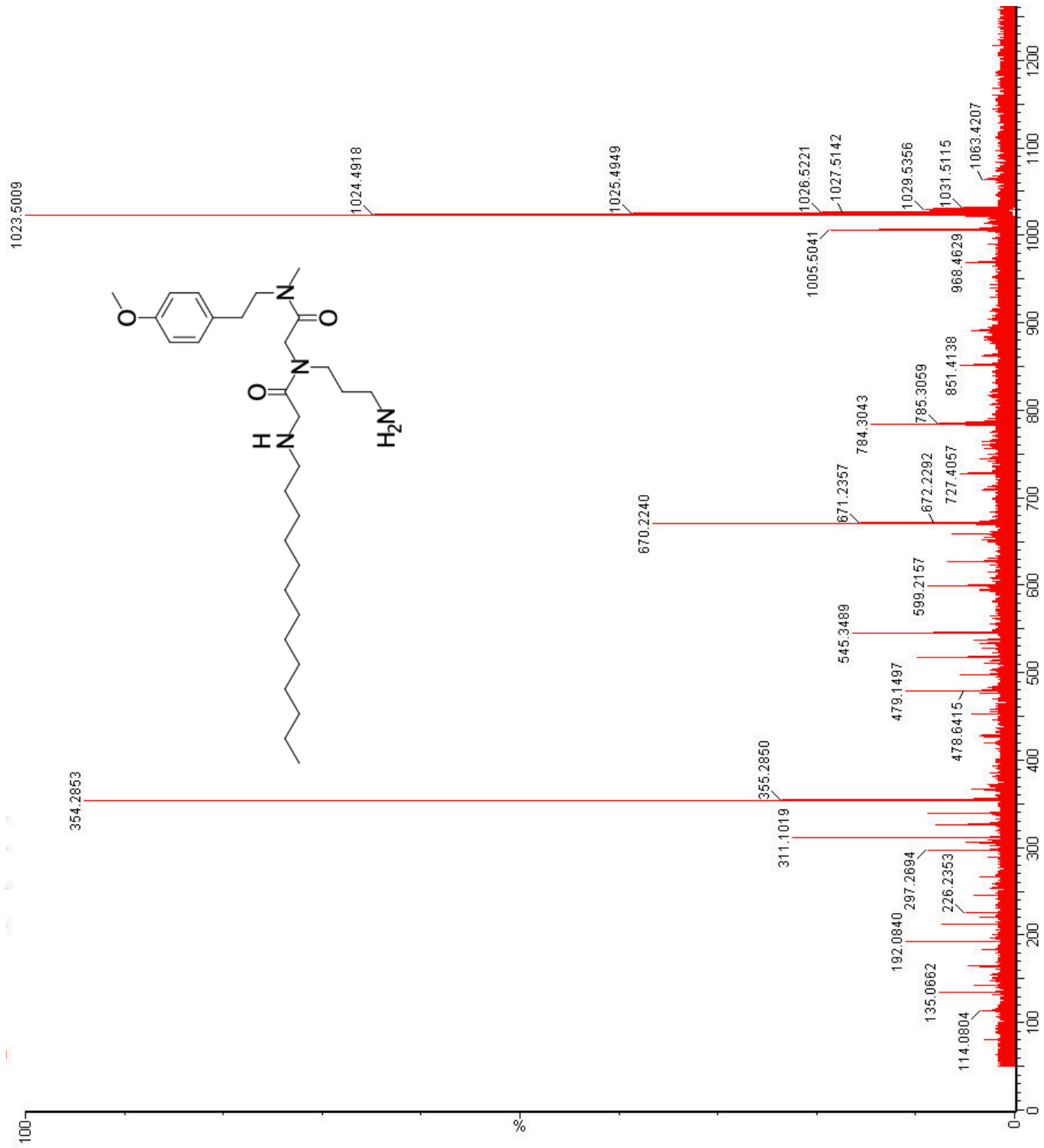


Figure 2.29. Tandem Mass Spectrometry of ALA2

Side chain length concerning cationic residues were explored to determine which length is optimal for biological activity. N_{ae} ,N_{pro} and N_{lys} were employed into libraries and abundance of cationic residues discovered in antimicrobial peptoids were noted. Sequences analyzed from library 004 consistently contained residues of shorter nature. In total, four sequences were detected, and it was concluded that none displayed a N_{lys} cationic residue in the backbone of their compound while N_{ae} has yet to be identified.

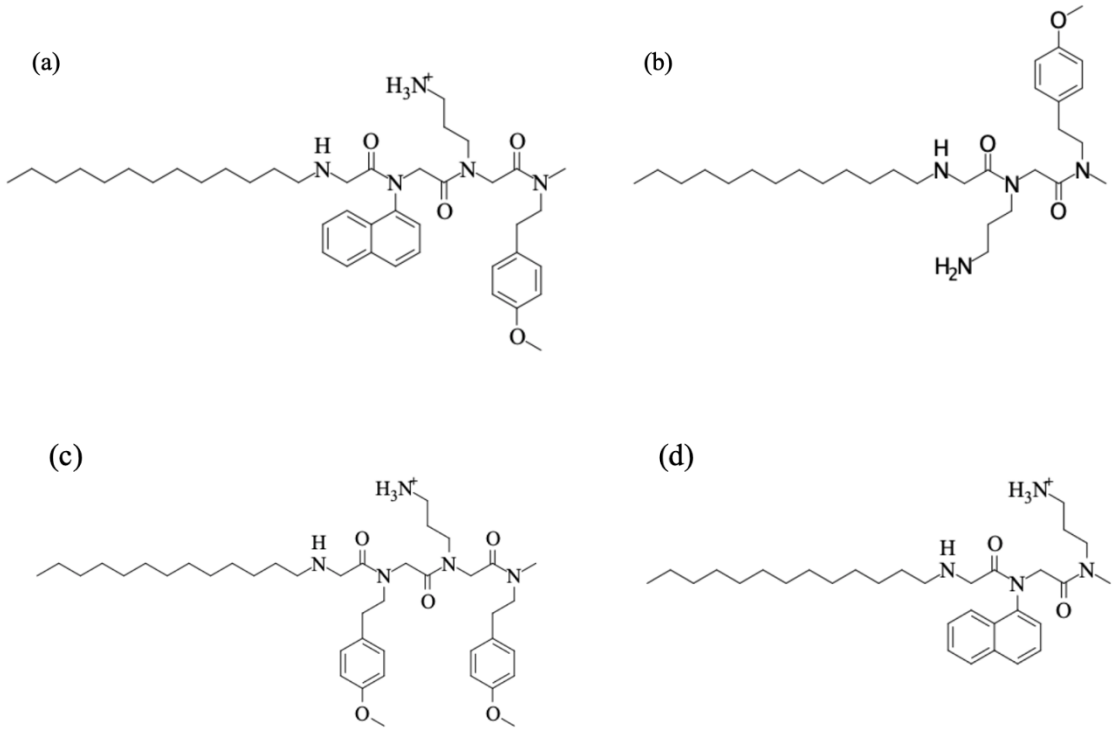


Figure 2.30: Sequences obtained from Library 004 Four sequences analyzed from single bead analysis. (a) ALA1 and (b) ALA2 used for further study (c) ALA3 and (d) ALA4 that were not selected for further study at this time.

Antimicrobial peptoids produced were labeled ALA1 ($N_{nap}N_{ap}N_{mpa}$), ALA2 ($N_{ap}N_{mpa}$), ALA3 ($N_{mpa}N_{pro}N_{mpa}$), and ALA4 ($N_{nap}N_{ap}$), all exhibiting similar structures (Figure 2.30).

Hits collected were analyzed and 18 potential compounds were properly sequenced (Table 2.3) out of 41 single beads tested. This equal outs to a 43.9% success rate. Compounds identified were consistent in nature with 5 single bead analyses maintaining ALA1's sequence, 5 maintaining ALA2's sequence, and 4 exhibiting both ALA3 and ALA4's sequence for a total of 18 confirmed sequences but 4 potential antimicrobial peptoids.

Lib 004 Hit	Zone of Inhibitions	Compound Identified
4 ▪ 1	0.965 nm	-
4 ▪ 2	0.827 nm	ALA1
4 ▪ 3	1.061 nm	ALA2
4 ▪ 4	1.009 nm	ALA3
4 ▪ 5	0.551 nm	-
4 ▪ 6	0.772 nm	ALA3
4 ▪ 7	0.839 nm	ALA1
4 ▪ 8	1.041 nm	ALA4
4 ▪ 9	0.935 nm	ALA4
4 ▪ 10	0.815 nm	-
4 ▪ 11	1.215 nm	ALA2
4 ▪ 12	0.693 nm	-
4 ▪ 13	0.768 nm	-
4 ▪ 14	0.674 nm	-
4 ▪ 15	1.187 nm	ALA2
4 ▪ 16	0.923 nm	ALA2
4 ▪ 17	0.690 nm	ALA4
4 ▪ 18	0.897 nm	ALA3
4 ▪ 19	0.720 nm	-

4 · 20	0.543 nm	ALA1
4 · 21	0.837 nm	ALA4
4 · 22	0.787 nm	ALA2
4 · 23	0.672 nm	ALA1
4 · 24	0.697 nm	-
4 · 25	0.726 nm	-
4 · 26	0.625 nm	-
4 · 27	0.553 nm	-
4 · 28	0.653 nm	-
4 · 29	0.574 nm	-
4 · 30	0.726 nm	-
4 · 35	0.957 nm	-
4 · 36	0.981 nm	ALA1
4 · 37	0.993 nm	-
4 · 38	0.695 nm	-
4 · 39	0.629 nm	-
4 · 40	0.799 nm	-
4 · 41	0.895 nm	-
4 · 42	0.754 nm	-
4 · 44	0.833 nm	ALA3
4 · 45	1.057 nm	-
4 · 46	0.812 nm	-

Table 2.3: Library 004 Hit Confirmations. 43.9% success rate for cleaving conditions. CNBr was cleaved in 5:4:1 ACN/AA/H_2O overnight and resuspended in

All four sequences obtained contain the three same submonomers, (1) 4-methoxyphenethylamine, (2) N-Boc-1,3-propanediamine, and (3) naphthylamine, in a variety of positions. The other four submonomers implemented were not identified in any compounds determined demonstrating high importance for (1), (2), and (3). Two of the four sequences were selected for further study, one of the three-residue compounds (ALA1) and one of the two-residue compounds (ALA2).

2.6 ZONE OF INHIBITION AND MINIMUM INHIBITORY CONCENTRATION STUDY

The zone of inhibition diameter could correlate with antimicrobial efficacy or compound diffusion properties. “Hits” collected from library 004 had varying zone of inhibition sizes but all exhibited some indication of antimicrobial efficiency. *K. pneumoniae* had slight zones of inhibition that displayed a less dense lawn of pathogen in the vicinity of the bead but not a complete clearance of growth. (Figure 2.31) Sequences were unable to be determined from the hits gathered from the lawn of *K. pneumoniae* preventing specific determination of minimum inhibitory concentration (MIC) values. The theoretical diversity for library 004 was 343 unique compounds which would suggest overlap with sequences obtained from other hits collected. In support of this theory, two of the four sequences obtained for *E. coli* were identified from hits four times each while the other two sequences were identified a total of five times each indicating the reproducible antibacterial activity of these peptoids. If this proved to be true, then the MIC values for the two sequences tested should be of higher value in respect to *K. pneumoniae* than would be observed for *E. coli* if zone of inhibition size correlates with efficacy. This would be consistent with descriptions of zones of inhibition that present with large and almost complete clearance of pathogen, as seen for *E. coli,* having lower MIC values than zones with minimal clearance observed, as seen for *K. pneumoniae.* Stronger antimicrobial activity, in theory, would suggest more success at inhibiting bacterial growth and would thus require a lower concentration to induce this effect.

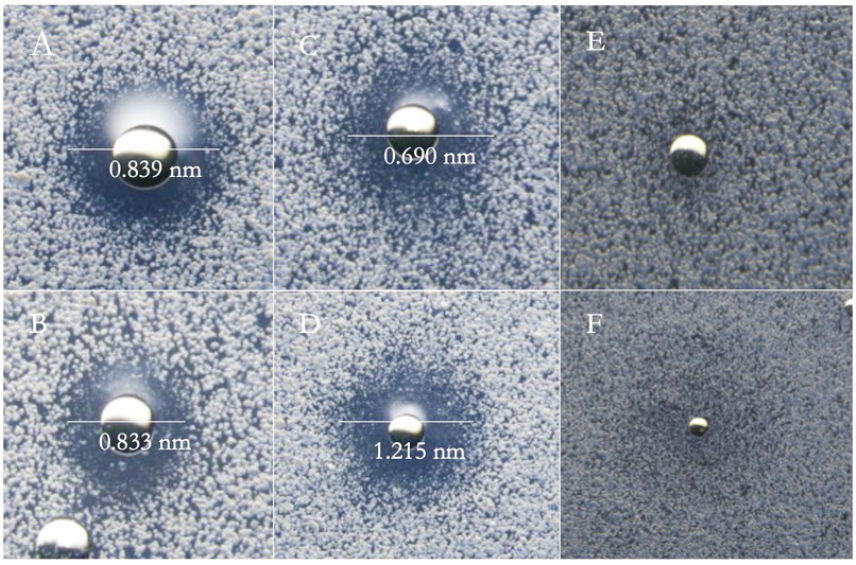


Figure 2.31: Measured zone of inhibitions for Library 004. (A) Zone of inhibition produced from ALA1 in a lawn of *E. coli.* (B) ALA3 produced zone of inhibition in *E. coli.* (C) ALA4 produced zone of inhibition in *E. coli.*(D) ALA2 with a large zone of inhibition in a lawn of *E. coli.* (E) Lawn of *K. pneumoniae* with mild zone of inhibition where the sequence was unable to be sequenced. (F) Lawn of *K. pneumoniae* with mild zone of inhibition where the sequence was unable to be sequenced.

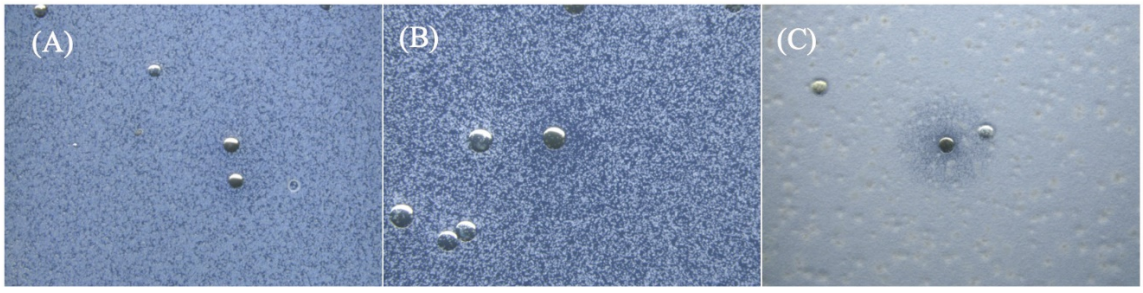


Figure 2.32: Quality of zone of inhibitions observed. "Hits" pulled for pathogens tested with various degrees of zone of inhibition clearance. (A) = *A. baumannii* (B) = *E. faecalis* (C) = *P. aeruginosa*

Minimum Inhibitory Concentration, defined as the lowest concentration required to inhibit growth of a microorganism, was tested for ALA1 and ALA2. ALA1 achieved better activity with lowered MIC values in five out of the seven pathogens tested. The compound displayed selectivity towards one Gram-negative with an MIC of 12.5 μg/mL for *E. coli* but ranged from 25-50 μg/mL against the other Gram-negative bacteria. The MIC, however, was lowered significantly from values obtained from compound ALA2 dropping from 100 μg/mL to 25 μg/mL for *A. baumannii* and from > 100 μg/mL to 50 μg/mL for *K. pneumoniae.* ALA2 demonstrated little antimicrobial activity towards the Gram-negatives tested with MIC values against *K. pneumoniae* of >100 μg/mL, *A. baumannii* of 100 μg/mL, and 50 μg/mL for both *P. aeruginosa* and *E. coli* (Table 2.4). This pattern could suggest antimicrobial strength is not responsible for zone of inhibition diameter as ALA2 exhibited larger zone of inhibition diameters but higher MIC values were obtained.

Minimum Inhibitory Concentration (μg/mL)		
	Sequence	
Pathogens Tested	ALA1	ALA2
E. faecalis	12.5	25
E. faecium	25	25
S. aureus	6.25	25
K. pneumoniae	50	>100
A. baumannii	25	100
P. aeruginosa	50	50
E. coli	12.5	50

Table 2.4: MIC Values. ALA 1 and ALA2 MIC values determined for the ESKAPE panel.

Zone of Inhibition Measurements (nm)		
	Sequence	
Labeled "Hits"	ALA1	ALA2
4 • 2	0.827	
4 • 3		1.061
4 • 7	0.839	
4 • 11		1.215
4 • 15		1.187
4 • 16		0.923
4 • 20	0.543	
4 • 22		0.787
4 • 23	0.672	
4 • 36	0.981	
Average	**0.772**	**1.035**

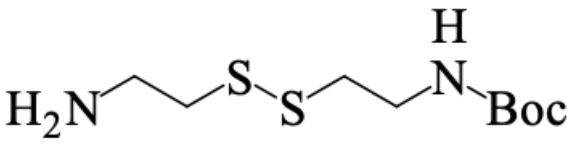
Table 2.5: Measurements of zone of inhibitions. Zone of inhibitions of possible hits were measured to determine if size correlated with biological strength or diffusion capabilities.

Another possibility concerning the significant diameter in correspondence with completely clear zones may be related to the compounds proposed action rather than depending on strength or mobility alone. A compound that produces this type of response might be bacteriostatic in nature rather than bactericidal. Overall, further studies need to be performed to correlate a compound's antimicrobial efficacy with its zone of inhibition size. However, ALA1 proved to exhibit much lower MIC values than were obtained from ALA2 significantly decreasing for Gram-negative pathogens. While values obtained were not sufficient for a potential clinical antibacterial agent, it does demonstrate that extension in length is a strong indication for increased activity. The only other difference is the addition of naphthylamine which could prove to be an important residue used in future libraries

2.7 EXPERIMENTAL METHODS:

Synthesis of Mono-N-cystamine:

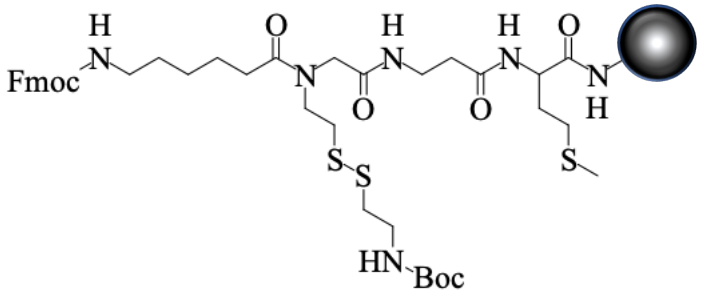
Cystamine dihydrochloride (5.9 g) was dissolved in methanol (300 mL) and placed on ice. Triethylamine (11.2 mL) was added and stirred for 30 minutes. Boc-anhydride (6.13 mL) was added dropwise and stirred overnight at room temperature. Solvent was evaporated *in vacuo* and then washed with diethyl ether (3x). Extraction was performed by addition of 1 M sodium hydroxide (NaOH) (100 mL) and dichloromethane (CH_2Cl_2) (100 mL). Organic layers were combined and washed with water and dried with calcium chloride. Solvent was evaporated *in vacuo* and silica gel column chromatography performed with 5% methanol/ 1% triethylamine in CH_2Cl_2.



Synthesis of PLAD Linker:

0.500 g of TentaGel macrobeads (0.25 mmol/g loading capacity) were transferred to a fritted column and swollen in dimethylformamide (DMF) for a 20-minute period. Fmoc-methionine-OH (0.186 g) was activated with 3-[Bis(dimethylamino)methyliumyl]-3H-benzotriazol-1-oxide hexafluorophosphate (HBTU, 0.190 g) in ~ 7 mL 5% N-methylmorpholine (NMM/DMF) for a 10-minute period. The solution was poured into the fritted column containing the Tentagel macrobeads and rocked gently for 1 hour to ensure coupling. This was removed by aspiration 4x, before and after being washed with DMF 3x.

A Kaiser test was performed to confirm coupling was successful. Fmoc protecting group were removed using ~ 8 mL of 20% piperidine/DMF 2x for 10 minutes each. A Kaiser test was performed to confirm the removal of the Fmoc protecting group and a free amine. Fmoc-β-alanine (0.156 g) was activated with HBTU (0.190 g) in ~ 7 mL 5% NMM/DMF for 10 minutes and then added to the vial for 1 hour of gentle rocking. The macrobeads were dried through house vacuum 4x, before and after being washed with DMF 3x. A Kaiser test was performed to confirm coupling was successful. Fmoc protecting group were removed using ~ 8 mL of 20% piperidine/DMF 2x for 10 minutes each. A Kaiser test was performed to confirm the removal of the Fmoc protecting group and a free amine. Acylation of the amine was conducted by the addition of 3 mL of 2 M bromoacetic acid in anhydrous DMF and 3 mL of N,N'-diisopropylcarbodiimide (DIC) in anhydrous DMF (50:50) to the column for a total of 6 mL. This was microwaved at 10% power (100 kW) 2x at 15 seconds each for a total of 30 seconds and placed on the rocker for a 20-minute period. Mono-*N*-Boc-cystamine (3.028 g) was dissolved in anhydrous DMF (6 mL), added to the column, and microwaved 2x for 15 seconds at 10% power (100 kW) for a total of 30 seconds. This was rocked overnight. Fmoc-6-aminohexanoic acid (0.176 g) was activated with HBTU (0.190 g) in ~ 7 mL 5% N-methylmorpholine (NMM/DMF) for a 10-minute period before being added to the column and gently rocked for 1 hour.



Combinatorial Library Synthesis:

TentaGel macrobeads with a synthesized PLAD linker underwent Boc deprotection achieved with a TFA cleavage cocktail of 7 mL 95% trifluoroacetic acid (TFA): 2.5% triisopropylsilane (TIS): 2.5% water (H_2O) for 1 hour at room temperature. After a DMF wash (3x), Fmoc deprotection was performed 2x for 20 minutes each with 20% piperdine in DMF. The resin was washed with DMF (3x). The resin was acylated with the addition of bromoacetic acid (0.833 g) in anhydrous DMF (3 mL) and DIC (1.5 mL) in anhydrous DMF (1.5 mL). This was microwaved at 10% power (100 kW) 2x at 15 seconds each for a total of 30 seconds and placed on the rocker for a 20-minute period. The solution was removed by aspiration, before and after the beads were washed with DMF (3x). The beads were resuspended in DMF, pipetted out, and placed into one of seven vials for displacement. Any excess DMF was pipetted out. Desired amines were added to each vial (2 mL, 2M in anhydrous DMF). The vials were microwaved at 10% power (100 kW) 2x at 15 seconds each for a total of 30 seconds and placed on the rocker for a 30-minute period. The resin was recollected into fritted column and again washed with DMF (3x). This split-and-pool method was continued with alternating steps of bromoacylation and amine displacement until the desired length was achieved. Boc deprotection was performed once more for cleavage of protection groups on side groups with the TFA cleavage cocktail (95%TFA: 2.5% TIS: 2.5% H_2O) for 1 hour on the rocker. Resin was washed with DMF (3x) followed by a wash with DIC (3x). The fritted column was left on the aspirator for 10 minutes to ensure complete drainage of solvent.

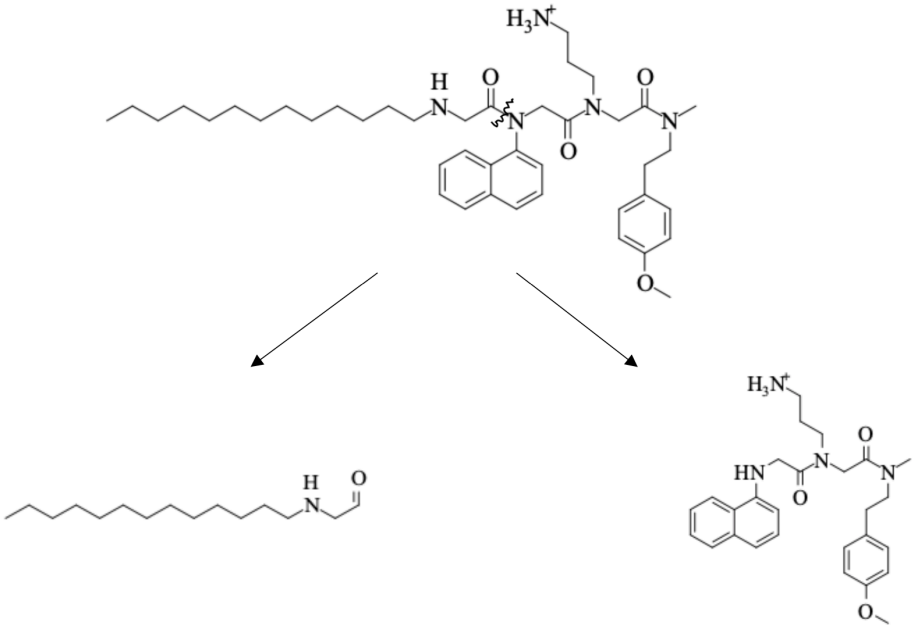
PLAD Screening:

Combinatorial library was deprotected with TFA cleavage cocktail (95% TFA: 2.5% TIS: 2.5% H_2O) for 1 hour and washed with DMF (3x). The resin was then washed CH_2Cl_2 (3x) and dried by aspiration for 10 minutes. Resin was separated into separate vials (between 3-5 mg), washed with phosphate buffered saline (PBS) (3x), and equilibrated overnight (500 µL). Bacterial colonies were collected with a sterile loop from overnight cultures and swabbed in 3 mL of tryptic soy broth (TSB). Aliquots of LB soft agar (3 mL) were heated to 100 °C on a hot plate until liquified and then placed in a hot bath at 47 °C. LB solid plates for screening were prepared by adding 570 µL of a reducing agent (Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) for *E. faecalis, E. faecium, S. aureus, K. pneumoniae,* and *E. coli.*; 2-mercaptoethanol (BME) for *A. baumannii* and. *P. aeruginosa*), 100 µL of combinatorial library, and 100 µL of bacterial inoculant (density 0.08-0.13) into 3 mL of LB soft agar. This was poured onto pre-labeled solid LB plates and incubated overnight at 37°C. Zone of inhibition were observed, images taken under a microscope, and hits collected for sequencing.

MS Sequencing:

100 µL of 1% SDS in PBS was added to each hit collected before incubating at 100°C for 20 minutes. Beads were washed with 1x PBS (3x) in preparation for cleavage. 75 µL of CNBr in 0.1 M HCl in H_2O was pipetted to each single bead and incubated in the dark overnight. Solvent was evaporated and released peptoid resuspended in 80:20

acetonitrile/water in 0.1% formic acid (FA). Tandem mass spectrometry was conducted on a Waters Synapt HDMS OToF with Ion Mobility, fragmentation occurred at each amide bond, and sequence determined by b and y -ions.



b-ion y-ion

Minimum Inhibitory Concentration (MIC) Determination against the ESKAPE Pathogens:

LB agar plates were streaked with frozen bacterial cultures from the ESKAPE family and incubated between 18-24 hours at 37º C. After incubation, a sterile loop was used to collect 2-3 colonies of each bacteria and resuspended in a culture tube of 4 mL Tryptic Soy Broth (TSB). Further incubation at 37º C for 2-9 hours, depending on bacterial strand,

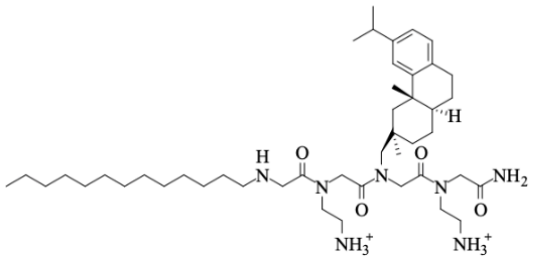
brought each broth solution within optimal optical density range. The solution containing broth and bacteria were further diluted with TSB if range was not within 0.08-0.13, measured at 600 nm on a spectrophotometer. 20 µL of each bacterial solution was further diluted in 380 uL Cation Adjusted Mueller-Hinton Broth (CAMHB) and set aside.

In a 96 well sterile tray, 90 µL of CAMHB was dispensed into each well leaving rows A and H broth free. To achieve a solution that consisted of 100 µg/mL K9, 4 µL of a 10 mg/mL stock solution was pipetted out and diluted in 356 µL of CAMHB. For each target organism, 180 µL of this 100 µg/mL solution was dispensed into three wells of Row A. By withdrawing 90 µL of row A and dispensing it into the next, 2-fold dilutions were performed down to row F giving final K9 concentrations of 100, 50, 25, 12.5, 6.3, and 3.13 µg/mL. 90 µL of row F were withdrawn and discarded to assure each well had a total volume of 90 µL. Peptoid free broth remained in row G to serve as a negative control. Each well attained a total volume of 100 µL with the addition of 10 µL of the diluted bacteria into rows A-G. 4 µL of a 2 mg/mL stock of a tetracycline control was pipetted out and diluted in 356 µL CAMHB where 40 µL of bacteria was also introduced. 100 µL of this solution was delivered in triplicates in row H for each bacterial strain tested.

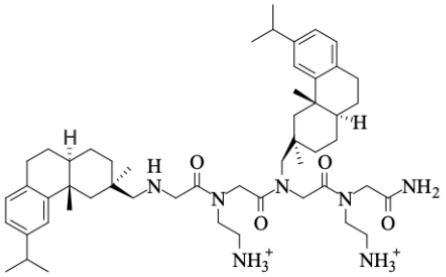
After an overnight incubation, for 18-24 hours at 37 º C, 10 µL of PrestoBlue was added to each well and further incubated for 1 hour. To determine the minimum inhibitory concentration (MIC), fluorescent intensity was tested with excitation at 555 nm, cut-off at 570 nm, and emission at 585 nm on a SpectroMax M5 Plate Reader. Each bacterial strain of the ESKAPE family underwent triplicate studies for MIC determination.

2.8 FUTURE WORK:

This study has provided significant insight in peptoid combinatorial library design selective for Gram-negative pathogens. Sequencing was accomplished for library 004 but further single bead analysis will be conducted to sequence compounds from library 003, library 005 and library 006. Analysis of Library 006 will determine if a modified linker will be continued for use in the future to enhance success rates. Additionally, structure-activity relationship studies will be conducted to further investigate various key features as presented below.



$N_{ae} - N_{daa} - N_{ae}$



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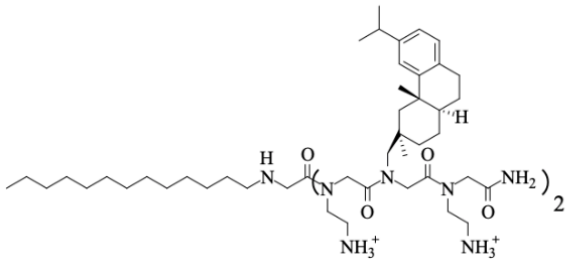
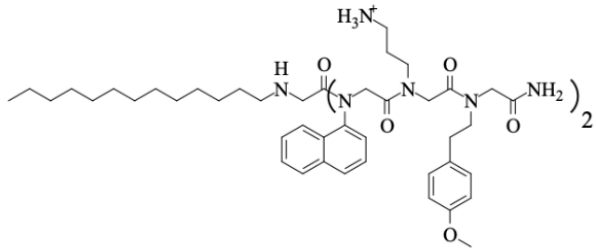
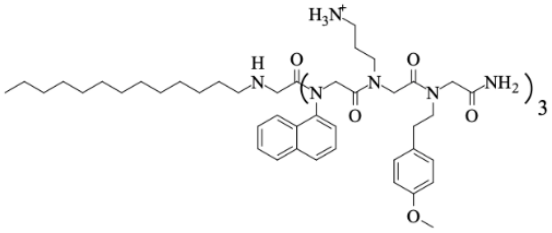


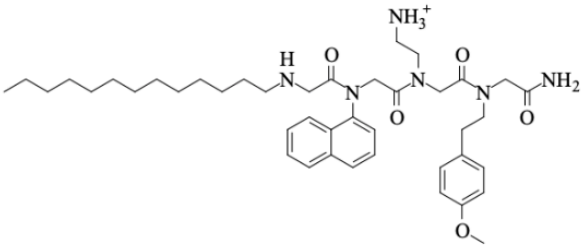
$(N_{ae} - N_{daa} - N_{ae})_2$



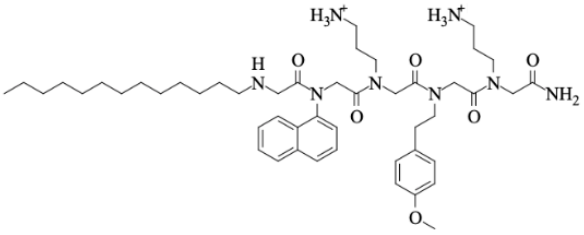
$(N_{nap} - N_{ap} - N_{mpa})_2$



$(N_{nap} - N_{ap} - N_{mpa})_3$



$N_{nap} - N_{ae} - N_{mpa}$



$N_{nap} - N_{ap} - N_{mpa} - N_{ap}$

CHAPTER 3: K9 AND K9 DERIVATIVES:

3.1. INTRODUCTION:

K9 (Figure 3.1) is a compound identified in our lab during a PLAD screening against *E. coli.* Structure-activity relationship studies were performed on K9 and two K9 derivatives with azole heterocycle backbones provided by Amelia Fuller at Santa Clara University to determine the antimicrobial efficacy of the Fuller lab's azole backbone modifications. All three were tested against the ESKAPE panel to obtain individual MIC values and determine the contribution of thiazole and oxazole groups.

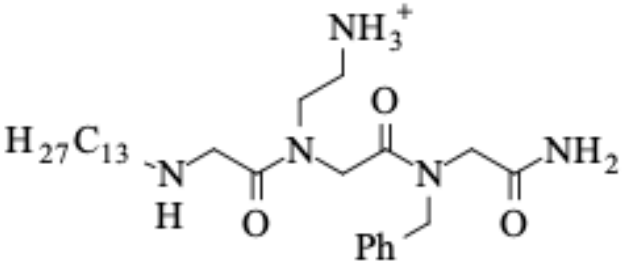
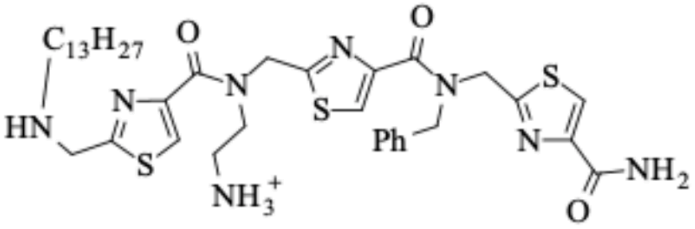


Figure 3.1: K9. The structure of K9 used for structure-activity study. MW = 503.73 g/mol

Additionally, toxicity levels correlated with each structure were determined against HepG2 cells. These cells are a common choice to utilize when testing toxicity levels of potential drugs and are a cell line that is derived from human liver carcinoma. Testing the potency and toxicity of the original K9 structure compared to the thiazole and oxazole derivatives will give insight to whether these groups are able to enhance antimicrobial properties. Thiazole and oxazole groups are heterocyclic aromatic compounds that have shown

antimicrobial activity and are present in common drugs. Each group consists of a five-membered unsaturated ring with a nitrogen in the 3rd position but differ in the heteroatom in the 1st position. Thiazole groups contain a sulfur in the 1st position while oxazole groups have an oxygen (Figure 3.2).

(a)



(b)

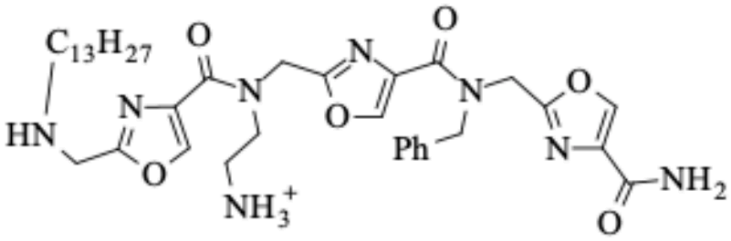


Figure 3.2: K9 Derivatives tested for structure-activity relationship study in comparison to the data obtained for the original K9 structure

(a) K9 Thiazole Derivative with a MW = 753.06 g/mol

(b) K9 Oxazole Derivative with a MW = 704.87. g/mol

3.2. RESULTS AND DISCUSSION:

The intended focus was to determine the potency and toxicity of a compound termed K9 and to compare those results with two K9 derivativities for structure activity relationship studies. Implementing the thiazole group into the structure of the peptoid proved to lower MIC values in four out of the seven pathogens tested when compared to K9. The results obtained also indicated that the MIC values for three species, *E. faecalis, S. aureus, and A. baumannii,* were lowest when utilizing the K9 thiazole derivative versus both K9 and the K9 oxazole derivative. In addition, this derivative was tied for lowest MIC value for two of the species, *K. pneumoniae* and *E. coli.* However, while there was a 50% decrease for MIC in *K. pneumoniae,* the concentration reduction from 100 µg/mL to 50 µg/mL is not promising for a potential drug candidate in its current state. Furthermore, it should be noted that even though the K9 thiazole derivative is tied for lowest MIC value regarding *E. coli,* there was no change from the MIC value obtained from the original K9 structure. Specifically, the addition of a thiazole group exhibited more impact towards Gram-positive bacteria, *E. faecalis* and *S. aureus,* than the Gram-negative pathogens of this panel. There is minimal reduction among the Gram-negative species with the exception of *A. baumannii* which lowers significantly by < 85%. The original K9 compound proved to be more effective in two of the seven species, *E. faecium* and *P. aeruginosa,* than its counterparts with no distinction between Gram-positive or Gram-negative bacteria. Notably, K9 showed increased efficiency when thiazole groups were implemented but exhibited less than promising results when oxazole groups were added instead. The K9 oxazole derivative expressed decreased MIC values for one species, *A. baumannii,* by 75% dropping from 100 µg/mL to 25 µg/mL but was further reduced with the addition of

thiazole groups. All other values increased from the original K9 data obtained with only one exception, *S. aureus,* where the value remained the same. However, the data regarding toxicity showed the K9 oxazole derivative had the highest IC_{50} obtained from all three groups, an indication of decreased toxicity. Both thiazole and oxazole groups showed increased IC_{50} values from an average of 13.64 µg/mL for K9 to 22.22 µg/mL for K9 thiazole and 47.96 µg/mL for K9 oxazole. The data obtained show that each of the three compounds are highly toxic and should be further studied with alternate options. While the oxazole groups demonstrated the highest IC_{50} values, it also provided no benefit towards MIC values. However, thiazole groups showed improvement in both categories and could be further addressed by utilizing different variations of thiazole functional groups.

	IC_{50} Average	Minimum Inhibitory Concentration						
		E. faecalis	*E. faecium*	*S. aureus*	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*	*E. coli*
K9	13.6	12.5	6.25	12.5	100	100	25	12.5
K9 Oxazole	47.9	25	12.5	12.5	50	25	> 100	25
K9 Thiazole	22.2	6.25	12.5	6.25	50	12.5	100	12.5

Table 3.1: Combined Results for potency and toxicity of K9 and K9 derivatives.

Blue = Lowest MIC value Green = A tie for lowest MIC value.

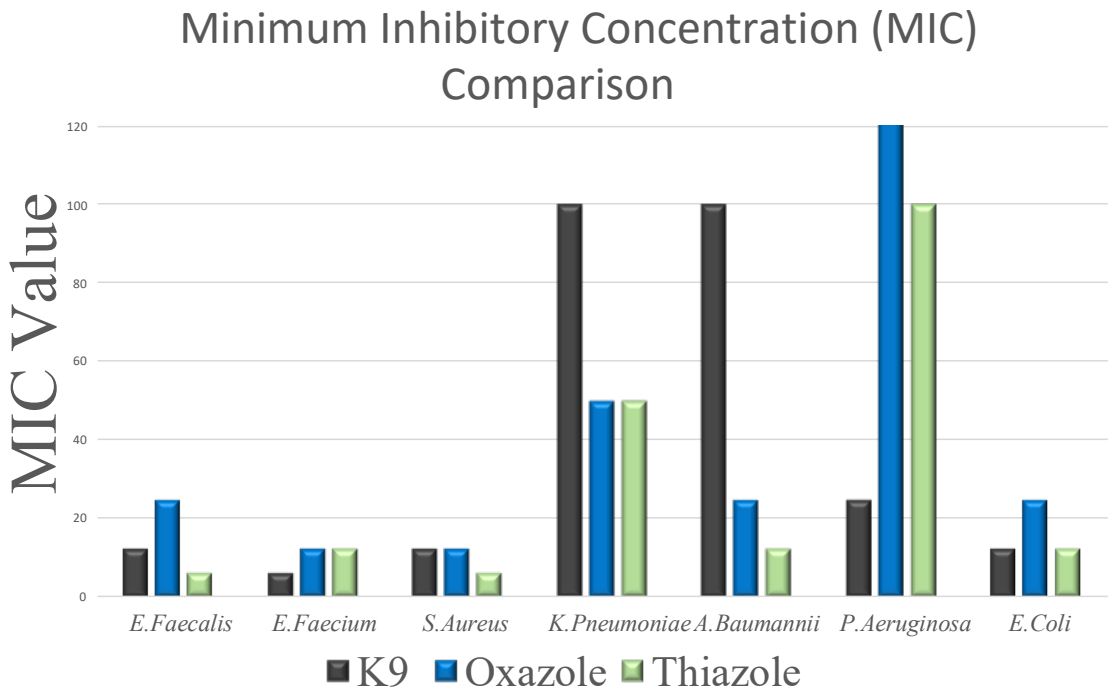


Figure 3.3: MIC values. Specific MIC values ranging from 6.25 µg/mL to <100 µg/mL. Comparison of MIC values obtained for each compound presents indication of which K9 derivative is most affective against each bacterial species tested.

All three compounds underwent further study to explore the relationship between activity and toxicity. Previous studies have shown that toxicity towards mammalian cells are enhanced alongside increased antimicrobial activity [32] requiring further knowledge to create an inverse rather than a direct relationship between the two variables. Based on this concept, it was theorized that the toxicity determined would be of moderate concern. This was considered as a particular outcome due to modest MIC values obtained with a 6.25 µg/mL being the lowest and >100 being the highest. Results indicated; however, significant toxicity was displayed with the original K9 (Figure 3.4).

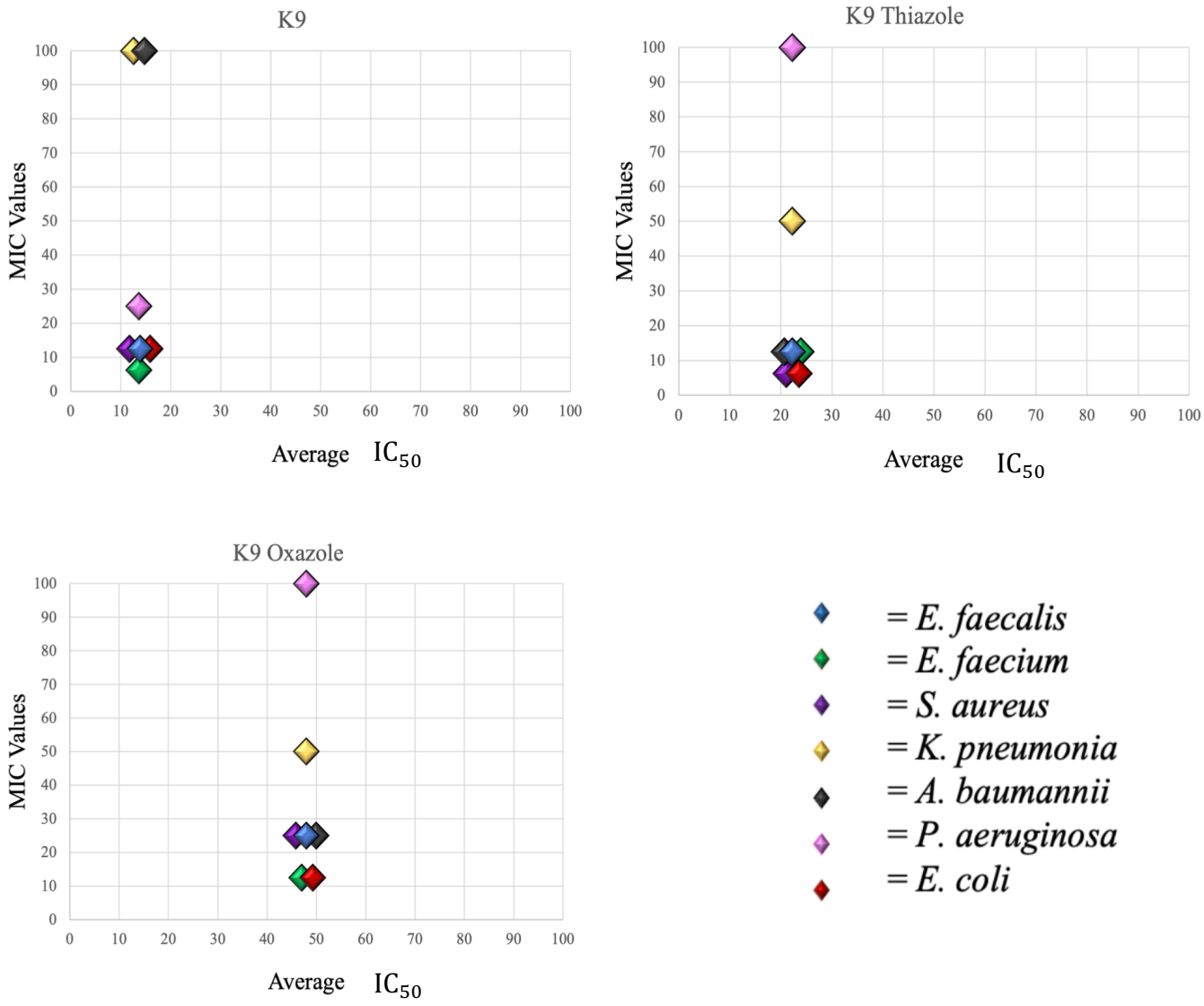


Figure 3.4: MIC and IC_{50}. In order of compounds from lowest to highest IC_{50}.

Selectivity Index (SI) was calculated (Table 3.2) with the IC_{50} value obtained by the MTT assays performed and the MIC's correlated with each bacterial species tested. A high SI would reveal a good candidate for future drug development due to indication of selectively towards the pathogen. A value lower than 10 demonstrates a non-selective compound [26] which highlights its ineffectiveness as a potential antimicrobial agent. Calculations revealed moderate to extreme cytotoxicity, not only in K9, but in the derivatives tested as well. SI values indicate. that the addition of azole heterocycle backbones improved the compounds cytotoxicity factor and selectivity ratio but to a minimal degree.

$$\text{Selectivity Index} = \frac{IC_{50}}{MIC}$$

The highest SI obtained for K9 was 2.17 for *E. faecium* and the lowest being 0.136. for *K. pneumoniae* and *A. baumannii.* Oxazole-derived compounds obtained a high value of 3.83 for *E. faecium* and *S. aureus* while displaying a low of 0.479 for *P. aeruginosa.* Thiazole-derived compounds exhibited a 3.55 for *E. faecalis* and a very low 0.22 for. *P. ae*. All compounds retained SI values significantly below a value of 10 indicating their insufficiency as potential clinical antimicrobial agents.

	SI for K9	SI for K9 Oxazole	SI for K9 Thiazole
E. faecalis	1.08	1.92	3.55
E. faecium	2.17	3.83	1.78
S. aureus	1.08	3.83	3.55
K. pneumoniae	0.136	0.958	0.44
A. baumannii	0.136	1.92	1.78
P. aeruginosa	0.544	0.479	0.22
E. coli	1.08	1.92	1.78

Table 3.2: SI for K9 and K9 Derivatives. SI values calculated indicated massive toxicity.

3.3 EXPERIMENTAL METHODS:

Minimum Inhibitory Concentration (MIC) Determination against the ESKAPE Pathogens:

LB agar plates were streaked with frozen bacterial cultures from the ESKAPE family and incubated between 18-24 hours at 37º C. After incubation, a sterile loop was used to collect 2-3 colonies of each bacteria and resuspended in a culture tube of 4 mL Tryptic Soy Broth (TSB). Further incubation at 37º C for 2-9 hours, depending on bacterial strand, brought each broth solution within optimal optical density range. The solution containing broth and bacteria were further diluted with TSB if range was not within 0.08-0.13, measured at 600 nm on a spectrophotometer. 20 µL of each bacterial solution was further diluted in 380 uL Cation Adjusted Mueller-Hinton Broth (CAMHB) and set aside.

In a 96 well sterile tray, 90 µL of CAMHB was dispensed into each well leaving rows A and H broth free. To achieve a solution that consisted of 100 µg/mL K9, 4 µL of a 10 mg/mL stock solution was pipetted out and diluted in 356 µL of CAMHB. For each target organism, 180 µL of this 100 µg/mL solution was dispensed into three wells of Row A. By withdrawing 90 µL of row A and dispensing it into the next, 2-fold dilutions were performed down to row F giving final K9 concentrations of 100, 50, 25, 12.5, 6.3, and 3.13 µg/mL. 90 µL of row F were withdrawn and discarded to assure each well had a total volume of 90 µL. Peptoid free broth remained in row G to serve as a negative control. Each well attained a total volume of 100 µL with the addition of 10 µL of the diluted bacteria into rows A-G. 4 µL of a 2 mg/mL stock of a tetracycline control was pipetted out and

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Cytotoxicity Testing Against HepG2 Cells

HepG2 hepatocarcinoma cells were grown in DMEM containing 10% FBS at 37 ˚C and 5% CO_2 atmosphere. For cytotoxicity testing, cells were seeded into 96-well plates in phenol red free DMEM containing 10% FBS and incubated for 2 h to allow cell attachment. Cells were then treated with varying concentrations of peptoid or vehicle control. After 72 h incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 20 µL; 5 mg/mL) was added to each well and the plate incubated for 3.5 h. Media was removed from each well and 100 µL of DMSO was added and the plate incubated at 37 ˚C for 10 min. The absorbance at 570 nm was then read on a spectrophotometer, percent inhibition determined, and TD_{50} calculated using GraFit. All triplicate assays were repeated twice on different days.

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