Colloidal and biological properties of triscationic amphiphiles with one or two tails

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Colloidal and Biological Properties of Triscationic Amphiphiles with One or Two Tails

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A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

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for the degree of

Master of Science

Department of Biology

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Dedication

I dedicate this Masters thesis to my Grandmother Antonetta P. Ranone who always believed in my potential. I especially thank my parents John T. Marafino and Janet R. Marafino for their continuing love and support. I am proud to be your son. Also to my sisters and brothers, Elizabeth, Laura, Catherine, Joel, Chad, and Peter for all your encouragement and understanding. I am truly blessed!

I further dedicate this thesis to my exquisite selection of friends. To Matthew Folmar and Josh Peters for their persistent friendship over long distances. To Craig and Sarah Parsons for your unwavering friendship and continued pursuit. To Quentin King for all his wisdom, apart from being a boss. To Seth Coggins for movie diversions. To Ashley McAdams, Wes Hedrick, Brittany Knopp, Kaitlin Knopp, Flosita Folmar, Artem Pekun, and the entire Mehegan family for your laughs, love, and food.

I also dedicate this thesis to my best friend Rebekah Ann Mehegan, who I will always hold close to my heart. Thank you for your encouragement, support, and understanding. Thank you for buying me groceries and bringing me supper in the office. Most of all, thank you for your love, which you have shown time and time again.

God, thank you for being the best. All of this belongs to you!
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make mistakes and constructively learn from them. There is no doubt in my mind that Dr. Kevin Caran is a Godsend and without him I may never have walked down this road.
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Abstract

The decline in the development of novel antimicrobials, combined with the misusage and over prescription of antibiotics, has contributed to the increasing prevalence of antimicrobial-resistant infections. Thus development of effective novel disinfectants could reduce the transmission of pathogens and decrease the risk of infection by antibiotic resistant organisms. The antimicrobial activity of amphiphiles, compounds with hydrophobic and hydrophilic regions, was first reported in 1935, and has influenced the synthesis of amphiphiles with variations in structure. In this study, three series of amphiphiles were synthesized by two subsequent Menshutkin reactions. Each amphiphile contains one or two hydrocarbon tails ranging from 8 to 22 carbons in their hydrophobic region and three cationic headgroups in their hydrophilic region. Using isothermal titration calorimetry (ITC) the critical micelle concentration (CMC) and thermodynamic parameters of micelle formation were measured. As tail length increases the CMC decreases where micelle formation is favorable (negative $\Delta G$) for all three series of amphiphiles. Micelle formation is both enthalpically and entropically favorable for short chained amphiphiles whereas micelle formation is enthalpically favorable and entropically disfavored for long chained derivatives. The minimum inhibitory concentration (MIC) of each compound was measured against 6 different strains of bacteria. As tail length increases the MIC decreases until an optimal tail length where antibacterial activity is lowest. The water solubility of an amphiphile decreases with increasing tail length; amphiphiles that have intermediate solubility, within a series, were found to have with lower MIC values.
I. Introduction

Over the last few decades, the overuse of antibiotics has decreased their effectiveness, contributing to bacterial acquired resistance. In addition, the production of novel antimicrobials continues to decrease due to low financial return. This decline in the development of novel antimicrobials, combined with the misusage and over prescription of antibiotics, has contributed to the increasing prevalence of antimicrobial-resistant infections (ARIs). ARIs have contributed to more than 25,000 deaths per year in member states of the European Union, Iceland, and Norway and 23,000 deaths per year in the United States. Hospitals and nursing homes are particularly prone to harboring antimicrobial-resistant organisms due to the frequent use of antimicrobial agents and influx of infected patients. Limiting the transmission of bacteria between individuals and contaminated equipment at these locations is critical to preventing hospital-acquired infections and reducing mortality rates. Furthermore, biofilm contamination hospital surfaces such as urinary catheters, central venous catheters, and dental syringes is also a growing concern. Thus, the development of potent novel disinfectants could reduce the transmission of pathogens and decrease the risk of infection by antibiotic resistant organisms. Amphiphiles have been used as antimicrobials since their first report of antimicrobial activity in 1935.

The structure of an amphiphile profoundly affects its properties, which in turn dictate its potential uses. This has led to a wide variety of useful applications, ranging from household, to scientific and medicinal applications. While amphiphiles are
found in many forms including phospholipids (the primary structural unit of a cell membrane), they are generally composed of two distinct regions: a hydrophobic water-insoluble tail and a hydrophilic water-soluble head group (Fig. 1). The hydrophobic region is often composed of a hydrocarbon chain typically consisting of 10 to 18 carbons. In contrast, the hydrophilic region is polar consisting of ionic or non-ionic bonds.  

Figure 1. Amphiphiles contain both hydrophilic and hydrophobic regions. The hydrophilic region, or head group, contains polar bonds and/or ionic charges. The hydrophobic region is often composed of a hydrocarbon tail and contains nonpolar bonds.
I.A. Colloidal Properties of Amphiphiles

Aggregation

The unique dual nature of amphiphiles (hydrophilic/hydrophobic) enables them to align at the interface of two dissimilar phases. In aqueous solutions, amphiphiles aggregate as a result of interactions between the hydrophobic tail and water, which impede interactions between water molecules. When a water molecule interacts with a hydrocarbon tail it has fewer hydrogen-bonding interactions with other water molecules relative to a water molecule surrounded only by other water. This causes it to form a hydrogen-bonded network with other interfacial water molecules in essence “freezing” and encasing the tail in a sheath of water (Fig. 2). As amphiphile concentration increases, amphiphiles begin to assemble into aggregates in which hydrocarbon tails interact with each other, thus releasing water that was formerly associated with non-polar regions.

Figure 2. Interactions between a hydrophobic tail and water. Water “freezes” and forms a sheath like structure around a hydrocarbon tail.

Amphiphiles typically assemble such that the orientations of hydrophilic head groups are exposed to the water while the hydrophobic tails interact with each other. However, amphiphiles that contain an ionic head group must also overcome the repulsion between head groups of the same charge in order to aggregate.
Amphiphiles assemble into different aggregates, such as micelles or bilayers—a process partially determined by overall molecular architecture and shape (Fig. 3). Single tailed amphiphiles are approximately cone-shaped, resulting in the formation of spherical aggregates called micelles in aqueous solution (Fig. 3a). The cone angle is in part determined by head group size and tail length. Amphiphiles with larger head groups and/or shorter tails have a larger cone angle causing the formation of micelles with a lower aggregation number (the total number of amphiphiles in an aggregate) (Fig. 3b).

Figure 3. How amphiphile shape affects aggregation. a) Single tailed amphiphiles have an overall cone shape and form spherical micelles in solution. b) Amphiphiles with larger head groups have a larger cone angle resulting in micelles with a lower aggregation number. c) Amphiphiles with two tails, such as phospholipids, have a cylindrical geometry forming bilayers. d) Adding cone shaped amphiphiles to bilayer aggregates leads to an increase in membrane curvature. e) Increasing the cone angle of an amphiphile added to a bilayer leads to more profound membrane curvature.
Many naturally occurring amphiphiles, such as phospholipids, contain a single head group and two hydrophobic tails imparting the amphiphile with an overall cylindrical shape, resulting in the formation of bilayers in solution (Fig. 3c). In a mixed aggregate (containing 2 or more structurally different amphiphiles), amphiphiles with a large head group relative to the hydrophobic tail contribute more to the aggregate curvature, decreasing its aggregate diameter and aggregation number (Fig. 3d,e).

**Critical Micelle Concentration**

The concentration of amphiphiles at which micelles begin to form is the critical micelle concentration (CMC). Micelles contain a hydrophobic core of interacting tails with head groups exposed to a polar solvent, such as water (Fig. 3a). Micelles typically consist of 40 - 100 amphiphiles that exist in equilibrium with dissolved amphiphiles in solution. Below the CMC, amphiphiles align at the air-water interface in equilibrium with monomers in solution. As the concentration reaches the CMC, micelles begin to form in solution (Fig. 4). The CMC is dependent on the number and length of hydrocarbon tails, type of head group, and overall amphiphile architecture, among other factors.

![Figure 4](image-url)  
*Figure 4*. Equilibria between amphiphiles in water. As the concentration of amphiphiles in water reaches the CMC micelles begin to form.
Increasing the tail length or the number of tails (total hydrophobicity) decreases an amphiphile’s solubility in water causing a decrease in CMC. In contrast, increasing the number of polar head groups on an amphiphile increases its solubility in water resulting in a higher CMC. Therefore as the number of head groups increases relative to the number of tails on an amphiphile, the dependence of CMC on tail length decreases. This is typically observed as a decrease in slope in a plot of log (CMC) versus tail length (Fig. 5). As stated above, the CMC can also be affected by other factors including the overall geometry of the amphiphile.

**Thermodynamics of Micelle Formation**

Micelle formation can also be characterized by thermodynamic properties such as the entropy ($\Delta S_{\text{mic}}$), the enthalpy ($\Delta H_{\text{mic}}$), and the Gibbs free energy ($\Delta G_{\text{mic}}$) of micelle formation. A combination of factors, listed below, dictate $\Delta S_{\text{mic}}$ and $\Delta H_{\text{mic}}$, which in turn determine $\Delta G_{\text{mic}}$, as calculated from the Gibbs-Helmholtz equation:

$$\Delta G_{\text{mic}} = \Delta H_{\text{mic}} - T\Delta S_{\text{mic}}$$

(1)

The $\Delta S_{\text{mic}}$ is dictated by a combination of entropic factors that favor or disfavor micelle formation. Upon micelle formation:
• Water molecules are released from the hydrophobic tails
  o Entropy of the water increases positively contributing to $\Delta S_{\text{mic}}$, thus favoring micelle formation

• Amphiphiles aggregate to form micelles
  o Entropy of the amphiphile decreases negatively contributing to $\Delta S_{\text{mic}}$, thus disfavoring micelle formation.

Likewise, the $\Delta H_{\text{mic}}$ is determined by a combination of enthalpic forces that favor or disfavor micelle formation. Upon micelle formation:

• Water molecules released from hydrophobic tails are free to form additional hydrogen bonds to other water molecules in the bulk
  o Exothermic process negatively contributing to $\Delta H_{\text{mic}}$, thus favoring micelle formation

• New interactions between hydrophobic chains are formed
  o Exothermic process negatively contributing to $\Delta H_{\text{mic}}$, thus favoring micelle formation

• Energy is required to break hydrogen bonds between water molecules within the “frozen” sheath
  o Endothermic process positively contributing to $\Delta H_{\text{mic}}$, thus disfavoring micelle formation

• Energy is required to disrupt the weak interactions between the water and hydrophobic tail
  o Endothermic process positively contributing to $\Delta H_{\text{mic}}$, thus disfavoring micelle formation
I.B. Biological Activity of Amphiphiles

Antimicrobial Activity below the CMC

As the concentration of amphiphile reaches or exceeds the CMC, amphiphiles act as detergents that indiscriminately solubilize cell membranes, a mechanism of action that could be broadly detrimental to cells, including bacteria.\textsuperscript{19,34,35} However, antimicrobial activity of amphiphiles is often observed below CMC suggesting that amphiphile aggregation is not required to kill bacteria.\textsuperscript{16,21,24,35} This suggests that there must be some intrinsic properties associated with an amphiphile’s structure that enables it to kill bacteria, independent of its ability to aggregate.

How Structure affects the Minimum Inhibitory Concentration

An amphiphile’s structure affects its antimicrobial activity. A reoccurring trend reported by multiple studies is the relationship between amphiphile tail length and the minimum inhibitory concentration (MIC), the lowest concentration at which an antimicrobial is able to inhibit bacterial growth.\textsuperscript{16-25} Typically, as tail length increases the MIC decreases until an optimal tail length is reached. Beyond this peak in antimicrobial activity, the MIC begins to increase for amphiphiles with longer tails (Fig. 6). This trend is consistent with the increased solubility of short-chained amphiphiles (which are too water soluble

\textbf{Figure 6.} Theoretical graph showing the trend between MIC and tail length.
to interact with membranes) and the decreased solubility of long-chained amphiphiles (which are too water-insoluble). To compensate for this decreased solubility of amphiphiles with long chains, amphiphiles with three head groups (tricephalic) allow for increased solubility in water. We hypothesize that these tricephalic amphiphiles would be more effective than their monocephalic or bicephalic counterparts, however this is not always the case. Headgroups can either be cationic, anionic, or nonionic. Two common cationic head groups found on amphiphiles are quaternary ammonium (QA) and pyridinium moieties. Previous studies indicate a slight decrease in the MIC of pyridinium head groups relative to QA but no significant difference has yet been reported.

The relative positioning of headgroups around an aromatic core can also affect the antimicrobial activity. In our previous study, a 2,4- or 3,5- substitution pattern around an aromatic ring (resulting in a 5-carbon spacer between cations) had the lowest MIC compared to other amphiphiles with different substitution patterns in the study. Each head group is further associated with a counter-ion (Cl⁻, Br⁻, or I⁻) where incorporation of an iodide counter-ion has been shown to cause a sharp increase in the MIC for amphiphiles with long hydrocarbon tails.

*Membrane Disruption*

It is hypothesized that amphiphiles intercalate into bacterial membranes eventually killing the cell (Fig. 7). Studies examining the effect of single tailed amphiphiles on phospholipid membranes show that these amphiphiles incorporate into
bilayer membranes making them permeable.\textsuperscript{38} At higher amphiphile concentrations, the bilayer membranes reorder into mixed micelles.\textsuperscript{36,38,39}

**Figure 7.** A diagram of amphiphiles intercalating into bacterial membranes.

Assuming amphiphiles do intercalate into membranes, amphiphile structure could be altered to preferentially exploit bacterial membranes over those of eukaryotic cells. Most bacteria possess an uneven number of positively and negatively charged amphiphiles in the membrane resulting in a net-negative charge.\textsuperscript{40-42} In contrast, most eukaryotic cell membranes are approximately neutral.\textsuperscript{40-42} Thus amphiphiles with cationic head groups may be able to exploit this difference and more readily interact with bacterial membranes.\textsuperscript{43}

**Rationale of Structure**

Here the synthesis, colloidal, antibacterial, and synergistic characteristics for three novel series of triple headed, double tailed amphiphiles (\textit{M-P}, \textit{M-I}, and \textit{M-I, I} series) were explored (\textbf{Fig. 8}). The \textit{M-P} and \textit{M-I} series of amphiphiles consist of three cationic head groups connected to a mesitylene core. Two of the head groups are quaternary ammoniums (QA) that further connect to linear hydrocarbon tails ranging in length from 8 to 16 carbons. Although both series of amphiphiles are similar in structure to conventional Gemini amphiphiles, they differ due to an additional pyridinium head group, for the \textit{M-P} series, or QA head group, for the \textit{M-I} series.\textsuperscript{16,17} The \textit{M-I, I} series also
consist of three cationic head groups (quaternary ammoniums) connected to a mesitylene core. However, only one of the head groups is further connected to a hydrocarbon tail ranging in length from 8 to 22 carbons.

Figure 8. Amphiphiles in the current study. All three series of amphiphiles are shown from left to right, M-P series, M-I series, and M-I,1 series.

Although there may be no direct relationship between an amphiphile’s colloidal and antimicrobial properties, both are clearly and profoundly affected by amphiphile structure. Developing a deeper understanding of these structure-function relationships may provide insight into the mechanism by which amphiphiles interact with and inhibit bacterial growth. These particular series of amphiphiles should create highly effective disinfectants that likely disrupt the bacterial membrane. The three cationic head groups should interact with the net-anionic bacterial membrane allowing subsequent intercalation of the amphiphile’s hydrophobic tail(s).
II. Methods and Materials

*Synthesis of Intermediates*

**19 (M-P)** (Preformed by Jhosdyn Barragan, Gabriel Fitzgerald, and Kristin McKenna)

1,3,5-tribromomethylbenzene (1.0 g, 2.8 mmol) was dissolved in acetone at room temperature in a round bottom flask (RBF). The solution was equipped with a stir-bar and attached to an addition funnel at room temperature. Pyridine (0.45 mL, 5.6 mmol) was dissolved in acetone and added drop-wise to the stirring solution of 1,3,5-tribromomethylbenzene overnight. A white precipitate containing a mixture of 19 (M-P) and side product formed. The crude reaction mixture was vacuum filtered, washed with acetone, and dried under vacuum yielding 943 mg (77.2%) of a tan solid, 19 (M-P). See supplemental information for analytical data.

**20 (M-I)**

1,3,5-tribromomethylbenzene (2.01 g, 5.6 mmol) was dissolved in acetone at room temperature in a round bottom flask (RBF). The solution was cooled on an ice bath for 30 minutes, equipped with a stir-bar, and attached to an addition funnel. An ethanolic trimethylamine solution (1.6 mL, 6.7 mmol) was dissolved in acetone and cooled on an ice bath for 30 minutes. The trimethylamine solution was added drop-wise to the stirring solution of 1,3,5-tribromomethylbenzene. The reaction was run overnight, and warmed slowly to room temperature. A white precipitate containing a mixture of 20 (M-I) and side product (21) formed. The crude reaction mixture was briefly heated to 50°C and vacuum filtered. The mother liquor was moved to a clean RBF and solvent was removed by rotary evaporation. The resulting solid was resuspended in room temperature acetone for 30 minutes, vacuum filtered, and dried under vacuum yielding 20 (M-I) (660 mg, 28%, white). Solid from the crude reaction mixture was resuspended in a solution of acetone and ethanol (100:3), heated to 60°C, stirred for at least 30 minutes, and vacuum filtered. The filtrate was transferred to a clean RBF and the solvent was removed by rotary evaporation. The resulting solid was resuspended in
room temperature acetone, vacuum filtered and dried yielding additional 20 (M-I) (200 mg, 37% total yield, white solid). See supplemental information for analytical data.

21 (M-I, I)

1,3,5-tribromomethylbenzene (1.0 g, 2.8 mmol) was dissolved in acetone at room temperature in a round bottom flask (RBF). The solution was cooled on an ice bath for 30 minutes, equipped with a stir-bar, and attached to an addition funnel. An ethanolic trimethylamine solution (1.2 mL, 5.6 mmol) was dissolved in acetone and cooled on an ice bath for 30 minutes. The trimethylamine solution was added drop-wise to the stirring solution of 1,3,5-tribromomethylbenzene. The reaction was run overnight, and warmed slowly to room temperature. A white precipitate containing a mixture of 21 (M-I, I) and side product (20) formed. The crude reaction mixture was vacuum filtered and resuspended in a solution of acetone and ethanol (100:3), heated to 60°C, stirred for at least 30 minutes, vacuum filtered, and dried under vacuum to produce 21 (M-I, I) (856.4 mg, 64.05%, white solid). See supplemental information for analytical data.

Synthesis of N,N-dimethylicosaneamine

1-bromoeicosane (1.00 g, 2.77 mmol) was dissolved in tetrahydrofuran (15 m) in a round bottom flask. The solution was cooled on an acetone bath (containing CO₂ pellets) to -78°C, equipped with a stir-bar, and attached to an addition funnel. Dimethylamine (40% in water, 26.3 mL, 207.75 mmol) was added dropwise (at -78°C) to the stirring solution in an inert atmosphere of N₂ and stirred for three days at room temperature. The reaction was dried with N₂ and rotary evaporated forming an oil. Diethyl ether was added to the crude product and combined with a 2 M aqueous solution of NaOH. The diethyl ether was separated, dried with Na₂SO₄, and gravity filtered. The remaining solvent was removed by rotary evaporation and the crude product was dried at 80°C under high power vacuum overnight yielding a clear oil (0.570 g, 63%).
Synthesis of N,N-dimethyldocosaneamine

1-bromoedocosane (1.01g, 2.57 mmol) was dissolved in tetrahydrofuran (20 mL) in a round bottom flask. The solution was cooled on an acetone bath (containing CO₂ pellets) to -78°C, equipped with a stir-bar, and attached to an addition funnel. Dimethylamine (40% in water, 28.0 mL, 193 mmol) was added dropwise (at -78°C) to the stirring solution in an inert atmosphere of N₂ and stirred for three days at room temperature. The reaction was dried with N₂ and rotary evaporated forming oil.

Diethyl ether was added to the crude product and mixed with a 2 M aqueous solution of NaOH. The diethyl ether was separated, dried with Na₂SO₄, filtered, and remaining solvent was removed by rotary evaporation. The crude product was dried at 80°C under a high power vacuum overnight yielding a clear oil (0.368 g, 40.4%).

General Protocol A [for installation of remaining cationic head group(s) and tail(s)]

19 (M-P), 20 (M-I), or 21 (M-I,1) was added to a two neck round bottom flask and dissolved in ethanol. The flask was equipped with a stir bar and attached to a water-cooled condenser. Alkyl amine (NMe₂(CH₂)ₙ₋₁CH₃, where n = 8, 10, 12, 14, 16, 18, 20, or 22) was added slowly to the flask using a syringe. The reaction was heated to 80°C and run overnight at reflux. Volatile materials were removed under a flow of N₂ (g). The resulting crude solid was resuspended in acetone, vacuum filtered and dried in under vacuum.

Isothermal Titration Calorimetry

The CMC and ΔHₘic were determined using a Nano-ITC (TA-Instruments). Prior to each experiment the sample cell was washed extensively with dH₂O (300 mL), ethanol (100 mL), dH₂O (300 mL) again, followed by nanopure water (200 mL). Next, 950 µL of nanopure water was added to the sample cell. A concentrated aqueous solution (>>CMC) of amphiphile was prepared and equilibrated at 37°C. A 250 µL syringe was filled with the aqueous solution, loaded into the Nano ITC, and continuously stirred at 300 rpm. Multiple single injections in aliquots of 5 µL were injected.
into the sample cell with time intervals varying from 300s to 1400s. The Nano-Analyze program (TA-Instruments) was used to analyze the data.

**Bacterial Strains and Growth Conditions**

The Gram-positive bacterial strains used in this study were *Staphylococcus aureus subsp. aureus* ATCC® 29213™, *Enterococcus faecalis* ATCC® 29212™, *Streptococcus agalactiae* J48, and *Bacillus subtilis*. The Gram-negative bacterial strains used were *Escherichia coli* ATCC® 25922™ and *Pseudomonas aeruginosa* ATCC® 27853™. All strains were grown in Mueller-Hinton Broth at 37 °C for 12-24 h. For the MIC and combination studies, bacterial suspensions were prepared by diluting overnight cultures in Mueller-Hinton Broth to 5 x 10⁶ CFU/mL.\(^{44}\)

**Minimum Inhibitory Concentration and Minimum Bactericidal Concentration**

The methods used to determine the MIC and MBC were performed as previously described and followed the standards set forth by the Clinical and Laboratory Standards Institute.\(^{16,45}\) Briefly, compounds were serially diluted and 100 µL of each dilution were added to the wells of a 96-well flat-bottomed plate in triplicate. After adding 100 µL of the bacterial cell suspension, 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. In order to determine the MBC, a 100 µl aliquot from each triplicate well was grown on Todd-Hewitt agar and incubated for 24 h at 37 °C. The MBC was defined as the concentration of the compound that resulted in a 99.9% reduction of the bacterial CFU/mL. The MIC was considered to be bactericidal if the MBC was the same concentration or one concentration higher in the dilution series as the MIC.\(^{46}\)
III. Results and Discussion

Synthesis

Each of the amphiphiles in this study was prepared by two subsequent Menshutkin reactions (Scheme 1). To prepare the $M$-$P$ series (1-5), 1,3,5-trisbromomethylbenzene was reacted with a slight excess of pyridine resulting in intermediate 19 ($M$-$P$). Selective reaction at just one of the three equivalent benzylic positions was aided by the decreased solubility of the desired product, which precipitates from the reaction mixture upon formation. Filtration of the reaction mixture followed by subsequent washes of acetone yields intermediate 19 ($M$-$P$). Substitution of the two remaining benzylic bromides on 19 ($M$-$P$) was accomplished using excess dimethylalkylamine (NMe$_2$(CH$_2$)$_n$-1CH$_3$, where $n$ = 8, 10, 12, 14 or 16) in ethanol at reflux producing the series of amphiphilic products 1-5.

Scheme 1. Synthesis of Amphiphiles
To prepare $M-I\, (6-10)$ or $M-I, I\, (11-18)$ series 1,3,5 trisbromomethylbenzene was reacted with an excess of trimethylamine resulting in intermediates $20\, (M-I)$ and $21\, (M-I, I)$ respectively. Selective reaction at just one of the three equivalent benzylic positions was aided by the decreased solubility of the desired products, which precipitate from the reaction mixture upon formation. To separate intermediate $20\, (M-I)$ from the reaction mixture, the reaction mixture is heated to 60°C and filtered. Rotary evaporation of the remaining filtrate yields a mixture of $20\, (M-I)$ and unreacted starting material. This crude mixture is subsequently suspended in RT acetone and filtered yielding $20\, (M-I)$. Filtration of the reaction mixture followed by subsequent washes with a solution of acetone and ethanol yields intermediate $21\, (M-I, I)$. Substitution of the two remaining benzylic bromides on $20\, (M-I)$ or $21\, (M-I, I)$ was accomplished using excess dimethylalkylamine ($\text{NMe}_2(\text{CH}_2)_n\text{CH}_3$, where $n = 8,\, 10,\, 12,\, 14,\, 16,\, 18,\, 20,$ or $22$) in ethanol at reflux producing the series of amphiphilic products $5-18$.

**Isothermal Titration Calorimetry**

A Nano ITC was used to determine the critical aggregation concentration (CMC) and the heat of micelle formation $\Delta H_{\text{mic}}$. Briefly, the Nano ITC is used to measure the heat change associated with the demicellization of amphiphiles via power compensation. A concentrated aqueous solution of amphiphile ($>>\text{CMC}$) is titrated into a thermally controlled sample cell, initially containing pure water, in a series of discrete injections. Throughout the experiment, three ranges of concentrations are found associated with the concentration of amphiphile in the sample cell below, during, and above the CMC (Fig. 9). During initial injections, or the first range, measured heat
changes are due to the dilution of micelles and the demicellization of amphiphiles. This is seen in the first 11 injections of compound 9 (\(M-1,14,14\)), where micelles absorbed heat and disassociate into dissolved monomers (Fig. 9a). The heat rate produced by each injection ranged from -230 µJ/s to -237 µJ/s while the concentration of amphiphile in the sample cell remained below the CMC (Fig. 9a,b). As the concentration in the sample cell approaches the second range of concentrations, not all aggregates dissociate reducing the total absorbed heat for each injection. Concentrations in this range span from

**Figure 9.** Isothermal titration calorimetry was used to determine the critical aggregation concentration (CMC). Ranges I and III divide graph a and b by the measured heat changes associated with either demicellization and micelle dilution (Range I) or solely micelle dilution (Range III). Range II indicates the range of concentrations associated with the CMC. A concentrated solution of amphiphile in water was injected into Nano-pure water. a) The heat associated with each injection over time for 9 (\(M-1,1,14\)). b) Integration of the heat per injection normalized by the number of moles of each injection verses the increasing concentration of amphiphile in the well. Inset) CMC is indicated by calculating the first derivative.

\(^a\) Note that the 1st injection is generally ignored. \(^b\)
approximately 0.50 - 0.70 mM indicating the CMC range for 9 (Fig. 9b). When the concentration of amphiphile in the well is above the CMC, the third range, any absorbed heat is due only to the dilution of aggregates. By plotting the first derivative of the titration curve the CMC is determined by the lowest point on the graph, in this case 0.64 mM (Fig. 9, inset). Similar results were seen for compounds 2-5, 7-10, and 14-18.

**Critical Micelle Concentration**

All three series of compounds exhibit a decrease in CMC as tail length increased (Table 1, Table 2). CMC values between the M-P and M-I series are approximately the same for each tail length. This results in a plot of log (CMC) versus tail length with approximately equivalent slopes of (-0.3 ± 0.01) for each series (Fig. 10a,b,c). Thus the dependence of CMC on tail length is consistent between double-tailed amphiphiles with a pyridinium or QA head group. The plot of log (CMC) versus tail length for the M-I,1 series produced a slope approximately half that of the M-P and M-I series (-0.16).

<table>
<thead>
<tr>
<th>M-P</th>
<th>M-I</th>
<th>Tail Length</th>
<th>CMC [mM]</th>
<th>( \Delta H_{mic} ) [kJ/mol]</th>
<th>( \Delta G_{mic} ) [kJ/mol]</th>
<th>( T\Delta S_{mic} ) [kJ/mol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>7</td>
<td>10,10</td>
<td>14</td>
<td>-11</td>
<td>-21</td>
<td>-10</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>12,12</td>
<td>2.4</td>
<td>-23</td>
<td>-26</td>
<td>-3.1</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>14,14</td>
<td>0.60</td>
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<td>-29</td>
<td>4.9</td>
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<tr>
<td>5</td>
<td>10</td>
<td>16,16</td>
<td>0.18</td>
<td>-42</td>
<td>-33</td>
<td>9.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M-I,1</th>
<th>Compound (#)</th>
<th>Tail Length (n for M-I,1)</th>
<th>CMC [mM]</th>
<th>( \Delta H_{mic} ) [kJ/mol]</th>
<th>( \Delta G_{mic} ) [kJ/mol]</th>
<th>( T\Delta S_{mic} ) [kJ/mol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>14</td>
<td></td>
<td>21</td>
<td>-10</td>
<td>-20</td>
<td>-10</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td></td>
<td>11</td>
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</tr>
<tr>
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<td>18</td>
<td></td>
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<td>-24</td>
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<td></td>
<td>0.99</td>
<td>-33</td>
<td>-28</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Table 1. CMC, \( \Delta H_{mic} \), \( \Delta G_{mic} \), and \( T\Delta S_{mic} \) for the M-P and M-I series. Experiments were performed at 37°C.

Table 2. CMC, \( \Delta H_{mic} \), \( \Delta G_{mic} \), and \( T\Delta S_{mic} \) for the M-I,1 series. Experiments were performed at 37°C.
The difference between the average heat of initial injections (representing the total heat due to the dilution of micelles and demicellization of amphiphiles) and the average heat of injections following the CMC range (representing the heat due solely to the dilution of micelles) gives the heat of demicellization, $\Delta H_{\text{demic}}$. The $\Delta H_{\text{demic}}$ for compound 9 ($M-1,14,14$), was 37 kJ/mol, corresponding to a heat of micelle formation, $\Delta H_{\text{mic}}$, of -37 kJ/mol ($\Delta H_{\text{mic}} = -\Delta H_{\text{demic}}$). The $\Delta H_{\text{mic}}$ was exothermic (negative) for all three series of compounds. Thus the heat released from the additional hydrogen bonding of water molecules and hydrocarbon tail interactions are greater than the heat required to break water/water and water/chain interactions in the ordered sheath. Generally, the release of heat due to micelle formation was found to increase as amphiphile tail length increases (Table 1, Table 2).
The $\Delta G_{\text{mic}}$ can be approximated for the micellization of nonionic amphiphiles by
the equation \(^{50,51}\)

$$
\Delta G_{\text{mic}} = -[-RT \ln (\text{CMC}/55.5)]
$$

(2)

where the CMC represents an equilibrium between monomers which associate to form micelles. The CMC is expressed as a molar fraction [molar units divided by the molar concentration of water (55.5 mol/L)]. However this equation slightly changes for ionic amphiphiles due to the presence of counterions and their degree of ionization to the micelle surface. Thus the equation can be approximated for a fully ionized amphiphile as \(^{50,51}\)

$$
\Delta G_{\text{mic}} = -\{RT \ln (\text{CMC}/55.5)\}
$$

(3)

where $m$ is the concentration of counterions that associate with a micelle and $n$ is the number of monomers that associate to form a micelle. Since this study did not include determination of the degree of ionization, the $\Delta G_{\text{mic}}$ was approximated using equation (2). The $\Delta G_{\text{mic}}$ for all compounds in the study is negative and becomes more negative as tail length increases (Table 1, Table 2). Thus the increase in chain length of amphiphiles within a series is consistent with the increased propensity to spontaneously form micelles.
The $\Delta S_{\text{mic}}$ can also be approximated using the Gibbs-Helmholtz equation (1). To aid intuitive comparison of all thermodynamic factors, the negative of $T\Delta S_{\text{mic}}$ was reported for all compounds. In both double tailed series, the $-T\Delta S_{\text{mic}}$ was negative for amphiphiles up to a chain length of 12. This indicates that the entropy gained from the release of water in the frozen sheath around compounds 2 ($M$-$P$,10,10), 3 ($M$-$P$,12,12), 7 ($M$-$I$,10,10), and 8 ($M$-$I$,12,12) is greater than the decrease in entropy of amphiphiles due to the formation of micelles. Thus amphiphiles with a shorter chain length contribute to the spontaneous formation (negative $\Delta G_{\text{mic}}$) of micelles. The $-T\Delta S_{\text{mic}}$ increases to a positive value for double tailed amphiphiles with tail lengths exceeding 12 carbons. This indicates that the entropy gained from the release of water in the frozen sheath around compounds 4 ($M$-$P$,14,14), 5 ($M$-$P$,16,16), 9 ($M$-$I$,14,14), and 10 ($M$-$I$,16,16) is smaller than the decrease in entropy of amphiphiles due to the formation of micelles. Thus amphiphiles with a longer chain length entropically disfavor formation of micelles making micelle formation of these derivatives an enthalpy-driven process. This decrease in entropy may be explained by the cone angle of amphiphiles with longer hydrophobic tails. As tail length increases in a series analogous amphiphiles, the cone angle decreases resulting in a higher aggregation number per micelle. This results in a decrease in the entropy of a larger number of amphiphiles upon micelle formation (Fig. 11). Similar results were measured for the $M$-$I$,1 series where compounds 14 ($M$-$I$,1,14), 15 ($M$-$I$,1,16), and 16 ($M$-$I$,1,18) all had negative $-T\Delta S_{\text{mic}}$ values. Chain lengths exceeding 18 carbons [17 ($M$-$I$,1,20), 18 ($M$-$I$,1,22)] had positive $-T\Delta S_{\text{mic}}$ values.
Figure 11. The effect of tail length and number of tails on the cone angle. As chain length and the number of tails increase on an amphiphile the cone angle (θ) decreases. a) Single tailed amphiphiles with short tail lengths relatively have the largest cone angle (θ). b) Double tailed amphiphiles with short tail lengths have larger cone angles (θ) than analogous double tailed amphiphiles with longer tails. c) Single tailed amphiphiles with long tails have a smaller cone angle (θ) than analogous single tailed amphiphiles with shorter tails. d) Double tailed amphiphiles with long tails relatively have the smallest cone angle (θ).

Minimum Inhibitory Concentration

The MICs of the M-P and M-1 series of compounds were determined for four Gram-positive (Staphylococcus aureus, Enterococcus faecalis, Streptococcus agalactiae, and Bacillus subtilis) and two Gram-negative (Escherichia coli and Pseudomonas aeruginosa) bacteria (Table 3). For all tested compounds, the MBC was the same concentration or a two-fold concentration higher than the MIC, indicating the amphiphiles are bactericidal. With only a few exceptions, the MIC was below the CMC demonstrating that amphiphile aggregation was not required to kill bacteria.
For the $M$-$P$ and $M$-$I$ series, the MIC decreases as tail length increases for amphiphiles with tail lengths ranging from 8-12 carbons (Fig. 12). The 12-carbon derivatives, compounds 3 ($M$-$P,12,12$) and 8 ($M$-$I,12,12$), have the lowest MICs against each strain with values ranging from 1-16 µM. The MIC then increases for amphiphiles with tail lengths exceeding 12 carbons. This trend is indicative of the relationship between solubility and bioactivity and is consistent with previous studies on other amphiphile series. $^{16,17,21}$

Table 3. MICs (µM) of $M$-$P$ and $M$-$I$ amphiphiles. G$^+$ = Gram-positive, G$^-$ = Gram-negative.

<table>
<thead>
<tr>
<th></th>
<th>$M$-$P$</th>
<th>$M$-$I$</th>
<th>Tail length</th>
<th>B. subtilis (G$^+$)</th>
<th>E. faecalis (G$^+$)</th>
<th>S. agalactiae (G$^+$)</th>
<th>S. aureus (G$^+$)</th>
<th>E. coli (G$^-$)</th>
<th>P. aeruginosa (G$^+$)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>6</td>
<td>8,8</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
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<td>32</td>
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<td>8</td>
<td>16</td>
</tr>
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</table>

Figure 12. MIC comparison of 6 bacterial strains with respect to tail length. Circles (○) indicate the M-$P$ series, while squares (■) indicate the M-$I$ series. # = MIC > 250 µM. G$^+$ = Gram-positive, G$^-$ = Gram-negative.
The MIC values of the $M\text{-}l,1$ series of compounds were also determined for four Gram-positive (Staphylococcus aureus, Enterococcus faecalis, Streptococcus agalactiae, and Bacillus subtilis) and two Gram-negative (Escherichia coli and Pseudomonas aeruginosa) bacteria (Table 4). As expected the MIC decreased as tail length increased for tail lengths ranging from 8-18 carbons, with one exception in S. agalactiae (Fig. 13). Generally, the 18 and/or 20-carbon derivatives, 16 ($M\text{-}l,1,18$) and 17 ($M\text{-}l,1,20$), had the

Table 4. MICs (µM) of $M\text{-}l,1$ amphiphiles. G$^+$ = Gram-positive, G$^-$ = Gram-negative.

<table>
<thead>
<tr>
<th>$M\text{-}l,1$ Compound # (n for $M\text{-}l,1,n$)</th>
<th>Tail length</th>
<th>B. subtilis (G$^+$)</th>
<th>E. faecalis (G$^+$)</th>
<th>S. agalactiae (G$^+$)</th>
<th>S. aureus (G$^+$)</th>
<th>E. coli (G$^-$)</th>
<th>P. aeruginosa (G$^-$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>8</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>125</td>
<td>125-250</td>
<td>63</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>13</td>
<td>12</td>
<td>31-63</td>
<td>250</td>
<td>125</td>
<td>63</td>
<td>&gt;250</td>
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<td>31</td>
<td>16</td>
<td>63</td>
<td>250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>16-31</td>
<td>31</td>
<td>&gt;250</td>
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<tr>
<td>16</td>
<td>18</td>
<td>2-4</td>
<td>4</td>
<td>2</td>
<td>16</td>
<td>16</td>
<td>125</td>
</tr>
<tr>
<td>17</td>
<td>20</td>
<td>4</td>
<td>2-4</td>
<td>4</td>
<td>16</td>
<td>31</td>
<td>250</td>
</tr>
<tr>
<td>18</td>
<td>22</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
</tbody>
</table>

Figure 13. MIC comparison with respect to Tail Length of the $M\text{-}l,1$ series. # = MIC > 250 µM. G$^+$ = Gram-positive, G$^-$ = Gram-negative
lowest MIC values against each strain. The MIC increases for the amphiphile with a 22 carbon chain (18, M-1,1,20). This increase ranges from modest to significant, depending on the bacterial strain.

The M-P and M-1 series allows for comparison between analogous double-tailed amphiphiles with different head group structures. MIC values between M-P and M-1 amphiphiles with the same tail length were approximately equivalent, suggesting that substituting one of the quaternary ammonium head groups on an M-1 amphiphile with a pyridinium does not significantly affect bioactivity. The M-1 and M-1,1 series allows for comparison of analogous single-tailed and double-tailed amphiphiles in two ways. First, amphiphiles with the same nominal tail length were compared (e.g., M-1,8,8 versus M-1,1,8). Second, amphiphiles with the same total number of carbons in their hydrophobic region were compared (e.g., M-1,8,8 versus M-1,1,16), each with a total of 16 carbons in their tail(s).

Compounds 6 (M-1,8,8) and 11 (M-1,1,8) both have MIC values higher than 250 μM (Fig. 14). This is attributed to high solubility, and thus a lower propensity for the amphiphile to partition into the hydrophobic bacterial membrane. For pairs of compounds with tails ranging from 10 to 14 carbons [7 (M-1,10,10) vs. 12 (M-1,1,10); 8 (M-1,12,12) vs. 13 (M-1,1,12); 9 (M-1,14,14) vs. 14 (M-1,1,14)], double tailed amphiphiles generally have a lower MIC. Comparing compounds 10 (M-1,16,16) and 15 (M-1,1,16), the single tail amphiphile had a lower MIC. The minimum MIC for all three series of amphiphiles is approximately the same. For example, in the two double-tailed series, the 12 carbon
chained derivatives \([3 \, (M-P, 12, 12), \, 8 \, (M-1, 12, 12)]\) have MIC values of 4 µM against \(E. \ faecalis\). The most effective single tailed amphiphiles also have MIC values ranging from 2-4 µM against \(E. \ faecalis\). This suggests that for each series of amphiphiles reaching a hydrophobic-lipophilic balance yields the most biologically active amphiphile.

**Figure 14.** Reaching a hydrophobic-lipophilic balance within a series results in the most biologically active amphiphile. The MIC for each tail length in a series was averaged for all six bacterial strains and plotted against the tail length.

Amphiphiles from the \(M-1\) and \(M-1,1\) series are also compared by the number of carbons in their hydrophobic region (**Fig. 15**). There is a profound difference in the MIC values between compounds 6 \((M-1,8,8)\) and 15 \((M-1,1,16)\) against all strains with the exception of \(P. \ aeruginosa\). Compound 6 \((M-1,8,8)\) has MIC values above 250 µM while compound 15 \((M-1,1,16)\) has MIC values ranging from 4-31 µM (excluding P. \(aeruginosa\) which was greater than 250 µM). This suggests that the double tailed amphiphiles are not as biologically active as single tailed derivatives with the same total
hydrocarbon chain length. There is also a profound decrease in the CMC between compounds 6 \((M-1,8,8)\) and 15 \((M-1,1,16)\). Using the log function of compounds 7-10, reported earlier, the CMC for compound 6 \((M-1,8,8)\) can be extrapolated to ~46 mM. This is significantly higher than the CMC of 15 \((M-1,1,16)\), at 11 mM. This suggests that double tailed derivatives have a higher solubility in solution than analogous single tailed amphiphiles with the same head group structure (likely due to additional hydrophobic surfaces exposed to the water). Thus, amphiphiles that have a high solubility are consistent with high MIC values and amphiphiles that are less soluble in solution, until an optimal tail length, are consistent with low MIC values.

<table>
<thead>
<tr>
<th></th>
<th>6 ((M-1,8,8))</th>
<th>11 ((M-1,1,16))</th>
<th>7 ((M-1,10,10))</th>
<th>17 ((M-1,1,20))</th>
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</thead>
<tbody>
<tr>
<td>CMC (mM)</td>
<td>46</td>
<td>250</td>
<td>12</td>
<td>69</td>
</tr>
<tr>
<td>MIC (μM)</td>
<td>10.8</td>
<td>55</td>
<td>2.05</td>
<td>52</td>
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</table>

**Figure 15.** Comparing amphiphiles that have the same number of carbons in the hydrophobic region. a) Compares the CMC and MIC of compounds 6 and 11. b) Compares the CMC and MIC of compounds 7 and 17.
For compounds 7 (M-1,10,10) and 17 (M-1,1,20), the CMC values were relatively similar (12 mM and 2.0 mM respectively) which is consistent with similar MIC values for all strains of bacteria [(B. subtilis: 8 µM and 4 µM respectively) (E. faecalis: 8 µM and 4 µM respectively) (S. agalactiae: 4 µM and 4 µM respectively) (S. aureus: 16 µM and 16 µM respectively) (E. coli: 125 µM and 31 µM respectively) (P. aeruginosa: >250 µM and 250 µM respectively)] (Fig. 15). This also suggests that an amphiphile’s solubility in water is related to its biological activity. Interestingly, amphiphiles that have the lowest MIC values in their respective series [3 (M-P,12,12), 8 (M-1,12,12), 16 (M-1,18,18), and 17 (M-1,1,20)] are also consistent with a $-T\Delta S_{\text{mic}}$ value close to 0.
IV. Conclusions

Here three novel series of amphiphiles were synthesized and structure-activity relationships were investigated. All three series show a consistent decrease in CMC as tail length increases. MIC values indicate an optimal tail length of 12 carbons for the $M$-$P$ and $M$-$I$ series with compounds $3$ ($M$-$P$,12,12) and $8$ ($M$-$I$,12,12) having the highest antibacterial activity against all strains tested. MIC values indicate an optimal tail length of 18 - 20 carbons for the $M$-$I$,1 series with compounds $16$ ($M$-$I$,1,18) and $17$ ($M$-$I$,1,20) having the highest antibacterial activity against all strains tested.

Short-chained amphiphiles that are highly soluble in water have high CMC values relative to those with larger chains. Aggregation of short-chained amphiphiles is both enthalpically and entropically favorable. These amphiphiles are also associated with high MIC values. Long-chained amphiphiles that have a lower solubility in water have lower CMC values. Aggregation of long-chained amphiphiles is enthalpically favored and entropically disfavored. As with short-chained amphiphiles, these amphiphiles are also associated with high MIC values.

Amphiphiles with intermediate chain lengths and water solubility (relative within a series) have intermediate CMC values. Aggregation of these amphiphiles is enthalpically favorable with little to no entropic contribution to $\Delta G_{\text{mic}}$. These compounds [$3$ ($M$-$P$,12,12), $8$ ($M$-$I$,12,12), $16$ ($M$-$I$,1,18), and $17$ ($M$-$I$,1,20)] are associated with low MIC values. It is interesting to note that in all three series the most effective amphiphiles
(lowest MIC) are those with $\Delta S_{\text{mic}} \approx 0$. At this point, it is not clear if there is a causal relationship between these two phenomena.

Notably, the MIC values of compounds 3 ($M-P,12,12$) and 8 ($M-1,12,12$) against *P. aeruginosa* are comparable to those of tobramycin (3µg/ml), commonly used to treat infection in cystic fibrosis patients, and cefepime, an antipseudomonal cephalosporin (6 µg/ml). 62,53 Many antibacterial agents are ineffective against *P. aeruginosa* due to its semipermeable outer membrane and production of efflux pumps and β-lactamases. 54 While other antibacterial agents fail to inhibit *P. aeruginosa*, amphiphiles such as compounds 3 ($M-P,12,12$) and 8 ($M-1,12,12$) kill this resilient bacteria at low concentrations (8 µM and 16µM, respectively) which may prove useful in a healthcare setting.
V. Experimental

(19) Intermediate M-P

Analytical data for compound 19 (Intermediate M-P): mp = 223.9 – 226.8°C (DEC). $^1$H NMR (DMSO, 300 MHz, 25°C) δ: 9.22 (d, $^3$J = 3.23 Hz, 2H, Pyr-H), 8.66 (t, $^3$J = 7.81 Hz, 1H, Pyr-H), 8.22 (t, $^3$J = 7.18, 2H, Pyr-H), 7.56 (s, 1H, Ar-H), 7.54 (s, 2H, Ar-H), 5.89 (s, 2H, Ar-CH$_2$), 4.69 (s, 4H, Ar-CH$_2$). For synthetic procedure see methods and materials.

(20) Intermediate M-I

Analytical data for compound 20 (Intermediate M-I): $^1$H NMR (DMSO, 400 MHz, 25°C) δ: 7.69 (s, 1H, Ar-H); 7.57 (s, 2H, Ar-H); 4.76 (s, 4H, Ar-CH$_2$); 4.57 (s, 2H, Ar-CH$_2$); 3.11 (s, 9H, N-(CH$_3$)$_3$). For synthetic procedure see methods and materials.

(21) Intermediate M-I,1

Analytical data for compound 21 (Intermediate M-I,1): $^1$H NMR (DMSO, 400 MHz, 25°C) δ: 7.79 (s, 2H, Ar-H); 7.68 (s, 1H, Ar-H); 4.82 (s, 2H, Ar-CH$_2$); 4.65 (s, 4H, Ar-CH$_2$); 3.09 (s, 18H, N-(CH$_3$)$_3$). For synthetic procedure see methods and materials.

(1) M-P,8,8

The product was synthesized via general protocol A. Compound (19) (2.900 g, 6.660 mmol) was dissolved in ethanol (50 mL) and reacted with N,N-dimethyoctylamine (ACROS, 97%, 2.74 mL, 13.3 mmol). Reaction yielded 4.4044 g (88.1 %) of a tan solid, mp = 147.3 – 154.0°C (dec). $^1$H NMR (DMSO, 400 MHz, 25°C) δ: 9.36 (d, $^3$J = 5.8 Hz, 2H, Pyr-H), 8.66 (t, $^3$J = 7.7 Hz, 1H, Pyr-H), 8.199 (t, $^3$J = 6.6, 2H, Pyr-H), 7.89 (s, 2H, Ar-H), 7.87 (s, 1H, Ar-H), 6.09 (s, 2H, Ar-CH$_2$), 4.62 (s, 4H, Ar-CH$_2$), 3.03 (s, 12H, Ar-CH$_2$), 1.77 (s, 4H, N-CH$_2$CH$_3$), 1.162-1.377 (m, 20H), 0.869 (t, $^3$J = 6.1 Hz, 6H, -CH$_2$CH$_3$). $^{13}$C NMR (DMSO, 100 MHz, 25°C) δ: 146.13, 145.22, 138.03, 135.19, 135.08, 129.69, 128.30, 65.34, 63.66, 62.26, 49.12, 31.20, 28.55, 28.50, 25.89, 22.06, 21.85, 13.97.
(2) M-P, 10, 10
The product was synthesized via general protocol A. Compound (19) (1.200 g, 0.275 mmol) was dissolved in ethanol (50 mL) and reacted with N,N-dimethyldecylamine (TCI, >93%, 1.24 mL, 0.61 mmol). Reaction yielded 1.40 g (63.3% yield) of an off-white solid, mp = 171.4 – 182.0°C (dec). 1H NMR (DMSO, 400 MHz, 25°C): δ: 9.30 (d, 3J = 5.9 Hz, 2H, Pyr-H), 8.67 (t, 3J = 7.7 Hz, 1H, Pyr-H), 8.21 (t, 3J = 7.0, 2H, Pyr-H), 7.85 (s, 2H, Ar-H), 7.81 (s, 1H, Ar-H), 6.05 (s, 2H, Ar-CH₃), 4.58 (s, 4H, Ar-CH₂), 3.00 (s, 12H, N-C₆H₃), 1.77 (s, 4H, N-CH₂CH₂), 1.135 – 1.38 (m, 28H), 0.85 (t, 3J = 6.1 Hz, 6H, -CH₂CH₃). 13C NMR (DMSO, 100 MHz, 25°C): δ: 146.11, 145.23, 138.03, 135.20, 135.08, 129.69, 128.29, 65.32, 63.61, 62.22, 49.12, 31.29, 28.95, 28.88, 28.69, 28.61, 25.90, 22.10, 21.86, 13.96.

(3) M-P, 12, 12
The product was synthesized via general protocol A. Compound (19) (0.250 g, 0.573 mmol) was dissolved in ethanol (5 mL) and reacted with N,N-dimethyldodecylamine (MP Biomedicals, 0.34 mL, 1.26 mmol). Reaction yielded 0.383 g (77.5% yield) of an off-white solid, mp = 192.2 – 195.7°C (dec). 1H NMR (DMSO, 400 MHz, 25°C): δ: 9.34 (d, 3J = 6.02 Hz, 2H, Pyr-H), 8.67 (t, 3J = 7.83 Hz, 1H, Pyr-H), 8.20 (t, 3J = 7.14, 2H, Pyr-H), 7.87 (s, 2H, Ar-H), 7.83 (s, 1H, Ar-H), 6.07 (s, 2H, Ar-CH₂), 4.60 (s, 4H, Ar-CH₂), 3.02 (s, 12H, N-CH₃), 1.76 (s, 4H, N-CH₂CH₂), 1.15-1.39 (m, 36H), 0.85 (t, 3J = 6.5 Hz, 6H, -CH₂CH₃). 13C NMR (DMSO, 75 MHz, 25°C): δ: 146.09, 145.23, 138.03, 135.20, 135.07, 129.69, 128.29, 65.32, 63.57, 62.20, 49.12, 31.29, 29.05, 29.02, 29.00, 28.88, 28.72, 28.61, 25.90, 22.08, 21.86, 13.94.

(4) M-P, 14, 14
The product was synthesized via general protocol A. Compound (19) (0.500 g, 1.150 mmol) was dissolved in ethanol (5 mL) and reacted with N,N-dimethyltetradecylamine (Aldrich, 95%, 0.77 mL, 2.52 mmol). Reaction yielded 0.920 g (87.0% yield) of an off-white solid, mp = 201.5 – 213.0°C (dec). 1H NMR (DMSO, 400 MHz, 25°C): δ: 9.23 (d, 3J = 5.5 Hz, 2H, Pyr-H), 8.68 (t, 3J = 7.7 Hz, 1H, Pyr-H), 8.21 (t, 3J = 6.81, 2H, Pyr-H), 7.8 (s, 2H, Ar-H), 7.75 (s, 1H, Ar-H), 6.01 (s, 2H, Ar-CH₂), 4.55 (s, 4H, Ar-CH₂), 2.98 (s, 12H, N-CH₃), 1.76 (s, 4H, N-CH₂CH₂), 1.17-1.35 (m, 44H), 0.856 (t, 3J = 6.5 Hz, 6H, -CH₂CH₃). 13C
NMR (DMSO, 100 MHz, 25°C) δ: 146.10, 145.24, 138.01, 135.21, 135.08, 129.69, 128.28, 65.34, 63.54, 62.21, 49.13, 31.29, 29.08, 29.02, 28.90, 28.71, 26.63, 25.91, 22.08, 21.86, 13.94.

(5) M-P,16,16
The product was synthesized via general protocol A. Compound (19) (0.250 g, 0.573 mmol) was dissolved in ethanol (5 mL) and reacted with N,N-dimethyldodecylamine (TCI, 98%, 0.42 mL, 1.26 mmol). Reaction yielded 0.512 g (91.6% yield) of an off-white solid, mp = 197.7 – 216.6°C (dec). 1H NMR (DMSO, 400 MHz, 25°C) δ: 9.32 (d, J = 6.11 Hz, 2H, Pyr-H), 8.67 (t, J = 7.79 Hz, 1H, Pyr-H), 8.20 (t, J = 7.19, 2H, Pyr-H), 7.86 (s, 2H, Ar-H), 7.81 (s, 1H, Ar-H), 6.06 (s, 2H, Ar-CH2), 4.60 (s, 4H, Ar-CH2), 3.01 (s, 12H, N-CH3), 1.76 (s, 4H, N-CH2CH2), 1.10 – 1.36 (m, 52H), 0.851 (t, J = 6.7 Hz, 6H, -CH2CH3).

13C NMR (DMSO, 75 MHz, 25°C) δ: 146.11, 145.22, 137.98, 135.20, 135.07, 129.68, 128.28, 65.36, 63.52, 62.23, 49.12, 31.27, 29.06, 29.00, 28.90, 28.69, 28.62, 25.91, 22.07, 21.86, 13.93.

(6) M-1,8,8
The product was synthesized via general protocol A. Compound (20) (502 mg, 1.20 mmol) was dissolved in ethanol (30 mL) and reacted with N,N-dimethyloctylamine (ACROS, 97%, 0.67 mL, 3.00 mmol). Reaction yielded 150 mg (19.8% yield) of a white solid, mp = 182.2 – 186.3°C (dec). 1H NMR (DMSO, 400 MHz, 25°C) δ: 7.86 (s, 1H, Ar-H); 7.83 (s, 2H, Ar-H); 4.62 (s, 2H, Ar-CH2); 4.59 (s, 4H, Ar-CH2); 3.11 (s, 9H, N-(C3H3)3); 3.02 (s, 12H, N-(C3H3)2); 1.80 (m, 4H, N-CH2-CH3); 1.20 – 1.39 (m, 20H); 0.88 (t, J = 6.9 Hz, 6H, -CH2-CH3). 13C NMR (DMSO, 100 MHz, 25°C) δ: 139.04, 138.81, 129.70, 129.51, 66.84, 65.36, 63.45, 51.81, 49.10, 31.19, 28.55, 22.92, 22.06, 21.86, 13.96.

(7) M-1,10,10
The product was synthesized via general protocol A. Compound (20) (100 mg, 0.30 mmol) was dissolved in ethanol (15 mL) and reacted with N,N-dimethyldecylamine (TCI, >93%, 150 mg, 0.75 mmol). Reaction yielded 156 mg (66% yield) of a white solid, mp = 189.6 – 191.8°C (dec). 1H NMR (DMSO, 400 MHz, 25°C) δ: 7.85 (s, 1H, Ar-H); 7.82 (s, 2H, Ar-H); 4.61 (s, 2H, Ar-CH2); 4.59 (s, 4H, Ar-CH2); 3.10 (s, 9H, N-(CH3)3); 3.02 (s, 12H, N-(CH3)2); 1.80 (m, 4H, N-CH3-CH3); 1.18 – 1.39 (m, 28H); 0.86 (t, J = 6.8 Hz,
$^1$H NMR (DMSO, 100 MHz, 25°C) $\delta$: 139.03, 138.80, 129.70, 129.51, 66.86, 65.37, 51.81, 49.10, 31.28, 28.94, 28.91, 28.69, 28.61, 25.93, 22.10, 21.86, 13.96.

**M-1,12,12**

The product was synthesized via general protocol A. Compound (20) (105 mg, 0.30 mmol) was dissolved in ethanol (15 mL) and reacted with N,N-dimethyldodecylamine (MP Biomedicals, 170 mg, 0.75 mmol). Reaction yielded 160 mg (63% yield) of a white solid, mp = 202.9 – 204.2°C (dec). $^1$H NMR (DMSO, 400MHz, 25°C) $\delta$: 7.84 (s, 1H, Ar-$H$); 7.81 (s, 2H, Ar-$H$); 4.61 (s, 2H, Ar-CH$_2$); 4.59 (s, 4H, Ar-CH$_2$); 3.10 (s, 9H, N-(CH$_3$)$_3$); 3.01 (s, 12H, N-(CH$_3$)$_2$); 1.80 (m, 4H, N-CH$_3$-CH$_2$); 1.16–1.40 (m, 36H); 0.86 (t, $^3$J = 6.8 Hz, 6H, -CH$_2$CH$_3$). $^{13}$C NMR (DMSO, 100 MHz, 25°C) $\delta$: 139.01, 138.79, 129.70, 129.51, 66.90, 65.39, 63.47, 51.82, 49.10, 31.29, 29.06, 29.02, 28.99, 28.92, 28.73, 28.62, 25.94, 22.09, 21.86, 13.95.

**M-1,14,14**

The product was synthesized via general protocol A. Compound (20) (105 mg, 0.30 mmol) was dissolved in ethanol (15 mL) and reacted with N,N-dimethyltetradecylamine (Aldrich, 95%, 195 mg, 0.75 mmol). Reaction yielded 202 mg (75% yield) of a white solid, mp = 207.0 – 212.4°C (dec). $^1$H NMR (DMSO, 400MHz, 25°C) $\delta$: 7.83 (s, 1H, Ar-$H$); 7.81 (s, 2H, Ar-$H$); 4.61 (s, 2H, Ar-CH$_2$); 4.58 (s, 4H, Ar-CH$_2$); 3.10 (s, 9H, N-(CH$_3$)$_3$); 3.01 (s, 12H, N-(CH$_3$)$_2$); 1.79 (m, 4H, N-CH$_3$-CH$_2$); 1.17–1.38 (m, 36H); 0.85 (t, $^3$J = 6.8 Hz, 6H, -CH$_2$CH$_3$). $^{13}$C NMR (DMSO, 100 MHz, 25°C) $\delta$: 139.01, 138.79, 129.70, 129.51, 66.88, 65.39, 63.41, 51.82, 49.10, 31.29, 29.08, 29.03, 28.93, 28.72, 28.63, 25.95, 22.08, 21.86, 13.95.

**M-1,16,16**

The product was synthesized via general protocol A. Compound (20) (100 mg, 1.30 mmol) was dissolved in ethanol (15 mL) and reacted with N,N-dimethylhexadecylamine (TCI, 98%, 200 mg, 0.75 mmol). Reaction yielded 170 mg (60% yield) of a white solid, mp = 209.3 – 211.1°C (dec). $^1$H NMR (DMSO, 400MHz, 25°C) $\delta$: 7.83 (s, 1H, Ar-$H$); 7.80 (s, 2H, Ar-$H$); 4.60 (s, 2H, Ar-CH$_2$); 4.58 (s, 4H, Ar-CH$_2$); 3.09 (s, 9H, N-(CH$_3$)$_3$); 3.00 (s, 12H, N-(CH$_3$)$_2$); 1.80 (m, 4H, N-CH$_3$-CH$_2$); 1.20–1.39 (m, 52H); 0.85 (t, $^3$J = 6.8 Hz, 6H, -CH$_2$CH$_3$). $^{13}$C NMR (DMSO, 100 MHz, 25°C) $\delta$: 139.01, 138.80, 129.70, 129.51, 66.88, 65.39, 63.41, 51.82, 49.10, 31.29, 29.08, 29.03, 28.93, 28.72, 28.63, 25.95, 22.08, 21.86, 13.95.
6.8 Hz, 6H, -CH₂CH₃).¹³C NMR (DMSO, 100 MHz, 25°C) δ: 139.00, 138.80, 129.70, 129.51, 66.91, 65.41, 63.40, 51.83, 49.11, 31.29, 29.08, 29.02, 28.94, 28.70, 28.64, 25.96, 22.09, 21.87, 13.95.

(11) M-1, 1, 8
The product was synthesized via general protocol A. Compound (21) (200 mg, 0.421 mmol) was dissolved in ethanol (20 mL) and reacted with N,N-dimethyloctylamine (ACROS, 97%, 0.104 ml, 0.505 mmol). Reaction yielded 183.8 mg (69% yield) of a white solid. ¹H NMR (DMSO, 400MHz, 25°C) δ: 7.93 (s, 2H, Ar-H); 7.89 (s, 1H, Ar-H); 4.71 (s, 4H, Ar-CH₂); 4.67 (s, 2H, Ar-CH₂); 3.17 (s, 18H, N-(CH₃)₃); 3.08 (s, 6H, N-(CH₃)₂); 1.80 (m, 2H, N-CH₃-CH₂); 1.19-1.40 (m, 10H); 0.869 (t, 3J = 6.6 Hz, 3H, -CH₂CH₃).

(12) M-1, 1, 10
The product was synthesized via general protocol A. Compound (21) (200 mg, 0.421 mmol) was dissolved in ethanol (15 mL) and reacted with N,N-dimethyldecylamine (TCI, >93%, 0.120 ml, 0.505 mmol). Reaction yielded 144 mg (43.5% yield) of a white solid. ¹H NMR (DMSO, 400MHz, 25°C) δ: 7.92 (s, 2H, Ar-H); 7.88 (s, 1H, Ar-H); 4.71 (s, 4H, Ar-CH₂); 4.67 (s, 2H, Ar-CH₂); 3.17 (s, 18H, N-(CH₃)₃); 3.08 (s, 6H, N-(CH₃)₂); 1.80 (m, 2H, N-CH₃-CH₂); 1.18-1.38 (m, 14H); 0.860 (t, 3J = 6.3 Hz, 3H, -CH₂CH₃).

(13) M-1, 1, 12
The product was synthesized via general protocol A. Compound (21) (200 mg, 0.421 mmol) was dissolved in ethanol (20 mL) and reacted with N,N-dimethyldecylamine (MP Biomedicals, 0.137 ml, 0.505 mmol). Reaction yielded 202 mg (69.7% yield) of a white solid. ¹H NMR (DMSO, 400MHz, 25°C) δ: 7.94 (s, 2H, Ar-H); 7.90 (s, 1H, Ar-H); 4.72 (s, 4H, Ar-CH₂); 4.68 (s, 2H, Ar-CH₂); 3.18 (s, 18H, N-(CH₃)₃); 3.08 (s, 6H, N-(CH₃)₂); 1.80 (m, 2H, N-CH₃-CH₂); 1.17-1.37 (m, 18H); 0.845 (t, 3J = 6.2 Hz, 3H, -CH₂CH₃).

(14) M-1, 1, 14
The product was synthesized via general protocol A. Compound (21) (200 mg, 0.421 mmol) was dissolved in ethanol (20 mL) and reacted with N,N-dimethyltetradecylamine (ALDRICH, 95%, 0.153 ml, 0.505 mmol). Reaction yielded 236 mg (78.2% yield) of a white solid. ¹H NMR (DMSO, 400MHz, 25°C) δ: 7.90
(15) **M-1,1,16**

The product was synthesized via general protocol A. Compound (21) (600 mg, 1.26 mmol) was dissolved in ethanol (15 mL) and reacted with N,N-dimethyhexadecylamine (TCI, 98%, 0.510 mL, 1.52 mmol). Reaction yielded 338 mg (36% yield) of a white solid. $^1$H NMR (DMSO, 400MHz, 25$^\circ$C) $\delta$: 7.93 (s, 2H, Ar-H); 7.89 (s, 1H, Ar-H); 4.71 (s, 4H, Ar-CH$_2$); 4.67 (s, 2H, Ar-CH$_2$); 3.17 (s, 18H, N-(CH$_3$)$_3$); 3.08 (s, 6H, N-(CH$_3$)$_2$); 1.80 (m, 2H, N-CH$_3$-CH$_2$); 1.18–1.37 (m, 26H); 0.853 (t, $^3$J = 6.5 Hz, 3H, -CH$_2$CH$_3$).

(16) **M-1,1,18**

The product was synthesized via general protocol A. Compound (21) (500 mg, 1.05 mmol) was dissolved in ethanol (60 mL) and reacted with N,N-dimethyloctadecylamine (TCI, 85%, 0.437 mL, 1.26 mmol). Reaction yielded 457 mg (43% yield) of a white solid. $^1$H NMR (DMSO, 400MHz, 25$^\circ$C) $\delta$: 7.91 (s, 2H, Ar-H); 7.90 (s, 1H, Ar-H); 4.71 (s, 4H, Ar-CH$_2$); 4.66 (s, 2H, Ar-CH$_2$); 3.16 (s, 18H, N-(CH$_3$)$_3$); 3.07 (s, 6H, N-(CH$_3$)$_2$); 1.80 (m, 2H, N-CH$_3$-CH$_2$); 1.12–1.45 (m, 30H); 0.847 (t, $^3$J = 6.3 Hz, 3H, -CH$_2$CH$_3$).

(17) **M-1,1,20**

The product was synthesized via general protocol A. Compound (21) (493 mg, 1.05 mmol) was dissolved in ethanol (100 mL) and reacted with N,N-dimethylicosanamine (410 mg, 1.26 mmol). Reaction yielded 715 mg (85.1% yield) of a white solid. $^1$H NMR (DMSO, 400MHz, 25$^\circ$C) $\delta$: 7.88 (s, 2H, Ar-H); 7.84 (s, 1H, Ar-H); 4.67 (s, 4H, Ar-CH$_2$); 4.64 (s, 2H, Ar-CH$_2$); 3.14 (s, 18H, N-(CH$_3$)$_3$); 3.05 (s, 6H, N-(CH$_3$)$_2$); 1.80 (m, 2H, N-CH$_3$-CH$_2$); 1.13–1.44 (m, 34H); 0.852 (t, $^3$J = 6.5 Hz, 6H, -CH$_2$CH$_3$).

(18) **M-1,1,22**

The product was synthesized via general protocol A. Compound (21) (99 mg, 0.21 mmol) was dissolved in ethanol (15 mL) and reacted with N,N-dimethydocosanylamine (89 mg, 0.252 mmol). Reaction yielded 87
mg (50% yield) of a white solid. $^1$H NMR (DMSO, 400MHz, 25°C) δ: 7.85 (s, 2H, Ar-H); 7.81 (s, 1H, Ar-H); 4.64 (s, 4H, Ar-CH$_2$); 4.61 (s, 2H, Ar-CH$_2$); 3.12 (s, 18H, N-(CH$_3$)$_3$); 3.02 (s, 6H, N-(CH$_3$)$_2$); 1.80 (m, 2H, N-CH$_3$-CH$_2$); 1.24–1.43 (m, 38H); 0.85 (t, $^3$$J$ = 6.6 Hz, 3H, -CH$_2$CH$_3$).
Figure 16. 19 (M-P) $^1$H NMR (DMSO, 300 MHz, 25°C)
Figure 17. 1 (M-P,8,8) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 18. 2 \( (M-P,10,10) \) \( ^1 \) H NMR (DMSO, 400 MHz, 25°C)
Figure 19. 3 (M-P,12,12) ¹H NMR (DMSO, 300 MHz, 25°C)
Figure 20. 4 (M-P,14,14) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 21. 5 (M-P,16,16) $^1$H NMR (DMSO, 300 MHz, 25°C)
Figure 22. 20 ($M$-$I$) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 23. 6 (M-1,8,8) $^1$H NMR (DMSO, 400 MHz, 25°C)
**Figure 24.** $7 (M-1,10,10)$ $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 25. 8 ($M$-$1,12,12$) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 26. 9 (M-1,14,14) \textsuperscript{1}H NMR (DMSO, 400 MHz, 25°C)
Figure 27. 10 (M-1,16,16) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 28. 21 (M-1) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 29. 11 (M-1,1,8) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 30. 12 (M-1,1,10) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 31. 13 (M-L, L,L) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 32. 14 (M-1, 1,14) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 33. 15 (M-1,1,16) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 34. 16 (M-1, 1, 18) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 35. 17 (M-1,1,20) $^1$H NMR (DMSO, 400 MHz, 25°C)
Figure 36. 18 $(M-1,1,22)$ $^1$H NMR (DMSO, 400 MHz, 25°C)
VII. References


