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# Antimicrobial activity of novel cationic amphiphiles

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Antimicrobial Activity of Novel Cationic Amphiphiles

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by Melanie Kusakavitch
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## Abstract

This project focused on the development of amphiphiles in order to prevent hospital-acquired infections before they have a chance to enter the host, thereby reducing the need for antibiotics. The antibiotic resistance crisis of the 21st century is a dangerous epidemic with global consequences. Therefore, there is a desperate need for novel approaches in antimicrobial research. This would decrease the overall length of stay in hospitals and costs associated with such a stay. In this study, tetracationic amphiphiles with two 12-carbon chains and an aliphatic linker were studied. The MIC value of each amphiphile and strain pair was determined and compared. Linker length, the distance between head groups attached to identical hydrophobic tails appears to affect antimicrobial activity. Amphiphiles with longer linkers tend to be more potent antibacterial agents than those with shorter linkers. Amphiphiles were also used in combination with antibiotics to determine whether the combinations interacted synergistically to kill bacteria. The most potent amphiphile was 12-B-10-B-12 against all bacteria. This amphiphile was longer in linker length relative to other derivatives, suggesting that there is a relationship between linker length and antimicrobial potency. The development of novel amphiphiles may be one way to reduce the need for antibiotics to treat hospital-acquired infections.

## Introduction

In 2013, the CDC reported more than two million Americans are infected with an antibiotic-resistant bacterial infection each year, contributing to more than 23,000 deaths in the U.S. per year (CDC, 2013)These infections span a diverse list of illnesses including skin and surgical site infections, urinary tract infections, pneumonia, bacteremia, and hospital-associated diarrhea. Approximately 2 million patients contract a healthcare-acquired infection each year (Klevens *et al.*, 2007) at a cost of $28 - $33 billion in healthcare annually (Scott, 2009). The cost of antibiotic-resistant infections to a single hospital has been estimated to be as high as $13.35 million a year (Scott, 2009). In the past 15 years, only four novel antimicrobial classes have been approved for clinical use. Resistance to these agents has already been observed in enterococci and staphylococci (Klevens *et al.*, 2007). Infections from antibiotic resistant bacteria have become more prevalent in the 20th century. There are growing challenges in treating these infections; therefore clinicians must work to limit the transmission of bacteria between contaminated equipment and/or staff to susceptible individuals. This is critical for preventing nosocomial infections and reducing mortality rates.

When the introduction of antibiotics, antiseptics, and disinfectants came into existence, public health was forever modified. These compounds were able to improve sanitation methods and reduce the spread of infectious diseases, most specifically in health care settings (Sköld, 2006). Communicable diseases, such as tuberculosis, pneumonia, and diarrhea, once ranked as top killers globally, now have been replaced by many chronic conditions due to the use of antimicrobials (Kochanek *et al.*, 2011).

Although this generation has seen many benefits from the use of antimicrobials in

controlling these infections, hospital-acquired infections (HAI) have posed a major threat to patients receiving health care. These infectious agents are dangerously gaining resistance to commonly used antimicrobials. HAIs frequently lead to increasing medical costs, longer hospital stays, increasing complication rates, and worsening overall morbidity and mortality (Klevens *et al.*, 2007). Many countries and hospitals have adopted policies and regulations in recent years attempting to decrease the impact of these healthcare-associated infections. Not only would reducing HAIs be beneficial to the patients themselves, it would also be highly beneficial to healthcare centers and insurance companies (Scott, 2009).

An increasing proportion of HAIs are resistant to various antibiotics. The rate of *Staphylococcus aureus* strains found in hospitals that were methicillin-resistant is increasing. MRSA, methicillin resistant *Staphylococcus aureus*, was once detected at a rate of 1-5% in the 1980's to 60-70% in recent years, proving greater incidence (Taubes, 2008). Multi-drug resistant *Pseudomonas aeruginosa* has become relatively common in Intensive Care Units (ICUs), accounting for 13.2% to 22.6% of HAIs reported in this environment (Driscoll *et al.*, 2007). Bacterial drug-resistance forces healthcare providers to resort to more expensive and potentially more harmful therapies (McGowan, 2001; Cosgrove & Carmeli, 2003). Patients with infections that are resistant to all available antibiotics often have to undergo surgical removal of the infection, which may include amputations (Cosgrove & Carmeli, 2003).

The rapid emergence of resistant bacteria has diminished the efficacy of antibiotics. The antimicrobial resistance of many pathogenic bacteria can be attributed to the regular overuse and misuse of antibiotics (Ventola, 2015). Unfortunately, resistance

has been documented for almost all antibiotics developed. Despite the known danger of antibiotic resistance, antibiotics are continually misused, over-prescribed, and abused in a number of industries including agriculture. Industrial use has contributed greatly to the vast increase in antibiotic resistance, particularly in human pathogens such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and multi-drug resistant *P. aeruginosa* and *Mycobacterium tuberculosis* (MDR-TB) (Gilbert & Moore, 2005). This increase has diminished the effectiveness of first- and second-line therapies for infectious disease, extending illness, and increasing the risk of mortality (Taubes, 2008).

Proper hygienic practices, including hand washing, are the easiest, most effective and cost efficient ways to prevent HAIs (Braine *et al*, 2009). Reducing surface contamination is one way that infections can be combated. Surface contamination can be extremely problematic when contaminated surfaces come in contact with a patient's open wound or surgical equipment, catheters, or ventilators used on a patient (Weber *et al.*, 2010). This potential contamination has increasingly negative effects on patients in hospitals, particularly those with compromised immune systems. Reducing potential cross contaminations between health care professionals and patients, whether it be by contact between persons or contact with hospital equipment prevents pathogen reservoirs from forming and reduces the risk of infection for patients. The need for novel antimicrobials is extremely important during this time of antibiotic resistance.

Cationic amphiphiles, compounds with at least one hydrophilic head group and hydrophobic tail (Figure 1), can have antibacterial properties. It is generally thought that amphiphiles work by targeting the cell membranes of bacteria. The amphiphile's positively charged head group interacts electrostatically with the negatively charged cell membrane of the bacterium, allowing hydrophobic tails to insert into the membrane and disrupt or destabilize it. When the membrane is disrupted, cytoplasmic materials leak out and cell lysis occurs. (Gilbert & Moore, 2005). Amphiphiles have served as antimicrobial agents in many industries. For example, benzalkonium chloride and chlorhexidine are active ingredients in topical antiseptics such as Neosporin®, mouthwash, and common disinfecting products, including Lysol® (Gilbert & Moore, 2005). These compounds have been successful for many decades and bacteria have no documented resistance to them (Gilbert & Moore, 2005). Therefore, there is great potential for cationic amphiphiles as antimicrobials.

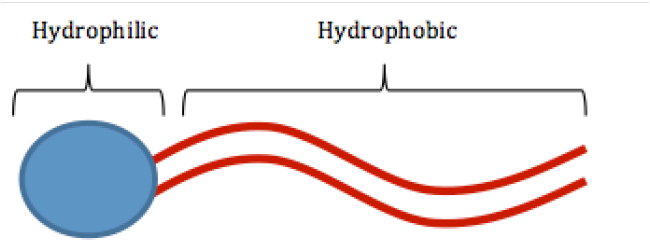



**Figure 1.** Generic amphiphile structure, including the hydrophilic head group (blue) and the hydrophobic hydrocarbon tail group (red).

Gemini amphiphiles, which contain four cationic head groups with two attached 12-carbon tails and a linkage group (Figure 2), are the main focus of this research because of their proven potent antimicrobial activity against Gram-negative and Gram-

positive bacteria. Dialkyl 4,4'- bipyridium amphiphiles with 12 carbon tails have been recognized as a potentially efficient group of membrane disruptors (Grenier *et al.* 2012). Dialkyl 4,4-bipyridium compounds, which include a gemini (two heads, two tails) structure (Figure 2), are effective against highly resistant bacterial strains such as *E. coli and P.aeruginosa* (Grenier *et al.* 2012). The compounds specifically used in this study all possess a 12-carbon tail. What is unique about each compound is the linker length varying from 6-12-carbons in length (Figure 2). Each of these amphiphiles possesses a tetracationic structure, with four head groups that possess a total of 4 positive charges.

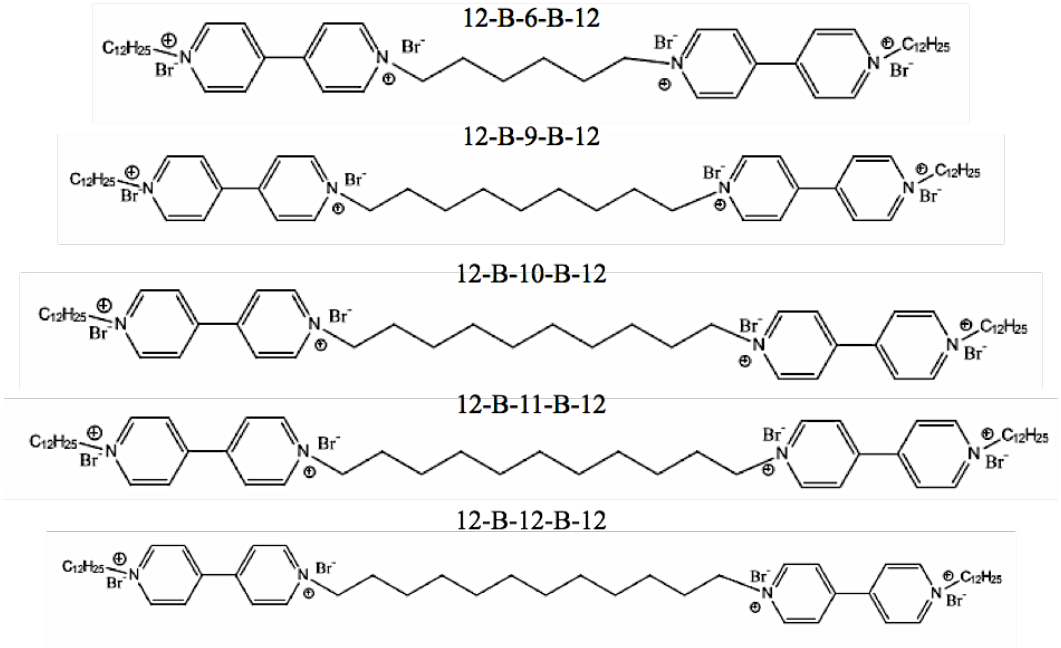



**Figure 2.** Dialkyl 4,4'-bipyridium amphiphiles.

There is much interest in developing single antimicrobials or combinations of drugs that have activity against multiple microorganisms (Giacometti *et al,* 2000. Tobramycin is an aminoglycoside antibiotic that is used to treat various types of bacterial infections, particularly Gram-negative infections. It is especially effective against species of *Pseudomonas*. Oxacillin is a penicillinase-resistant antibiotic that is

widely used clinically in the United States to treat penicillin-resistant bacterial infections, such as ones caused by *S. aureus* (Jones *et al,* 2002). In this study, novel amphiphiles were used in combination with different antibiotics, tobramycin and oxacillin, to determine if the combinations interacted synergistically to kill bacteria.

The antibacterial activity of a series of 4,4'-bipyridinium compounds against Gram-positive and Gram-negative pathogens was investigated to further analyze the effects of various structural features on antibacterial activity. In this study, antimicrobial properties of novel amphiphiles were explored by determining MIC values. These MIC values were determined and analyzed in order to determine the relationship between linker length and antimicrobial activity of amphiphiles. Amphiphile 12-B-10-B-12 was a potent killer against all Gram-positive and Gram-negative bacteria.

Combination assays were then performed in order to detect possible synergistic relationships between other novel amphiphiles and tobramycin and oxacillin. Although no significant results were obtained with these specific combinations, preliminary experiments suggest that amphiphiles in this study are potent antimicrobials.

## Materials and Methods

### *Bacterial Strains and Growth Conditions*

The Gram-negative bacterial strains used in this study were *Escherichia coli* ATCC® 25922™, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC® 27853™ for experiments with planktonic cells, and hyper-biofilm forming strain PAO2 for biofilm disruption studies (Holloway, 1955). The Gram-positive bacterial strains used were *Staphylococcus aureus* subsp. *aureus* ATCC 29213™, *Enterococcus faecalis* ATCC® 29212™, *Bacillus anthracis* Sterne, *Streptococcus agalactiae* J48 (Seifert *et al*., 2006). All bacterial strains, except for PAO2 were grown in MHB. For biofilm studies, PAO2 was grown in Luria-Bertani (LB) broth.

### *Broth Microdilution Method for Determining the Minimum Inhibitory Concentration*

Students from James Madison University under the direction of Dr. Kevin Caran synthesized the amphiphiles in this study. The Minimum Inhibitory Concentration (MIC) was determined for each amphiphile against four Gram-positive and three Gram-negative bacterial strains as previously described (CLSI, 2012). The MIC was determined using the broth microdilution method (Wayne, 2009). Bacterial cultures were grown overnight at 37°C and diluted in MHB to a concentration of $5x10^6$ cells/mL. Aliquots of 100µL of bacteria were added to 96-wells plates along with 100µL of amphiphile being tested at 2-fold dilutions, from 500µM to 2µM, in triplicate. The plates were incubated at 37°C for 72 hours. The wells with the lowest concentration of amphiphile without visible growth were considered the MIC. Each amphiphile was tested a minimum of 2 times.

***Checkerboard Assay***

A checkerboard assay using 96-well microtiter plates was used to determine if synergy exists when amphiphiles are combined with other compounds against *E.coli* ATCC® 25922™ and *S. aureus* ATCC® 29213™ (ATCC, Manassas, VA, USA). Bacteria diluted to $5x10^6$ cell/mL was added to each well yielding $5x10^5$ cell/mL in each well. Wells with sterile deionized $H_2O$ treatment were used as a control for bacterial growth. In the checkerboard section of the assay, 50µl of each compound at 2-fold dilutions at concentrations below the previously determined MIC of each compound was added to individual wells, such that multiple combinations of the two compounds are tested on a single plate. The Fractional Inhibitory Concentration (FIC) was determined by comparing the MIC value of each compound alone and in combination using the following equation:

$FIC = FIC_A + FIC_B$

$FIC_A = A/MIC_A$

$FIC_B = B/MIC_B$

$MIC_A$ and $MIC_B$ represent the MIC of compound A and B alone and A and B represent the MIC values of compound A and compound B combined. Synergy was defined as an FIC value of $<0.5$, and antagonism was defined as an FIC value of $>4$. Synergy assays for each combination was performed at least 3 times.

## Results

### *Effect of Varying Linker Length of Gemini Bipyridium Amphiphiles on antibacterial activity*

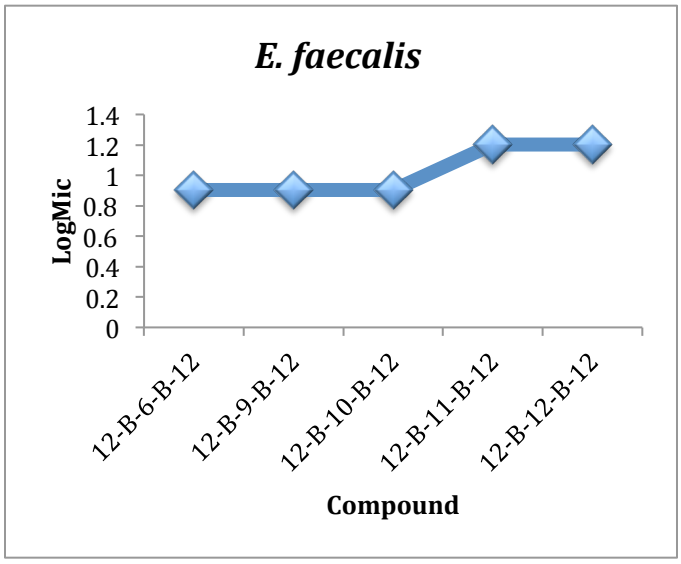
To determine the effect of varying linker length on antimicrobial activity, MIC assays were performed with dialkyl 4,4'-bipyridium amphiphiles that had varying linker lengths ranging from 6-12 carbons (Table 1, 2). The variation in linker length resulted in a trend that as linker length increases, MIC values decrease for species such as Gram-negative, *K. pneumonia* (Table 2). As linker length increased, MIC values increased, for Gram-positive species such as *E. faecalis* and *S. aureus* (Table 1), showing that, in this case, shorter linker lengths were more effective. When 4,4'-bipyridium amphiphiles treated Gram-positive *S. agalactiae* and *B. anthracis* there showed promising results but no definitive trends (Table 1). When 4,4'-bipyridium amphiphiles treated Gram-negative *P. aeruginosa* and *E.coli* again, there showed no definitive trends (Table 2). Overall, the most effective amphiphile was 12-B-10-B-12 against all bacteria (Figure 3,4), showing that a linker length of 10-carbons was optimal. Amphiphiles were generally more effective against Gram-positive bacteria than they were against Gram-negative bacteria. *B.anthracis* appears to be most sensitive to all amphiphiles tested (Figure 3).

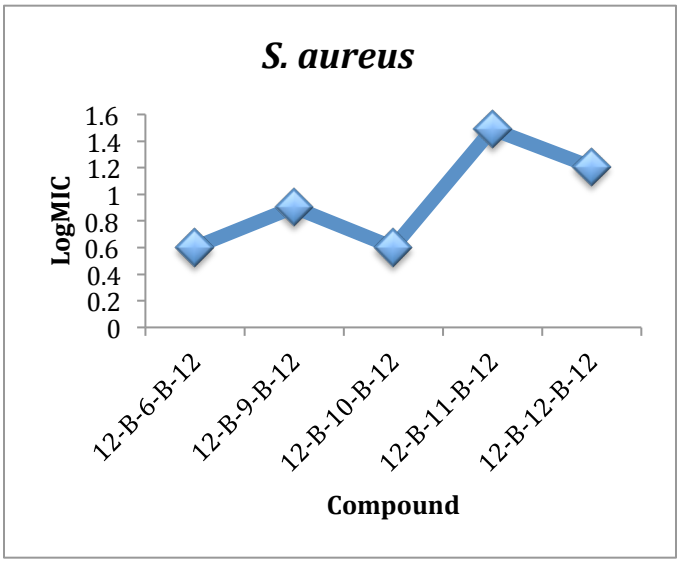
**Table 1.** Minimal Inhibitory Concentrations (μM) for amphiphiles against Gram-positive bacteria.

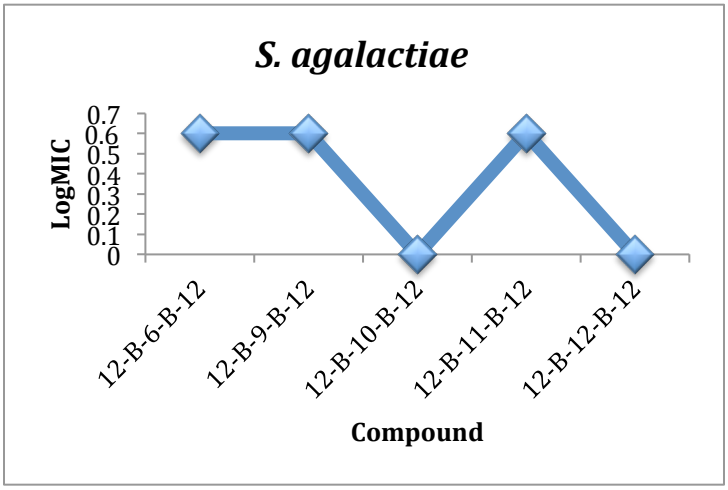
| n-B-n-B-n | *E. faecalis* | *S. aureus* | *S. agalactiae* | *B. anthracis* |
|---|---|---|---|---|
| **12-B-6-B-12** | 8 | 4 | 4 | 2 |
| **12-B-9-B-12** | 8 | 8 | 4 | 2 |
| **12-B-10-B-12** | 8 | 4 | 1 | 1 |
| **12-B-11-B-12** | 16 | 32 | 4 | 1 |
| **12-B-12-B-12** | 16 | 16 | 1 | 4 |

**Table 2**. Minimal Inhibitory Concentrations (μM) for amphiphiles against Gram-negative bacteria.

| n-B-n-B-n2 | *P. aeruginosa* | *E. coli* | *K. pneumoniae* |
|---|---|---|---|
| **12-B-6-B-12** | 32 | 16 | 32 |
| **12-B-9-B-12** | 16 | 16 | 16 |
| **12-B-10-B-12** | 16 | 8 | 8 |
| **12-B-11-B-12** | 8 | 16 | 8 |
| **12-B-12-B-12** | 16 | 16 | 4 |



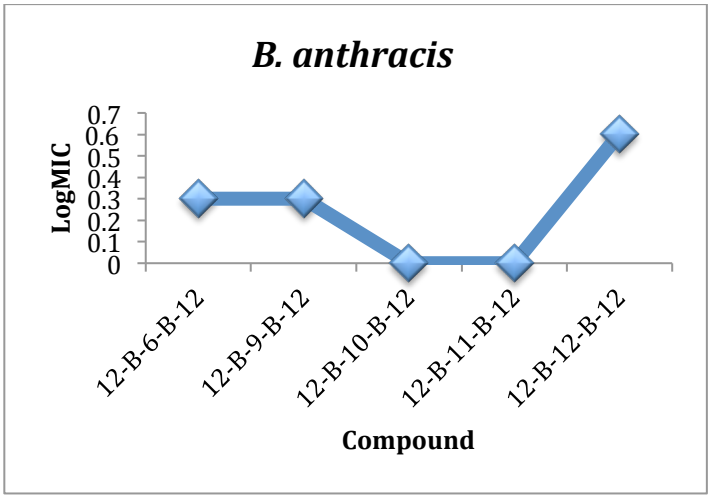
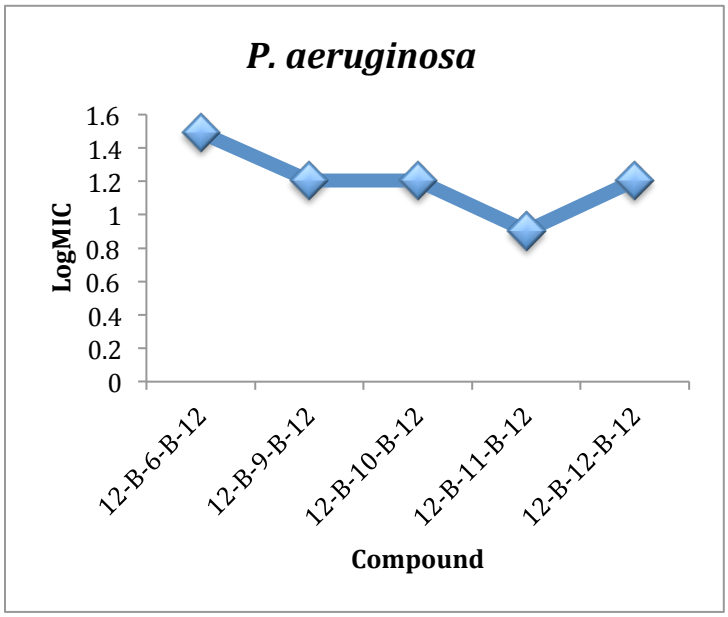
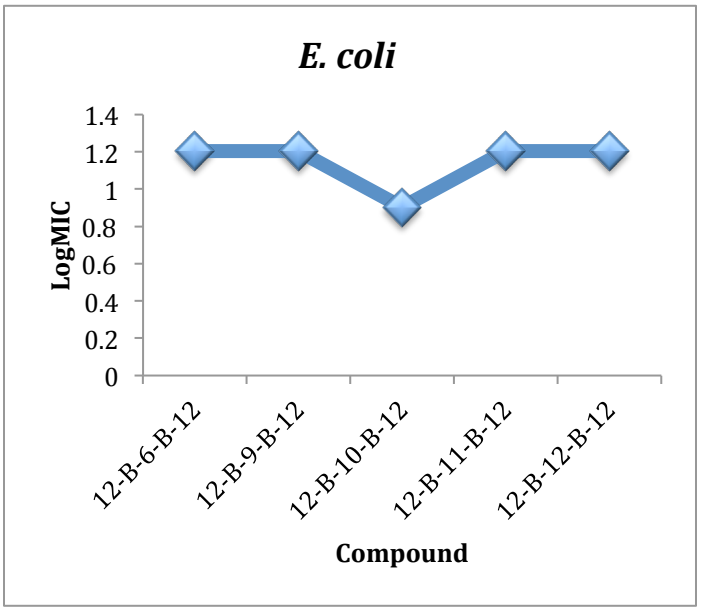




**Figure 3.** Log [MIC(μM)] values of dialkyl 4'4 bipyridium amphiphiles for Gram-positive bacteria.



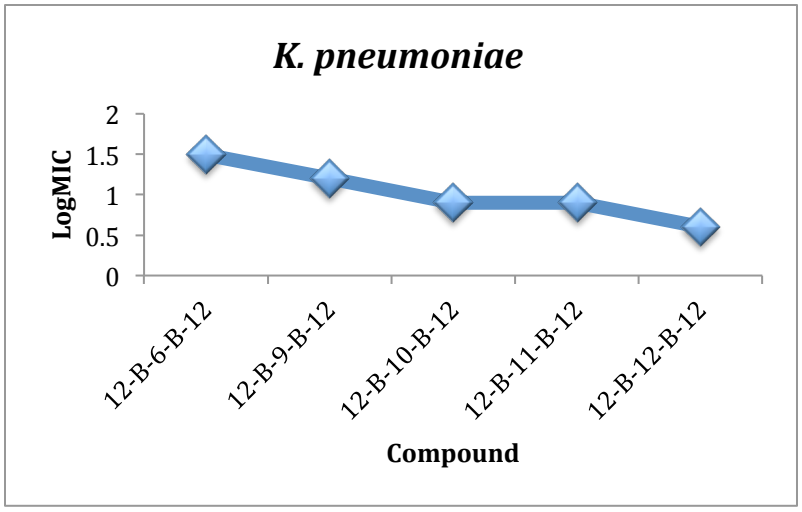
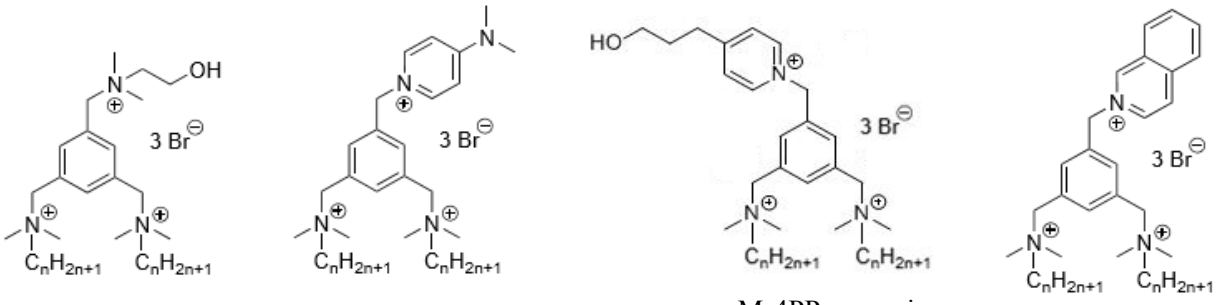




**Figure 4**. Log [MIC(μM)] values values of dialkyl 4'4 bipyridium amphiphiles for Gram-negative bacteria.

***Effect of Synergy between Cationic Amphiphiles and Antibiotics on E. coli***

Four series of double tailed tris-cationic amphiphiles were synthesized by the Caran lab (James Madison University). For each series, the amphiphiles consisted of a mesitylene core (M) with three attached positively charged head groups. Two of these head groups were dimethylalkylammonium groups with attached hydrocarbon tails. The third head group was either ethanolammonium for the M-E,n,n series, dimethylaminopyridinium for the M-DMAP,n,n series, isoquinolinium for the M-IQ,n,n series, and 4-pyridine propanol for the M-4PP,n,n series. The hydrocarbon tails were linear and symmetrical, and consisted of 12 carbons for all of the amphiphiles in this series.




M-E,n,n series
n = 12

M-DMAP,n,n series
n = 12

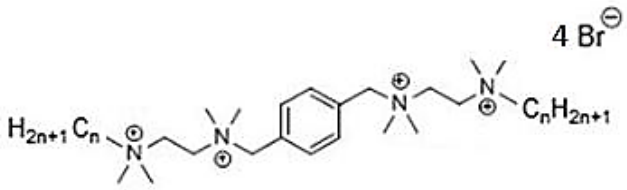
M-4PP,n,n series
n = 12

M-IQ,n,n series
n = 12

**Figure 5.** Structure of amphiphiles. *M* represents the Mesitylene core; *n* represents the number of carbons in each symmetrical hydrocarbon tail. *E* = ethanolammonium, *DMAP* = dimethylaminopryidinium, *IQ* = isoquinolinium, and *4PP* = 4-pyridine propanol.

The tetra-cationic amphiphile series were structured similarly, however two dimethylalkylammonium head groups were attached to each of the same positions on the central ring, connected by a 2-carbon linker. The linear hydrocarbon tail connected to the

head group in each of the tetra-cationic series contained 8 or 10 carbons for the pX-(2,n)$_2$ series (Figure 6).




pX-(2,n)$_2$ series
n = 8,10

**Figure 6.** Structure of tetra-cationic amphiphiles. *n* represents the number of carbons in each symmetrical hydrocarbon tail.; *pX* = para-orientation.

Tobramycin and oxacillin are both known to be effective antibiotics and were combined with these amphiphiles to determine if they acted synergistically when used on *E. coli.* Based on the FIC values, there was not synergy with any of these combinations (Table 3), though it was close in some cases (e.g., PX(2,8)2).

**Table 3**. FIC values for amphiphile and antibiotic synergy assays. A value of <0.5 indicates synergy while a value of >4 indicates antagonism. "ND" represents "Not Done."

| | **Tobramycin** | **Oxacillin** |
|---|---|---|
| M-E,12,12 | 0.708 | 1.09 |
| M-DMAP,12,12 | 0.917 | 0.909 |
| M-IQ,12,12 | 0.625 | 0.704 |
| M-4PP,12,12 | 0.664 | 0.915 |
| PX(2,8)2 | 0.501 | 0.506 |
| PX(2,10)2 | 0.69 | ND |

## Discussion

Dialkyl 4,4'-bipyridium amphiphiles with linker lengths varying from 6-12 carbons, were studied to determine if linker length affected MIC values. As linker length increased, MIC values increased, for *E. faecalis* and *S. aureus*. This type of relationship has been described with other amphiphiles as well (Palermo *et al,* 2012).

Since linker length is correlated with activity of the amphiphile, it is reasonable to assume that as the linker length increases, the flexibility of the amphiphile increases, thus allowing the amphiphile to penetrate the cell membrane of the bacteria more effectively. As the head groups attach to the membrane of the bacterial cell, the linkers may help drive the amphiphile through the membrane to induce cell lysis of the bacterial membrane (Lombardo *et al,* 2015).

The compounds with longer linker lengths were more effective at disrupting the Gram-positive membranes, resulting in lower MICs. Gram-negative bacteria are generally less penetrable by antimicrobials because they have two lipid bilayer membranes with a tightly packed lipid polysaccharide (LPS) layer in the outer membrane (Khalil *et al.*, 2008), consistent with generally higher MIC values.

Synergy between cationic amphiphiles and antibiotics on *E. coli* was investigated. Although there was not synergy with any of these combinations, synergy has been observed with other compounds of similar structure. Other studies have indicated that amphiphiles can increase effectiveness of antibiotics (Purdy *et al,* 2009), and will be investigated further.

There are several avenues of investigation that could broaden this study. First, studies on increased linker length would be reasonable. Combination therapies were explored in this study, but further investigation should be done because synergy between amphiphiles and antibiotics has been documented (Purdy *et al,* 2009). The rate of killing of these amphiphiles is also worth further research because developing a potent amphiphile that works quickly would be preferable for product development. Such endeavors would help expand the understanding of structural features that optimize the antibacterial activity of dialkyl 4,4'-bipyridium amphiphiles.

When developing antimicrobials for disinfectant purposes, it is very important to consider the factors that affect resistance in order to combat healthcare-acquired infections. It is important that the antibacterial agent does not harm eukaryotic cells. A parallel avenue to study would be to determine the toxicity of these amphiphiles on eukaryotic cells. Ideally, a disinfectant would selectively target bacterial membranes, which would be of great interests to hospitals in preventing healthcare-acquired infections. Medical professionals must stop over-prescribing antibiotics that are ineffective against bacteria and embrace effective antiseptics and disinfectants such as amphiphiles. While bacteria are becoming increasing resistant to commercially available antimicrobials, research is moving towards novel approaches to combat antibiotic resistance and amphiphiles are one avenue worth exploring.

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