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Antibiotics

Tobramycin and Nebramine as Pseudo-oligosaccharide Scaffolds for the Development of Antimicrobial Cationic Amphiphiles

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Abstract: Antimicrobial cationic amphiphiles derived from aminoglycoside pseudo-oligosaccharide antibiotics interfere with the structure and function of bacterial membranes and offer a promising direction for the development of novel antibiotics. Herein, we report the design and synthesis of cationic amphiphiles derived from the pseudo-trisaccharide aminoglycoside tobramycin and its pseudo-disaccharide segment nebramine. Antimicrobial activity, membrane selectivity, mode of action, and structure–activity relationships were studied. Several cationic amphiphiles showed marked antimicrobial activity, and one amphiphilic nebramine derivative proved effective against all of the tested strains of bacteria; furthermore, against several of the tested strains, this compound was well over an order of magnitude more potent than the parent antibiotic tobramycin, the membrane-targeting antimicrobial peptide mixture gramicidin D, and the cationic lipopeptide polymyxin B, which are in clinical use.

Introduction

Bacterial cell membranes offer important drug targets,[1] and yet only a limited number of clinically approved antibiotics act by affecting membrane structure and function.[2] Antimicrobial agents capable of damaging the bacterial membrane should maintain similar levels of efficacy during each bacterial cell cycle stage and, hence, should prove useful for the treatment of infections caused by slowly dividing bacteria. These infections are challenging to eradicate by using the currently available repertoire of antibiotics.[1] To date, cationic antimicrobial peptides (CAPs) and non-cationic antimicrobial peptides are the best characterized families of antimicrobial agents that act by disrupting the structures and functions of bacterial membranes. These peptides are produced by numerous organisms from bacteria and fungi to mammals.[3,4] Natural antimicrobial peptides such as the non-cationic peptide mixture gramicidin D (GRM D) and the CAP gramicidin S have been approved for clinical use and are frequently used for the treatment of external infections.[5]

Several mechanisms of resistance to CAPs have evolved in bacteria. Chemical modifications that reduce the overall negative charge of the bacterial cell surface reduce affinity to CAPs, and this type of resistance is prevalent amongst CAP-resistant bacteria.[6,7] For example, the incorporation of D-alanyl units into the negatively charged teichoic acids found in Gram-positive bacterial cell walls through an ester bond neutralizes the cell wall charge due to the positively charged amine group of the attached amino acid residue (Figure 1 A).[8] In Gram-negative bacteria, a similar negative-charge reduction takes place through the attachment of a positively charged 4-aminoarabinopyranose to phosphate groups on the lipid A part of the lipopolysaccharides (LPS) that compose the outer leaflet of the outer membrane (Figure 1 B).[9,10] This reduction in the overall negative charge results in CAP resistance. An additional mechanism of CAP resistance is based on molecular trapping: Bacterial surface-associated and secreted proteins bind to CAPs and prevent them from reaching the lipid bilayer and disrupting the bacterial cell membrane.[6,11] A third type of resistance to CAPs involves proteolytic degradation of the CAPs by proteases or peptidases; however, this mechanism is ineffective against cyclic CAPs or those rich in prolines or arginines.[12] Finally, although cationic antimicrobial peptides act on the cell membrane, resistance to these antimicrobials is also associated with the presence of active efflux proteins.[13]

Inspired by the chemical structure of CAPs and in search of strategies to overcome the limitations of resistance, safety, and antimicrobial activity of CAPs, several types of synthetic antimicrobial cationic amphiphile have been developed in recent years.[14–18] We and other groups have demonstrated that aminoglycoside antibiotics (AGs), which primarily act by perturbing bacterial protein synthesis and are highly positively charged under physiological conditions, can be used as scaffolds for the development of potent unnatural cationic antimicrobial amphiphiles.[19,20] Attachment of one or more hydro-

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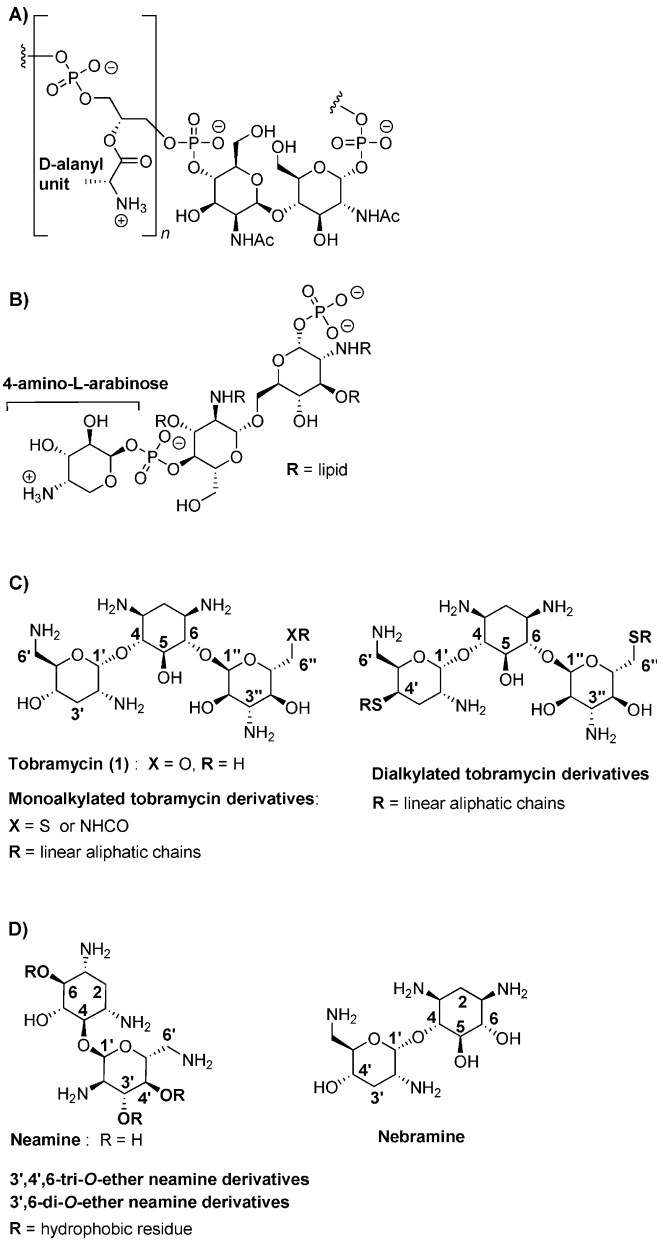


Figure 1. A) Structure of teichoic acid segment bearing a D-alanyl modification on a glycerol phosphate unit. B) LPS lipid A core structure modified by a 4-amino-L-arabinose unit. C) Tobramycin and mono- and dialkylated tobramycin derivatives that act as cationic antimicrobial amphiphiles. D) Neamine and di- and tri-*O*-ether neamine-derived cationic antimicrobial amphiphiles and the structure of nebramine.

phobic residues to various AGs results in potent cationic antimicrobial amphiphiles. We previously designed and synthesized several subsets of cationic antimicrobial amphiphiles derived from the AG tobramycin (**1**; Figure 1C) and linear aliphatic chains. This pseudo-trisaccharide AG contains five amine groups that are positively charged under physiological conditions. Biological evaluation of amphiphilic tobramycin derivatives indicated that the number and length of the linear aliphatic chains, the chemical group that connects the hydrophobic chains to the AG scaffold, and the positioning of the aliphatic chains are structural parameters that affect the antimicrobial activities of these compounds (Figure 1C).[21–24]

The same structural parameters also affect the selectivity of these compounds for bacterial rather than mammalian erythrocyte cell membranes. In general, all of the previously reported AG-derived antimicrobial cationic amphiphiles exhibit potency against a variety of Gram-positive bacteria; however, their efficacy against Gram-negative strains was poor. An exception was reported by Mingeot-Leclercq and co-workers. This group developed several di- and tri-*O*-alkylated neamine-derived cationic amphiphiles (Figure 1D) that exhibit potent antimicrobial activity against pathogenic Gram-negative bacteria with minimum inhibitory concentration (MIC) values in the range 4–16 $\mu g\,mL^{-1}$.[25,26]

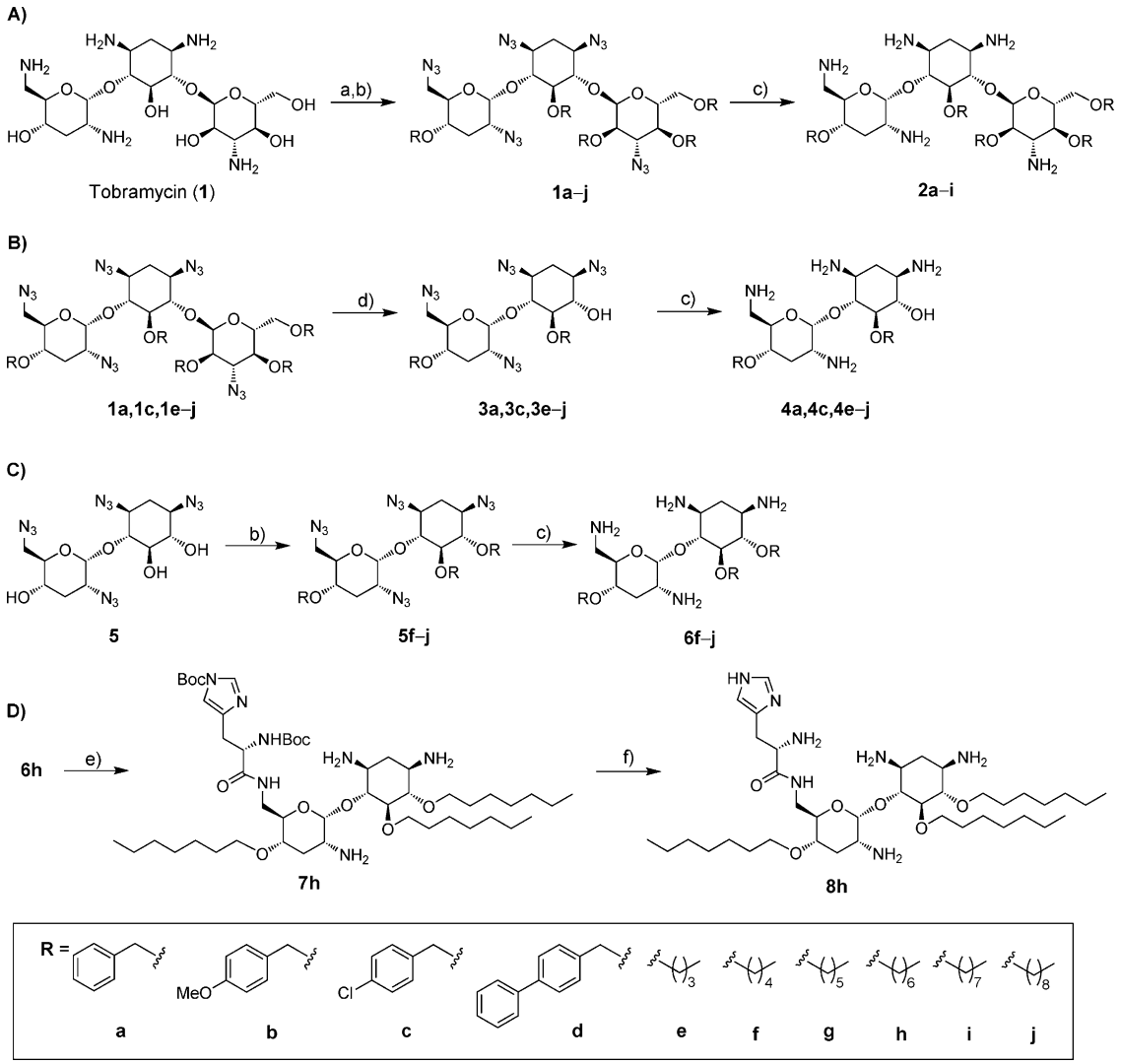
We have sought to determine whether the antimicrobial-activity spectrum against Gram-positive and Gram-negative bacteria can be associated with general molecular descriptors of AG-derived antimicrobial cationic amphiphiles or whether the activity depends on the specific AG scaffold. We prepared 23 cationic antimicrobial amphiphiles derived from tobramycin or its pseudo-disaccharide segment nebramine (Figure 1B). Each of the 23 amphiphilic AGs were biologically evaluated by means of several biological assays, including antimicrobial-activity tests and hemolysis tests, and the results were analyzed to identify the determinants of antimicrobial activity and specificity.

Results and Discussion

Molecular design and synthesis

In designing the cationic amphiphiles for this study, we focused on varying the following parameters: the numbers of hydrophobic residues, the types of hydrophobic residue, and the hydrophobicity/hydrophilicity ratio. Tobramycin has five amine and five alcohol groups, whereas its pseudo-disaccharide fragment nebramine has only four amine and three alcohol groups. Hence, etherification by similar aliphatic residues of all five alcohol groups of tobramycin or all three alcohol groups of nebramine will result in more hydrophobic cationic amphiphiles in the case of tobramycin than nebramine. Tobramycin-derived cationic antimicrobial amphiphiles **2a**–**i** (Scheme 1A) were prepared in three steps from the parent AG. Commercially available tobramycin was transformed into the penta-azido tobramycin following a previously reported procedure (Scheme 1A).[27] Etherification of all five alcohol groups of the azide-protected tobramycin resulted in **1a**–**j** in 68–99% yield of the isolated products. Reduction of the azido groups of **1a**–**i** by using the Staudinger reaction gave the tobramycin-derived antimicrobial cationic amphiphiles **2a**–**i** in 77–98% yield of the isolated products. The free amine form of **1j** was not tested due to the very poor solubility of the deprotected product in aqueous media.

Heating **1a**, **1c**, and **1e**–**j** to reflux in a 1.5 M solution of H_2SO_4 in methanol resulted in selective cleavage of the protected 3-deoxy-3-amino-D-glucose ring of the tobramycin pseudo-trisaccharide to yield the corresponding pseudo-disac-



Scheme 1. Synthesis of tobramycin- and nebramine-derived cationic amphiphiles. Reagents and conditions: a) TfN_3, $ZnCl_2$, Et_3N, H_2O/MeOH/CH_2Cl_2 (3:10:3), 95%; b) alkyl bromide, alkyl chloride, or alkyl iodide; NaH; TBAI; DMF; 68–99%; c) 1.0 M $P(Me)_3$ in THF, H_2O/THF (1:9), 0.1 M NaOH; 77–99%; d) 1.5 M H_2SO_4 in MeOH, reflux, 40–96%; e) *N*,*N*-Bis-Boc-L-histidine *N*-hydroxysuccinimide ester, K_2CO_3, MeOH; 75%; f) 95% TFA, quantitative yield. TBAI = tetrabutylammonium iodide, TFA = trifluoroacetic acid.

charide nebramine derivatives **3a**, **3c**, and **3e–j** in 40–96% yield of the isolated products (Scheme 1B).[28] Reduction of the azide groups of compounds **3a**, **3c**, and **3e–j** using the Staudinger reaction conditions gave the desired subset of 4′,5-di-*O*-ether nebramine derivatives **4a**, **4c**, and **4e–j** in 79–99% yield of the isolated products. An additional subset of nebramine-derived cationic amphiphiles was generated by *n*-alkylation ($n = 5$–9 carbon atoms in the chain) of each of the three alcohol groups of tetra-azido nebramine **5** (Scheme 1C) to yield **5f–j**, which were deprotected to yield 4′,5,6-tri-*O*-*n*-alkyl nebramine derivatives **6f–j**.

Finally, to test the effect of the number of positively charged amine groups on the biological performance of AG-derived antimicrobial cationic amphiphiles, we treated **6h** with *N*-hydroxysuccinimide (NHS) ester-activated and di-Boc-protected histidine (Boc-His(Boc)-NHS) to yield **7h** (72% yield) with high chemoselectivity to the 6′-amine position of **6h** (Scheme 1D).

A short treatment of **7h** with TFA resulted in **8h**, the 6′-NH-histidine analogue of **6h**. Compound **6h** has four positively charged amine groups under physiological conditions, whereas **8h** has four primary amine groups and a histidine imidazole ring that are positively charged under physiological conditions. Hence, biological evaluation of **6h** and **8h** enabled us to determine the effects of the addition of the histidine unit on the biological activity of these nebramine-derived cationic amphiphiles. The synthetic antimicrobial cationic amphiphiles were treated with TFA to yield the corresponding ammonium TFA salts. The exception was **2b**, which was TFA labile and therefore used in its free amine form. All of the compounds were characterized by means of ^1H and ^{13}C NMR spectroscopic and low-resolution (R) mass-spectrometric analysis. Proton assignments were achieved by 1D TOCSY experiments. The molecular weights of all the compounds that were subjected to biological evaluation were confirmed by high-resolution (HR) mass-

spectrometric analysis, and their purity was greater than 95%, as determined by LCMS (see the Experimental Section and Supporting Information).

Antimicrobial activity

The MIC values of the 23 cationic amphiphiles against five Gram-positive and five Gram-negative bacterial strains were determined by using the standard double-dilution method (Table 1).[21] The chosen bacteria represent a diverse selection of pathogens with drug resistance to the parent AG tobramycin and/or to the clinically used membrane-disrupting antibiotics that were used as controls in this study, the non-cationic antimicrobial peptide mixture GRM D and the cationic lipopeptide polymyxin B (PMX). Amongst the five Gram-positive pathogens were the antibiotic-resistant and pathogenic methicillin-resistant *Staphylococcus aureus* (MRSA, strain A) and *Staphylococcus aureus Cowan* (strain B).

The panel of five Gram-negative bacteria included two strains of *Klebsiella pneumonia* (strains G and H), which are encapsulated by negatively charged uronic acid-rich polysaccharides and are therefore resistant to several CAPs.[10,29]

Table 1. MIC values of evaluated compounds against Gram-positive (+) and Gram-negative (−) strains.[a,b]

Cpd.	A (+)	B (+)	C (+)	D (+)	E (+)	F (−)	G (−)	H (−)	I (−)	J (−)
PMX	64	64	64	64	64	2	16	>64	2	4
GRM D	>64	>64	8	0.5	2	>64	>64	>64	>64	>64
1	>64	4	16	4	32	8	2	4	1	>64
2a	16	8	4	4	8	>64	>64	>64	>64	>64
2b	32	>64	4	8	8	>64	>64	>64	>64	>64
2c	8	8	4	8	16	>64	>64	>64	>64	>64
2d	>64	>64	>64	>64	64	>64	>64	>64	>64	>64
2e	>64	64	32	16	>64	>64	>64	>64	>64	>64
2f	4	16	2	2	4	>64	>64	32	>64	>64
2g	8	64	4	4	8	>64	64	64	64	64
2h	>64	>64	>64	>64	64	>64	>64	>64	>64	>64
2i	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
4a	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
4c	>64	>64	32	32	>64	>64	>64	>64	>64	>64
4e	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
4f	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
4g	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
4h	64	>64	8	8	32	>64	>64	>64	>64	>64
4i	32	16	4	4	16	64	>64	>64	>64	>64
4j	4	4	4	4	4	>64	64	64	32	64
6f	>64	>64	32	16	64	>64	>64	>64	>64	>64
6g	8	4	2	2	4	64	>64	32	32	>64
6h	2	2	4	1	2	4	8	8	4	8
6i	8	4	4	4	16	>64	>64	32	32	>64
6j	32	32	64	32	32	>64	>64	>64	>64	>64
8h	4	4	2	4	4	32	64	32	>64	>64

[a] MIC values are presented in units of $\mu g\,mL^{-1}$. [b] Strain A: MRSA; strain B: *S. aureus* Cowan ATCC 12598; strain C: *S. pyogenes* glossy; strain D: *S. pyogenes* JRS75; strain E, *S. epidermidis* RP62A ATCC 35984; strain F: *E. coli* ATCC 25922; strain G: *K. pneumoniae* K36; strain H; *K. pneumoniae* K21; strain I, *P. aeruginosa* PAO1; and strain J, *P. aeruginosa* ATCC 33347. All the experiments were performed in triplicate, and the results were obtained from two independent experiments. MRSA = methicillin-resistant *S. aureus*.

Of the subset of tobramycin derivatives **2a–d** containing the substituted benzyl ether cationic amphiphiles, only the benzyl ether derivative **2a** and the 4-chlorobenzyl derivative **2c** demonstrated antimicrobial activity against all the tested Gram-positive strains; the compounds in this subset were ineffective against the tested Gram-negative strains (Table 1). Of the subset of compounds **2e–i** containing the tobramycin core that were substituted with five linear alkyl ether chains, the penta-*O*-*n*-pentyl derivative **2f** demonstrated the most potent antimicrobial activity against the tested Gram-positive strains (MIC = 2–16 $\mu g\,mL^{-1}$); however, this compound was ineffective against the Gram-negative strains. Moreover, of this subset, compounds with alkyl chains longer or shorter than *n*-pentyl were less effective antimicrobial agents than those with the *n*-pentyl chains. MIC tests of the subset containing the 4′,5-di-*O*-ether nebramine derivatives **4a**, **4c**, and **4e–j** revealed that only those compounds with linear *n*-alkyl ether chains longer than six carbon atoms were potent against the tested Gram-positive strains; again, none were active against the tested Gram-negative strains. The di-*n*-nonyl derivative **4j** was the most potent antimicrobial agent of this subset with MIC values of 4 $\mu g\,mL^{-1}$ against all of the tested Gram-positive bacterial strains.

Finally, of the 4′,5,6-tri-*O*-*n*-alkyl ether nebramine subset **6f–j**, the optimal antimicrobial activity was observed for the tri-*O*-*n*-heptyl nebramine **6h**, which had MIC values of 1–4 $\mu g\,mL^{-1}$ against all of the tested Gram-positive strains. Moreover, **6h** was the only compound with potent antimicrobial activity against all of the tested Gram-negative strains (MIC = 4–8 $\mu g\,mL^{-1}$). Compound **8h**, the more hydrophilic and positively charged analogue of **6h**, was ineffective against the tested Gram-negative bacteria. Compound **8h** had slightly reduced efficacy against the tested Gram-positive bacteria relative to the parent compound **6h**.

Connection between LPS affinity and antimicrobial-activity spectrum

Compound **6h** was the only one that demonstrated potent antimicrobial activity against all five tested Gram-negative pathogens (Table 1). Therefore, we examined whether the potency of **6h** against Gram-negative bacteria was associated with an affinity for LPS, which is a major component of the outer leaflet of the outer membrane of Gram-negative strains. Hence, we initially tested the MIC values of **6h** against the Gram-negative *E. coli* ATCC 25922 (strain G) and against the Gram-positive *S. pyogenes* glossy (strain C) in the presence of LPS in a range of concentrations. The LPS was purified from *E. coli* (serotype O111:B4) by means of ion-exchange chromatography and evaluated for binding to a toll-like receptor (TLR). The anti-Gram negative cationic lipopeptide antibiotic PMX that acts by binding to the lipid A core of LPS was tested as a control. An increase in LPS concentration led to an increase in the MIC values of PMX against *E. coli* ATCC 25922 of up to eightfold (Table 2). A similar effect was observed for **6h** against both tested bacteria. We tested the effect of added LPS on the antimicrobial activity of **8h**, the analogue of **6h** that demonstrated

Table 2. Effect of LPS on the MIC values of PMX, **6h**, and **8h**.[a]

Bacterial strain	*E. coli* (ATCC 25992)		*S. pyogenes* glossy	
***E. coli* LPS**[b]	**6h**[b]	PMX[b]	**6h**[b]	**8h**[b]
0	4	2	4	2
10	8	4	4	4
50	16	8	16	8
100	32	16	32	16

[a] LPS was purified by ion-exchange chromatography from *E. coli* (serotype O111:B4) grown in BHI broth. Test compounds were preincubated for 1 h at room temperature with LPS and then the bacteria were added. The results were obtained after 18 h of incubation, and repeated in two independent experiments. [b] Values are in units of $\mu g\,mL^{-1}$.

potent antimicrobial activity against all of the tested Gram-positive strains but not against the tested Gram-negative strains. As observed for **6h**, increased concentrations of LPS led to an increase in the MIC value of **8h** against *S. pyogenes* glossy (Table 2).

These findings can be rationalized by interactions between LPS and either PMX or **6h** or **8h** that effectively reduce the concentration of the free test compound available to interact with the bacterial cell membrane, thus leading to a decrease in their antimicrobial activity.[30]

The fact that added LPS had a similar deactivating effect on the antimicrobial activities of PMX and compounds **6h** and **8h** suggested that these compounds interact with LPS regardless of their potency against Gram-negative bacteria. To test this hypothesis, the interactions between LPS and the ribosome-targeting tobramycin (negative control), LPS-targeting PMX (positive control), **6h**, and **8h** were quantitatively evaluated by measuring the increase in fluorescence as a result of the competitive displacement of the LPS binding fluorescent dye BODIPY-cadaverine by the tested molecules.[31] As expected, the parent ribosome-targeting AG tobramycin demonstrated a very low LPS displacement effect. Interestingly, the maximal displacement effects (Y_{max}) of both **6h**, which demonstrated anti-Gram-negative activity, and **8h**, which was effective only against Gram-positive strains, were significantly higher than that of PMX (Table 3). There was little difference between the EC_{50} values of **6h** and **8h**, but the EC_{50} value of PMX was approximately threefold lower than **6h** and **8h** (Table 3). The results of the competitive-displacement assay clearly demonstrated that **6h** and **8h** interacted with LPS; hence, that in addition to LPS affinity, the anti-Gram negative activity of **6h** is likely to result from interactions with additional Gram-negative membrane determinants.

Table 3. BODIPY-cadaverine fluorescent displacement assay.[a]

Compound	EC_{50} [μM]	Hill coefficient	Y_{max} [%]
tobramycin	12.3	1.2	8
PMX	7.1	3.4	43
6h	25.8	1.4	100
8h	21.3	1.2	71

[a] LPS of *E. coli* (serotype O111:B4) was incubated with BODIPY-cadaverine and a range of concentrations of the tested compound. Fluorescence was measured at $\lambda = 580$ nm. Maximal probe displacement (Y_{max}) of **6h** was defined as 100%. The Hill coefficients *n* (in arbitrary units) were calculated by using OriginLab software. The experiments were carried out in triplicate.

Evaluation of membrane selectivity

The selectivity of the AG-derived cationic amphiphiles for bacterial cell membranes relative to mammalian cell membranes was evaluated by measuring the hemolytic effect of the compounds on isolated rabbit or rat erythrocytes. Erythrocytes are frequently used as models for the evaluation of mammalian cell-membrane damage. Of the tobramycin derivatives **2a–d** that were substituted with aromatic ether functionalities, benzyl ether derivative **2a**, and 4-methoxybenzyl ether derivative **2b** caused almost no measurable hemolysis at a concentration of 128 $\mu g\,mL^{-1}$ (2–4%; Table 4). In contrast, 4-chlorobenzyl ether derivative **2c** caused significant hemolysis in the same range as the MIC value of this compound against several of the tested Gram-positive strains (8±2% at 8 $\mu g\,mL^{-1}$). Of the *n*-alkyl ether tobramycin derivatives **2e–i**, those that were potent antimicrobial agents were also highly hemolytic (Table 4); for example, **2f** (MIC = 2–4 $\mu g\,mL^{-1}$ against four of the five tested Gram-positive strains) caused 100% hemolysis at a concentration of 64 $\mu g\,mL^{-1}$. A similar trend was observed in the case of the 4′,5-di-*O*-ether nebramine subset **4a**, **4c**, and

Table 4. Hemolysis of rabbit erythrocytes [%] in the presence of the indicated concentrations of the evaluated compounds.[a]

	Concentration [$\mu g\,mL^{-1}$]						
Cpd.	128	64	32	16	8	4	2
PMX	0	0	0	0	0	0	0
GRM D	52±8	39±6	30±4	20±4	10±2	5±2	2±1
2a	4±1	0	0	0	0	0	0
2b	2±1	0	0	0	0	0	0
2c	98±2	89±9	47±9	17±4	8±2	3±1	1±0
2d	26±2	16±1	9±1	3±1	0	0	0
2e	0	0	0	0	0	0	0
2f	100	100	67±13	30±13	3±1	0	0
2g	99±1	99±1	89±4	57±3	16±2	2±1	0
2h	70±1	43±10	19±4	8±1	4±1	1±1	0
2i	14±2	8±2	4±1	2±1	1±1	0	0
4a	1±0	1±1	1±0	1±0	0	0	0
4c	2±0	1±1	1±1	1±0	0	0	0
4e	0	0	0	0	0	0	0
4f	0	0	0	0	0	0	0
4g	0	0	0	0	0	0	0
4h	37±4	4±0	0	0	0	0	0
4i	100	25±6	5±0	3±1	3±0	1±0	0
4j	100	100	95±5	25±5	7±1	2±1	1±0
6f	4±1	0	0	0	0	0	0
6g	100	100	61±2	4±1	0	0	0
6h	100	100	100	58±3	7±1	0	0
6i	100	99±1	62±9	12±5	5±2	0	0
6j	69±7	28±3	12±1	6±1	3±1	1±1	0
8h	95±5	82±4	28±2	9±1	4±1	2±1	2±1

[a] Rabbit erythrocytes were incubated with the test compounds for 1 h at 37 °C. All the experiments were performed in triplicate, and the results are the average of three independent experiments on blood samples from three different laboratory rabbits.

4e–j. For example, 4′,5-di-*O*-*n*-nonyl nebramine derivative **4j** was the most potent of this subset against the tested Gram-positive bacteria and was also the most hemolytic, thus causing 95±5% hemolysis at a concentration of 32 μg mL^{-1}. Finally, of the 4′,5,6-tri-*O*-*n*-alkyl ether nebramine subset **6f–j**, those with linear aliphatic chains with 6–8 carbon atoms were highly hemolytic (Table 4). Compound **6h**, the most potent antimicrobial in this study, was also the most hemolytic; that is, this compound caused 58±3% hemolysis at a low concentration of 16 μg mL^{-1}. Compound **8h**, the more hydrophilic and positively charged analogue of **6h**, caused significantly less hemolysis than **6h**. Exposure of rabbit erythrocytes to 32 μg mL^{-1} of **6h** resulted in 100% hemolysis, whereas exposure to **8h** at a similar concentration led to only 28±2% hemolysis.

The standard MIC tests were conducted in a brain–heart infusion (BHI) broth, which contains a yeast extract that is rich in negatively charged molecules, such as nucleotides and proteins, which may interact with antimicrobial cationic amphiphiles and reduce their antimicrobial activity. In contrast, the hemolysis tests were performed in a phosphate-buffered saline (PBS) solution. Therefore, we evaluated the hemolytic effect of AG-derived antimicrobial agents in BHI broth to determine whether the media affected the membrane selectivity. For this analysis, we chose the tobramycin-derived **2f** and nebramine-derived **6h**, which were potent antimicrobial agents that were also highly hemolytic. The hemolysis tests, carried out in a BHI broth identical to that used for the MIC tests, showed that **2f** and **6h** were significantly less hemolytic than in PBS (Table 5).

For example, **2f** caused **89.8**±8.0% hemolysis in PBS at a concentration of 32 μg mL^{-1}, whereas this compound was approximately one order of magnitude less hemolytic (**8.3**±0.8%) at the same concentration in BHI. The significance of the media in which the hemolysis tests were conducted was diminished at higher concentrations of the test compounds (Table 5). The antimicrobial cationic amphiphiles likely interact with negatively charged molecules found in the rich media. Saturation of these interactions at higher concentrations of the antimicrobial cationic amphiphiles reduced this rich-media effect and left sufficient concentration of the free compounds to act on the membranes.

Table 5. The effect of the BHI media on the hemolytic activity.[a]

Cpd.	BHI [%]	Concentration [μg mL^{-1}] 128	64	32	16	8	4
2f	0	99±1	97±3	90±8	16±3	2±0	0
	100	93±7	52±8	8±1	1±1	0	0
6h	0	100	100	95±1	50±8	4±0	0
	100	100	100	78±19	16±1	1±0	0

[a] Rat erythrocytes were incubated with the test compounds for 1 hour at 37 °C. All the experiments were performed in triplicate, and the results are the average of three independent experiments on blood samples from two individual laboratory rats.

Correlation of partition coefficient values with biological activity

To test whether the biological activities of the tobramycin- and nebramine-derived antimicrobial cationic amphiphiles were correlated with their hydrophobicity/hydrophilicity ratios, we calculated the partition coefficients (log *P*) for each of the 23 compounds in this study (see Table 1S in the Supporting Information). The calculated log *P* values ranged from −0.67 to 13.60. All of the most potent antimicrobials in this study fell into a narrow log *P* range of 5.36–6.08 (Table 6), thus suggesting that log *P* may be used as a general molecular-design consideration for the development of this type of antimicrobial cationic amphiphile.

Table 6. Hemolysis, MIC range, and log *P* values of selected compounds.

Compound	Hemolysis [%][a]	MIC range [μg mL^{-1}] Gram (+)	Gram (−)	log *P*[b]
2a	0	4–16	>64	5.36
2f	100	2–16	32–>64	5.58
6h	100	1–4	4–16	5.30
4j	100	4	32–>64	6.08

[a] Hemolysis was determined after exposure of rabbit erythrocytes to 64 μg mL^{-1} of the test compound. [b] The log *P* values were calculated for the free-base form of the compound by using the MarvinSketch software (Marvin 6.3.1).

Log *P* was not predictive, however, of the hemolytic activity or antimicrobial-activity spectrum. For example, **2a** has a log *P* value of 5.36 and was the most potent antimicrobial of the subset of aromatic ether-substituted tobramycin derivatives against Gram-positive bacteria, but this compound was inactive against all of the tested Gram-negative strains and was the least hemolytic of all of the cationic amphiphiles in this study. In contrast, **6h**, with a similar log *P* value of 5.30 and was the most potent antimicrobial of the 4′,5,6-tri-*O*-*n*-alkyl ether nebramine derivatives, was active against all Gram-positive and Gram-negative strains and was one of the most hemolytic compounds tested.

Evaluation of bacterial-membrane disruption effect

The reduced hemolytic activity of the penta-*O*-benzyl tobramycin derivative **2a** relative to all of the other compounds in this study suggests that this compound may act on bacteria through a different mode of action than the other compounds evaluated. To test this possibility, we monitored the lytic effect of **2a** on bacteria that express cytosolic yellow fluorescent protein (YFP) and compared it to that of penta-*O*-*n*-pentyl tobramycin **2f**.[21,32] Similar to **2a**, **2f** demonstrated antimicrobial efficacy against all five tested Gram-positive strains; however, unlike **2a**, **2f** demonstrated poor membrane selectivity when an exposure of rat erythrocytes at a concentration of 16 μg mL^{-1} of **2f** led to 30±13% hemolysis.

A culture of untreated YFP-expressing *Bacillus subtilis* (Figure 2 A–C) was treated with **2 a** and **2 f** at concentrations that were twofold the MIC value (2 μg mL^{-1}). Although most of the bacterial cells in the untreated sample were viable and fluorescently labeled, a significant drop in the bacterial cell number and in cytosolic fluorescence was observed in bacterial culture containing **2 a** or **2 f** (Figure 2 D–I). Similar to the loss of hemoglobin during the hemolysis tests, the observed loss of cytosolic fluorescent protein content likely resulted from a disruption in the membrane integrity caused by **2 a** and **2 f**, thus leading to bacterial cell lysis. We concluded that even though **2 a** has relatively low hemolytic activity, it appears to be toxic to bacteria due to membrane disruption and not from a different mode of antimicrobial action.

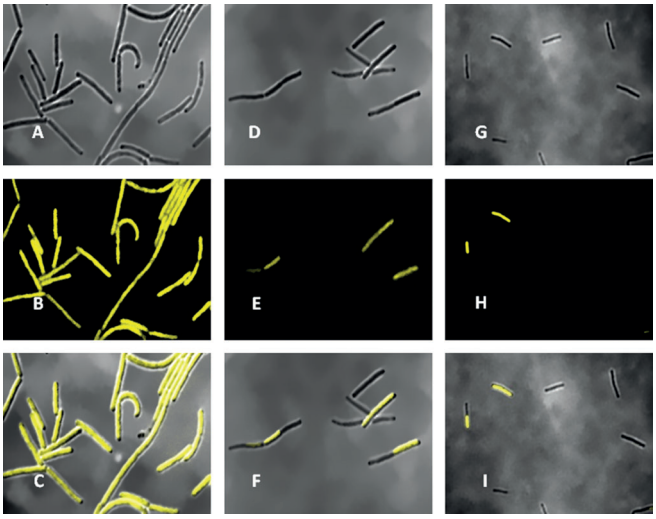


Figure 2. Bright-field and epi-fluorescence microscopy. *B. subtilis* (PY79) that constitutively express the YFP gene were imaged after cells had been incubated for 30 min with **2 a** or with **2 f** at 4 μg mL^{-1} (2×MIC). A) Control (untreated cells), bright field; B) control, fluorescent field; C) control, merge, D) **2 a**, bright field; E) **2 a**, fluorescent field; F) **2 a**, merge; G) **2 f**, bright field; H) **2 f**, fluorescent field; I) **2 f**, merge.

Conclusion

Four defined subsets of novel cationic amphiphiles derived from the AGs tobramycin and its pseudo-disaccharide segment nebramine were synthesized by etherification of some or all of the alcohol groups on the parent compounds. Antimicrobial activity tests revealed that the most potent antimicrobial cationic amphiphiles were compounds **2 a**, **2 f**, **4 j**, and **6 h**. Biological evaluation of the cationic amphiphiles in this study focused on four properties: First, we performed antimicrobial tests to determine the potency and efficacy spectrum of the compounds. Second, we assessed the selectivity of the cationic amphiphiles for bacterial membranes by evaluating the damage that these compounds caused to red blood cells, which served as a model for the evaluation of mammalian cell-membrane damage. We also evaluated the significance of performing the hemolysis assay in the same rich BHI broth used for the antimicrobial activity tests. Third, we investigated whether there is a general parameter that can be used to design AG-derived antimicrobial cationic amphiphiles by calculating the partitioning coefficient for each of the cationic amphiphiles. As a fourth direction, we studied the mode of action of the potent antimicrobial agents.

Compound **6 h**, the most potent antimicrobial cationic amphiphile in this study, had a significantly broader spectrum of antimicrobial activity relative to the antimicrobial activity of the parent AG tobramycin, the membrane-disrupting antimicrobial peptide-mixture gramicidin D, and the cationic lipopeptide PMX, all of which are clinically used antimicrobial agents. Compound **6 h** was at least 32 times more effective than the ribosome-targeting antibiotic tobramycin and the membrane-targeting antibiotic gramicidin D against several of the tested Gram-positive and Gram-negative bacteria and, unlike PMX, was effective against the tested Gram-positive pathogens. MIC experiments revealed that compounds that exhibited potent antimicrobial activity against the tested Gram-positive bacteria were not necessarily active against the tested Gram-negative strains. On the other hand, our observations suggest that activity against Gram-negative strains serves as an indication of potency against Gram-positive bacteria.

In general, potent antimicrobial activity was accompanied by higher erythrocyte hemolytic activity. Erythrocyte membrane damage caused by the AG-derived antimicrobial cationic amphiphiles was significantly diminished in rich physiological environments relative to PBS solutions in which it is usually evaluated. An exception was observed in the case of penta-*O*-benzyl tobramycin **2 a** because this compound had potent antimicrobial activity against the tested Gram-positive strains, yet caused almost no measurable hemolysis at a concentration range of 16–32-fold greater than its effective MIC range (4–8 μg mL^{-1}).

The calculated log *P* values of the four most potent antimicrobials, which vary in their molecular structures, fell into a narrow range of 5.36–6.08. This outcome suggests that this parameter may be used as a consideration for the design of other AG-derived antimicrobial cationic amphiphiles. However, log *P* values did not correlate with the antimicrobial-activity spectrum or the extent of the hemolytic activity of the compounds in this study and cannot be used to predict these biological activities.

Finally, we investigated whether there was a connection between the anti-Gram negative activity of **6 h** and its affinity to LPS as a possible mode of action. We concluded that the anti-Gram negative activity of **6 h** is likely to result from interactions with LPS and with additional Gram-negative membrane determinants. Second, we investigated if the antimicrobial cationic amphiphiles in this study (represented by **2 a** and **2 f**) induce bacterial cell-membrane damage, hence causing the loss of intracellular contents. This investigation was accomplished by observing the loss of fluorescent cytosolic protein from cytosolic YFP-expressing bacteria after incubation with the tested cationic amphiphiles. From these experiments, we concluded that **2 a** disrupts bacterial membrane structure, even though it did not cause significant red-blood-cell lysis at concentrations near the MIC values.

To conclude, the AG-derived antimicrobial cationic amphiphiles, such as those presented herein, offer a promising direction for the development of novel antibiotics for the treatment of topical infection caused by pathogens with resistance to the currently available repertoire of clinically used antimicrobial agents.

Experimental Section

General methods

^{1}H NMR spectra (including 1D TOCSY) were recorded on Bruker Avance 400 or 500 spectrometers, and chemical shifts (reported in ppm) were calibrated to CD_3OD, D_2O, or $CDCl_3$(δ = 3.33, 4.63, and 7.27 ppm, respectively). ^{13}C NMR spectra were recorded on Bruker Avance 400 or 500 spectrometers at 100.6 or 125 MHz, respectively. Multiplicities are reported with the following abbreviations: b = broad, s = singlet, d = doublet, t = triplet, dt = doublet of triplets, dd = doublet of doublets, ddd = doublet of doublet of doublets, m = multiplet, eq = equatorial, ax = axial. Coupling constants (*J*) are given in Hertz. Low-resolution electron spray ionization mass spectra were measured on a Waters 3100 mass detector. High-resolution electron spray ionization mass spectra were measured on a Waters Synapt instrument. Chemical reactions were monitored by TLC analysis (Merck, Silica gel 60 F254). Visualization was achieved by using a cerium molybdate stain ($(NH_4)_2Ce(NO_3)_6$ (5 g), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (120 g), H_2SO_4 (80 mL), and H_2O (720 mL)). All the reactions were carried out in an argon atmosphere with anhydrous solvents, unless otherwise noted. All chemicals, unless otherwise stated, were obtained from commercial sources. Compounds were purified by means of flash chromatography (SiO_2, Merck, Kieselgel 60).

General procedure for etherification of azide-protected aminoglycoside scaffolds

Benzyl bromide (1.2 mL, 10.0 mmol, 6.0 equiv), TBAI (617 mg, 1.67 mmol, 1.0 equiv), and NaH (60%, 402 mg, 10.0 mmol, 6.0 equiv) to a solution of penta-azidotobramycin[27] (1.0 g, 1.67 mmol) dissolved in dry DMF (10 mL) under argon. The reaction mixture was stirred at ambient temperature overnight. Progress of the reaction was monitored by TLC analysis (petroleum ether/EtOAc 75:25). Upon completion, the reaction mixture was diluted with EtOAc (100 mL), and the organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by flash column chromatography (SiO_2, petroleum ether/EtOAc 99:1→75:25) gave **1a** (1.7 g, 97%) as a colorless oil. The ^{1}H NMR spectra was in full agreement with the previously reported spectra.[28]

General procedure for reduction of azide protecting groups

Compound **1a** (130 mg, 0.12 mmol) was dissolved in THF (4.0 mL) and H_2O (0.5 mL). The mixture was added to NaOH (0.1 N, 0.2 mL) and trimethylphosphine in THF (1 M, 1.24 mL, 1.24 mmol, 10.0 equiv). Progress of the reaction was monitored by TLC analysis (2.8% NH_4OH solution in MeOH/dichloromethane 10:90). After 24 h the reaction mixture was evaporated under reduced pressure. Purification by flash column chromatography (SiO_2, 2.8% NH_4OH solution in MeOH/dichloromethane 0:100→10:90) gave **2a** (100 mg, 88%). The pure residue was dissolved in 95% TFA (0.5 mL), which was removed after 2 min under reduced pressure. The residue was dissolved in a minimal volume of H_2O and freeze-dried to afford the TFA salt of **2a**. HRMS (ESI): *m/z* calcd for $C_{53}H_{68}N_5O_9$: 918.5017 $[M+H]^+$; found, 918. 5015; ^{1}H NMR (400 MHz, D_2O) δ = 7.45–7.25 (m, 18H; benzyl), 7.21–7.15 (m, 3H; benzyl), 7.11–7.05 (m, 4H; benzyl), 5.19 (d, *J* = 3.0 Hz, 1H; H-1″), 5.05 (d, *J* = 2.1 Hz, 1H; H-1′), 4.85 (d, *J* = 11.9 Hz, 1H; CH_2 benzyl), 4.77–4.70 (m, 2H; CH_2 benzyl), 4.66–4.55 (m, 3H; CH_2 benzyl), 4.57–4.48 (m, 1H; CH_2 benzyl), 4.40 (bd, *J* = 10.2 Hz, 1H; H-5′), 4.30 (d, *J* = 11.1 Hz, 1H; CH_2 benzyl), 4.16 (d, *J* = 11.8 Hz, 1H; CH_2 benzyl), 4.11–4.03 (dd, *J* = 8.9, 9.1 Hz 1H; H-4), 4.00 (d, *J* = 11.8 Hz, 1H; CH_2 benzyl), 3.83 (m, 1H; H-5″), 3.81–3.75 (m, 2H; H-5, H-6), 3.74–3.54 (m, 4H; H-4′, H-2″, H-3″, H-4″), 3.52–3.36 (m, 2H; H-1, H-3), 3.40 (dd, *J* = 11.6, 2.4 Hz, 1H; H-6″), 3.21 (m, 1H; H-2′), 3.18–3.05 (m, 2H; H-6′, H-6″), 2.99 (dd, *J* = 14.0, 4.1 Hz, 1H; H-6′), 2.38 (dt, *J* = 12.6, 4.3 Hz, 1H; H-2_{eq}), 2.18 (m,1H; H-3_{eq}), 1.89–1.71 ppm (m, 2H; H-2_{ax}, H-3_{ax}); ^{13}C NMR (100 MHz, D_2O) δ = 163.09 (q, *J* = 35.4 Hz; CF_3CO_2H), 137.28, 136.88, 136.30, 136.19, 135.44, 129.44, 129.35, 129.08, 129.05, 128.84, 128.79, 128.64, 128.49, 128.40, 128.21, 126.67, 116.34 (q, *J* = 291.8 Hz, CF_3CO_2H), 98.28, 92.90, 81.80, 81.54, 78.14, 75.35, 75.04, 74.66, 74.38, 73.74, 73.06, 71.59, 71.23, 69.88, 66.56, 52.74, 49.56, 48.17, 46.72, 37.78, 30.22, 27.26, 25.59 ppm.

General procedure for the preparation of nebramine derivatives from the corresponding tobramycin derivatives

Concentrated sulfuric acid (1.5 N, 2.5 mL) was added to **1a** (200 mg, 0.19 mmol) was dissolved in MeOH (60 mL). The reaction mixture was heated to reflux for 48 h. Progress of the reaction was monitored by TLC analysis (petroleum ether/EtOAc 92:8). Upon completion, the reaction mixture was quenched with saturated sodium bicarbonate and extracted with ethyl acetate (3×100 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by flash column chromatography (SiO_2, petroleum ether/EtOAc 99:1→92:8) gave **3a** (100 mg, 88%) as a colorless oil. LRMS (ESI): *m/z* calcd for $C_{26}H_{30}N_{12}O_5$: 613.24 $[M+Na]^+$; found 613.16; ^{1}H NMR (500 MHz, CD_3OD) δ = 7.41 (m, 2H; benzyl), 7.38–7.23 (m, 8H; benzyl), 5.54 (d, *J* = 3.4 Hz, 1H; H-1′), 5.12 (d, *J* = 10.2 Hz, 1H; CH_2 benzyl), 4.79 (d, *J* = 10.2 Hz, 1H; CH_2 benzyl), 4.68 (d, *J* = 11.6 Hz, 1H; CH_2 benzyl), 4.50 (d, *J* = 11.6 Hz, 1H; CH_2 benzyl), 4.22 (m, 1H; H-5′), 3.66–3.42 (m, 8H; H-1, H-3, H-4, H-5, H-6, H-2′, H-4′, H-6′), 3.28 (m, 1H; H-6′), 3.15–3.05 (m, 1H; H-3′$_{eq}$), 2.43 (dt, *J* = 11.0, 4.3 Hz, 1H; H-2_{eq}), 2.25 (dt, *J* = 12.3, 4.0 Hz, 1H; H-3′$_{ax}$), 2.01 ppm (m, 1H; H-2_{ax}); ^{13}C NMR (125 MHz, CD_3OD) δ = 138.38, 138.09, 128.05, 127.91, 127.71, 127.62, 127.47, 127.24, 96.45, 84.73, 77.36, 77.23, 74.60, 72.36, 71.10, 70.45, 60.80, 59.98, 55.99, 51.04, 31.80, 27.75 ppm.

Synthesis of 7h

K_2CO_3 (25.7 mg, 0.18 mmol, 1.1 equiv) and bis-Boc-L-histidine *N*-hydroxysuccinimide ester (84.15 mg, 0.18 mmol, 1.1equiv) were added to **6h** (110 mg, 0.17 mmol) in MeOH (5 mL). The reaction mixture was stirred at room temperature for 24 h. Progress of the reaction was monitored by TLC analysis (2.8% NH_4OH solution in MeOH/dichloromethane 10:90). Upon completion, the reaction mixture was concentrated under reduced pressure and further purified by flash chromatography (SiO_2, 2.8% NH_4OH solution in MeOH/ dichloromethane 0:100→10:90). The fractions containing the pure product were concentrated under reduced pressure. The residue was dissolved in a minimal volume of H_2O and freeze-dried to afford the pure product **7h** (112 mg, 75%) as a white powder. LRMS (ESI): *m/z* calcd for $C_{49}H_{91}N_7O_{10}$: 838.38 $[M-CO_2C(CH_3)_3+2H]^+$; found 838.74; ^{1}H NMR (500 MHz, CD_3OD) δ = 7.64 (s,1H; histidine), 6.89 (s, 1H; histidine), 4.93 (s, 1H; H-1′), 4.32 (dd, *J* = 9.0, 5.2 Hz, 1H; CH-α-histidine), 3.89–3.79 (m, 2H; *n*-heptyl (2H)), 3.74 (dt, *J* =

9.2, 4.6 Hz, 1H; H-5′), 3.73–3.55 (m, 3H; *n*-heptyl (3H)), 3.52–3.42 (m, 2H; H-6′ (2H)), 3.37 (m, 1H; *n*-heptyl (1H)), 3.29 (t, $J=9.3$ Hz, 1H; H-4), 3.21 (t, $J=9.3$ Hz, 1H; H-5), 3.12–3.03 (m, 2H; H-4′, CH-β-histidine (1H)), 2.93 (t, $J=9.5$ Hz, 1H; H-6), 2.90–2.72 (m, 3H; H-3, H-2′, CH-β-histidine (1H)), 2.67 (ddd, $J=12.2$, 9.8, 4.1 Hz, 1H; H-1), 2.17 (dt, $J=11.6$, 4.2 Hz, 1H; H-3′$_{eq}$), 1.95 (dt, $J=12.9$, 4.2 Hz 1H; H-2$_{eq}$), 1.72–1.55 (m, 6H; *n*-heptyl (6H)), 1.50 (m, 1H; H-3′$_{ax}$), 1.45–1.28 (m, 42H; *Boc*-histidine (18H), *n*-heptyl (24H)), 1.20 (m, 1H; H-2$_{ax}$), 0.97–0.81 ppm (m, 6H; *n*-heptyl (9H)); ^{13}C NMR (125 MHz, CD_3OD) $\delta=173.01$, 156.24, 134.80, 99.93, 86.82, 86.14, 83.73, 79.20, 73.91, 73.38, 73.30, 71.06, 68.66, 54.89, 51.40, 50.69, 49.75, 39.88, 35.98, 31.87, 31.76, 30.20, 30.16, 29.76, 29.11, 29.05, 28.88, 27.33, 25.91, 25.85 ppm.

Synthesis of 8h

Compound **7h** (50 mg, 0.05 mmol) was treated at room temperature with 95% TFA (1 mL) for 3 min. The TFA was removed under reduced pressure, and the residue was dissolved in a minimal volume of H_2O and freeze-dried to afford **8h** (39 mg, quantitative yield) as a white foam. HRMS (ESI): *m/z* calcd for $C_{39}H_{76}N_7O_6$: 738.5857 $[M+H]^+$; found, 738.5861; ^{1}H NMR (500 MHz, CD_3OD) $\delta=$ 8.61 (s, 1H; histidine), 7.38 (s, 1H; histidine), 5.28 (d, $J=1.8$ Hz, 1H; H-1′), 4.33 (dd, $J=8.3$, 5.7 Hz, 1H; CH-α-histidine), 4.23 (t, $J=$ 7.1 Hz, 1H; H-5′), 4.00 (dd, $J=10.0$, 9.2 Hz, 1H; H-4), 3.85–3.78 (m, 2H; *n*-heptyl (2H)), 3.74 (m, 1H; *n*-heptyl (1H)), 3.66–3.52 (m, 5H; H-2′, H-6′ (2H), *n*-heptyl (2H)), 3.50–3.34 (m, 6H; H-3, H-5, H-6, H-4′, *n*-heptyl (1H), CH-β-histidine (1H)), 3.30–3.20 (m, 2H; H-1, CH-β-histidine), 2.46 (dt, $J=12.3$, 4.2 Hz, 1H; H-2$_{eq}$), 2.40–2.21 (m, 2H; H-3′$_{eq}$, H-3′$_{ax}$), 1.93 (m, 1H; H-2$_{ax}$), 1.72–1.57 (m, 6H; *n*-heptyl (6H)), 1.43–1.27 (m, 24H; *n*-heptyl (24H)), 1.00–0.83 ppm (m, 9H; *n*-heptyl (9H)); ^{13}C NMR (100 MHz, CD_3OD) $\delta=168.33$, 161.83 (q, $J=$ 34.7 Hz, CF_3CO_2H)), 135.05, 128.45, 118.08, 117.07 (p, $J=292.6$ Hz; CF_3CO_2H)), 93.53, 83.25, 80.42, 78.41, 75.77, 74.06, 73.90, 71.12, 69.80, 52.39, 49.52, 48.63, 38.33, 31.80, 31.75, 31.72, 30.37, 29.79, 29.66, 29.22, 29.16, 29.09, 28.16, 27.40, 26.29, 26.24, 25.85, 25.75, 22.41, 13.13 ppm.

Protocol for minimal inhibitory-concentration tests

MIC values were determined as reported previously.[23] In each experiment, the samples were analyzed in triplicate, and the results were obtained from two independent experiments. MIC values (μg mL^{-1}) were determined as the lowest concentration at which no bacterial growth was observed.

Protocol for MIC experiments with added LPS

Starter cultures of *E. coli* ATCC 25922 (Strain G) and *S. pyogenes* glossy were incubated for 24 h (37 °C, 5% CO_2, aerobic conditions) and then diluted in fresh BHI medium to obtain an optical density of 0.004 (OD_{600}). Tested compounds were diluted by using the double-dilution method starting at 64 μg mL^{-1} into 96-well plates (Corning) at a volume of 100 μL. The LPS (50 μL) was prepared from *E. coli* 0111:B4, purified by ion-exchange chromatography, and tested to ensure that it could serve as a TLR ligand. The LPS was added in final concentrations of 0, 10, 50, and 100 μg mL^{-1}. The compounds and LPS were incubated for 1 h at room temperature. Diluted bacteria solution (50 μL) was added to each well to obtain a final volume of 200 μL. This mixture was incubated for 18 h (37 °C, 5% CO_2, aerobic conditions) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 50 μL, 1 mg mL^{-1} in H_2O) was added to each well, followed by an additional incubation at 37 °C for 30 min.

Protocol LPS BODIPY-cadaverine displacement assay

Stock solutions of LPS from *E. coli* 0111:B4 purified as above (5 mg mL^{-1}) and BODIPY-TR-cadaverine (500 μM) were prepared in tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4, 50 mM). The LPS stock (1 mL) and BODIPY-TR-cadaverine stock (1 mL) were mixed and diluted in Tris buffer to a final volume of 100 mL, thus yielding final concentrations of 50 μg mL^{-1} of LPS and 5 μM BODIPY-TR-cadaverine. BODIPY-TR-cadaverine/LPS mixture (40 μL) was added to each well of the 96-well plate. The test compound was added and diluted with Tris buffer to a final volume of 60 μL. Fluorescence measurements were made at 25 °C on a TECAN microplate reader (infinite F200 PRO). The BODIPY-TR-cadaverine excitation wavelength was $\lambda=580$ nm. Emission spectra were taken at $\lambda=620$ nm.

Protocol for erythrocyte hemolysis assay

The hemolysis assay was performed by following a previously reported protocol.[23] These experiments were done in triplicate, and the results reported are the averages of three independent experiments on three different rabbit blood samples. Hemolysis assays conducted in BHI broth were performed as follows: A sample of rat red blood cells in BHI medium (2% w/w) were incubated with each of the tested compounds for 1 h at 37 °C in 5% CO_2 by using the double-dilution method and starting at concentration of 128 μg mL^{-1}. The negative control was BHI, and the positive control was 1% v/v solution of Triton X100 in BHI (100% hemolysis). Following centrifugation (2000 rpm, 10 min, ambient temperature), the supernatant was removed and absorbance at $\lambda=550$ nm was measured by using a microplate reader (SpectraMax-M2). The results are expressed as percentage of hemoglobin released relative to the positive control (Triton X100). The experiments were performed in triplicate, and the results are an average of experiments on blood samples taken from two rats.

Protocol for epi-fluorescence microscopy

The epi-fluorescence microscopy assay was performed as previously reported[21] with the following changes: *B. subtilis* PY79 cells that constitutively express YFP were used. *B. subtilis* PY79 cells carrying YFP taken from a freshly streaked plate were grown (37 °C, ≈14 h) in lysogeny broth (LB; 5 mL) supplemented with erythromycin (3 μg mL^{-1}). The overnight culture (0.06 mL) was diluted into fresh LB broth (6 mL) and grown to $OD_{600}=0.3$. The cells were treated with **2a** or **2f** at 8 μg mL^{-1} (4×MIC) and incubated at 37 °C. After 30 min in the presence of **2a** or **2f**, aliquots (1 μL) were transferred to agarose slabs made of LB. Snapshot fluorescence images were taken with a 100× lens of an inverted epi-fluorescence microscope (Nikon TiE).

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Keywords: cationic amphiphiles · antibiotic resistance · antibiotics · bacterial membranes · glycosides

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