

Spring 2015

# The synthesis and study of the biological and colloidal properties of bolaamphiphiles

Louis M. Damiano  
*James Madison University*

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# The Synthesis and Study of the Biological and Colloidal Properties of Bolaamphiphiles

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A Project Presented to  
the Faculty of the Undergraduate  
College of Science and Mathematics  
James Madison University

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in Partial Fulfillment of the Requirements  
for the Degree of Bachelor of Science

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by Louis Damiano

May 2015

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Accepted by the faculty of the Department of Chemistry and Biochemistry, James Madison University, in partial fulfillment of the requirements for the Degree of Bachelor of Science.

FACULTY COMMITTEE:

HONORS PROGRAM APPROVAL:

---

Project Advisor: Kevin L. Caran, Ph.D.,  
Associate Professor, Chemistry and Biochemistry

---

Philip Frana, Ph.D.,  
Interim Director, Honors Program

---

Reader: Kyle Seifert, Ph.D.,  
Associate Professor, Biology

---

Reader: Scott Lewis, Ph.D.,  
Associate Professor, Chemistry and Biochemistry

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### **Acknowledgments**

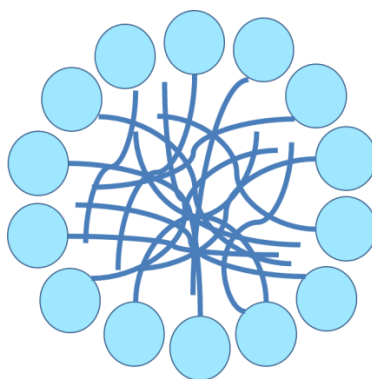
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## Abstract

Over the past decade, antibiotic resistant bacteria have caused infections in patients throughout the world.<sup>[1]</sup> The rise in antibiotic resistance is primarily due to the misuse and overuse of antibiotics.<sup>[1]</sup> To counter the increase in antibiotic resistance, infection control mechanisms have been aggressively researched in recent years. In particular, drug delivery has become a focal point to fight antibiotic resistant infections.<sup>[2]</sup> Amphiphiles have a wide range of applications in the clinical setting, including the ability to inhibit bacterial transference because of their bactericidal activity.<sup>[3]</sup> Bolaamphiphiles are a subclass of amphiphiles that possess two or more hydrophilic heads on either side of hydrophobic linker (typically a hydrocarbon chain). Altering the length of the hydrophobic linker or structure of hydrophilic heads can change their biological and colloidal properties. This study includes the synthesis as well as the colloidal and biological study of a novel hexacationic bolaamphiphile with three cationic groups on each end of an intervening twelve carbon tail. The critical micelle concentration (CMC) and minimum inhibitory concentration (MIC) have been determined. In addition preliminary studies on interactions between the hexacationic bolaamphiphile and a hexaanionic salt will be presented.

## Introduction

An amphiphile is a compound consisting of one or more polar hydrophilic regions (water soluble) and one or more non-polar hydrophobic regions (not water soluble).<sup>[2]</sup> These two divergent properties of an amphiphile are the reason they form micelles in aqueous solution.<sup>[2]</sup> A micelle is a dynamic, roughly spherical aggregate of amphiphiles arranged to minimize hydrophobic/water interactions (Figure 1).



**Figure 1:** Cross section of micelle, with hydrophilic head groups and hydrophobic tails. <sup>[6]</sup>

Micelle formation allows the hydrophilic head groups to interact with the water molecules and provide the hydrophobic tails a water excluded environment. This type of aggregation is due to what is commonly referred to as the hydrophobic effect. Micelle formation is affected by the size, shape, and the relative number of the amphiphiles' hydrophobic and hydrophilic groups.

The world has seen a rise in antibiotic resistance due to the misuse and overuse of common antibiotic drugs.<sup>[1]</sup> There has been a substantial increase in the number of patients with antimicrobial resistant infections in the United States and abroad during this time.<sup>[1]</sup> Amphiphiles are a potential antimicrobial to combat antibiotic resistance. Much energy has been expended exploring amphiphiles possessing a range of structural attributes including various numbers of hydrophilic head portions and hydrophobic tail portions, that make them useful in the medical field.<sup>[2]</sup> Developing novel disinfectants

will decrease the spread of bacterial infections as well as mortalities from antimicrobial resistant infections. The purpose of this senior honors project is to synthesize and study the properties of bolaamphiphiles as well as their aggregates. Bolaamphiphiles consist of two or more polar groups on either side of a hydrophobic chain. Oftentimes, bolaamphiphiles form small spherical vesicles when in an aqueous environment to allow the hydrophilic head groups on both sides of the hydrophobic linker to interact with water.<sup>[3]</sup> Some bolaamphiphiles are used to disrupt biological membranes in bacteria or to serve as vehicles for drug delivery.<sup>[4]</sup> This has brought considerable attention to the synthesis and study of the chemical, physical, and biological properties of bolaamphiphiles in recent years.<sup>[4]</sup> By understanding the relationship between amphiphile structure and bioactivity, this study can provide insight into the potential use of bolaamphiphiles in the medical field. The bolaamphiphiles in this study consist of six hydrophilic head groups bonded to a single hydrophobic linker. Three of the polar head groups reside on each end of the non-polar hydrocarbon linker to obtain the bolaamphiphile architecture (Figure 2).



**Figure 2:** Schematic drawing of **(M-1,1)<sub>2</sub>-12**.

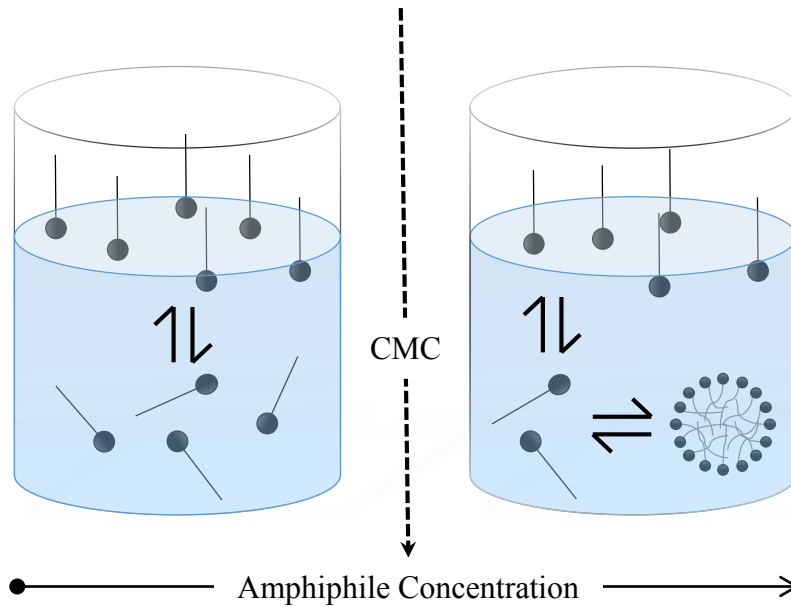
The first half of the project focused on the synthesis, purification, and spectroscopic analysis of the bolaamphiphile. The project then turned to the examination of the colloidal properties of the novel bolaamphiphile by measuring the critical micelle concentration (CMC). In addition, the project in collaboration with the Seifert lab (James Madison University Biology Department) explored the



biological properties of this bolaamphiphile by measuring the minimum inhibitory concentrations (MIC) for a variety of Gram positive and Gram negative bacteria.

### **Colloidal and Biological Properties of Amphiphiles**

The structure of amphiphiles allows for unique interactions in aqueous solution. At low concentrations, amphiphiles are in equilibrium between being located at the air-water interface and in solution. When in solution, the hydrophobic tails interact with surrounding water molecules. A sheath of hydrogen-bonded water molecules surround the hydrophobic tails, which decreases the entropy (S) or disorder of the system.<sup>[5]</sup> Once the amphiphile concentration increases above a critical point, the hydrophobic tails from several amphiphiles start interacting with one another in solution due to the hydrophobic effect. Accompanying this process, the water molecules surrounding the hydrophobic tails are released to the bulk, increasing the entropy in the system.<sup>[5]</sup> The favorable interaction between hydrophobic tails, in combination with the favorable entropy of aggregation, overcomes the repulsion of similarly charged hydrophilic head groups, thus forming micelles.<sup>[3]</sup> A micelle contains a large number of amphiphiles arranged with interacting hydrophobic tails while the hydrophilic heads interact with a polar solvent. The concentration of amphiphiles in solution above which micelles form is the critical micelle concentration (CMC). Above the CMC, an equilibrium is established between dissolved monomers in solution and aggregated amphiphiles in micelles (Figure 3).

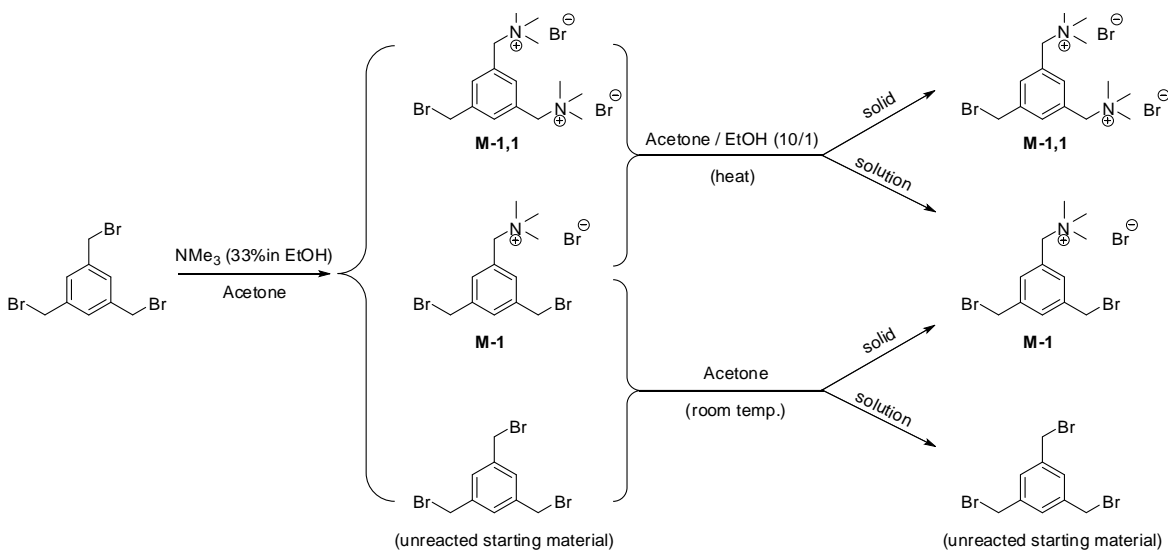


**Figure 3.** Micelles form in solution at concentrations above the CMC.<sup>[9]</sup>

Amphiphiles can incorporate into a bilayer membrane of bacteria and disrupt concentration gradients, potentially killing the bacteria. However, disruption of bilayer membranes has been recorded at concentrations below the CMC, indicating that amphiphiles do not have to aggregate in order to kill bacteria.<sup>[5]</sup> The minimum inhibitory concentration (MIC) is the smallest concentration of amphiphiles needed to inhibit bacterial growth.<sup>[4]</sup> Therefore, amphiphiles with low MIC values may prove useful in the medical field to inhibit the growth of antibiotic resistant strains of bacteria.<sup>[4]</sup>

## Methodology

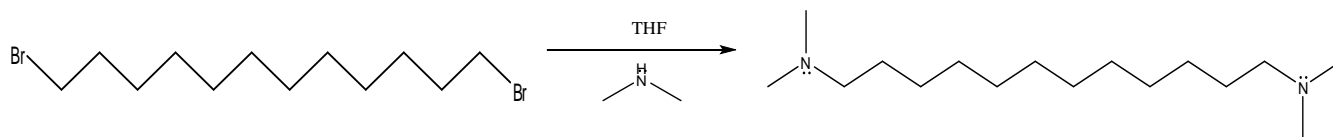
The synthesis of our target bolaamphiphile involved three reactions. The first two reactions produced intermediates that were subsequently combined in the third reaction to make the final product. The first reaction used 1,3,5-tris(bromomethyl)benzene which was reacted with two equivalents of trimethylamine (33% ethanolic solution) in acetone to obtain the bis-trimethylammonium bromide intermediate (**M-1,1**) (Scheme 1). This reaction also yields a second side product, in which only one of the bromine atoms was substituted with trimethylamine (**M-1**). To accomplish the synthesis, 1.00 grams (2.80mmol) of 1,3,5-tris(bromomethyl)benzene was added to 200mL of acetone in a 500mL round bottom flask. The reaction was cooled in an ice bath for approximately 30 minutes. Approximately 0.55 grams (3.08mmol) of Trimethylamine (33% ethanolic solution, 2eq) was added to 100mL of acetone and cooled (0°C) for 30 minutes. The solution of trimethylamine and acetone was then added drop-wise using an addition funnel. The reaction was stirred for approximately 24hr during which it was allowed to come to room temperature.



**Scheme 1:** Reaction of 1,3,5-tris(bromomethyl)benzene with trimethylamine in EtOH/acetone produces a mixture of mono- and bis-trimethylammonium bromide intermediates (**M-1** and **M-1,1**, respectively), which can be separated based on solubility.

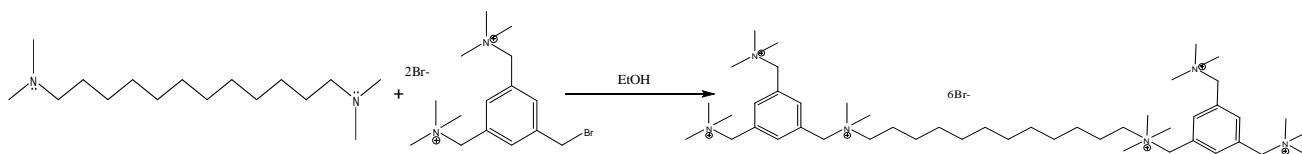
After the reaction was complete, the product mixture was collected by filtration and washed with acetone. The filtrate generally contained the mono-trimethylammonium bromide intermediate (**M-1**) along with unreacted starting material, and the solid contains a mixture of **M-1** and **M-1,1** intermediates. A mixture of 10:1 acetone to ethanol was heated and mixed with the solid from the filtration. This solution was filtered again. This second solid contains **M-1,1**, which was the desired product of the reaction, and the filtrate contained a solution of **M-1** (side product) dissolved in the acetone and ethanol mixture. The filtrate was subsequently concentrated via rotary evaporation and resuspended in room temperature acetone to collect the **M-1** product for use in other syntheses. The percent yield for this reaction was on average 82%.

The synthesis of 1,12-bis(*N,N*-dimethylamino)dodecane was based on slightly modified literature procedure (Scheme 2).<sup>[5]</sup> 1,12-dibromododecane (0.25g, 0.762mmol) was added to a two necked round bottom flask containing approximately 3-5mL of THF. The solution was cooled to -78°C in a dry ice/acetone bath. Dimethylamine (14.7mL, 40% in water, 0.114mol) was added drop-wise to the solution, which was subsequently allowed to warm to room temperature for 24 hours. Upon completion, the solution was exposed to a flow of nitrogen gas in order to remove excess dimethylamine and solvent. The remaining solution was concentrated via rotary evaporation to remove the solvent. The mixture was re-dissolved in a mixture of 15mL diethyl ether and 15mL 2.0M NaOH (aq) for 30 min. The diethyl ether layer was then dried with Na<sub>2</sub>SO<sub>4</sub>, which was subsequently removed by filtration. The filtrate was concentrated via rotary evaporation. Approximately 0.195g of 1,12-bis(*N,N*-dimethylamino)dodecane was obtained, giving a 76% yield for this reaction.



**Scheme 2:** 1,12-dibromododecane reaction with dimethylamine (40% aqueous solution) in THF in a substitution reaction to afford 1,12-bis(*N,N*-dimethylamino)dodecane.

The final reaction involved the **M-1,1** product (0.340g, 0.715mmol) from the first reaction and the 1,12-bis(*N,N*-dimethylamino)dodecane (0.09mg, 0.358mmol) from the second reaction. The two reactants were combined in ethanol at room temperature. Two and a half equivalents of **M-1,1** and one equivalent of 1,12-bis(*N,N*-dimethylamino)dodecane were required for the highest yield of the final product (**M-1,1**)<sub>2</sub>-**12**. Once the reaction was completed after 24 hours, the mixture was cooled overnight. The solution was filtered in order to obtain the solid bolaamphiphile. The final product was dried on vacuum for 24 hours. Approximately 0.120g of (**M-1,1**)<sub>2</sub>-**12** was obtained, giving a 28% yield for the one step reaction.



**Scheme 3:** 1,12-bis(*N,N*-dimethylamino)dodecane is reacted with **M-1,1** to form (**M-1,1**)<sub>2</sub>-**12**.

After the synthesis of (**M-1,1**)<sub>2</sub>-**12**, the structure of the bolaamphiphile was confirmed using HRMS, <sup>1</sup>H NMR, <sup>13</sup>C NMR and <sup>13</sup>C DEPT 135 NMR. Then, the biological and colloidal properties of (**M-1,1**)<sub>2</sub>-**12** were studied. Critical micelle concentration (CMC) was determined using <sup>1</sup>H NMR and conductivity. In collaboration with James Madison University's Biology Department under Dr. Kyle Seifert's laboratory, the minimum inhibitory concentrations (MIC) of the bolaamphiphiles were determined to study the effects of bolaamphiphiles on bacteria.

### CMC Studies

#### <sup>1</sup>H NMR

A stock solution of bolaamphiphile in D<sub>2</sub>O (5.00 mL) was prepared in a volumetric flask, from which diluted samples (each 0.70 mL total volume) of various concentrations were prepared in standard

5 mm width NMR tubes by adding known volumes of stock solution and additional D<sub>2</sub>O . NMR spectra were recorded at 298 K using a 400 MHz spectrometer. For the highly concentrated samples only 16 scans were needed for adequate quality <sup>1</sup>H NMR. However, the lower concentrated samples required 48 scans.

#### *Conductivity*

A 6.50-mL solution of bolaamphiphile at a concentration 4x the estimated CMC was prepared and equilibrated at 25°C. After stirring, the conductivity was recorded for 10 seconds, and the average value was recorded. The sample was diluted by repeatedly removing 0.65mL (10%) of the solution with an Eppendorf pipette and then adding 0.65mL of temperature-equilibrated pure water. The solution was thoroughly mixed before recording the next conductivity value. The process was then repeated so that a broad range of measurements were recorded above and below the estimated CMC.

#### **MIC Study – Performed by Dr. Seifert Lab, James Madison University Biology Department**

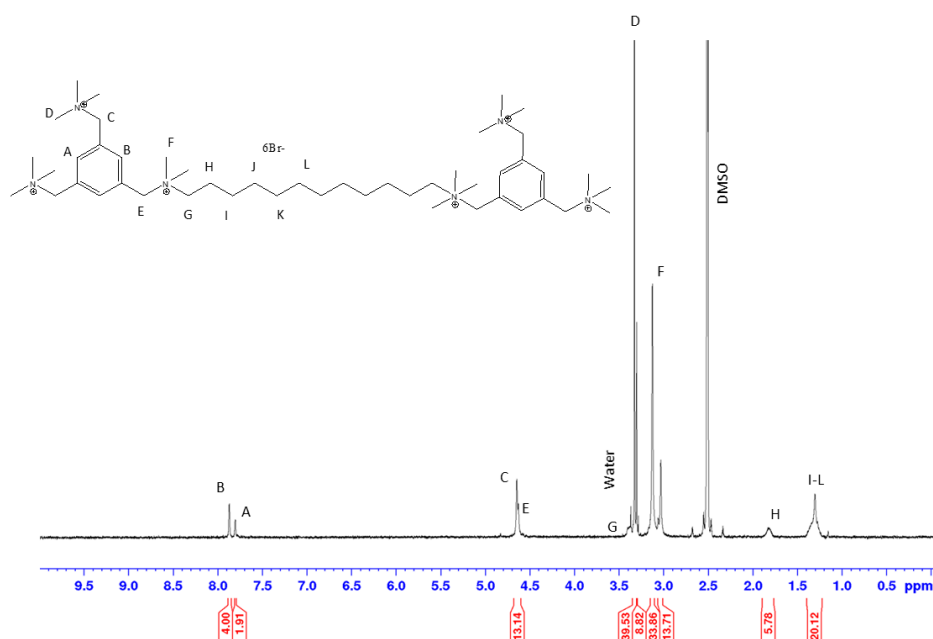
##### *MIC Determination*

The Gram-positive bacterial strains used in this study were Staphylococcus aureus subspecies aureus, Enterococcus faecalis, Streptococcus agalactiae J48, and Bacillus subtilis. The Gram-negative bacterial strains used were Escherichia coli and Pseudomonas aeruginosa. The bacteria was grown in Mueller-Hinton Broth at 37 °C for 24 hours. The bolaamphiphile was serially diluted and 100 µL was added to the wells of a 96-well flat-bottomed plate. The plates were incubated at 37 °C for 72 h. The MIC of the compound was the minimum concentration that resulted in visible inhibition of bacterial growth.

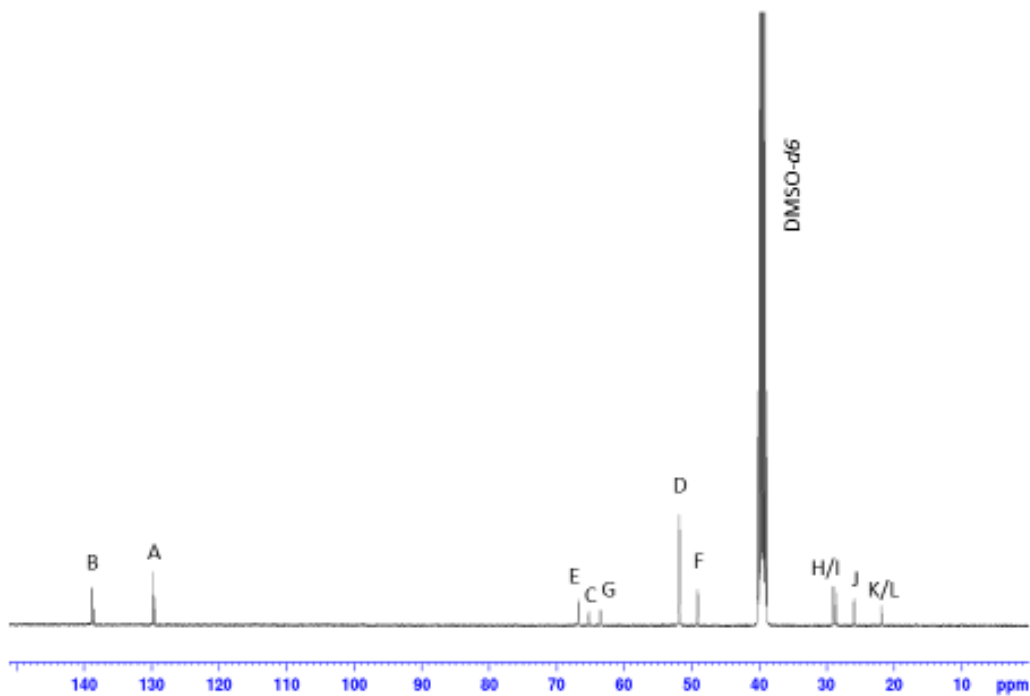
## Results and Discussion

### Confirmation of Structure: NMR and HRMS

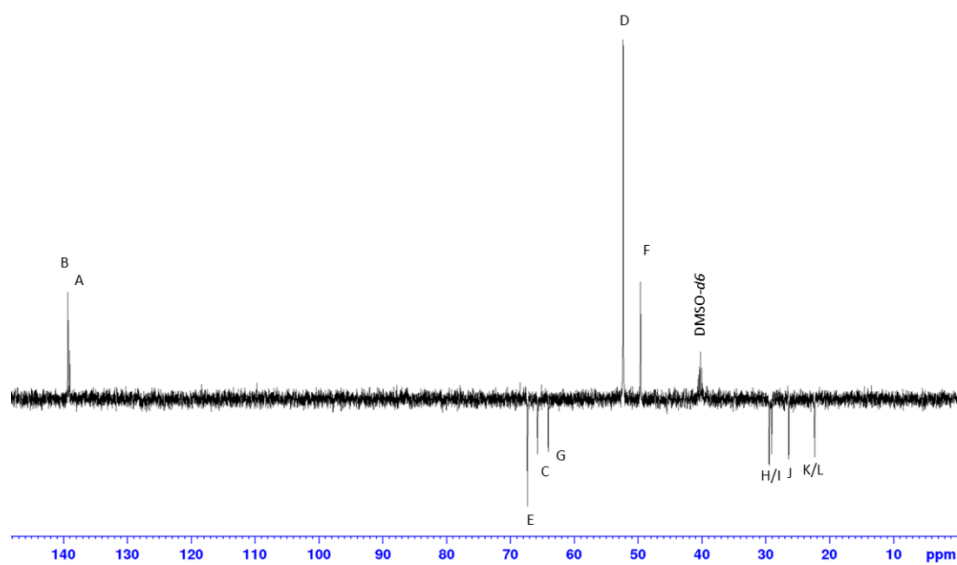
In order to confirm the structure of **(M-1,1)<sub>2</sub>-12**, Proton (<sup>1</sup>H) and Carbon (<sup>13</sup>C DEPT 135) Nuclear Magnetic Resonance Spectroscopy and High Resolution Mass Spectroscopy were performed. The spectra confirm the successful synthesis of the **(M-1,1)<sub>2</sub>-12** bolaamphiphile. Nuclear magnetic resonance spectra were recorded using the following instrument: Bruker-Spectrospin 400 (<sup>1</sup>H: 400 MHz, <sup>13</sup>C: 100 MHz). The solvent peak dimethylsulfoxide (DMSO-d<sub>5</sub> for <sup>1</sup>H NMR and DMSO-d<sub>6</sub> for <sup>13</sup>C NMR) was used as a reference (Figure 4, 5, and 6). NMR studies were conducted at 25°C.



**Figure 4:** <sup>1</sup>H NMR of **(M-1,1)<sub>2</sub>-12**. The <sup>1</sup>H spectrum confirms the structure of the bolaamphiphile. The spectrum is annotated with structural assignments for each signal.



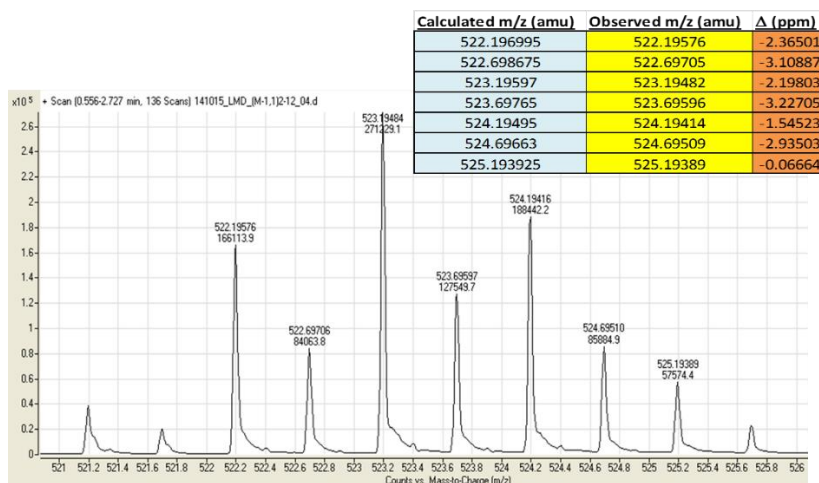
**Figure 5:**  $^{13}\text{C}$  NMR of  $(\text{M-1,1})_2\text{-12}$ . The data confirms the structure of the bolaamphiphile. The spectrum is annotated with structural assignments for each signal according to the bolaamphiphile in Figure 4.



**Figure 6:**  $^{13}\text{C}$  DEPT 135 NMR of  $(\text{M-1,1})_2\text{-12}$ . The data confirms the structure of the bolaamphiphile. The spectrum is annotated with structural assignments for each signal according to the bolaamphiphile in Figure 4.



The HRMS confirms the molecular weight of **(M-1,1)<sub>2</sub>-12** [M-2Br]<sup>2+</sup> (6 most abundant species labeled). The multiple peaks (each within 4% of calculated values) are consistent with molecules with various isotopes of bromine and carbon (Figure 7). The molecular weight of **(M-1,1)<sub>2</sub>-12** is 1206.67 g/mol.



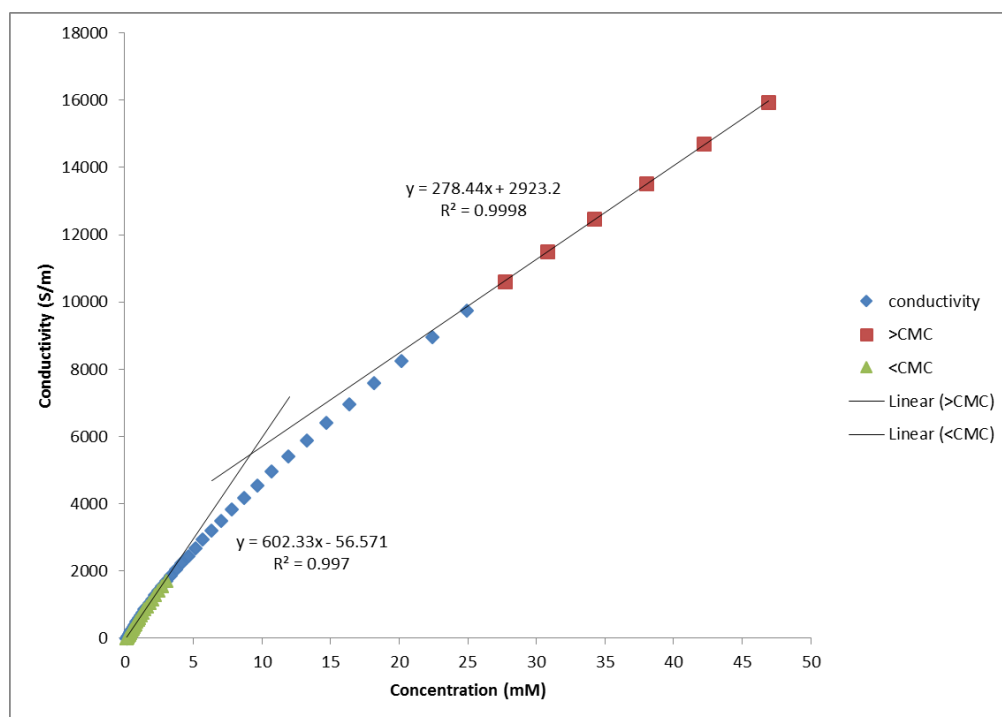
**Figure 7:** HRMS of **(M-1,1)<sub>2</sub>-12** confirming the molecular weight of **(M-1,1)<sub>2</sub>-12**. Each observed value is within 4% of the calculated value.

## CMC Determination

### Conductivity

Conductivity studies were performed by preparing a highly concentrated stock solution and taking conductivity measurements of a serially diluted temperature-equilibrated solution at 25°C. The conductivity decreased throughout the experiment as bolaamphiphile concentration decreased. This is due to the decrease in free ions in solution. Initially, the concentration of the sample was well above the estimated CMC value. When micelles are in solution, counter-ion concentration is higher in the region of the micelle than in the bulk solution due to the higher concentration of cationic charge on the outer surface of the micelles. Once the concentration of the sample was below the CMC, the distribution of counter-ions in solution was dispersed. The change in counter ion distribution causes a change in slope in the concentration vs. conductivity plot. Lines of best fit were extended from highest concentration

points as well as the lowest concentration points. The intersection of the two lines was assigned as the CMC (Figure 8). The CMC in the conductivity study was determined to be 9.06mM.



**Figure 8:** Conductivity data for **(M-1,1)<sub>2</sub>-12**. The intersection of the linear lines of best fit corresponds to the critical micelle concentration value of 9.06mM.

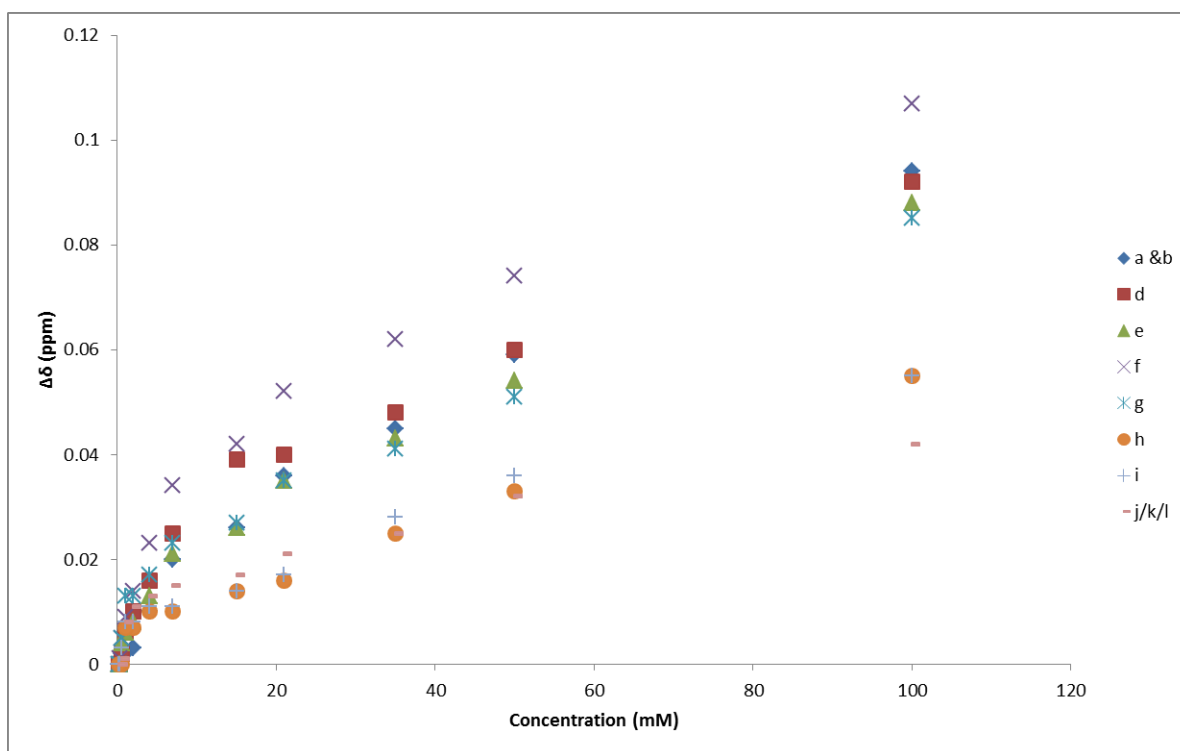
### <sup>1</sup>H NMR

Serial dilutions of **(M-1,1)<sub>2</sub>-12** in D<sub>2</sub>O were made in order to determine the change in chemical shift ( $\Delta\delta$ ) due to the change in concentration. After determining the change in chemical shift of all spectra, a linear plot of  $1/\text{concentration (mM}^{-1}\text{)}$  vs. change in chemical shift (ppm) was used to determine the CMC for each peak. The highest four concentrations and lowest five concentrations were used to plot the two lines of best fit to determine the CMC (Figure 10). The highest data points represented concentrations above the CMC while the lowest data points represent concentrations in which compounds remain as monomers (below the CMC). Table 1 shows the average CMC calculated for

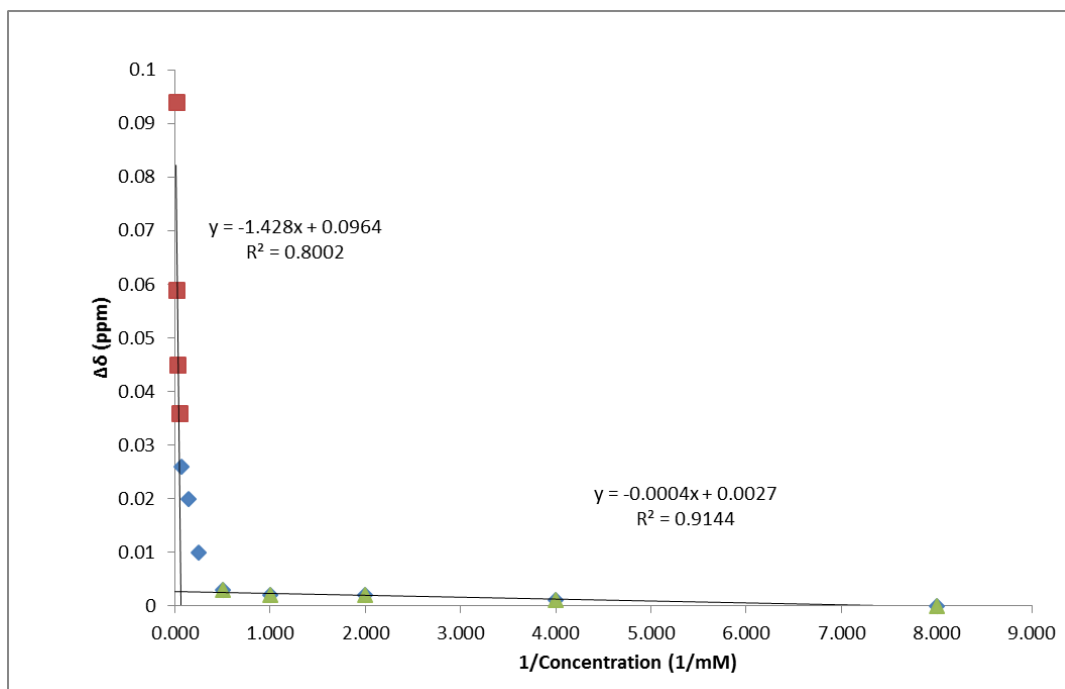
each position A-L in the bolaamphiphile. The average CMC from the study was determined to be 15.6mM.

| 1H NMR signal | A/B  | D    | E    | F    | G    | H    | I    | J/K/L | Average CMC |
|---------------|------|------|------|------|------|------|------|-------|-------------|
| CMC (mM)      | 15.2 | 13.9 | 15.6 | 13.3 | 16.4 | 18.4 | 18.3 | 13.8  | 15.6        |

**Table 1:** Calculated CMC values (mM) for each designated position due to the change in concentration vs. the change in chemical shift. Letters correspond to the labeled positions on the structure in Figure 4.



**Figure 9:** Critical Micelle determination for  $(\mathbf{M-1,1})_2\mathbf{-12}$  using  $^1\text{H}$ NMR. Each data series is labeled according to the schematic drawing in Figure 4. The average CMC was calculated to be 15.6mM



**Figure 10:** Critical Micelle Concentration determination using  $^1\text{H}$ NMR. The CMC for the aromatic protons (A/B) was calculated to be 15.2mM, as determined by the intersection of the two best fit lines in this plot of 1/concentration vs. change in chemical shift. The average CMC was calculated to be 15.6mM

### MIC Study

After testing **(M-1,1)<sub>2</sub>-12** against both Gram positive and Gram negative bacteria, results from the Seifert lab concluded that the bolaamphiphile did not possess antibacterial activity. A possible explanation of the lack of antibacterial activity is due to the length of the twelve carbon linker, relative to the two higher hydrophilic terminal groups, rendering the molecule very water-soluble. The lack of a significant hydrophobic section may lead to a decreased ability to interact with bacterial membranes. Bolaamphiphiles with various carbon linkers will be investigated by the Caran and Seifert labs in the future for antibacterial activity.

### Colloidal Properties with Mellitate

To study the colloidal properties of bolaamphiphiles, various ratios **(M-1,1)<sub>2</sub>-12** to mellitate in DMSO-d<sub>6</sub>/D<sub>2</sub>O (7/3) were studied using  $^1\text{H}$  NMR. A stock solution of mellitic acid was deprotonated

using 6eq. NaOD in order to make the hexaanionic salt mellitate. Samples contained a total of 1mL. The overall concentration of [bolaamphiphile] + [mellitate] was 4.2mM in each sample. A color change in multiple samples indicated that there was a charge transfer between **(M-1,1)<sub>2</sub>-12** and mellitate (Figure 11). Therefore, we conclude that there is an interaction between the hexacationic bolaamphiphile and the hexaanionic salt. We plan to investigate this system further using UV/Vis spectroscopy. The <sup>1</sup>H NMR study is also under further investigation to define a more precise relationship between **(M-1,1)<sub>2</sub>-12** and mellitate.



**Figure 11:** Different ratios of **(M-1,1)<sub>2</sub>-12** to mellitate in DMSO-d<sub>6</sub>/D<sub>2</sub>O (7/3). From left to right the [bolaamphiphile]:[mellitate] decreases, while [bolaamphiphile] + [mellitate] is kept constant at 4.2mM.

## Conclusion

The purpose of this senior honors project was to synthesize and study the colloidal and biological properties of a novel bolaamphiphile as well as its aggregates. The project resulted in the successful synthesis of a novel bolaamphiphile and produced a reliable synthetic approach to synthesizing bolaamphiphiles. Secondly, we determined the approximate critical micelle concentration of **(M-1,1)<sub>2</sub>-12**. Through <sup>1</sup>HNMR and conductivity studies, the average critical micelle concentration for **(M-1,1)<sub>2</sub>-12** was calculated to be 15.6mM using <sup>1</sup>HNMR and 9.06mM using conductivity. The hexacationic bolaamphiphile and hexaanionic salt colloidal study provides evidence for an interaction between the two molecules due to the color change attributed to the formation of a charge transfer complex. Further investigation into the relationship between hexacationic bolaamphiphiles and hexaanionic salts will be an emphasis of future work in the Caran lab. Finally, although **(M-1,1)<sub>2</sub>-12** did not possess any significant antibacterial activity, similar bolaamphiphiles may play a promising role in drug delivery in the future. Overall, the synthesis was successful and laid the ground work for future discovery into biological and colloidal properties of bolaamphiphiles.

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