Colloidal and Antibacterial Properties of Novel Triple-Headed, Double-Tailed Amphiphiles: Exploring Structure-Activity Relationships and Synergistic Mixtures

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Abbreviations: MBC, Minimum Bactericidal Concentration; MIC, Minimum Inhibitory Concentration; CAC, Critical Aggregation Concentration; ARI, Antimicrobial-Resistant Infections; ITC, Isothermal Titration Calorimetry.

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Abstract:

Two novel series of tris-cationic, triple-headed, double-tailed amphiphiles were synthesized and the effects of tail length and head group composition on the critical aggregation concentration (CAC), thermodynamic parameters, and minimum inhibitory concentration (MIC) against six bacterial strains were investigated. Synergistic antibacterial combinations of these amphiphiles were also identified. Amphiphiles in this study are composed of a benzene core with three benzylic ammonium bromide groups, two of which have alkyl chains, each 8 to 16 carbons in length. The third head group is a trimethylammonium or pyridinium. Log of critical aggregation concentration ($\log[\text{CAC}]$) and heat of aggregation (ΔH_{agg}) were both inversely proportional to the length of the linear hydrocarbon chains. Antibacterial activity increases with tail length until an optimal tail length of 12 carbons per chain, above which, activity decreased. The derivatives with two 12 carbon chains had the best antibacterial activity, killing all tested strains at concentrations of 1-2 μM for Gram-positive and 4-16 μM for Gram-negative bacteria. The identity of the third head group (trimethylammonium or pyridinium) had minimal effect on colloidal and antibacterial activity. The antibacterial activity of several binary combinations of amphiphiles from this study was higher than activity of individual amphiphiles, indicating that these combinations are synergistic. These amphiphiles show promise as novel antibacterial agents that could be used in a variety of applications.

1. Introduction

Over the past few decades, the overuse of antibiotics has resulted in an increase in antibiotic-resistant bacteria.¹⁻⁵ In addition, the production of novel antimicrobials continues to decrease, primarily due to low financial return.^{5,6} This combination of circumstances has contributed to the increasing prevalence of antimicrobial-resistant infections (ARIs), especially in the hospital setting. ARIs have contributed to tens of

thousands of deaths per year in the European Union and United States alone.^{7,8} Hospitals and nursing homes are particularly prone to harboring antimicrobial-resistant organisms due to the frequent use of antimicrobial agents and an influx of infected, and frequently, immunocompromised patients.⁹ Limiting the transmission of bacteria from contaminated equipment and individuals to a susceptible population is critical to reducing the risk of hospital acquired infections by antibiotic resistant organisms.^{9,10} The development of effective novel antibacterials could be of benefit in the healthcare setting by decreasing the cost associated with treating infected patients, and ultimately, decreasing mortality due to these types of infections.¹¹

The use of cationic amphiphiles as antimicrobials was first reported in 1935.¹² Amphiphiles continue to be utilized as antimicrobial agents in detergents, disinfectants, cosmetics, and other common household products.¹³ A large variety of novel amphiphiles has been synthesized in an effort to increase effectiveness and specificity.¹⁴⁻²⁴

Amphiphile structure, including size and relative number of hydrophobic tails and hydrophilic head groups, governs colloidal characteristics including the critical aggregation concentration (CAC) and thermodynamic properties.²⁵ At concentrations below the CAC, amphiphiles tend to align at the air-water interface in equilibrium with dissolved monomers in solution.^{25,26} At concentrations above the CAC, amphiphiles form aggregates in which hydrocarbon tails interact with each other releasing water that was formerly associated with the tails, resulting in a second equilibrium between monomers and aggregates in solution.^{25,27} An increase in entropy typically associated with aggregate formation is generally attributed in large part to the release of water molecules surrounding hydrophobic units to the bulk water that accompanies this process.²⁵ Increasing amphiphile hydrophobicity (for example by increasing the length or number of tails) decreases water solubility, thus decreasing CAC. In contrast, additional head groups can increase amphiphile solubility, typically resulting in a higher CAC.²⁸⁻³¹

Amphiphile structure also affects antimicrobial effectiveness. A 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 microbial growth. Typically, as tail length increases the MIC decreases until an optimal tail length is reached. The MIC then increases for amphiphiles with tail lengths exceeding this optimal length.¹⁴⁻²¹ Variations in spacing between head groups can also impact the MIC. For example, studies conducted by our labs show that amphiphiles with a 5-carbon spacer between two cationic head groups are the most biologically active.¹⁴ The amphiphiles in the current study also have a 5-carbon spacer between cationic groups.

Some mixtures of two or more amphiphiles exhibit synergy – the inhibition of bacteria at lower concentrations than when each amphiphile is used separately.³² By decreasing the required concentration of

compounds, combination therapy reduces the potential for, or degree of, adverse side effects and increases the effectiveness of antibacterial agents. The improved efficacy of synergistic combinations has contributed to the improvement of hand disinfectants and effective treatment for patients with ARIs.³³⁻³⁶

In this report, the synthesis and colloidal, antibacterial, and synergistic characteristics for two novel series of triple-headed, double-tailed amphiphiles are reported (**Fig. 1**). Amphiphiles in this study consist of three cationic head groups connected to a mesitylene (*M*) core. Two of the head groups are dimethylalkylammonium groups with linear hydrocarbon tails from 8 to 16 carbons in length. The third head group is either pyridinium (*M-P* series) or trimethylammonium (*M-I* series).

2. Results and Discussion

2.1 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-trisubstituted benzene was reacted with a slight excess of pyridine resulting in intermediate **11** (77% yield). 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 and washing with acetone provides compound **11** in sufficient purity for the next synthetic step. Substitution of the two remaining benzylic bromides on **11** was accomplished using excess dimethylalkylamine ($\text{NMe}_2(\text{CH}_2)_n\text{CH}_3$, where $n = 8, 10, 12, 14$ or 16) in ethanol at reflux producing the *M-P* series of amphiphilic products **1-5** (63 – 92% yield).

To prepare the *M-I* series (**6-10**), 1,3,5 trisubstituted benzene was reacted with a slight excess of trimethylamine resulting in intermediate **12** (37% yield). This reaction produces primarily **12**, along with a highly insoluble biscationic byproduct (a result of two benzylic bromides being substituted with trimethylamine), which can be effectively removed by an extraction protocol as detailed in the experimental section. Substitution of the two remaining benzylic bromides on **12** was accomplished using excess dimethylalkylamine ($\text{NMe}_2(\text{CH}_2)_n\text{CH}_3$, where $n = 8, 10, 12, 14, 16$) in ethanol at reflux producing the *M-I* series of amphiphilic products **6-10** (20 – 75% yield).

2.2 Critical Aggregation Concentration and Heat of Aggregate Formation

Isothermal titration calorimetry (ITC) was used to determine the critical aggregation concentration (CAC) and the thermodynamic parameters associated with aggregate formation for each amphiphile (Table 1). Briefly, the Nano ITC was used to measure the heat change associated with the deaggregation of amphiphiles via power compensation.³⁷⁻⁴⁰ A concentrated aqueous solution of amphiphile ($\gg \text{CAC}$) was titrated into a thermally controlled sample cell, initially containing pure water, in a series of discrete injections. Typical ITC results can be divided into three ranges associated with the concentration of

amphiphile in the sample cell below, at, and above the CAC, as seen for compound **9** (*M-I,14,14*, **Fig. 2**). During initial injections (Range I), measured heat changes are due to both the dilution of aggregates and deaggregation. This can be observed in the first 10 injections, where aggregates absorbed heat and disassociated into dissolved monomers.^a The heat change produced by each injection was approximately constant in this range. In Range II, injected aggregates don't fully dissociate, reducing the total absorbed heat for each subsequent injection. At concentrations well above the CAC (Range III), absorbed heat is attributed only to the dilution of aggregates and is approximately constant. The CAC was determined by the inflection point in Range II, as measured by the minimum value in the plot of CAC versus the first derivative of the heat (**Fig. 2**, inset). Analogous results were recorded for compounds **2-5** and **7-10**, the results of which are presented in table 1. CAC and thermodynamic data were not determined for the amphiphiles with the shortest tails [**1** (*M-P,8,8*) and **6** (*M-I,8,8*)] because the heat evolved during injection was above the threshold of the instrument.

Both series of compounds exhibit a decrease in CAC as tail length increases (Table 1). CAC values for the *M-P* and *M-I* series are approximately equivalent for each tail length, following a linear plot of log(CAC) versus tail length (**Fig. 3a**) according to the equation:

$$\log(\text{CAC}) = A - Bn \quad (1)$$

where *A* and *B* are constants and *n* is the number of carbons in each hydrocarbon tail.^{29, 41, 42} The dependence of CAC on tail length is the same for each series (*B* = 0.31).

The difference between the average heat of injections in Range I, representing the total heat due to the dilution of aggregates and deaggregation, and the average heat of injections in Range III, representing the heat due solely to the dilution of aggregates, gives the heat of deaggregation, ΔH_{deagg} . The ΔH_{deagg} for compound **9** (*M-I,14,14*) is 37 kJ/mol, corresponding to a heat of aggregate formation, ΔH_{agg} , of -37 kJ/mol ($\Delta H_{\text{agg}} = -\Delta H_{\text{deagg}}$). The negative ΔH_{agg} values indicate that aggregation is an exothermic process for all of the amphiphiles in the study (Table 1). This is attributed to the release of heat accompanying aggregate formation from additional hydrogen bonding of water molecules joining the bulk, and from hydrocarbon tail interactions in the newly formed aggregate. Furthermore, ΔH_{agg} becomes more negative as tail length increases for both series of amphiphiles, following a linear trend (**Fig. 3b**) according to the equation:

$$\Delta H_{\text{agg}} = C - Dn \quad (2)$$

^a Typically the 1st injection is ignored.⁴³

where C and D are constants and n is the number of carbons in each hydrocarbon tail.^{29, 41, 42} The dependence of ΔH_{agg} on tail length is similar for each series ($D = -5.1$ for the *M-P* series; $D = -5.8$ for the *M-I* series). The inverse relationship between ΔH_{agg} and amphiphile tail length is consistent with a larger amount of water released per molecule of amphiphile, as well as greater van der Waals interactions between the longer tails.

The ΔG_{agg} can be approximated for the aggregation of nonionic amphiphiles by the equation

$$\Delta G_{\text{agg}} = RT \ln(\text{CAC}/55.5) \quad (3)$$

where the CAC represents an equilibrium between monomeric and aggregated amphiphiles.^{43, 44} The CAC is expressed as a molar fraction [molar units divided by the molar concentration of water (55.5 mol/L)]. This equation changes for ionic amphiphiles due to the presence of counterions and the degree of ionization at the aggregate surface. Thus the equation is modified for an ionic amphiphile as^{43, 44}

$$\Delta G_{\text{agg}} = RT [1+(m/n)] \ln(\text{CAC}/55.5) \quad (4)$$

where m is the concentration of counterions that associate with a aggregate and n is the number of monomers that associate to form an aggregate.^{43, 44} Since this study did not include determination of the degree of ionization or aggregate aggregation, ΔG_{agg} was approximated using equation (3), as has been done by others.^{28, 39, 40} The ΔG_{agg} for all compounds in the study is negative and becomes more negative as tail length increases (Table 1). Thus the increase in chain length within a series of amphiphiles is consistent with the increased propensity to spontaneously form aggregates.

The entropy of aggregation (ΔS_{agg}) is dictated by a combination of entropic factors that favor or disfavor aggregate formation and can be approximated using the Gibbs-Helmholtz equation:

$$\Delta G_{\text{agg}} = \Delta H_{\text{agg}} - T\Delta S_{\text{agg}} \quad (5)$$

To aid the reader, the entropic contribution to ΔG_{agg} , is presented as the negative of $T\Delta S_{\text{agg}}$ (Table 1). In both series, the $-T\Delta S_{\text{agg}}$ term is negative for amphiphiles with chain lengths of up to 12 carbons [**2** (*M-P*, 10, 10), **3** (*M-P*, 12, 12), **7** (*M-I*, 10, 10), and **8** (*M-I*, 12, 12)]. This suggests that the entropy gained from the release of water from hydrophobic sections is greater than the decrease in entropy of amphiphiles due to the formation of aggregates. Thus the entropic factor, for amphiphiles with shorter chain lengths, contributes to the spontaneous formation (negative ΔG_{agg}) of aggregates.

The $-T\Delta S_{\text{agg}}$ term is positive for double tailed amphiphiles with tail lengths exceeding 12 carbons [**4** (*M-P,14,14*), **5** (*M-P,16,16*), **9** (*M-I,14,14*), and **10** (*M-I,16,16*)]. This suggests that the entropy gained from the release of water from hydrophobic units is smaller than the decrease in entropy of amphiphiles due to the formation of aggregates. Thus amphiphiles with longer chains entropically disfavor formation of aggregates making aggregate formation of these derivatives solely an enthalpy-driven process. This decrease in entropy may be explained by considering the effect of an amphiphile's cone angle on aggregate size.⁴² As tail length increases in a series of analogous amphiphiles, the cone angle decreases resulting in a higher aggregation number per aggregate. Formation of larger aggregates results in a larger decrease in entropy upon aggregate formation due to the greater number of amphiphiles per aggregate.

2.3 Minimum Inhibitory Concentration

The MIC values of all compounds from both series were determined for four Gram-positive (*Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus agalactiae*, and *Bacillus subtilis*) and two Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) strains (Table 2). For both series, the MIC decreases with tail length until an optimal tail length of 12 carbons per chain, above which the MIC increases (**Fig. 4**). The derivatives with two 12-carbon chains, compounds **3** (*M-P,12,12*) and **8** (*M-I,12,12*), have the lowest MIC values against each strain with values ranging from 1-2 μM for Gram-positive bacteria and 4-16 μM for Gram-negative bacteria. This trend is indicative of the relationship between solubility and bioactivity and is consistent with previous studies on other amphiphile series.^{14, 15, 19} Higher MIC values against Gram-negative strains may be due to their outer membrane, which is not present in Gram-positive bacteria.

Many antibacterial agents are ineffective against *P. aeruginosa* due to its semipermeable outer membrane and production of efflux pumps and β -lactamases.⁴⁵ The contamination of medical equipment with *P. aeruginosa* biofilms contributes to hospital-acquired infections, particularly caused by antibiotic-resistant strains.⁴⁶⁻⁵⁰ While other antibacterial agents fail to inhibit *P. aeruginosa*, two amphiphiles in this study [**3** (*M-P,12,12*) and **8** (*M-I,12,12*)] kill this organism at relatively low concentrations, which may prove useful in a healthcare setting. Notably, MIC values of compounds **3** (*M-P,12,12*) and **8** (*M-I,12,12*) against *P. aeruginosa* (8 μM and 16 μM , respectively) are comparable to those of tobramycin (6.4 μM), commonly used to treat infection in cystic fibrosis patients, and cefepime (12.5 μM), an antispseudomonal cephalosporin.^{51, 52}

MIC values between respective *M-P* and *M-I* tail lengths were generally similar, indicating that replacing the trimethylammonium head group with a pyridinium did not significantly affect bioactivity. A notable exception is observed between the two derivatives with 16-carbon chains, where the trimethylammonium derivative (**10**, *M-I,16,16*) was more effective than the pyridinium derivative (**5**, *M-P,16,16*) for all strains except *P. aeruginosa*. For all tested compounds, the MBC was the same concentration or a two-fold

concentration higher than the MIC, indicating the amphiphiles are bactericidal, consistent with previous studies on other multiheaded amphiphiles.^{14, 53}

With only a few exceptions, MIC values are significantly below CAC values, demonstrating that amphiphile aggregation is not required to kill bacteria.¹⁹ At concentrations near or above the CAC the amphiphile may act as a detergent, solubilizing the cell membrane – a mechanism of action that could be detrimental to prokaryotic cells.^{17, 19} Amphiphiles at sub-CAC levels are potent antibacterials at concentrations where detergent effects are not observed. It is recognized CAC values reported here (measured in pure water) may differ to some degree from the CAC values in the medium used for MIC studies.

2.4 Synergistic combinations

In addition to determining the antibacterial activity of individual amphiphiles, combination studies were performed in order to determine if binary mixtures of amphiphiles demonstrate synergy in killing representative Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) organisms (**Fig. 5**). A combination is considered synergistic if a mixture of amphiphiles has greater antimicrobial activity than simply an additive effect. The fractional inhibitory concentration (FIC) was used to indicate whether two chemicals were synergistic (FIC<0.5), indifferent (FIC 0.5-4.0), or antagonistic (FIC>4.0).³² Two amphiphile combinations are synergistic against *S. aureus*: combinations of compounds **3** and **4** (FIC=0.31) and compounds **3** and **10** (FIC=0.38). In addition, one combination is synergistic against *E. coli*: compound **3** and **9** (FIC=0.38). All three synergistic combinations contain compound **3** (*M-P, 12, 12*). The use of synergistic antibacterial combinations could be beneficial in a health-care setting, where frequent antimicrobial use has contributed to the presence of antibiotic-resistant organisms.^{33, 34, 36, 54-57}

3. Conclusions

Two novel series of amphiphiles were synthesized and structure-activity relationships were investigated. Log (CAC) and ΔH_{agg} each decrease linearly with increasing tail length for both series. For most of the amphiphiles in this study, the MIC is significantly below the CAC, indicating amphiphile aggregation is not necessary for antibacterial activity. MIC values also indicated an optimal tail length of 12 carbons for each series as compounds **3** and **8** (*M-P, 12, 12* and *M-I, 12, 12*) have the lowest MIC against all strains tested. Furthermore, three binary combinations of amphiphiles have been identified that exhibit synergistic relationships. These amphiphiles could prove useful in the medical field especially when treating or preventing infection with antibiotic-resistant organisms.

4. Methods and Materials

4.1 Synthesis

Synthesis of **11**

1,3,5-tribromomethylbenzene (1.0 g, 2.8 mmol, Sigma-Aldrich) was dissolved in acetone (200 mL) at room temperature in a round bottom flask. The solution was equipped with a stir-bar and attached to an addition funnel. Pyridine (0.45 mL, 5.6 mmol, Sigma-Aldrich) was dissolved in acetone (100 mL) and added drop-wise to the stirring solution of 1,3,5-tribromomethylbenzene at room temperature and allowed to react overnight. A white precipitate formed. The crude reaction mixture was vacuum filtered, washed with acetone, and dried under vacuum yielding 943 mg (77%) of a tan solid, **11**. See Supporting Information for analytical data.

Synthesis of **12**

1,3,5-tribromomethylbenzene (2.01 g, 5.6 mmol) was dissolved in acetone (400 mL) at room temperature in a round bottom flask. 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, ACROS) was diluted with acetone (200 mL) 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 allowed to warm slowly to room temperature. A white precipitate containing a mixture of **12** and a biscationic side product formed. The crude reaction mixture was briefly heated to 50°C and vacuum filtered. The mother liquor (which contained a mixture of **12** and unreacted starting material) was transferred to a clean RBF and the 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 **12** (white solid). The solid filtered 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 flask and the solvent was removed by rotary evaporation yielding additional **20** (white solid, total yield = 860 mg, 37%). See Supporting Information for analytical data.

Synthesis of **1-10**: *General Protocol A*

Compound **11** or **12** 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. Excess dimethylalkylamine ($\text{NMe}_2(\text{CH}_2)_{n-1}\text{CH}_3$, where $n = 8, 10, 12, 14$ or 16) was added slowly to the flask via syringe. The reaction was heated to reflux and allowed react overnight. Volatile materials were removed under a flow of N_2 (g). The resulting crude solid was resuspended in acetone to dissolve any residual dimethylalkylamine and vacuum filtered to yield the solid amphiphile product (**1-10**). Details for each product and analytical data are provided in the Supporting Information.

4.2 Isothermal Titration Calorimetry

CAC and ΔH_{agg} were determined using a Nano-ITC (TA-Instruments). Prior to each experiment the sample cell was washed with dH₂O (300 mL), ethanol (100 mL), dH₂O (300 mL) and nanopure water (200 mL). Next, 950 μL of nanopure water was added to the sample cell. A concentrated aqueous solution ($\gg\text{CAC}$) of amphiphile was prepared and equilibrated at 37 °C. A 250 μL syringe was filled with the aqueous solution, and loaded into the Nano ITC. Multiple single injections in aliquots of 5 μL were injected into the sample cell with time intervals varying from 300s to 1400s. Samples were continuously stirred (300 rpm) throughout the titration. The Nano-Analyze program (TA-Instruments) was used to analyze the data. CAC and ΔH_{agg} values reported are the average of two or more repeat experiments for each amphiphile.

4.3 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™, *Bacillus subtilis*, and *Streptococcus agalactiae* J48.⁵⁸ The Gram-negative bacterial strains used were *Escherichia coli* ATCC® 25922™ and *Pseudomonas aeruginosa* ATCC® 27853™. All strains were grown in 1X Mueller-Hinton Broth at 37 °C for 12-24 h. For the MIC and combination studies, bacterial suspensions were prepared by diluting overnight cultures to 5×10^6 CFU/mL in 2X Mueller-Hinton Broth, so that when amphiphile solutions are added the final broth strength is 1X.

4.4 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.^{14, 59} Briefly, compounds were serially diluted and 100 μL of each dilution were added to the wells of a 96-well flat-bottomed microtiter 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.⁵³

4.5 Combination Studies

To determine if two amphiphiles act synergistically to kill *E. coli* or *S. aureus*, combination studies were performed using the checkerboard technique as described.³² Amphiphiles with MIC values higher than the maximum concentration used ($>250 \mu\text{M}$) were excluded from the combination studies. The amphiphile concentrations used in the combination studies ranged from 1/16 to 2 x the MIC. Fifty microliters of each amphiphile dilution and 100 μL of the bacterial suspension were added to the wells of a 96-well flat-bottomed microtiter plate. Control wells consisted of the bacterial suspension treated with media alone and

bacteria treated with individual amphiphiles. Plates were incubated at 37 °C for 72 h. The FIC index was calculated using the following formula: $FIC = FIC_A + FIC_B = A / MIC_A + B / MIC_B$. A and B are the MIC values of compound A and compound B when combined, and MIC_A and MIC_B are the MIC of compound A and B alone.³² A combination was considered to be synergistic if the FIC was less than 0.5; a combination was considered indifferent with an FIC of 0.5-4; and a combination was considered antagonistic with an FIC > 4. The FIC indices of synergistic combinations were confirmed in two separate experiments.

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Figure 1. Structures of triple-headed, double-tailed amphiphiles in this study. Compounds are named as follows: $M-X,n,n$ where M refers to the Mesitylene core, and X and n represent the attached groups: P indicates a pyridinium; a number indicates the number of carbon atoms in the alkyl group of a dimethylalkyl ammonium (e.g., 1 = trimethylammonium; 8 = octyldimethylammonium).

Scheme 1. Synthesis of triple-headed, double-tailed amphiphiles.

Figure 2. Isothermal titration calorimetry was used to determine the critical aggregation concentration (CAC) and ΔH_{agg} . a) The heat rate associated with each injection versus time for **9** ($M-1,14,14$). b) Integration of the heat per injection normalized by the number of moles of each injection versus the increasing concentration of amphiphile in the well. Inset: CAC is determined by calculating the first derivative the heat curve.

Figure 3. Plots of $\log(\text{CAC})$ (a) and ΔH_{agg} (b) versus tail length at 37 °C for the $M-P$ (●) and $M-I$ (■) series.

Figure 4. Effect of tail length and head group on $\log(\text{MIC})$ ($M-P$ series = ●; $M-I$ series = ■). # = MIC > 250 μM . G^+ = Gram-positive; G^- = Gram-negative.

Figure 5. Synergy studies for $M-P$ and $M-I$ amphiphiles tested against *E. coli* and *S. aureus*. The FIC value for each combination is given. Shaded blocks indicate synergistic combinations (FIC < 0.5). Numbers below the diagonal represent FIC values against *S. aureus*. Numbers above the diagonal represent FIC values against *E. coli*.

Table 1. Critical aggregation concentration (CAC) and thermodynamic parameters at 37 °C for the *M-P* and *M-I* series.

<i>M-P</i>	<i>M-I</i>	Tail Length (<i>n</i> for <i>M-X,n,n</i>)	CAC (mM)		ΔH_{agg} (kJ/mol)		ΔG_{agg} (kJ/mol)		$-T\Delta S_{\text{agg}}$ (kJ/mol)	
			<i>M-P</i>	<i>M-I</i>	<i>M-P</i>	<i>M-I</i>	<i>M-P</i>	<i>M-I</i>	<i>M-P</i>	<i>M-I</i>
2	7	10,10	14	12	-11	-15	-21	-22	-10	-6.6
3	8	12,12	2.4	2.5	-23	-26	-26	-26	-3.1	-0.082
4	9	14,14	0.60	0.61	-34	-37	-29	-29	4.9	8.0
5	10	16,16	0.18	0.16	-42	-55	-33	-33	9.2	22

Table 2. MIC values (μM) for *M-P* and *M-I* amphiphiles. G⁺ = Gram-positive, G⁻ = Gram-negative.

<i>M-P</i>	<i>M-I</i>	Tail length (<i>n</i> for <i>M-X,n,n</i>)	<i>B. subtilis</i> (G ⁺)		<i>E. faecalis</i> (G ⁺)		<i>S. agalactiae</i> (G ⁺)		<i>S. aureus</i> (G ⁺)		<i>E. coli</i> (G ⁻)		<i>P. aeruginosa</i> (G ⁻)	
			<i>M-P</i>	<i>M-I</i>	<i>M-P</i>	<i>M-I</i>	<i>M-P</i>	<i>M-I</i>	<i>M-P</i>	<i>M-I</i>	<i>M-P</i>	<i>M-I</i>	<i>M-P</i>	<i>M-I</i>
1	6	8,8	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
2	7	10,10	8	8	8	8	8	4	2	16	16	125	63	>250
3	8	12,12	2	2	2	2	1	2	2	2	4	4-8	8	16
4	9	14,14	8	4	4	4	4	2-4	4	4-8	16	8	63	125
5	10	16,16	>250	16	>250	32	>250	8	>250	16	>250	63	>250	>250

Figure 1

Scheme 1

Figure 2

Figure 3

Figure 4ew

Figure 5

M-1,12,12

M-P,12,12