Biscationic Tartaric Acid-Based Amphiphiles: Charge Location Impacts Antimicrobial Activity

Allison Faig,† Timothy D. Arthur,‡ Patrick O. Fitzgerald,§ Michael Chikindas,§∥ Evan Mintzer,⊥§ and Kathryn E. Uhrich*†

†Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, New Jersey 08854, United States
‡Department of Microbiology and Biochemistry and §School of Environmental and Biological Sciences, Rutgers University, New Brunswick, New Jersey 08901, United States
∥Center for Digestive Health, New Jersey Institute for Food, Nutrition and Health, New Brunswick, New Jersey 08901, United States
⊥Lander College of Arts and Sciences, Touro College, 1602 Avenue J, Brooklyn, New York 11230, United States
§Department of Chemistry and Biochemistry, Stern College, 245 Lexington Ave., Room 552, New York, New York 10016, United States

Supporting Information

ABSTRACT: Cationic amphiphiles have received increasing attention as antimicrobials given their unique ability to disrupt bacteria cell membranes. While extensive research has demonstrated that amphiphiles’ hydrophobic-to-charge ratio significantly modulates antibacterial activity, less work has focused on elucidating the specific impact of charge location on amphiphile bioactivity. In this study, two series of cationic amphiphiles, termed bola-like and gemini-like, were synthesized with analogous hydrophobic-to-charge ratios yet differing charge location, and their resulting antibacterial activity was assessed. Bola-like amphiphiles exhibited preferential activity against two Gram-positive bacteria, with activity increasing with increasing hydrophobicity, whereas gemini-like amphiphiles were active against both Gram-positive and Gram-negative bacteria, with activity decreasing with increasing hydrophobicity. After identifying lead compounds from each amphiphile series (bola- and gemini-like), biophysical experiments indicated that both amphiphiles were membrane-active; notably, the lead gemini-like amphiphile exhibited a strong dependence on electrostatic interactions for membrane interaction. In contrast, the lead bola-like amphiphile exhibited a reliance on both hydrophobic and electrostatic contributions. These results demonstrate that charge location significantly impacts cationic amphiphiles’ antibacterial and membrane activity.

1. INTRODUCTION

The development of antibiotic-resistant bacteria is a prevalent concern that has prompted the development of new antimicrobial agents.1−4 As an alternative to conventional antibiotics, antimicrobial peptides (AMPs) have received widespread attention. Many of the naturally occurring AMPs elicit antibacterial activity by targeting the cellular membrane.1−3 Although these peptides have diverse primary structures, many exhibit a net cationic charge and a facial amphiphilic secondary structure in which hydrophobic and hydrophilic domains exist on opposite “faces” of the molecule;5 it is the cationic amphiphilic character that appears to give rise to AMPs’ unique mechanism of action.1−3 These AMPs first interact with negatively charged bacterial membranes via electrostatic bonding.2,5 After the initial interaction, AMPs’ hydrophobic domains interact with the hydrophobic membrane interior, ultimately disrupting the membrane and resulting in cell death.2,5 Owing to their membrane-targeting activity, AMPs exhibit reduced instances of bacterial resistance and are promising antibiotic alternatives.2,5,6,7,8 High production costs and instability in the presence of proteases, however, have limited their clinical application.3,9,10

Received: March 16, 2015
Revised: September 29, 2015
Published: October 21, 2015
In an effort to overcome AMPs’ current drawbacks, many researchers have synthesized peptidomimetic compounds containing AMPs’ key physicochemical properties, namely, a net cationic charge and amphiphilic structure. LaDow et al. developed a series of aryl-based bicephalic amphiphiles (two cationic heads, one hydrophobic tail, Figure 1A) of varying hydrocarbon tail length and determined that bicephalic compounds were more likely to be effective against both Gram-positive and Gram-negative bacteria than conventional monocationic amphiphiles.\(^{11}\) Building upon this work, Grenier et al. designed a series of bipyridinium-based gemini amphiphiles (two cationic heads, two hydrophobic tails, Figure 1A) that demonstrated improved antimicrobial activity over bicephalic amphiphiles, with optimum activity occurring at intermediate hydrocarbon tail lengths.\(^{7}\) Further, Mondal et al. conjugated cationic lysine residues onto glucose to generate bicephalic amphiphiles that may mimic peptide post-translational modifications of AMPs.\(^{12}\) In addition to investigating small-molecule amphiphiles as antimicrobial agents, researchers have also studied oligomers\(^ {13}\) and polymers\(^ {9,14−16}\) in an attempt to develop potent bioactives. Paslay et al., for instance, developed a series of poly(methacrylamide) (co)polymers which demonstrated increasing antimicrobial activity with increasing primary amine content.\(^ {14}\)

In evaluating the diverse array of antimicrobial peptides and amphiphiles that have been developed, one trend becomes apparent: antimicrobial activity is largely influenced by a molecule’s hydrophobic-to-charge ratio.\(^ {2,7,11,15,16}\) Very few studies, however, have compared amphiphiles possessing identical hydrophobic-to-charge ratios with varying charge locations. Studies by LaDow et al. revealed that the spacing between cationic charges on structurally similar bicephalic amphiphiles, containing the same hydrophobic-to-charge ratio, does influence the antimicrobial activity.\(^ {11}\) Within our work, we expanded the approach by not only exploring the specific impact of charge location on cationic amphiphiles’ antimicrobial activity but also delving into amphiphiles’ specific membrane activity.

To investigate this correlation, two series of sugar-based biscationic amphiphiles were synthesized with varying charge locations and varying, yet equivalent, hydrophobic-to-charge ratios. Each series had differing amphiphile architectures as a result of their charge location. Whereas one series more closely resembled gemini amphiphiles (two heads, two tails), which have been widely investigated for antimicrobial applications,\(^ {7,17,18}\) the other was more bolaamphiphilic (two heads connected via one tail) in nature (Figure 1B). We hypothesized that the gemini-like amphiphiles would exhibit improved antimicrobial activity compared to the bola-like amphiphiles due to their more facially amphiphilic structure and that each series’ antimicrobial activity would increase with an increasing hydrophobic-to-charge ratio due to enhanced hydrophobic interactions, leading to membrane permeabilization. Upon successful synthesis of all amphiphiles, their antimicrobial activity was assessed against Gram-negative and Gram-positive bacteria. The lead compounds were further evaluated; specifically, their interactions with model membranes via Langmuir monolayer techniques and isothermal titration calorimetry (ITC) were measured. These studies served to elucidate whether charge location influences amphiphile antibacterial and membrane activity.

2. MATERIALS AND METHODS

2.1. Materials. All reagents and solvents were purchased from Sigma-Aldrich (Milwaukee, WI) and used as received unless otherwise noted. Hydrochloric acid (HCl, 1 N), concentrated ammonium hydroxide, deuterated methanol (CD\(_3\)OD), Petri dishes, and cotton swabs were purchased from Fisher Scientific (Fair Lawn, NJ). Muller-Hinton agar and blank paper disks were purchased from Becton Dickinson (Franklin Lakes, NJ). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. N,N-Dipropyl tartramide (PT) was prepared according to published procedures.\(^ {19}\)
For this microfiltration assays, bacterial strains Escherichia coli ATCC 43895, Pseudomonas aeruginosa ATCC 14213, Listeria monocytogenes ATCC 49594, and Staphylococcus aureus Rosenbach ATCC 29213 were received from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). These strains were chosen because they are representative of Gram-negative and Gram-positive pathogens.

2.2. Characterization. Proton (1H) and carbon (13C) nuclear magnetic resonance (NMR) spectra were obtained using a Varian 400 or 500 MHz spectrometer. Samples were dissolved in deuterated chloroform (CDCl3), dimethyl sulfoxide (DMSO-d6), or CD3OD using trimethylsilane or a deuterated solvent (DMSO-d6 or CD3OD) as an internal reference. Fourier transform infrared (FT-IR) spectra were obtained using a Thermo Scientific Nicolet iS10 spectrophotometer equipped with OMNIC software. FT-IR samples were either pressed into potassium bromide (KBr) discs (1 wt % sample) or solvent-cast onto sodium chloride plates; each spectrum was an average of 32 scans. Molecular weights were determined using a ThermoQuest Finnigan LCQ-DUO system equipped with an atmospheric pressure ionization (API) source, a mass spectrometer (MS) detector, and the Xcalibur data system. Samples were prepared at a concentration of 10 μg/mL in methanol (MeOH) or 50:50 MeOH/dichloromethane (DCM).

2.3. Synthesis of Bola-like Amphiphiles. 2.3.1. Synthesis of tert-Butyloxycarbonyl-(Boc)-Protected Alanic Acids (3). Following modified literature procedures,23,30,31 bromo-terminated alanic acid (1, 3.62 mmol) was either dissolved (1a) or suspended (1b, 1c) in concentrated ammonium hydroxide (10–100 mL) and stirred for 24–48 h. Upon complete consumption of the starting material (monitored by thin layer chromatography, 75:25 hexanes/ethyl acetate with acetic acid), the reaction mixture was concentrated in vacuo to isolate an amine-terminated alanic acid intermediate (2). The intermediate was then suspended in a 1:1 mixture of dioxane and 10% sodium carbonate (14 mL each) and gently warmed to 30 °C. If necessary, additional water (5 mL) was added to improve stirring. Di-tert-butyl dicarbonate (3.98 mmol) was added, and the reaction was stirred at reflux temperature (65 °C) overnight. The reaction mixture was concentrated in vacuo, and the resulting crude mixture was reconstituted in 1 N HCl and diethyl ether and subsequently extracted with diethyl ether (4 × 80 mL). The combined organic layers were washed with 1:1 brine/water (80 mL total) and dried over magnesium sulfate (MgSO4), and the product (3) was isolated in vacuo.

2.3.1.1. 8-Bocaminocarboxylic Acid (3a). Yield: 1.67 g, 78% (off-white solid).

2.3.1.2. 10-Bocaminocarboxylic Acid (3b). Yield: 1.09 g, 97% (off-white solid).

2.3.1.3. 12-Bocaminocarboxylic Acid (3c). Yield: 1.00 g, 97% (off-white solid).

2.3.2. Synthesis of 2,3-Bis(Boc-Protected Alanyl) PTS (4). PT (1.36 mmol), 3 (2.99 mmol), and catalytic dimethylaminopyridine (DMAP, 0.57 mmol) were dissolved in anhydrous DCM (27 mL) and dimethylformamide (DMF, 13 mL) and nitrogen. Upon complete dissolution, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 5.71 mmol) was added, and the reaction was stirred overnight under nitrogen. The reaction mixture was concentrated in vacuo, reconstituted in DCM, and washed with aqueous solutions of 10% potassium bisulfite (KHSO5, 3 × 80 mL) and saturated sodium bicarbonate (NaHCO3, 3 × 80 mL). The organic layer was then washed with brine (80 mL) and dried over MgSO4, and the product (4) was isolated in vacuo.

2.3.2.1. B-Bocaminocarboxyl PT (4a). Yield: 1.25 g, 95% (pale-yellow solid).

2.3.2.2. 10-Bocaminocarboxyl PT (4b). Yield: 0.81 g, 95% (pale-yellow solid).

2.3.2.3. 12-Bocaminocarboxyl PT (4c). Yield: 0.79 g, quantitative (pale-yellow solid).

2.3.3. Synthesis of Bola-like Amphiphiles (5). Boc groups were deprotected following modified procedures.23 In brief, HCl (4 M in dioxane, 50.78 mmol) was cooled to 0 °C under nitrogen, 4 was added (1.27 mmol), and the reaction was stirred at 0 °C for 30 min. The reaction mixture was then warmed to room temperature, stirred an additional 3 h, and concentrated in vacuo. The crude product was dissolved in minimal methanol (10 mL), and aliquots (1 mL) were added to ten 50 mL centrifuge tubes containing diethyl ether (45 mL each), resulting in the precipitation of 5. 5 was isolated via centrifugation (Hettich EBA 12, Beverly, MA: 1370 g, 5 min) and decanting the ether. Bola-like amphiphiles will be referred to as Bx, where B denotes bola-like and x refers to the number of methylene in the acyl arms.

2.3.3.1. B7 (5a). Yield: 0.55 g, 96% (off-white solid).

2.3.3.2. B9 (5b). Yield: 0.72 g, 95% (off-white solid).

2.3.3.3. B11 (5c). Yield: 0.86 g, 97% (off-white solid).

2.4. Synthesis of Gemini-like Amphiphiles. 2.4.1. Synthesis of 2-Bocaminotetramide (2-Boc-AET) (7). In brief, dimethyl tartrate (6, 2.43 mmol) was dissolved in anhydrous tetrahydrofuran (7.5 mL) under nitrogen. N-Boc-ethylenediamine (6.79 mmol) was added, and the reaction mixture was stirred at 40 °C overnight. The crude reaction mixture was concentrated in vacuo, and pure product
(7) was triturated in diethyl ether (25 mL) and isolated via vacuum filtration. To improve yields, the filtrate was reconstituted in vacuum, triturated, and vacuum filtered to isolate additional pure product. Yield: 1.93 g, 91% (white solid). 1H NMR (400 MHz, DMSO-d6): δ 7.79 (br, 2H), 6.81 (br, 2H), 5.43 (d, 2H), 4.20 (d, 2H), 3.12 (m, 4H), 2.60 (m, 4H), 1.36 (s, 18H). 13C NMR (100 MHz, DMSO-d6): δ 172.92, 165.35, 73.22, 40.43, 39.28, 29.02, 13.28. IR (cm−1, KBr): 3356 (NH), 1687 (C=O, amide), 1629 (C=O, amide). ESI-MS m/z: 457.2 [M + 23].

2.4.2. Synthesis of 2,3-Bis(alcanoyl) Boc-AET (8). Following methods similar to those described for the synthesis of 4, alkanic acid (2.53 mmol), and DMAP (0.48 mmol) were dissolved in anhydrous DCM (50 mL) and anhydrous DMF (25 mL) under nitrogen. EDCI (4.83 mmol) was added, and the reaction was stirred overnight and concentrated in vacuo. The crude product was dissolved in minimal methanol (6 mL), and methylenes in the acyl arms.

2.4.3.1. G7 (9a). Yield: 0.40 g, 94% (white solid). 1H NMR (500 MHz, CD3OD): δ 8.62 (br, 2H), 5.75 (s, 2H), 3.52 (m, 4H), 3.09 (m, 4H), 2.49 (m, 4H), 1.62 (m, 4H), 1.29 (br, 2H), 0.90 (t, 6H). 13C NMR (500 MHz, CD3OD): δ 172.91, 168.95, 72.47, 39.46, 36.92, 33.43, 31.90, 29.57, 29.48, 29.30, 29.01, 24.62, 22.56, 13.27. IR (cm−1, KBr): 3435 (NH), 1739 (C=O, ester), 1652 (C=O, amide). ESI-MS m/z: 571.3 [M + 1].

2.4.3.2. G7 (9b). Yield: 0.53 g, 98% (white solid). 1H NMR (500 MHz, CD3OD): δ 8.62 (br, 2H), 5.75 (s, 2H), 3.52 (m, 4H), 3.09 (m, 4H), 2.49 (m, 4H), 1.62 (m, 4H), 1.29 (br, 2H), 0.90 (t, 6H). 13C NMR (500 MHz, CD3OD): δ 172.91, 168.95, 72.47, 39.46, 36.92, 33.43, 31.90, 29.57, 29.48, 29.30, 29.01, 24.62, 22.56, 13.27. IR (cm−1, KBr): 3448 (NH), 1744 (C=O, ester), 1641 (C=O, amide). ESI-MS m/z: 3144.4 [M + 2]/2.}

2.5. Antimicrobial Screening. Amphiphiles’ antimicrobial activity against Gram-negative (E. coli) and Gram-positive (S. aureus) bacteria was first screened using the disk diffusion method.11 Bacteria inocula were grown overnight in nutrient broth (EMD Chemicals, Gibbstown, NJ) at 37 °C under shaking conditions to give a bacterial count of approximately 108 CFU/mL. Muller-Hinton agar was poured into sterile Petri dishes to a thickness of 4 mm. The agar plate was then inoculated with the bacteria broth culture using a sterile cotton swab. Separately, amphiphiles were dissolved in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM, pH 7.4) at concentrations ranging from 0.8 to 100 mM. Sterile paper disks (6 mm diameter) were impregnated with 20 μL of test solution, and the disks were placed onto the inoculated agar plates. Plates were incubated at 37 °C for 20 h, after which zones of inhibition were measured with a ruler. HEPES buffer served as a negative control.

2.6. Broth Microdilution Assay. The broth microdilution method was modified from previous studies.11 Briefly, amphiphiles were serially diluted 2-fold in tryptic soy broth (TSB), and 100 μL aliquots of each dilution were transferred to a 96-well microtiter plate in triplicate. S. aureus, L. monocytogenes, E. coli, and P. aeruginosa were grown on tryptic soy agar (TSA) at 37 °C for 24 h, and sterile doubly distilled water was inoculated with isolated colonies from these overnight plates. The inoculum concentration was adjusted to 5 × 108 CFU/mL with ultraviolet–visible (UV–vis) spectroscopy at 600 nm. Aliquots (100 μL) were transferred to the 96-well microtiter plate to achieve a final concentration of 5 × 106 CFU/well. Plates were incubated at 37 °C for 24 h. The lowest amphiphile concentration that yielded no visible growth was recorded as the minimum inhibitory concentration (MIC). Cetyltrimethylammonium bromide (CTAB), a cationic amphiphile, served as a positive control that could mimic the proposed bactericidal mechanism of the newly synthesized tartaric acid-based compounds.

2.7. Langmuir Monolayer Studies. The ability of amphiphiles to penetrate lipid monolayers was analyzed using a Langmuir surface balance equipped with a custom-built microtitrator from KSV-Nima (Biolin Scientific, Espoo, Finland). Lipid solutions were prepared by dissolving DOPC, DOPG, or DOPC/DOPG (1:1 mole ratio), and sonicated in HPLC-grade CHCl3 (∼1.2 mg/mL total lipid). After rinsing with an ethanol/methanol mixture, the trough was filled with HEPES buffer and the surface was aspirated to remove surface-active particles. Using a Hamilton syringe (Renov, NV), small aliquots of lipid solution were applied to the air/buffer interface to obtain varying initial surface pressures ranging from approximately 17 to 38 mN/m. After solvent evaporation and monolayer equilibration (at least 500 s), 5 μL of B11 or G7 dissolved in HEPES buffer (5 mM initial amphiphile) was injected into the aqueous phase via a side port to avoid puncturing the monolayer, and the surface pressure increase was monitored over time. Data were collected and processed using KSV Nima and Origin software.

2.8. Isothermal Titration Calorimetry. High-sensitivity isothermal titration calorimetry (MicroCal VP-ITC, Malvern Instruments, Westborough, MA) was used to assess the energetics of amphiphile interactions with lipid vesicles. Large unilamellar vesicles (LUVs) composed of DOPC or DOPC/DOPG (1:1 mole ratio) were prepared following a published extrusion method.12 In brief, dried lipid films (pure DOPC or DOPC/DOPG (1:1 mole ratio), and reconstituted in 0.1 M sodium phosphate, pH 7.4, 100 mM NaCl) were extruded through 100 nm polycarbonate filters 10 times using a nitrogen-driven device (Lipex Biomembranes, Vancouver, BC, Canada).

The ITC sample cell (∼1.4 mL) was filled with solutions of 25 μM B11 or G7 dissolved in HEPES buffer, and the reference cell was filled
Scheme 1. Synthesis of Bola-like Amphiphiles with Alkyl Chains of Varying Lengths

Scheme 2. Synthesis of Gemini-like Amphiphiles with Alkyl Chains of Varying Lengths

with the same buffer. The syringe (250 μL) was filled with LUV dispersions containing 5 mM total lipid. All solutions were degassed for 10 min prior to each experiment. Upon system equilibration and a 1 μL preinjection, 5 μL aliquots were injected into the sample cell every 11 min for the first four injections, after which time aliquots were injected in 8 min intervals. Data were collected and processed using proprietary software from MicroCal. All experiments were performed at least in triplicate. Titrations of LUVs into buffer were conducted as negative controls and subtracted from experimental data.

3. RESULTS AND DISCUSSION

3.1. Amphiphile Synthesis. To explore the impact of charge location on antimicrobial activity, two series of cationic amphiphiles (gemini- and bola-like) were synthesized with equivalent hydrophobic-to-charge ratios (Figure 1B). Both series employed tartaric acid, an inexpensive naturally occurring compound produced in fruits, as a backbone that could provide two distinct chemical moieties for further modification. By altering the charge location on these tartaric acid-based molecules, two structurally diverse amphiphile series were developed.

Bola-like amphiphiles resulted when cationic charges were incorporated at the terminal ends of hydrophobic acyl arms (Scheme 1). This series was synthesized by first reacting bromo-containing alkanoic acids (1) with concentrated ammonium hydroxide to generate amine-terminated alkanoic acid intermediates (2). The amine-terminated alkanoic acids were then Boc-protected (3) using di-tert-butyl dicarbonate and subsequently conjugated to a PT backbone using carbodiimide coupling to generate 4. Following successful acylation, 4 was deprotected using HCl in dioxane to generate the final bola-like...
amphiphiles (S) as chloride salts. All amphiphiles’ and intermediates’ chemical structures were confirmed via NMR and FT-IR spectroscopies and mass spectrometry.

A series of gemini-like amphiphiles were synthesized by incorporating cationic charges at the tartaric acid backbone. These amphiphiles possessed analogous molecular weights, chemical moieties (e.g., number of amine moieties or methylene units), and hydrophobic-to-charge ratios as the bola-like amphiphiles, differing only in their charge location (Figure 1B). To synthesize these molecules, dimethyl tartrate was first reacted with N-Boc-ethylenediamine via an aminolysis reaction to generate 7 (Scheme 2). 7 was then acylated with alkanolic acids of varying hydrophobic chain lengths using carbodiimide coupling, and the Boc protecting groups were removed using HCl in dioxane to generate the final amphiphile structures (9). Successful synthesis of the gemini-like amphiphiles and their intermediates was confirmed as described above.

3.2. Antimicrobial Activity. Antimicrobial activity was first screened using the disk diffusion method, a qualitative assay which indicated that all amphiphiles except G11, the most hydrophobic gemini-like amphiphile, exhibited activity against *S. aureus* and *E. coli* in the millimolar range (4−100 mM, Figure S1−2). While this method is excellent for screening, it is not always suitable for the assessment of hydrophobic compounds as they diffuse more slowly through the agar and may not accurately depict bioactivity. Consequently, a broth microdilution assay was carried out to quantitatively assess amphiphile activity. Amphiphiles were incubated with *S. aureus, L. monocytogenes, E. coli,* or *P. aeruginosa* in TSB; the lowest amphiphile concentrations that yielded no visible bacterial growth were taken as the MIC values. With the exception of G11, whose antibacterial assessment was hampered by poor aqueous solubility, all amphiphiles exhibited MICs within the low micromolar to low millimolar range (Table 1).

**Table 1. MICs (μM) of Amphiphiles**

<table>
<thead>
<tr>
<th>amphiphile</th>
<th><em>S. aureus</em> (G+)</th>
<th><em>L. monocytogenes</em> (G+)</th>
<th><em>E. coli</em> (G−)</th>
<th><em>P. aeruginosa</em> (G−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B7</td>
<td>500</td>
<td>500</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>B9</td>
<td>125</td>
<td>125</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td>B11</td>
<td>25±</td>
<td>12.5±</td>
<td>100</td>
<td>50±</td>
</tr>
<tr>
<td>G7</td>
<td>62.5±</td>
<td>62.5±</td>
<td>62.5±</td>
<td>125</td>
</tr>
<tr>
<td>G9</td>
<td>500</td>
<td>250</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td>G11</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>CTAB</td>
<td>4</td>
<td>8</td>
<td>32</td>
<td>16</td>
</tr>
</tbody>
</table>

“Lead cationic amphiphile treatments possessing MIC values lower than 50 μg/mL.

In comparing amphiphiles’ antibacterial activity, it became apparent that the hydrophobic-to-charge ratio, which was investigated by varying the number of methylene units present in amphiphiles’ hydrophobic domains, significantly influenced the amphiphile bioactivity (Table 1). Within the bola-like series (B7, B9, and B11), amphiphiles exhibited increasing antibacterial activity as the number of methylene units increased, with B11 demonstrating the highest potency against Gram-positive (MIC: 25 and 12.5 μM against *S. aureus* and *L. monocytogenes*, respectively) and Gram-negative (MIC: 100 and 50 μM against *E. coli* and *P. aeruginosa*, respectively) bacteria. These results align with previous findings, which indicate that increasing acyl chain lengths can result in enhanced bioactivity as long as the solubility is not drastically diminished.

Furthermore, a recent study by Palermo et al. indicated that antimicrobial activity increases as the spacer length between ammonium ions and a methacrylate polymer backbone increases. Given that the methylenes of the bola-like amphiphiles’ acyl arms are analogous to such spacer units, these compounds may behave similarly, with longer acyl arms allowing for enhanced membrane penetration and increased bioactivity. In contrast to the trends noted for the bola-like amphiphiles, gemini-like amphiphiles (G7, G9, and G11) exhibited decreased antimicrobial activity with increasing acyl chain length. Previous studies have indicated that amphiphiles with poor solubility exhibit decreased antibacterial activity because they are incapable of reaching the bacterial membrane. Upon increasing gemini-like amphiphiles’ acyl chain length to G11, the amphiphile could not dissolve above 200 μM in TSB. It is plausible that gemini-like amphiphiles’ decreased solubility in TSB compromised their antibacterial activity. Although solubility effects may have influenced the gemini-like amphiphile series, G7 exhibited high efficacy against *S. aureus, L. monocytogenes,* and *E. coli* (MICs: 63 μM). Because compounds that exhibit MIC values ≤50 μg/mL are commonly considered to be antimicrobial, broth microdilution studies enabled us to identify two lead compounds, B11 and G7, that possessed micromolar MIC values corresponding to values ranging from 8.7 to 37.0 μg/mL (Table 1).

When the two series’ effects on Gram-positive and Gram-negative bacteria were compared, diverging trends emerged. Bola-like amphiphiles exhibited higher activity against Gram-positive organisms *S. aureus* and *L. monocytogenes,* which may result from the bola amphiphiles’ tendency to penetrate membranes without causing membrane disruption.

Given that Gram-negative bacteria contain an additional outer membrane, this potential mechanism of action could have rendered bola-like amphiphiles less active against Gram-negative bacteria. Furthermore, as Gram-positive and Gram-negative bacteria possess different types and ratios of lipids within their cell membranes, it is plausible that the bola-like amphiphiles’ enhanced activity against *S. aureus* and *L. monocytogenes* results from interactions with specific lipid components.

Gemini-like amphiphiles exhibited no definitive trends against the different bacteria classes; however, G7’s high activity against *S. aureus, L. monocytogenes,* and *E. coli* indicates that gemini-like amphiphiles may possess broader activity against both Gram-positive and Gram-negative bacteria. Given that the bola-like and gemini-like amphiphiles were influenced differently by their hydrophobic-to-charge ratios and exhibited varying activities against Gram-positive and Gram-negative bacteria, the two series apparently act via different bactericidal mechanisms. Nonetheless, future studies investigating a more expansive series of Gram-positive and Gram-negative bacteria will be necessary to fully understand the potential relationship between bacteria classes and antibacterial activity.

While the newly synthesized tartaric acid-based compounds did not outperform the positive control, CTAB, these novel compounds were effective in assessing the importance of charge location in amphiphile bioactivity. Bola-like amphiphiles were more active against two Gram-positive microorganisms, and their activity increased with increasing hydrophobicity. In contrast, gemini-like amphiphiles were active against both Gram-positive and Gram-negative bacteria, and their activity decreased with increasing hydrophobicity. Through further
analyzing the lead compounds’ specific membrane activity, we elucidate the structure–function relationship of the bola- and gemini-like amphiphiles and thereby enable a more targeted design for next-generation antimicrobial compounds.

3.3. Biophysical Assessment. Two amphiphiles, B11 and G7, were identified as lead antimicrobial agents. As many AMPs interact with bacterial membranes,1,2 we hypothesized that these lead compounds may also interact with bacterial membranes as part of their bactericidal mechanisms. To this end, Langmuir monolayer assays and ITC experiments were conducted to ascertain how the lead compounds interact with model membrane systems. Given that bola-like and gemini-like amphiphiles exhibited different trends in antibacterial activity, we sought to understand further whether B11 and G7 would exhibit different interactions with model membranes.

3.3.1. Langmuir Monolayer Studies: B11 and G7 Can Preferentially Penetrate Anionic Biomembranes. Langmuir monolayer techniques were employed to understand amphiphile/lipid interactions. Within these studies, neutral DOPC monolayers served to mimic eukaryotic membranes, whereas anionic DOPG or DOPC/DOPG (1:1 mole ratio) monolayers served to mimic bacterial membranes and elucidate the influence of charge on membrane interactions. Monolayers of varying initial surface pressure were spread at the air/ buffer interface, and the surface pressure increase was monitored upon injection of either B11 or G7 into the aqueous subphase. By plotting the change in surface pressure as a function of initial surface pressure, the x intercept, corresponding to the amphiphiles’ maximum insertion pressure (MIP), was extrapolated.31 MIP values denote the maximum pressure at which insertion into the monolayer is favorable and provide a quantitative means to compare amphiphile/lipid interactions. As MIP values higher than 30–35 mN/m are indicative of biomembrane penetration,31 this methodology provides insight into B11 and G7 interactions with eukaryotic and/or bacterial membranes.

Figure 2. Raw Langmuir monolayer data depicting the surface pressure increase upon injection of B11 (panel A) or G7 (panel B) into the aqueous subphase of a trough containing DOPC (black), DOPG (red), or DOPC/DOPG (1:1 mole ratio, green) monolayers at initial surface pressures of approximately 26 mN/m.

Figure 3. Interaction of B11 (triangles, panel A) and G7 (diamonds, panel B) with DOPG (black) or DOPC/DOPG (1:1 mole ratio, light gray) lipid monolayers indicated by a change in surface pressure as a function of initial surface pressure.
B11 and G7 exhibited no significant incorporation into neutral DOPC monolayers (Figure 2), with negligible changes in surface pressure and no linear regression with increasing initial surface pressures. In contrast, both amphiphiles interacted with anionic monolayers (Figure 2), exhibiting a surface pressure increase which decreased with higher initial surface pressures (Figure 3). This enhanced membrane activity in the presence of anionic lipids has been previously reported and may indicate that the amphiphiles behave similarly to cationic AMPs, which initially interact with bacterial membranes via electrostatic interactions. In DOPG and DOPC/DOPG monolayers, B11 exhibited MIP values of 40 and 42 mN/m, respectively, whereas G7 exhibited MIP values of 46 and 52 mN/m, respectively (Figure 3). In general, G7’s higher MIP values indicate enhanced interactions with anionic monolayers. As all MIP values were greater than the biomembrane lateral pressure and comparable to MIP values of known AMPs, it is expected that both B11 and G7 are capable of intercalating within anionic bacterial membranes, suggesting that these amphiphiles may behave similarly to AMPs and may target the bacterial membrane as part of their bactericidal mechanism. In comparing amphiphile interactions with the two different anionic lipid systems, both amphiphiles exhibited higher MIP values in the presence of DOPC/DOPG monolayer mixtures. This phenomenon could result from DOPC’s smaller headgroup area enabling a more favorable insertion of amphiphiles into the lipid monolayer.

### 3.3.2. Langmuir Monolayer Studies: Electrostatic Contributions in Membrane Interaction Differ for B11 and G7.

In addition to extrapolating MIP values, a second parameter that provides useful information for analyzing membrane interaction is the maximum surface pressure increase measured during Langmuir monolayer studies. Through comparing the amphiphiles’ maximum surface pressure increase in the presence of both DOPG and DOPC/DOPG, we better understand the influence of monolayer charge on amphiphile adsorption. This value is typically obtained by comparing adsorption curves with the same initial surface pressure; however, both amphiphiles exhibited a plateau in the maximum surface pressure increase at lower initial surface pressures, likely due to their equilibrium with the bulk aqueous phase. Consequently, the lowest initial surface pressures plotted in Figure 3 correspond to the maximum surface increase for a given amphiphile/lipid system.

G7 exhibits a maximum surface pressure increase of 24 mN/m in the presence of pure DOPG, which decreases to 12 mN/m in the presence of DOPC/DOPG (Figure 3B). This dependence of the maximum surface pressure increase on the mole fraction of anionic lipid has been reported and indicates an electrostatic contribution in membrane binding. B11 also exhibits a decrease in the maximum surface pressure increase when changing the lipid system from DOPG to DOPC/DOPG yet to a smaller extent (20 to 15 mN/m, Figure 3A) than for G7; this result reflects a lesser dependence on electrostatic interactions. These results are further emphasized in Figure 2; B11 behaves similarly in the presence of both DOPG and DOPC/DOPG, whereas G7 exhibits a drastic decrease in the surface pressure increase upon changing the lipid system from DOPG to DOPC/DOPG. The notable difference in electrostatic contribution suggests that B11 relies on a combination of electrostatic and hydrophobic interactions to elicit bacterial death, whereas G7’s bactericidal mechanism may be largely driven by electrostatic interactions. B11’s reliance on both hydrophobic and electrostatic interactions for monolayer intercalation could suggest that the bola-like compounds are drawn to the bacterial membrane via an initial electrostatic interaction followed by intercalation into the hydrophobic membrane interior via hydrophobic interactions. G7’s strong electrostatic interaction with anionic monolayers suggests that gemini-like compounds may interact predominantly with anionic components of bacterial membranes, including PG headgroups, lipopolysaccharide (LPS, found in Gram-negative
bacteria), and lipoteichoic acids (LTA, found in Gram-positive bacteria). A primarily electrostatic mechanism of action could be hampered by increasing hydrophobic content, potentially resulting in gemini-like amphiphiles’ decreased activity with increasing hydrophobic-to-charge ratio.

3.3.3. ITC Studies: B11 and G7 Operate via Different Bactericidal Mechanisms. While Langmuir monolayer studies provided valuable insight into amphiphile’s interactions with biomembranes, ITC was used to investigate amphiphiles’ interactions with bilayers, a more relevant model membrane system. LUVs composed of pure DOPC or DOPC/DOPG (1:1 mole ratio) were prepared to mimic eukaryotic and bacterial membranes, respectively; these LUVs were titrated into a sample cell containing amphiphile solution (i.e., B11 or G7 dissolved in HEPES buffer). Both B11 and G7 exhibited no interactions with neutral DOPC LUVs, as evidenced by negligible heat signals during the titration (Figure S3). As eukaryotic membranes also exhibit a net neutral charge, these results may indicate that both amphiphiles would interact minimally with eukaryotic cells, a correlation that has been previously depicted by Epand et al. In investigating anionic LUVs (i.e., DOPC/DOPG), both lead amphiphiles exhibited binding interactions, and the heats associated with these binding interactions generally decreased as the titrations progressed. As LUVs were added to the titration cell, amphiphiles would bind to LUVs, leaving fewer amphiphiles available for binding and resulting in smaller heat signals in subsequent LUV injections until all amphiphiles were removed from the bulk solution. B11 exhibited endothermic binding interactions, indicated by a positive enthalpy change (Figure 4A). Such binding interactions often result from the displacement of counterions or water molecules as a result of the hydrophobic effect, suggesting that B11’s hydrophobic domain may penetrate the hydrophobic membrane interior of the anionic LUVs and that binding is largely influenced by hydrophobic interactions. As B11 did not interact with DOPC (i.e., neutral) LUVs yet did interact with DOPC/DOPG (i.e., anionic) LUVs, we hypothesize that an initial electrostatic interaction occurred. Although electrostatic binding results in exothermic heat signals, it is plausible that B11’s stronger dependence on the hydrophobic effect resulted in the observed positive enthalpy change.

In contrast to B11, G7 exhibited a negative enthalpy change under identical conditions (Figure 4B), which suggests an exothermic, electrostatic interaction between G7 and anionic LUVs. This exothermic interaction supports Langmuir monolayer data, which indicated that G7’s membrane insertion activity involved a larger electrostatic contribution than did B11.

The diverging energetics of binding indicate that B11 and G7 may act via different bactericidal mechanisms. Gemini-like amphiphiles demonstrated activity against both Gram-positive and Gram-negative organisms. As G7 exhibits electrostatic binding interactions with anionic LUVs, these amphiphiles may interact favorably with the negatively charged lipid components of Gram-positive and Gram-negative bacteria (e.g., lipid headgroups, LPS, or LTA), enabling specific activity against bacteria. For instance, G7 may interact with LPS on the outer membrane of E. coli, potentially neutralizing LPS or displacing divalent cations associated with LPS and ultimately distorting the outer membrane. This electrostatic interaction may have been hampered upon increasing compounds’ hydrophobic-to-charge ratio, resulting in decreased activity. After this initial electrostatic interaction, gemini-like compounds likely insert their hydrophobic tails into the hydrophobic membrane interior; however, this interaction was not observed during biophysical studies. In contrast, bola-like amphiphiles exhibited preferential activity against Gram-positive bacteria with B11 demonstrating endothermic binding interactions with anionic LUVs, indicative of the entropically driven hydrophobic effect. These molecules likely rely on an initial electrostatic interaction, with the negatively charged peptidoglycan matrix of Gram-positive bacteria, followed by intercalation into the membrane’s hydrophobic domain, potentially adopting a U-shaped or membrane-spanning conformation. This reliance on hydrophobic interactions could explain why bola-like amphiphiles exhibited enhanced activity upon increasing their hydrophobic-to-charge ratio. Modeling studies are currently underway to understand this conformation. Over time, this intercalation may result in membrane destabilization through various potential mechanisms, such as membrane thinning or pore formation. Although the specific bactericidal mechanisms of G7 and B11 require further elucidation, and antimicrobial studies in conjunction with biophysical experiments indicate the significant influence of charge location on amphiphile activity.

4. CONCLUSIONS
Bola-like and gemini-like amphiphiles were synthesized to understand the specific influence of charge location on antibacterial activity. Bola-like amphiphiles exhibited increased activity with increasing hydrophobic-to-charge ratios, likely resulting from a combination of both hydrophobic and electrostatic interactions with the bacterial membranes. Gemini-like amphiphiles demonstrated a different trend, with antibacterial activity increasing as the hydrophobic-to-charge ratios decreased. This phenomenon may have resulted from the decreased solubility of more hydrophobic gemini-like amphiphiles or from gemini-like amphiphiles relying primarily on electrostatic interactions in their bactericidal mechanism. Additionally, both amphiphiles exhibited differences in bioactivity against the tested Gram-positive and Gram-negative bacteria, further suggesting that the two amphiphile series possess different bactericidal mechanisms and may interact with different components of bacteria membranes. These studies reveal that, in addition to the hydrophobic-to-charge ratio, charge location significantly modulates the cationic amphiphiles’ antibacterial activity and bactericidal mechanism. Through understanding this influence of charge location, antimicrobial agents could be designed to target different bacteria types and/or membrane structures.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.5b03347.
Disk diffusion assay results and representative ITC data for B11 and G7 interactions with DOPC (PDF)

AUTHOR INFORMATION
Corresponding Author
*Tel: (848) 445-0361. Fax: (732) 445-7036. E-mail: keuhrich@rutgers.edu.
Notes
The authors declare no competing financial interest.

Acknowledgments
We thank Dr. Susan Skelly (Rutgers University) for assistance with disk diffusion assays and D’Vora Feinblum ( Stern College for Women) for intellectual discussions. We thank Stern College for Women for their support and access to ITC and Langmuir troughs (E.M.). This study was supported by a U.S. Department of Education fellowship for Graduate Assistance in Areas of National Need (GAANN, A.F.). This study was also supported in part by the National Institutes of Health (NIH R01 HL107913, K.E.U.).

References