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The antimicrobial and biofilm disruption activity of novel amphiphiles

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The Antimicrobial and Biofilm Disruption Activity of Novel Amphiphiles

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A thesis submitted to the Graduate Faculty of
JAMES MADISON UNIVERSITY
In
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Dedication

This thesis is dedicated to my friends and family who have supported me throughout my time at James Madison University. In particular, I would like to dedicate this work to my mother, Mary Rogers, and my father, Malcolm Rogers, for instilling in me a strong sense of curiosity and a desire to learn. I would also like to thank Scott Oslin for being a constant friend and confidant for the past six years and offering endless encouragement when things got hard.
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Abstract

Antibiotic resistant infections are responsible for approximately 23,000 deaths every year in the United States alone. The formation of bacterial biofilms makes resistant bacteria difficult to eliminate completely using chemical treatment. Therefore, novel antimicrobial compounds such as amphiphiles are essential to slow or stop the spread of resistant bacteria. Several novel series of amphiphiles were synthesized, and discrete aspects of their chemical structure were altered to investigate the relationship between structure and antibacterial activity. Minimum inhibitory concentration (MIC) assays were used to measure antibacterial activity against two Gram-negative and five Gram-positive bacteria, and the most effective compounds were tested for biofilm disruption activity against *Pseudomonas aeruginosa* using a Crystal Violet assay. For the tris-cationic M-E,n,n; M-DMAP,n,n; M-IQ,n,n; and M-4PP,n,n series, twelve carbons per tail was the ideal tail length for antibacterial activity, having MIC values as low as 4-8 μM against Gram-negative species and 2-4 μM against Gram-positive species. The 12-carbon derivatives also disrupted up to 70% of established *P. aeruginosa* biofilms at concentrations comparable to tobramycin and benzalkonium chloride. Hofmeister counterion substitution of one single-tailed and one double-tailed compound (M-1,1,18 and M-1,12,12, respectively) resulted in a notable 8-fold decrease in the MIC against *P. aeruginosa* when the bromide counterion was replaced with chloride, and a 16-fold decrease when the counterion was substituted with iodide. Finally, in order to determine the effect of charge distribution in the head group, three bis-cationic (oX-n,n; mX-n,n; and pX-n,n) and three tetra-cationic series (oX-(2,n)_2; mX-(2,n)_2; and pX-(2,n)_2) of double-tailed amphiphiles of varying tail lengths were synthesized and tested for their
MIC values. For all three series of bis-cationic amphiphiles, as well as the mX-(2,n)₂ and pX-(2,n)₂ series, the 12-carbon derivatives had the lowest MIC values. However, the oX-(2,n)₂ series had an ideal tail length of 8 carbons per tail. This further understanding of the relationship between structure and function of antimicrobial amphiphiles can be used to create more effective disinfectants and antibiotics. Continuing research into novel antibacterial compounds and biofilm disrupting agents is essential to combat the growing problem of antibiotic resistance.
Chapter 1. Introduction

1.1 Antibiotic- and Disinfectant-Resistant Bacteria

Bacteria that are resistant to commonly used antibiotics and disinfectants cause over 2 million illnesses and 23,000 deaths each year in the United States alone (CDC, 2013). Several strains of resistant bacteria have emerged since the development of antibiotics. *Staphylococcus aureus* is well-known for its ability to resist killing by antibiotics, and Methicillin-resistant strains of *S. aureus* (MRSA) have been implicated in community-acquired infections after decades of being historically limited to a healthcare settings (Chambers and DeLeo, 2009). Gram-negative bacteria can resist antibiotics by, among other methods, altering the makeup and permeability of their outer membrane (Sawai et al. 1979). *Pseudomonas aeruginosa* is a ubiquitous organism that has demonstrated resistance to certain compounds partly due to its ability to enzymatically break down these compounds after they enter the cell (Ali et al. 2015; Ma et al. 1998). Bacteria such as *Escherichia coli* further resist centimide and other antibiotics by forming highly resilient biofilms (Evans et al. 1990).

The development of resistance can be affected – and often accelerated – by human activity. The overuse of antibiotics has been shown to lead to an increase in the incidence of highly resistant organisms, particularly in hospitals (Dancer et al. 2009). Resistance to some of the most effective antibiotics such as vancomycin and colistin, often used to treat highly resistant infections, has also recently begun to emerge (Liu et al. 2016; Cardile et al. 2015; Schrenk et al. 2015). Despite this increasing emergence of resistant infections, antibiotics continue to be overprescribed in the United States and worldwide. The consumption of antibiotic drugs increased 36% worldwide between 2000 and 2010,
including increases in the prescription of last-resort antibiotics such as polymixins and carbapenems (van Boeckel et al. 2014). Additionally, as many as 60% of the antibiotics prescribed in United States intensive care units may not be the optimal agent for eradication of the infection that is being treated, and the choice of treatment may be incorrect in as many as 50% of cases (CDC, 2013; Luyt et al. 2014). In fact, in many cases of infection, the causative agent is not positively identified at all (Bartlett et al. 2013), increasing the risk of inappropriate therapy contributing to the development of resistance.

The majority of antibiotics sold in the United States, however, are used in agriculture rather than in medicine in order to increase the growth and yield of domesticated animals (Bartlett et al. 2013). The first case of plasmid-mediated resistance to the last-resort antibiotic, colistin, carried on the mcr-1 gene, was traced back to the antibiotic’s use in Chinese pig farms as a growth promoting treatment (Liu et al. 2016). These resistant bacteria can reach consumers through animal products such as meat (Bartlett et al. 2013), but can also be dispersed through secretion in urine and stool into groundwater and fertilizer (Bartlett et al. 2013; CDC 2013). The same gene has been found in multiple pathogenic bacteria such as *Klebsiella pneumoniae* (Nicolet et al. 2016) and *E. coli* (Poirel et al. 2016). The dispersal of resistance genes between different species and even genera or families leaves some pathogens unable to be treated with any known antibiotic.

The widespread and inappropriate use of antibiotics has led to an increase the demand for novel antimicrobial compounds, particularly in hospital settings where resistant infections can spread rapidly within immunocompromised populations. These healthcare-associated, or nosocomial infections, are often associated with invasive medical devices
such as ventilators and catheters, and can be extremely difficult to treat due to several bacterial resistance genes and virulence factors that decrease susceptibility to common antibacterial agents (CDC, 2014).

1.2 Biofilms and Transmission of Resistant Bacteria – Implications for Nosocomial Infection

    Many diverse virulence factors may contribute to the resistance of pathogenic bacteria to eradication by standard antimicrobials, but one that is particularly significant to the spread of infections within hospitals is the formation of biofilms. A biofilm is a collection of bacterial cells that have adhered irreversibly to a surface within a heterogeneous extracellular polysaccharide matrix (Figure 1), which may compose as much as 90% of the organic material of the biofilm. Although the physical characteristics of the biofilm itself are highly variable, bacteria living within a biofilm tend to have a decreased metabolic rate, as well as increased resistance to antibacterial compounds and the host immune response (Donlan, 2002; Pawar et al. 2015). In addition, bacteria within biofilms have also been shown to acquire new resistance at a faster rate than planktonic cells (Gillings et al. 2009).

    Biofilm formation begins when bacteria attach at first reversibly, and later irreversibly, to a surface. At this stage, characteristics of the surface itself have significant

![Figure 1. Scanning electron microscope image of Staphylococcus aureus biofilm. Bacteria are surrounded by a web-like extracellular matrix which protects them from desiccation and chemical stressors. (Photo from the CDC Public Health Image Library)](image-url)
influence on the rate of biofilm development, with rougher and more hydrophobic materials generally fostering more rapid growth (Fletcher et al. 1979; Quirynen et al. 2000). Characteristics such as the presence of fimbriae (Rosenberg et al. 1982) or flagella (Korber et al. 1989) also affect the initial attachment of bacteria to surfaces during biofilm formation. Irreversibly attached cells form highly hydrated extracellular polysaccharides, forming a heterogeneous matrix that includes filter-like channels which transport nutrients throughout the biofilm (Lewandowski, 2000).

Established biofilms are difficult to remove using established chemical cleaning protocols, often requiring extensive physical means of dispersal or harsh chemical sterilization for complete removal (Vickery et al. 2004, 2012; O’Donnell et al. 2005). Failure to completely remove these biofilms from hospital equipment such as catheters can contribute to nosocomial infection by providing a route of infection to an opportunistic pathogen. Cancer patients, for example, are particularly susceptible to *Staphylococcus* infection through semi-permanent catheters such as peripherally inserted central catheter (PICC) lines (Schrenk et al. 2015). Endoscopes serve as a major route of nosocomial infection due to biofilm adherence; *P. aeruginosa* is often transmitted between patients following incomplete cleaning of endoscopes between procedures. However, although human error may contribute to a large portion of infection cases, even strict compliance with cleaning protocol has been found to result in roughly a 2% bacterial contamination rate in endoscopes deemed ready for use in patients (Vickery et al. 2004). Therefore, current decontamination procedures are unable to completely eradicate all biofilms or prevent transmission of infection between patients, even without the addition of human error.
P. aeruginosa and S. aureus, two opportunistic pathogens with high affinity for biofilm formation, are also associated with additional complications following surgery. Bacteria with higher capacity for biofilm formation tend to be associated with longer recovery time and more complications following endoscopic surgical procedures (Bendouah et al. 2006). S. aureus has also been implicated in cases of septic arthritis and graft failure following ligament reconstruction surgery, as well as surgical site and bone infections (Hiller et al. 2015; Wright and Nair, 2010; Humphreys et al. 2016). It is important to note, however, that S. aureus and even P. aeruginosa have been observed as part of the normal skin and nasal flora in healthy individuals, suggesting that other factors influence virulence in addition to high affinity for biofilm formation (Al-Shemari et al. 2007).

P. aeruginosa is a prominent pathogen in hospital settings, and is responsible for most cases of hospital-associated pneumonia (Brewer et al. 1996, Richards et al. 1999). Pneumonia, along with surgical site infection, is one of the leading causes of morbidity and mortality associated with nosocomial infection (CDC, 2014; Figure 2). Additionally, P. aeruginosa is highly resistant to many disinfectant compounds largely due to its

Figure 2. Type distribution of reported nosocomial infections in the United States (CDC, 2014).
production of efflux pumps and β-lactamases (Li et al. 1994). Contamination of hospital equipment such as ventilators and endoscopes can increase hospital-associated morbidity and mortality by fostering the spread of bacteria between patients, causing infections such as ventilator-associated pneumonia (VAP; Brewer et al. 1996).

Another prominent cause of nosocomial infection is *Escherichia coli*, which, along with *P. aeruginosa* and *Enterococcus* species such as *E. faecalis* and *E. faecium*, is one of the most common causes of bloodstream infections (Peleg, 2010, Olawale et al. 2011). *E. coli* is also the most common Gram-negative species implicated in urinary tract infections associated with urethral catheters (Hidron et al. 2008), and uropathogenic strains possess a variety of virulence factors aiding in persistence and infection, including several associated with biofilm formation (Jacobsen et al. 2008).

*Streptococcus agalactiae*, also commonly known as Group B streptococcus, is associated with severe meningitis in newborns, with onset possible as early as within one week of birth, or as late as 3 months of age (Guiltbert et al. 2010). Although nosocomial transmission has been mostly attributed to limited contamination by medical workers, rare outbreaks have occurred where no common health care workers could be identified, and were linked to environmental reservoirs within the neonatal intensive care unit (MacFarguhar et al. 2010).

### 1.3 Antibacterial Development

Although the demand for antibiotic drugs is high, most of the world’s most prominent scientific funding agencies allocate very little grant money to antibiotic discovery and development (Speck, 2013). Funding institutions in the United Kingdom, for example,
allocate only approximately 3.9% of total funds to antimicrobial resistance research (Head et al. 2013). In the United States, the NIH allocated slightly over 1% of its budget to antimicrobial resistance research, less than a tenth of what was allocated to biotechnology and a seventh of what was allocated to research into aging (NIH, 2016).

Additionally, the development of antibiotics with novel targets and mechanisms has slowed considerably. Two truly novel antibiotic classes with new microbial targets – daptomycin and linezolid – have been introduced in the past decade (IDSA, 2010; 2011). This is due in large part financial limitations, as research into antibiotics – which are relatively cheap and most often used short-term – is not viewed as economically profitable for pharmaceutical companies when compared to research into other drugs for chronic health conditions (Piddock, 2012; Bartlett et al. 2013). Additionally, regulatory barriers prevent the rapid delivery of novel drugs to market. Implementation of new drugs can take up to 2 decades from drug discovery to use in the general public (Figure 3). This is in part because human trials comparing the efficacy of new compounds to a placebo are often considered unethical, and so studies must rely on comparisons to existing antibiotics, necessitating large sample sizes and therefore high cost (Wright and Nair, 2014).

![Figure 3](image)

**Figure 3.** Timeline of novel drug discovery and development. Novel drug development can take up to 2 decades to complete, and involve proving safety and efficacy in humans before reaching the majority of patients in need.
One promising area of study is in the activity of antimicrobial peptides (AMPs), which are short, positively charged, amphiphilic amino acid chains produced by a wide array of organisms as part of the innate immune response (Jenssen et al. 2006). These peptides have broad-spectrum antibacterial activity, affecting the bacterial cytoplasmic membrane and causing depolarization and cell lysis (Hancock and Rozek, 2002). The bactericidal activity of these peptides relies on both the charge of the molecule and hydrophobic interactions for insertion into the membrane (Dathe and Wieprecht, 1999). Many bacteria remain sensitive to killing by AMPs despite frequent exposure to these peptides, likely due to the fact that many mutations conferring resistance to AMPs require a high metabolic cost (Nizet, 2006). Despite the broad spectrum of efficacy of AMPs, the widespread implementation of AMPs in a clinical setting is limited by the high cost of large-scale AMP production (Marr et al. 2006; Findlay et al. 2010). The development of wholly synthetic AMPs provides a potential way to circumvent the high cost of AMP production, and amphiphilic AMP mimics lacking natural amino acids still have broad-spectrum antibacterial activity against both Gram-negative and Gram-positive organisms (Mowery et al. 2007; Scott et al. 2008). Quaternary ammonium compounds are simple amphiphilic mimics of AMPs that are easier to produce, but still retain broad-spectrum antibacterial and anti-biofilm activity (Jennings et al. 2014).

1.4 Amphiphiles

Amphiphiles are compounds that possess both a hydrophobic and a hydrophilic portion on a single molecule (LaDow et al. 2011; Figure 4). The hydrophilic portion of the molecule is charged, and cationic (positively-charged) amphiphiles have been the focus of study for purposes of biofilm disruption due to the potential for interference with
the negatively charged extracellular matrix produced by bacteria (Jennings et al. 2014). Such interactions would likely be species-independent and broad-spectrum, allowing for a wide range of uses. Additionally the sheer number of possible structures available would promote a higher diversity of effective compounds to protect against the development of resistance resulting from the overuse of any single compound.

The antibacterial activity of these types of compounds was first noted in 1935, and they are still commonly used as disinfecting agents and detergents (Gerhard, 1935). Amphiphiles have a wide array of possible structures, and some of the most promising are quaternary ammonium compounds (Forman et al, 2016). Ample research exists investigating the relationship between amphiphile structure and antibacterial activity (LaDow et al. 2011; Marafino et al. 2015; Song et al. 2011; Palermo et al. 2009). Previous studies have provided information about the effect of cationic head group substitution and hydrophobic hydrocarbon chain length on the minimum inhibitory concentration (MIC) of certain compounds on bacteria including *P. aeruginosa* and *S. aureus*. Structural changes such as hydrocarbon tail length variation, tail symmetry, and changes in the charge and structure of the hydrophilic region have been studied for their effects on antibacterial activity (Marafino et al. 2015; LaDow et al. 2011; Paniak et al. 2014). In particular, the size of the hydrophobic region appear to be the strongest indicator of membrane permeability and antibacterial activity, with changes in the charge
and size of the hydrophilic region having less of an effect on the effectiveness of QACs (Paniak et al. 2014).

Cationic amphiphiles interact strongly with the negatively charged phospholipids that make up 20-25% of the membrane (Glukov et al. 2005). The primary mechanism of action of these amphiphiles is disruption of the bacterial cell membrane, leading to disruption of transport proteins and proton motive force, cell lysis and death (Hoque et al. 2012; Thiyagarajan et al. 2014; Ladow et al. 2011). As the lipid membrane is ubiquitous and energetically costly to repair, QACs offer broad-spectrum efficacy and little potential for the development of total resistance (Vudumula et al. 2012). This is particularly promising for the treatment of Gram-negative pathogens, whose additional outer membrane can decrease their susceptibility to several antibiotics unable to pass through the additional barrier (Bola et al. 2011).

The biofilm disruption capabilities of amphiphilic compounds have also been the focus of a large amount of research. Previous work has suggested that amphiphilic polymers containing amide side chains are not only effective bactericidal agents against antibiotic-resistant strains of bacteria such as MRSA and vancomycin-resistant Enterococcus faecium (VRE), but were also more effective at disrupting both non-resistant and multidrug-resistant Acinetobacter baumanii biofilms than established antibiotics such as erythromycin, tobramycin, and colistin (Uppu et al. 2016). Amphiphiles with different structures, including QACs, have also exhibited biofilm-disruption activity. The likely mechanism of action of biofilm disruption by these compounds involves biofilm dispersal by electrostatic interactions which allow the
compounds to lyse the planktonic cells released from the biofilm, leading to biofilm disruption due to the death of bacterial cells (Jennings et al. 2014).

1.5 Amphiphiles Used in the Current Study

*Head group substitution and hydrocarbon tail length variation*

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 dimethylethanolammonium 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-propanol pyridinium for the M-4PP,n,n series. The hydrocarbon tails were linear and symmetrical, and consisted of 8-16 carbons each for the

![Structure of amphiphiles.](image)

*Figure 5. Structure of amphiphiles. M represents the Mesitylene core; n represents the number of carbons in each symmetrical hydrocarbon tail. E = dimethylethanolammonium, DMAP = dimethylaminopyridinium, IQ = isoquinolinium, and 4PP = 4-propanol pyridinium.*
M-E,n,n series, 10-16 carbons each for the M-DMAP,n,n series, 10-12 carbons each for the M-IQ series, and 10-14 carbons each for the M-4PP,n,n series (Figure 5).

Counterion exchange

Two tris-cationic amphiphiles were synthesized by the Caran lab in order to determine the effects of counterion substitution on antibacterial activity. One single-tailed compound (M-1,1,18) and one double-tailed compound (M-1,12,12), which have both been previously studied (Marafino et al 2015), were used. Both compounds consisted of three positively charged dimethylalkylammonium residues attached to a mesitylene core, with a linear 18-carbon chain attached to one of these groups in M-1,1,18, and a linear 12-carbon chain attached to each of two of the groups in M-1,12,12 (Figure 6). The standard bromide counterions were substituted with carbonate, acetate, nitrate, chloride, or iodide for M-1,1,18, and substituted with acetate, chloride, or iodide for M-1,12,12. The structure of the amphiphiles themselves was not changed with these substitutions.

![Figure 6. Structure of amphiphiles.](image)

Spacer variation and hydrocarbon tail length variation
Three series of bis-cationic and three series of tetra-cationic double-tailed amphiphiles were synthesized by the Caran lab in order to determine the effects of varied placement of the headgroups and tails on the central mesitylene ring. The bis-cationic oX-, mX-, and pX-n,n series consisted of two dimethylalkylammonium residues attached to the central ring in either the ortho (oX-n,n series), meta (mX-n,n series) or para orientation (pX-n,n series). One linear hydrocarbon chain was attached to each of the two dimethylalkylammonium groups, and contained 8-14 carbons for the oX-n,n series, 8-12 carbons for the mX-n,n series, and 8-16 carbons for the pX-n,n series (Figure 7).

![Figure 7. Structure of bis-cationic amphiphiles.](image)

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 8-12 carbons for the oX-, mX-, and pX-(2,n)2 series (Figure 8).
Additional compounds

One series of amphiphiles was synthesized by the Caran lab including 6 total dimethylalkylammonium head groups and three linear hydrocarbon tails of 8, 10, or 12 carbons in length (Figure 9). The hexa-cationic M-(2,n)_3 series was tested for its antibacterial activity with tail length variation.

The final compound synthesized by the Caran lab was 10-14-AO, a mono-cationic fluorescent compound derived from acridine orange that included a single 14-carbon tail (Figure 10).
Figure 9. Structure of the 6-headed, triple-tailed M-(2,n) series. n represents the number of carbons per tail (8,10,12).

Figure 10. Structure of the fluorescent acridine orange derivative, 10-14-AO.
Chapter 2: The effect of tail length variation and head group substitution on minimum inhibitory concentration and biofilm disruption.

2.1 Introduction.

Bacteria that are resistant to commonly used antibiotics and disinfectants cause over 2 million illnesses and 23,000 deaths each year in the United States alone (CDC, 2013). The development of resistance can be affected – and often accelerated – by human activity. The overuse of frequently used antibiotics has led to an increase in the incidence of highly resistant organisms, particularly in hospitals (Dancer et al. 2009). Resistance to powerful antibiotics such as vancomycin and colistin, often used to treat highly resistant infections, has also recently emerged (Liu et al. 2016; Cardile et al. 2015; Schrenk et al. 2015).

The accelerated development of resistance in ubiquitous organisms has contributed to the increased prevalence of nosocomial infections, which spread rapidly within immunocompromised populations often found in hospitals and nursing homes (Johnston and Bryce, 2009). Nosocomial infections such as pneumonia, bloodstream infections, and urinary tract infections are responsible for approximately 75,000 patient deaths every year (Magill et al. 2014) and place a significant economic burden on the United States healthcare system (CDC, 2013). Gram-negative bacteria can further resist antibiotics by altering the makeup and permeability of their outer membrane in addition to having other adaptations for resistance (Sawai et al. 1979). *Pseudomonas aeruginosa* is a ubiquitous organism that has demonstrated resistance to certain compounds partly due to its ability to enzymatically break down these compounds after they enter the cell (Ali et al. 2015; Ma et al. 1998). Bacteria such as *Escherichia coli* resist centimide and other
antibiotics by forming biofilms (Evans et al. 1990). The development of novel antibacterial compounds is essential to curb the spread of resistant infections between patients and to reduce hospital-associated morbidity and mortality.

Amphiphiles are a diverse class of compounds with well-documented antibacterial effects (Marafino et al. 2015; Ladow et al. 2011; Goswami et al. 2015; Thiyagarajan et al. 2014; Song et al. 2011). The antibacterial activity of these compounds was first noted in 1935, and they are still commonly used as disinfecting agents and detergents (Gerhard, 1935). Amphiphiles have a wide array of possible structures, and some of the most promising are quaternary ammonium compounds, which are molecules containing positively charged nitrogen residues (Forman et al. 2016). In addition to the charged hydrophilic head group, amphiphiles also contain a hydrophobic portion, usually consisting of one or more hydrocarbon tails. Structural changes such as hydrocarbon tail length variation, symmetry, and changes in the charge and structure of the hydrophilic region have been studied for their effects on antibacterial activity (Marafino et al. 2015; Ladow et al. 2011; Paniak et al. 2014). In particular, the size of the hydrophobic region appear to be the strongest indicator of membrane penetration and antibacterial activity, with changes in the charge and size of the hydrophilic region having less of an effect on the effectiveness of QACs (Paniak et al. 2014). Increased understanding of the effects of amphiphile structure on bactericidal activity is essential for the development of more effective antibacterial compounds.

Four novel series of amphiphiles were synthesized, and the antibacterial effect of changes in head group structure and tail length was determined. All four series consisted of three cationic head groups connected to a mesitylene core. For each series, two of the
head groups were dimethylalkylammonium groups, each connected to a linear hydrocarbon tail. The hydrocarbon tails were symmetrical and ranged in length from 8-16 carbons for the M-E series, 10-16 carbons for the M-DMAP series, 10-12 carbons for the M-IQ series, and 10-14 carbons for the M-4PP series. The third head groups were an dimethylethanolammonium (M-E series), dimethylaminopyridinium (M-DMAP series), isoquinolinium (M-IQ series), or 4-propanol pyridinium (M-4PP series; Figure 5).

2.2 Results and Discussion.

Critical Aggregation Concentration

The critical aggregation concentration (CAC) refers to the concentration at which the molecules aggregate in solution. Below the CAC, the amphiphile tends to behave as a monomer, while above the CAC, the amphiphile behaves as a micelle. The CAC was determined for the 10-16 carbon chain length derivatives of the M-E series. Consistent with previous studies of amphiphiles with similar structures (Marafino et al. 2015), the CAC decreases with increasing tail lengths, suggesting that a longer pair of hydrophobic hydrocarbon tails encourages aggregation into micelles at lower concentrations (Figure 11).
Information about the CAC can also be used to determine more about the mechanism of action of these compounds, namely whether they act as monomers to exert a bacteriocidal effect, or if they act more similarly to detergents (as micelles). Because the MIC of all of the M-E series derivatives tested was below the CAC (Table 1, Table 2), it is likely that these compounds are acting as monomers at bactericidal concentrations, and do not have a mechanism of action similar to detergents. This supports previous research on similar compounds, where tris-cationic double-tailed amphiphiles with trimethylammonium or pyridinium head groups also had MICs that fell far below their respective CAC values (Marafino et al. 2015).

**Minimum Inhibitory Concentration**

The minimum inhibitory concentration (MIC) was determined for two Gram-negative bacterial species (*P. aeruginosa* and *E. coli*) and five Gram-positive species (*S. aureus, E. faecalis, S. agalactiae, B. subtilis, and B. anthracis*; Table 1, Figure 12). For
all series (with only one exception) the MIC was lowest at a tail length of 12 carbons per tail. Above or below this optimal tail length, the MIC increased. This trend was observed for both Gram-negative and all Gram-positive bacteria tested. A similar trend was observed for all head group substitutions, with 12 carbons being the ideal tail length for structures with an dimethylethanolammonium, dimethylaminopyridinium, isoquinolinium, or 4-propanol pyridinium head group (Figure 12).

The 12-carbon compound M-E,12,12 had MIC values ranging from 2-4 μM for Gram-positive bacteria, and 4-8 μM for Gram-negative bacteria. M-DMAP,12,12 had an MIC of 8 μM for both Gram-negative species, and MIC values ranging from 2-8 μM for Gram-positive species. MIC values for M-4PP,12,12 ranged from 2-4 μM for all bacteria tested, and those for M-IQ,12,12 ranged from 4-8 μM. Both M-4PP,12,12 and M-IQ,12,12 were able to effectively inhibit the growth of *P. aeruginosa* at concentrations as low as 4 μM, a concentration comparable to the antibiotic tobramycin, an aminoglycoside widely used to treat chronic *P. aeruginosa* infection (Shawar et al. 1999).

The trends observed for these compounds are consistent with previous research on structurally similar amphiphiles. Derivatives with two 12-carbon chains in the tail region have previously been reported to have lower MIC values than those with longer or shorter tails (Marafino et al. 2015).
Table 1. MIC values for all compounds, benzalkonium chloride (BZK), and tobramycin. Values were confirmed with 2-3 independent experiments. X = head group (E = dimethylethanolammonium; DMAP = dimethylanminopyridinium; IQ = isoquinolinium; 4PP = 4-propanol pyridinium) and n = the number of carbons per tail. G+ = Gram-positive and G- = Gram-negative.

<table>
<thead>
<tr>
<th>Compound (M-X,n,n)</th>
<th>Minimum Inhibitory Concentration (μM)</th>
<th>P. aeruginosa (G-)</th>
<th>E. coli (G-)</th>
<th>E. faecalis (G+)</th>
<th>S. aureus (G+)</th>
<th>B. subtilis (G+)</th>
<th>S. agalactiae (G+)</th>
<th>B. anthracis (G+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-E,8,8</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>63</td>
<td>250</td>
<td>250</td>
<td>125</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>M-E,10,10</td>
<td>250</td>
<td>125</td>
<td>4</td>
<td>125</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>M-E,12,12</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>M-E,14,14</td>
<td>125</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>M-E,16,16</td>
<td>125</td>
<td>63</td>
<td>63</td>
<td>63</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td>M-DMAP,10,10</td>
<td>63</td>
<td>63</td>
<td>8</td>
<td>63</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>M-DMAP,12,12</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>2</td>
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<tr>
<td>M-DMAP,14,14</td>
<td>16</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
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<tr>
<td>M-DMAP,16,16</td>
<td>250</td>
<td>31</td>
<td>8</td>
<td>16</td>
<td>16</td>
<td>8</td>
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</tr>
<tr>
<td>M-4PP,10,10</td>
<td>8</td>
<td>4</td>
<td>4</td>
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<td>2</td>
<td>2</td>
<td>2</td>
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<tr>
<td>M-4PP,12,12</td>
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<tr>
<td>M-4PP,14,14</td>
<td>31</td>
<td>16</td>
<td>4</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>M-IQ,10,10</td>
<td>125</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>M-IQ,12,12</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>BZK</td>
<td>8</td>
<td>31</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Replacing the hydrophilic dimethylethanolammonium residue with dimethylaminopyridinium, isoquinolinium, or 4-propanol pyridinium did not significantly change the trend in MIC. Other structurally similar 12-carbon derivatives with different head groups (trimethylammonium and pyridinium) studied previously have had comparable antibacterial activity, further illustrating the effect of tail length on the efficacy of these double-tailed amphiphiles (Marafino et al. 2015). Taken together, these data suggest that substitution with equally charged head groups of comparable size does not have a considerable effect on antibacterial activity, and that tail length has a much larger effect on the ability of these compounds to inhibit bacterial growth.
Structurally, these compounds are similar to benzalkonium chloride (BZK), a synthetic quaternary ammonium biocide with broad-spectrum antibacterial activity (Carson et al. 2008; Fazlara and Ekhtelat, 2012). Interestingly, all of the 12-carbon derivatives, as well as some 10- and 14-carbon derivatives tested have greater antibacterial activity than BZK against Gram-negative and Gram-positive species (Fazlara and Ekhtelat, 2012).

All four series of tris-cationic, double-tailed amphiphiles presented in this study follow the previously illustrated trend suggesting a strong relationship between hydrocarbon tail length and antibacterial activity. Amphiphiles with 12 carbons in each symmetrical tail were the most effective at inhibiting all species of bacteria tested, supporting the findings of previous work (Marafino et al. 2015).
Figure 12. The effect of tail length on antibacterial activity of the M-E,n,n; M-DMAP,n,n; M-IQ,n,n; and M-4PP,n,n series. G- = Gram-negative and G+ = Gram-positive. Bacterial strain names are...
Biofilm disruption.

Established *P. aeruginosa* biofilms were exposed to two-fold dilutions M-E,12,12; M-DMAP,12,12; M-IQ,12,12; and M-4PP,12,12. The percentage of biofilm disruption increased in a concentration-dependent manner from 2-63 µM for all amphiphiles tested, but at concentrations above 63 µM, increasing the compound concentration did not increase biofilm disruption. The amphiphiles tested disrupted a maximum of approximately 65% of established biofilm, with the exception of M-4PP,12,12, which had a maximum disruption of approximately 40% (Figure 13).

This disruption activity was compared to that of BZK and the aminoglycoside tobramycin. At similar concentrations, tobramycin disrupted approximately 66% of established biofilm, while BZK disrupted approximately 35-40% (Figure 13).

Bacterial biofilms are commonly associated with greatly increased resistance to biocides due to the formation of a protective extracellular matrix and reduced metabolic rate of biofilm-associated cells (van der Veen and Abee, 2010; Costerton et al. 1999). Due to this increased resistance compared to planktonic cells, it is unsurprising that the concentration of biocide necessary to attain maximum biofilm disruption is several dilutions higher than the MIC, which was determined against planktonic cells.
Figure 13. *P. aeruginosa* biofilm disruption by (A) M-E,12,12; (B) M-DMAP,12,12; (C) M-4PP,12,12; (D) M-IQ,12,12; (E) tobramycin and (F) BZK. Error bars represent standard deviation of three independent trials.
2.3 Conclusions.

These data further support the relationship between solubility and antibacterial activity, suggesting that there is an ideal level of solubility that may maximize bactericidal efficiency. Of particular note are the MIC values of M-E,12,12; M-DMAP,12,12; M-4PP,12,12; and M-IQ,12,12. These compounds are effective at inhibiting the growth of *P. aeruginosa* at concentrations as low as 4-8µM (Table 1). *P. aeruginosa* is a prominent pathogen in hospital settings, and is responsible for most cases of hospital-associated pneumonia (Brewer et al. 1996; Richards et al. 1999). Additionally, *P. aeruginosa* is highly resistant to many disinfectant compounds due to its semipermeable outer membrane and the production of efflux pumps and β-lactamases (Li et al. 1994). Contamination of hospital equipment such as ventilators and endoscopes can increase hospital-associated morbidity and mortality by fostering the spread of bacteria between patients, causing infections such as ventilator-associated pneumonia (VAP; Brewer et al. 1996). Four compounds mentioned effectively inhibit the growth of *P. aeruginosa* at concentrations comparable to the MIC of tobramycin, an antibiotic used to treat *P. aeruginosa* infection in cystic fibrosis patient (Oliver et al. 2000; Singh et al. 2000; Vidya et al. 2016). Given this, as well as the fact that the MIC values of the most effective compounds were lower than those of the disinfectant, benzalkonium chloride, these compounds may prove useful in a hospital setting as novel disinfectants to prevent the spread of nosocomial infection due to *P. aeruginosa*. Because biofilm contributes to the spread and pathogenesis of *P. aeruginosa*, the potential for synergistic interaction between the compounds presented in this study and biofilm matrix-degrading enzymes such as dispersin B warrants future study (Kaplan, 2010).
Further research is needed to understand the mechanism of action of these compounds against bacterial biofilms, as it is uncertain whether they are actively interacting with the extracellular biofilm matrix or merely penetrating the matrix to target the bacterial cells embedded in the biofilm. The solubility and hydrophobicity of the EPS is highly variable, and it likely possesses localized hydrophilic and hydrophobic regions, making it unclear to what extent these amphiphiles are able to interact with the matrix effectively (Sutherland, 2001).

Given the increasing incidence of hospital acquired infection, particularly by highly resistant strains of bacteria, the need for novel antibacterial compounds is paramount, and understanding structure-function relationships of bactericidal molecules is necessary for the development of more effective disinfectants. Several compounds presented in this study show potential as novel disinfectants, either alone or in synergistic combination with other disinfecting agents or antibiotics. It is likely that these compounds behave similarly to other amphiphiles against planktonic cells by targeting the bacterial membrane (Goswami et al. 2015; Thiyagarajan et al. 2014; Song et al. 2011). Gram-negative bacteria are particularly difficult to eradicate due to the presence of an outer membrane serving as a selectively permeable barrier to chemical stressors (Bolla et al. 2011). Membrane-permeabilizing agents, therefore, may lead to enhanced susceptibility to antibiotics not normally able to bypass this barrier. The results presented in this work provide information on the relationship between amphiphile structure and function, and may lead to the development of novel disinfecting agents that could decrease hospital-associated morbidity and mortality as a result of bacterial transmission.
2.4 Methods

Colloidal properties

A conductivity probe was used to measure the critical aggregate concentration of the amphiphile. The solution was heated to 37°C. The probe was mixed in the solution and the measurements were collected. Ten percent of the solution was pipetted out and replaced with nanopure water. The solution was mixed and the conductivity measurement recorded. These steps were repeated for the entirety of the experiment.

Bacterial strains and growth conditions.

The two Gram-negative bacterial strains used were *Escherichia coli* ATCC® 25922™ and *Pseudomonas aeruginosa* ATCC® 27853™, and the five Gram-positive bacterial strains used in this study were *Staphylococcus aureus subsp. aureus* ATCC® 29213™, *Enterococcus faecalis* ATCC® 29212™, *Bacillus subtilis*, *B. anthracis* Sterne, and *Streptococcus agalactiae* J48 (Seifert et al. 2006). All strains were grown in 1x Mueller-Hinton Broth at 37°C for 12–24 h. For the MIC studies, bacterial suspensions were prepared by diluting overnight cultures to 5x10⁶ CFU/mL in 2X Mueller-Hinton Broth, so that when amphiphile solutions were added the final broth strength was 1X.

Minimum inhibitory concentration.

The methods used to determine the MIC were performed as previously described and followed standards of the Clinical and Laboratory Standards Institute (Ladow et al. 2011; CLSI, 2012). Briefly, compounds were serially diluted in sterile deionized water and 100µL of each dilution were added to the wells of a 96-well flat-bottomed microtiter plate in triplicate. After adding 100µL of the bacterial cell suspension (5x10⁶ CFU/mL),
the plates were incubated at 37°C for 72 h. The MIC of the compound was defined as the minimum concentration that resulted in visible inhibition of bacterial growth.

**Biofilm disruption**

Biofilm disruption was determined as previously described (O’Toole, 2011). Briefly, 100µL of 5x10^6 CFU/mL *P. aeruginosa* suspended in Luria Broth (LB) were added to a 96-well flat-bottomed microtiter plate. Plates were incubated at 37°C for 24 h to allow the biofilm to form. Sterile dd H_2O served as a negative control for biofilm disruption, and 100µL of each dilution of the compounds were added in triplicate to the remaining wells. Plates were incubated at 37°C for another 24 h, after which the plates were emptied and rinsed with sterile dd water and allowed to dry. Wells were stained with 100µL crystal violet, rinsed with gently running water, and allowed to dry. Finally, 100µL of 95% ethanol was added to each well for 1 h. The ethanol-crystal violet solution was transferred to a fresh 96-well microtiter plate, and the absorbance value at 570 nm was determined.
Chapter 3: The effect of Hoffmeister series counterion exchange on minimum inhibitory concentration of single- and double-tailed amphiphiles.

3.1 Introduction.

Counterions accompany charged molecules in solution in order to maintain electric neutrality. These counterions can have an effect on several aspects of the interaction between molecules in solution, most notably on micellization of surfactants (Naskar et al. 2012). The self-assembly of amphiphiles in solution is a thermodynamically driven process that can be affected by differences in the hydrocarbon tail length and the charge and size of the hydrophilic head group. Ions in solution interact with the head group, and thereby have an effect on the assembly of monomers into micelles (Rosholm et al. 2010). These interactions are of particular interest to medicinal chemistry and drug discovery, since variation of the counterion paired with antibacterial compounds may affect antibacterial activity.

Of particular interest are counterions from the well-studied Hofmeister series (Figure 14). The Hofmeister series was originally observed by Franz Hofmeister in 1888, and was reported to be a series of salts ordered according to their ability to precipitate proteins out of solution (Hofmeister, 1888; Kunz et al. 2004; Lo Nostro et al. 2012). More recently, ions in the Hofmeister series have been correlated with several

![Figure 14. Selected counterions from the Hofmeister used in this study, presented in order from strongly hydrated kosmotropes (left) to weakly hydrated chaotropes (right).](image-url)
chemical properties of amphiphiles in solution (Rosholm et al. 2010), as well as having a documented effect on the growth of both Gram-positive and negative bacteria including *S. aureus* and *P. aeruginosa* (Lo Nostro et al. 2005).

Interestingly however, relatively little research has been done on the effects of Hofmeister series counterion substitution on the antibacterial activity of amphiphiles and similar membrane-active compounds. Therefore, the relationship between the known properties of Hofmeister series counterion exchange and amphiphile activity remains largely unknown.

It was hypothesized that changes in micelle formation and aggregation due to counterion substitution could lead to a change in MIC correlated with the chemical changes associated with the Hofmeister series. Therefore, one single-tailed amphiphile (M-1,1,18) and one double-tailed amphiphile (M-1,12,12) were selected for counterion substitution (Figure 6). These amphiphiles were selected due to their low MIC values compared to other compounds with different hydrocarbon chain lengths in their respective series (Marafino et al. 2015). Counterions from the Hofmeister series were substituted for bromide (3Br⁻) without altering the structure of either amphiphile in order to determine whether counterion exchange on its own was sufficient to cause changes in MIC.

### 3.2 Results and Discussion.

*Critical aggregation concentration*

CAC, predictably, increased with increasing chaotropicity for single-tailed derivatives paired with different counterions in the Hofmeister series. When paired in solution with iodide, M-1,1,18 formed micelles at a minimum concentration
approximately ten times lower than when paired with acetate, a more weakly hydrated kosmotrope (CAC table).

Nitrogen-based cations such as those found in these amphiphiles have been categorized previously as largely chaotropic. Chaotropes interact most strongly with other chaotropes in solution, which may explain the decrease in CAC when M-1,1,18 was paired with other more chaotropic Hofmeister ions in solution (Abezgauz et al. 2009).

Table 2. CAC values of M-1,1,18 paired with six selected Hofmeister anions.

<table>
<thead>
<tr>
<th>Counterion (M-1,1,18 + X)</th>
<th>CAC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_3^{2-}$</td>
<td>N/A</td>
</tr>
<tr>
<td>C$_2$H$_3$O$_2^{-}$</td>
<td>10.10</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>5.96</td>
</tr>
<tr>
<td>Br$^-$</td>
<td>3.13</td>
</tr>
<tr>
<td>NO$_3^{-}$</td>
<td>2.07</td>
</tr>
<tr>
<td>I$^-$</td>
<td>1.03</td>
</tr>
</tbody>
</table>

Minimum inhibitory concentration

Previously, all compounds tested in this study had bromide as the counterion; however, the effects of counterion substitution with other Hofmeister series anions had not been tested. Six Hofmeister anions (bromide, carbonate, acetate, nitrate, chloride, and iodide) were selected for counterion exchange with the single-tailed compound, M-1,1,18 (Table 3). Substitution with carbonate, acetate, or nitrate resulted in slightly increased MIC values, particularly against Gram-positive bacteria, suggesting that these counterions may cause decreased bacteriocidal efficacy for this compound. Interestingly, while acetate and carbonate are both more kosmotrophic than bromide in the Hofmeister series, nitrate is more chaotropic. It is likely that the change in antibacterial activity can
therefore not be attributed to the chemical attributes of the Hofmeister series alone when these ions are paired with these antibacterial compounds.

Chloride and iodide were also investigated as possible counterions for these amphiphiles. When paired with both of these anions, M-1,1,18 had much lower MIC values compared to M-1,1,18 paired with the other anions studied. Most notably, substitution with chloride and iodide resulted in a sixteen-fold and eight-fold decrease in MIC compared to bromide for *P. aeruginosa*, respectively, and an eight-fold decrease in MIC for *S. aureus*. While M-1,1,18 paired with bromide had an MIC of 125 µM against *P. aeruginosa*, the same compound was able to inhibit the growth of *P. aeruginosa* at 8 µM when paired with chloride and at 16 µM when paired with iodide. Counterion exchanged M-1,1,18 paired with chloride and iodide were, with very few exceptions, more effective than M-1,1,18 paired with carbonate, acetate, or nitrate (Table 3).

**Table 3.** MIC values of M-1,1,18 paired with six selected Hofmeister series anions (X): bromide, carbonate, acetate, nitrate, chloride, and iodide. Values were confirmed with 2-3 independent experiments. G+=Gram-positive and G-=Gram-negative.

<table>
<thead>
<tr>
<th>Counterion (M-1,1,18 + X)</th>
<th>Minimum Inhibitory Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td>(G-)</td>
</tr>
<tr>
<td>3 Br⁻</td>
<td>125</td>
</tr>
<tr>
<td>1.5 CO₃²⁻</td>
<td>250</td>
</tr>
<tr>
<td>3 C₂H₃O₂⁻</td>
<td>250</td>
</tr>
<tr>
<td>3 NO₃⁻</td>
<td>250</td>
</tr>
<tr>
<td>3 Cl⁻</td>
<td>8</td>
</tr>
<tr>
<td>3 I⁻</td>
<td>16</td>
</tr>
</tbody>
</table>

Despite notable changes in MIC with counterion exchange, no clear trend between Hofmeister series order and antibacterial activity was observed. The observed increase in CAC with increasing chaotropicity was not an accurate predictor of
antibacterial activity. It is possible that other unknown factors, independent of Hofmeister series attributes, may contribute to antibacterial activity for these counterion exchanged amphiphiles, especially since somewhat different trends were observed for Gram-negative versus Gram-positive species tested.

To determine whether similar trends existed with counterion exchange for double-tailed amphiphiles, the compound M-1,12,12 was paired with four different Hofmeister series anions: bromide, acetate, chloride, and iodide. Very little difference in MIC was observed for Gram-positive species, although M-1,12,12 paired with iodide did have lower MIC values compared with the compound paired with bromide. A four-fold decrease in MIC was observed against *P. aeruginosa* for the chloride- and iodide-paired compound compared to the compound with bromide serving as the counterion. Interestingly, M-1,12,12 paired with acetate had similar antibacterial activity when compared with the compound with the chloride and iodide counterions, a trend that was not observed for the single-tailed M-1,1,18 (Table 3, Table 4). Once again, no clear trend relating the Hofmeister series to antibacterial activity of counterion exchanged compounds was observed, suggesting that other factors may influence changes in antibacterial activity than colloidal or chemical attributes of Hofmeister series anions paired with antibacterial amphiphiles.
Table 4. MIC values of M-1,12,12 paired with four selected Hofmeister series anions (X): bromide, acetate, chloride, and iodide. Values were confirmed with 2-3 independent experiments. G+ = Gram-positive and G- = Gram-negative.

<table>
<thead>
<tr>
<th>Counterion (M-1,12,12 + X)</th>
<th>Minimum Inhibitory Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td>(G-)</td>
</tr>
<tr>
<td>3 Br⁻</td>
<td>16</td>
</tr>
<tr>
<td>3 C₂H₃O₂⁻</td>
<td>4</td>
</tr>
<tr>
<td>3 Cl⁻</td>
<td>2</td>
</tr>
<tr>
<td>3 I⁻</td>
<td>2</td>
</tr>
</tbody>
</table>

Previous research has suggested that more chaotropic ions may be more effective at penetrating lipid monolayers than kosmotropic ions (Zhang and Cremer, 2006), and may suggest that direct interactions between the ions and the bacterial membrane itself may be at least partially responsible for the changes in MIC, in addition to interactions between the counterions and the amphiphiles in solution. Dissolved Hofmeister anions have also been shown to affect the absorption of cationic polyions onto hydrophobic surfaces. The addition of more kosmotrophic salts such as NaCl increase electrostatic screening and allow polycations to move closer to the interface with the hydrophobic surface. Chaotropic salts such as NaI allow for significant polycationic absorption at low concentrations, but the amount of polyion absorbed at the hydrophobic interface decreases with increasing concentrations due to increased electrostatic screening of the attraction between the anions and the polyelectrolytes (dos Santos and Levin, 2013). Although these findings were limited to simulation, they support the conclusion that chaotropic Hofmeister anions such as I⁻ may increase the efficiency of the interaction between the cationic hydrophilic head residue of these amphiphiles and the lipid membrane of bacteria. It is also important to note that this does not account for the
hydrophobic tail residues, which have been shown to have a strong effect on antibacterial activity. Therefore, it is likely that several aspects of structure contribute to the overall antibacterial activity of these amphiphiles, and the ideal combination of head group, tail length, counterion, etc. may differ depending on different attributes of the bacteria such as membrane structure (Lo Nostro et al. 2005).

Continued research is necessary to fully understand how counterion exchange affects the antibacterial activity of these amphiphiles. Although a clear trend was not observed, chloride and iodide may have promise as counterions for future compounds, especially those intended for use against *P. aeruginosa*. Further study of these effects is necessary to fully understanding the mechanisms of this change in antibacterial activity, as it is possible that counterion exchange with chloride and iodide may influence other aspects of antibacterial activity, such as biofilm disruption.

3.3 Methods.

*Synthesis of M-1,1*

Preparation of M-1,1 was completed with a 1:1.8 molar equivalence of 1,3,5-Trisbromomeythylbenzene (0.0056mol, 350mL) to trimethylamine (0.01009mol, 150mL) in acetone. Both solutions are chilled on ice for 15min. After chilling, the trimethylamine solution was added drop wise to the 1,3,5-Trisbromomeythylbenzene solution overnight, while on ice. The product of the reaction was then filtered and recrystallized using a 100:3 solution of acetone to ethanol (100mL per 27mg M-1,1). Synthesis of the product confirmed using $^1$HNMR.
Synthesis of $M$-1,1,18

Preparation of $M$-1,1,18 was completed using a 1:1.5 mole ratio of $M$-1,1 to dimethyl-octadecylamine respectively. The reaction was completed in ethanol and left at 80°C under reflux overnight. The product was dried under N$_2$ gas, re-suspended in acetone, and filtered. The resulting solid was recrystallized using ethanol and acetone. Synthesis of the product was confirmed using $^1$HNMR.

Counterion exchange of $M$-1,1,18

Monovalent counter ion exchange was done with a 1:3.6 molar equivalence of $M$-1,1,18 to the corresponding AgX salt (X=NO$_3^-$, C$_2$H$_5$O$_2^-$, I$,^-$, and SCN$^-$). While divalent counter ion exchange was done with a 1:1.8 molar equivalence of $M$-1,1,18 to the corresponding Ag$_2$X salt (X=CO$_3^{2-}$). The reaction was done in methanol (45mL per 250mg $M$-1,1,18), stirring overnight under light free conditions. The resulting product was filtered through a celite packed filter and rinsed with 50 mL of excess methanol. The filtrate was rotovapped down at temperatures below 50°C. The resulting solid was resuspended in 5mL methanol and centrifuged for 3min. A small partition of the supernatant was combined with AgNO$_3$ and the resulting precipitate was tested on the PXRD. The absence of major peaks at 31 and 44.3 indicated that no remaining AgBr was present in solution. The supernatant was then rotovapped down and the resulting solid was put in the desiccator with P$_2$O$_5$ to remove any water present. The presence of the correct ion exchanged compound was confirmed via HRMS and $^1$HNMR.

Critical aggregation concentration
A conductivity probe was used to measure the critical aggregate concentration of the amphiphile. The solution was heated to 37°C. The probe was mixed in the solution and the measurements were collected. Ten percent of the current solution was pipetted out and replaced with nanopure water. The solution was mixed and the conductivity measurement recorded. These steps were repeated for the entirety of the experiment.

Bacterial strains and growth conditions

The two Gram-negative bacterial strains used were *Escherichia coli* ATCC® 25922™ and *Pseudomonas aeruginosa* ATCC® 27853™, and the five Gram-positive bacterial strains used in this study were *Staphylococcus aureus* subsp. aureus ATCC® 29213™, *Enterococcus faecalis* ATCC® 29212™, *Bacillus subtilis*, *B. anthracis* Sterne, and *Streptococcus agalactiae* J48 (Seifert et al. 2006). All strains were grown in 1X Mueller-Hinton Broth at 37°C for 12–24 h. For the MIC studies, bacterial suspensions were prepared by diluting overnight cultures to 5x10^6 CFU/mL in 2X Mueller-Hinton Broth, so that when amphiphile solutions were added the final broth strength was 1X.

Minimum inhibitory concentration

The methods used to determine the MIC were performed as previously described (Ladow et al. 2011; CLSI, 2012). Briefly, compounds were serially diluted in sterile dionized water and 100µL of each dilution were added to the wells of a 96-well flat-bottomed microtiter plate in triplicate. After adding 100µL of the bacterial cell suspension (5x10^6 CFU/mL), the plates were incubated at 37°C for 72 h. The MIC of the compound was defined as the minimum concentration that resulted in visible inhibition of bacterial growth.
Chapter 4: The effect of spacer variation on the antibacterial activity of bis- and tetra-cationic double-tailed amphiphiles.

4.1 Introduction.

The relative size of the hydrophobic region of QACs relative to the hydrophilic region is one of the greatest predictors of membrane permeability and antibacterial activity (Paniak et al. 2014). While changing the length of the hydrocarbon tails alters the size of the hydrophobic region relative to the charged head group and alter characteristics of membrane insertion activity, this effect may also be achieved by changing the way the hydrocarbon tails are positioned on the central ring of the amphiphile. It is therefore possible that changing the number of carbons separating two symmetrical hydrocarbon tails on a central Mesitylene ring may affect antibacterial activity.

Three series of bis-cationic amphiphiles and three series of tetra-cationic amphiphiles were synthesized in order to determine differences in the orientation of the hydrocarbon tails relative to one another on the central ring affects antibacterial activity. The bis-cationic amphiphiles included one series with the symmetrical tails in an ortho orientation (oX-n,n), one series with the tails in a meta orientation (mX-n,n), and one series with the tails in a para orientation (pX-n,n; Figure 7). The oX-n,n series included four tail length derivatives ranging from 8 to 14 carbons per tail. The mX-n,n series included three tail length derivatives ranging from 8 to 12 carbons per tail, and the pX-n,n series included five derivatives ranging from 8 to 16 carbons per tail.

Tetra-cationic amphiphiles each had two head groups, each containing two dimethylammonium residues separated by a chain of two carbons. Each head group had a single attached hydrocarbon tail. The two tails were symmetrical and varied in length.
from 8-12 carbons for each of the three series: oX-(2,n)_2, mX-(2,n)_2, and pX-(2,n)_2 (Figure 8).

Additionally, one series of three triple-tailed amphiphiles was also tested for its antibacterial activity. The M-(2,n)_3 series consists of amphiphiles with six positive charges and three tails, each of varying lengths (Figure 9).
3.2 Results and Discussion.

The antibacterial activity of the bis-cationic series of amphiphiles followed the same trend as previous amphiphile series according to tail length, with 12 carbon derivatives having the lowest MIC values (Table 5, Figure 15). This trend was seen for all three series, suggesting that, similar to series with changes in head group discussed in Chapter 1, the length of the hydrocarbon tails is the most accurate predictor of antibacterial activity. Changing the spacer length between the tails on the central ring did not appear to have a significant effect on which hydrocarbon tail length was most effective.

The 12-carbon derivatives of the bis-cationic series had broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria. Interestingly, oX-12,12 had the highest level of bacteriocidal activity against *P. aeruginosa*, with an MIC of 2 μM, two- to four-fold lower than that of the Gram-positive species tested. pX-12,12 had an MIC of 2-4 μM against Gram-positive bacteria, and 4 μM against both *P. aeruginosa* and *E. coli* (Table 5).

**Table 5.** Antibacterial activity of bis-cationic compounds in the oX-*n,n*, mX-*n,n*, and pX-*n,n* series with different hydrocarbon tail lengths. oX = ortho orientation; mX = meta orientation; pX = para orientation and *n* = the number of carbons per tail. G- = Gram-negative and G+ = Gram-positive.

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>P. aeruginosa</em> (G-)</th>
<th><em>E. coli</em> (G-)</th>
<th><em>E. faecalis</em> (G+)</th>
<th><em>S. aureus</em> (G+)</th>
<th><em>B. subtilis</em> (G+)</th>
<th><em>S. agalactiae</em> (G+)</th>
<th><em>B. anthracis</em> (G+)</th>
<th>Minimum Inhibitory Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oX-8,8</td>
<td>250</td>
<td>250</td>
<td>125</td>
<td>250</td>
<td>&gt;250</td>
<td>&gt;250</td>
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<td></td>
</tr>
<tr>
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<td>4</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
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<td>4</td>
<td>8</td>
<td>8</td>
<td>4</td>
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<tr>
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<td>63</td>
<td>8</td>
<td>63</td>
<td>4</td>
<td>16</td>
<td>4</td>
<td></td>
</tr>
<tr>
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<td>250</td>
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<td>4</td>
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<tr>
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<td>&gt;250</td>
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<td>31</td>
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</table>
Figure 15. Antibacterial activity against seven bacterial species of bis-cationic compounds in the oX-n,n (diamonds), mX-n,n (squares), and pX-n,n (triangles) series with different hydrocarbon tail lengths. G- = Gram-negative and G+ = Gram-positive.
Three series of tetra-cationic amphiphiles were also evaluated for their antibacterial activity (MIC) at three different hydrocarbon tail lengths for three spacer sizes (ortho, meta, and para orientation). For the compounds with the tails in the meta and para orientations, the same trend was observed as the bis-cationic amphiphiles discussed earlier. Compounds with the ideal 12 carbon chain length had the lowest MIC values compared to other tail lengths in the series. However, for tetra-cationic amphiphiles with the tails in the ortho orientation relative to one another, 8 hydrocarbons per tail appeared to be the most effective, with oX-(2,8)_2 having the lowest MIC values (4 μM against Gram-negative bacteria, and 2 μM against Gram-positive bacteria) compared to oX-(2,10)_2 and oX-(2,12)_2 (Table 6, Figure 16).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum Inhibitory Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aeruginosa (G-)</td>
</tr>
<tr>
<td>oX-(2,8)_2</td>
<td>4</td>
</tr>
<tr>
<td>oX-(2,10)_2</td>
<td>125</td>
</tr>
<tr>
<td>oX-(2,12)_2</td>
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</tr>
<tr>
<td>mX-(2,8)_2</td>
<td>&gt;250</td>
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<tr>
<td>mX-(2,10)_2</td>
<td>16</td>
</tr>
<tr>
<td>mX-(2,12)_2</td>
<td>8</td>
</tr>
<tr>
<td>pX-(2,8)_2</td>
<td>&gt;250</td>
</tr>
<tr>
<td>pX-(2,10)_2</td>
<td>16</td>
</tr>
<tr>
<td>pX-(2,12)_2</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 6. Antibacterial activity of tetra-cationic compounds in the oX-(2,n)_2, mX-(2,n)_2, and pX-(2,n)_2 series with different hydrocarbon tail lengths. oX = ortho orientation; mX = meta orientation; pX = para orientation and n = the number of carbons per tail. G- = Gram-negative and G+ = Gram-positive.
Figure 16. Antibacterial activity against seven bacterial species of tetra-cationic compounds in the oX-(2, n)_2 (diamonds), mX-(2, n)_2 (squares), and pX-(2, n)_2 (triangles) series with different hydrocarbon tail lengths. G- = Gram-negative and G+ = Gram-positive.
A single triple-tailed series, M-(2, n)_3, was also tested for its antibacterial activity. This series of compounds contains six positive charges and three tails of equal length, each with 8, 10, or 12 carbons. The compound with 10 carbons per tail (M-[2,10]_3) had the lowest MIC values of any of the three derivatives (Table 7. Figure 17). Similar to the trend observed for the tetra-cationic oX-(2, n)_2 series, the ideal tail length for the M-(2, n)_3 series was not 12 carbons, as it was for the majority of the other compounds tested. The 8-carbon and 12-carbon tail length derivatives of this series both had higher MIC values on the whole than the 10-carbon derivative.

Table 7. Antibacterial activity of the triple-tailed M-(2, n)_3 series. n = the number of carbons per tail. G- = Gram-negative and G+ = Gram-positive.

<table>
<thead>
<tr>
<th>Compound M-(2, n)_3</th>
<th>Minimum Inhibitory Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aeruginosa (G-)</td>
</tr>
<tr>
<td>M-(2,8)_3</td>
<td>16</td>
</tr>
<tr>
<td>M-(2,10)_3</td>
<td>8</td>
</tr>
<tr>
<td>M-(2,12)_3</td>
<td>16</td>
</tr>
</tbody>
</table>

Figure 17. Antibacterial activity of compounds in the M-(2, n)_3 series with different hydrocarbon tail lengths. G- = Gram-negative and G+ = Gram-positive.
The MIC of M-(2,10)$_3$ was notably as low as 1 μM against several Gram-positive species, including *S. aureus*, and was as low as 2 μM against the Gram-negative *E. coli* and 8 μM against *P. aeruginosa* (Table 7). These MIC values are comparable to the MIC values of 12-carbon derivatives of the double-tailed, triscationic series studied previously.

These data taken together suggest that the ideal length of the hydrocarbon tails of these amphiphiles may change depending on other structural characteristics. Changes in the distribution of charge within the head group of these molecules likely affect electrostatic interactions between the amphiphiles and the largely negative lipid membrane of bacteria. Because electrostatic interactions and hydrophobic interactions both contribute to the overall antimicrobial mechanism of action (Ramos et al. 2007), changing the distribution of charge in relation to the size of the hydrophobic region may have significant effects on the ability of these molecules to insert into the membrane. Because the oX-(2,*n*)$_2$ series has four positive residues compared to two in the oX-(*n*,*n*) series, this effect may be more pronounced, resulting in the change in the ideal size of the hydrophobic tails for insertion into the bacterial membrane.

### 3.3 Methods.

**Bacterial strains and growth conditions.**

The two Gram-negative bacterial strains used were *Escherichia coli* ATCC® 25922™ and *Pseudomonas aeruginosa* ATCC® 27853™, and the five Gram-positive bacterial strains used in this study were *Staphylococcus aureus subsp. aureus* ATCC® 29213™, *Enterococcus faecalis* ATCC® 29212™, *Bacillus subtilis*, *B. anthracis* Sterne, and *Streptococcus agalactiae* J48 (Seifert et al. 2006). All strains were grown in 1x
Mueller-Hinton Broth at 37° C for 12–24 h. For the MIC studies, bacterial suspensions were prepared by diluting overnight cultures to 5x10^6 CFU/mL in 2X Mueller-Hinton Broth, so that when amphiphile solutions were added the final broth strength was 1X.

**Minimum inhibitory concentration.**

The methods used to determine the MIC were performed as previously described (Ladow et al. 2011; CLSI, 2012). Briefly, compounds were serially diluted in sterile dionized water and 100µL of each dilution were added to the wells of a 96-well flat-bottomed microtiter plate in triplicate. After adding 100µL of the bacterial cell suspension (5x10^6 CFU/mL), the plates were incubated at 37°C for 72 h. The MIC of the compound was defined as the minimum concentration that resulted in visible inhibition of bacterial growth.
Chapter 5: Conclusions and Future Research.

5.1 Conclusions

The minimum inhibitory concentration was determined for all amphiphiles against two Gram-negative and five Gram-positive organisms. Tail length affects antibacterial activity, with one tail length being optimal for each series. Consistent with previous studies (Marafino et al. 2015), compounds with twelve carbons in each of two symmetrical hydrocarbon tails are the most effective, compared to compounds with longer or shorter tails. This trend was observed for four different amphiphile series, each with a differently substituted third head group residue, suggesting that head group substitution does not have as large of an effect on antibacterial activity as tail length, provided that the head group residues are of comparable size and charge.

All of the amphiphiles tested (M-E,12,12; M-DMAP,12,12; M-IQ,12,12; and M-4PP,12,12) were capable of disrupting established biofilm. Despite having MIC values as low as 4-8 µM, significant biofilm disruption was only observed at much higher concentrations, approximately 31-63 µM. This was unsurprising, considering the fact that bacteria in biofilms tend to be highly resistant to antibiotics and biocides (Lewis, 2001). M-E,12,12; M-DMAP,12,12; and M-IQ,12,12 were able to disrupt a similar amount of established P. aeruginosa biofilm as the antibiotic tobramycin at comparable concentrations. The amphiphile M-4PP,12,12 disrupted a similar amount of biofilm as benzalkonium chloride at comparable concentrations, though the amount of biofilm removed by M-4PP,12,12 was lower than that removed by the other 12-carbon derivative compounds tested. Due to their ability to remove established biofilms, these compounds may have potential to be used as novel disinfectants, especially in combination with other
antimicrobial agents. Further research is necessary to determine the exact mechanism of action of biofilm removal, and what aspects of amphiphile structure are most important for variations in anti-biofilm activity.

Different counterions also affect antibacterial activity. Substitution of bromide with different ions from the Hofmeister series led to changes in MIC, particularly against *P. aeruginosa*. The single-tailed amphiphile M-1,1,18 and the double-tailed amphiphile M-1,12,12 were more effective at inhibiting the growth of *P. aeruginosa* with chloride and iodide counterions than with bromide. Other counterions such as acetate, carbonate, and nitrate had little effect, or even increased the MIC for these amphiphiles. Despite notable differences in the MIC for amphiphiles paired with different Hofmeister counterions, no clear trend was observed between the MIC and any chemical attributes of the Hofmeister series itself. Although use of different counterions such as chloride and iodide appear to have potential to increase the efficacy of charged cationic antimicrobials, particularly against Gram-negative pathogens such as *P. aeruginosa*, more research is needed to determine the mechanism by which these different counterions increase the efficacy of quaternary ammonium amphiphiles.

Finally, the orientation of tails relative to each other on the mesitylene core of tetra-cationic amphiphiles also has an effect on the optimal tail length. The amphiphiles with tails in the *para* or *meta* orientation on the central ring followed a similar trend to the tris-cationic double-tailed amphiphiles, with 12 carbons being the ideal length per tail for the most efficient antibacterial activity. However, amphiphiles with the two tails in the *ortho* orientation on the central ring had an optimal tail length of 8 carbons per tail.
No shift in the optimal tail length was observed for the bis-cationic amphiphiles. Bis-cationic amphiphiles with the two tails in the ortho, meta, and para orientation, all had an ideal tail length of 12 carbons per tail.

The shift in optimal tail length specific to tetra-cationic amphiphiles bears further study, particularly the possibility that compounds in the oX-(2,n)_2 series with shorter hydrocarbon tails may have even more efficient antibacterial activity. If 8 carbons is indeed the ideal tail length for amphiphiles in this series, then it is more likely that the MIC will increase with shorter tail lengths, similar to the trend observed for previously tested compounds, whose ideal tail length is 12 carbons per tail.

All of these data can be used to synthesize more effective compounds in the future. Understanding effects of tail length, counterion, and charge distribution on antibacterial activity, biofilm disruption, and cytotoxicity will be useful for developing amphiphilic compounds that may serve as novel antibiotics or disinfectants. The development of novel compounds is essential to prevent the spread of resistant infections in the population, particularly in people with compromised immune systems, such as the elderly or those with chronic diseases, and especially in hospitals.
5.2 Future Research

10-14-AO – Fluorescent Compound

The fluorescent compound 10-14-AO was derived from acridine orange. A 50 μM solution of 10-14-AO was incubated at room temperature for one hour with an overnight liquid culture of *Streptococcus agalactiae* and visualized using a 60X objective on a confocal microscope.

The compound 10-14-AO was successfully taken up into the bacterial cells and fluoresced at approximately 540 nm, allowing for the visualization of intact *S. agalactiae* cells (Figure 18, Figure 19). This compound may have applications in the determination of mechanism of action, or in the study of the movement of amphiphiles through bacterial biofilm over time. The

![Figure 18](image1)

**Figure 18.** Excitation and emission spectra for 10-14-AO. Excitation was determined by irradiating the sample with light from 400-520 nm and monitoring emitted light at 53 nm. Emission was determined by irradiating the sample with light at 493 nm and monitoring emitted light from 500-750 nm.

![Figure 19](image2)

**Figure 19.** *S. agalactiae* incubated with fluorescent compound 10-14-AO, visualized at 60X magnification using confocal microscopy.
antibacterial activity of this compound has yet to be determined, and further studies will determine the effects of structural changes on fluorescence and uptake into the cells, as well as biofilm penetration.

Activity against other E.S.K.A.P.E. pathogens

E.S.K.A.P.E. pathogens refer to the most relevant antibiotic-resistant bacteria (Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumanii, P. aeruginosa, and Enterobacter species; Rice, 2008). Because of their promising level of activity against prominent pathogens such as P. aeruginosa and S. aureus, these amphiphiles should be tested for their ability to kill other as-yet-untested pathogenic bacteria such as Acinetobacter baumannii and Klebsiella pneumoniae. While these pathogens pose little risk to healthy people, they are common causes of healthcare-associated wound and bloodstream infection, and are often resistant to many commonly used antibiotics (CDC, 2010; 2015). K. pneumoniae, in particular, has been implicated in the increasing spread of carbapenem-resistant Enterobacteriaceae (CRE) due to its ability to produce a potent carbapenemase (CDC, 2015). Due to their broad spectrum of activity against Gram-negative and Gram-positive pathogens notorious for having high levels of resistance to antibacterial compounds, the compounds presented in this study may also be effective against K. pneumoniae and A. baumannii, presenting an opportunity for the development of new compounds to prevent the spread of these and other prominent opportunistic pathogens.
**Mechanism of action**

Given ample previous research into the mechanism of action of amphiphilc AMPs and structurally similar amphiphiles, it is likely that these compounds are membrane-active, causing depolarization and lysis of bacterial cells (Dathe and Wieprecht, 1999; Thiyagarajan et al. 2014; Goswami et al. 2015). Propidium iodide permeability assays can be used in order to gain more information about the membrane-permeabilizing activity of these compounds (Rieger et al. 2011). Further studies to determine the biofilm disruption mechanism will be explored such as fluorescence microscopy.

**Cytotoxicity**

Because the mode of action of these compounds is likely membrane permeabilization, depolarization and lysis, it is possible that these amphiphiles may not be compatible with human cells. The cytotoxicity of amphiphiles is largely structure-dependent, with some amphiphiles having cytotoxic concentrations many times higher than the MIC (Goswami et al. 2015). It is also possible that fundamental differences between the mammalian and bacterial cell membranes may increase the therapeutic potential of these compounds. Namely, unlike the net negatively charged bacterial membrane, the outer leaflet of the mammalian plasma membrane is neutral, with negatively charged phospholipids largely localized to the cytosolic leaflet (Cooper, 2000). This may decrease the effects of electrostatic interactions between cationic membrane-active compounds and the membrane by making hydrophobic interactions the largest driving force behind insertion into the membrane. Determination of cytotoxicity
through hemolysis assays and testing using epithelial cells is necessary to confirm the safety of these compounds for therapeutic use.

**Synergistic combinations and pre-treatment**

Synergistic combinations of some similar amphiphiles have been observed in prior studies (Marafino et al. 2015). Of particular interest is the potential for synergy between antibacterial amphiphiles and sodium metaperiodate (NaIO₄), which is used to disrupt extracellular polysaccharides, particularly against established biofilms (Gawande et al. 2007; Gutiérrez et al. 2013). Since these compounds likely disrupt bacterial biofilms by infiltrating the matrix and lysing cells within the biofilm, without disrupting the matrix itself directly, pre-treatment or co-incubation with NaIO₄ may increase the biofilm disruption ability of QACs.

Some research also suggests that synergistic interactions with conventional antibiotics may increase bactericidal activity. Membrane permeabilizing agents have been used with antibiotics such as erythromycin in order to make Gram-negative bacteria susceptible to otherwise ineffective treatments by breaking down their outer membrane (Goswami et al. 2015). Similar studies to characterize any synergistic combinations of these compounds and a variety of antibiotics could further characterize the method of action of the compounds being studied, as well as provide a proof of concept for these compounds being used as adjuvants in treatment with conventional antibiotics.
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National Institutes of Health. 2016. Estimates of funding for various research, condition, and disease categories (RDCD).


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