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Synthesis and antibacterial properties of carbohydrate-templated lysine surfactants

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ABSTRACT

The synthesis of four dicationic glucose-templated D-lysine-derived surfactants and their two unmodified D-lysine-analogs is described. Replacement of D-lysine by D-glucose-templated-D-lysine provides a general tool to introduce chemical diversity into the side chain of lysine. The presence of the polyfunctional D-*gluco*-configured polyol scaffold provides rich opportunities to study structure–activity relationships in lysine–lipid conjugates. All cationic lipids were tested for inhibition of bacterial growth using a panel of clinically relevant Gram-positive and Gram-negative strains. Our results show that substitution of D-lysine by D-glucose-templated D-lysine surfactants retains, but does not improve, the antibacterial activity. Similarly, conversion of the D-*gluco*-based polyol scaffold into a hydrophobically enhanced -tri-*O*-phenylcarbamate scaffold does not further enhance the antibacterial activity of the cationic lipid. However, improvements in the antibacterial activity were observed by guanidinylation of the two lysine-based amino groups.

1. Introduction

Multidrug-resistant bacteria are becoming more prevalent in both the hospitals and community settings. This has resulted in an emerging crisis where an increasing number of antibiotics cease to be of clinical usefulness.[1] As a result, there is a pressing need for novel classes of antibacterial agents with new or combined mechanisms of action and reduced likelihood for the development of resistance.

Oligocationic amphiphilic antibacterials (OAAs) containing multiple positively charged amino or other cationic groups define a structurally diverse class of antibacterials with broad-spectrum activity and different modes of action.[2–4] This class of antibacterial agents are comprised of the naturally occurring cationic antimicrobial peptides,[5] synthetic mimics of antimicrobial peptides (SMAMPs),[6] synthetic oligocationic lipopeptides,[7] oligocationic lipids,[8] and polymers.[9] The cationic charges of the OAAs ensure accumulation at polyanionic microbial cell surfaces that contain acidic polymers, such as lipopolysaccharides and wall-associated teichoic acids in Gram-negative and Gram-positive bacteria, respectively.[5a] Antimicrobial peptides (polymyxin B, defensins, gramicidin S variants, and others) transit the outer membrane by interacting at sites at which divalent cations crossbridge adjacent polyanionic polymers. This causes a destabilization of the outer membrane that is proposed to lead to self-promoted uptake of the OAAs and/or other extracellular molecules.[5a,10] After transit through the outer membrane OAAs contact the anionic surface of the cytoplasmic membrane. Here they can insert themselves into the cytoplasmic membrane thereby either disrupting the physical integrity of the bilayer, via membrane thinning, transient poration and/or disruption of the barrier function or translocation across the membrane and act on internal targets.[5a] This mode of action has been shown to limit the risk of cross resistance.[5,11]

Another class of OAAs are cationic lipids, which have been developed as antibacterial agents, antiseptics, and disinfectants but also as drug delivery systems against diseases[14,15] and gene transfection.[16] For instance, cationic lipids including dodine,[17] benzalkonium chlorides,[18] sphingosine[19], and fatty amines,[20] chlorohexidine,[21] cationic polymers[22] exhibit broad spectrum antibacterial activities (Fig. 1). Many of these are in clinical use as antiseptics, disinfectants for several decades with little or no occurrence of resistance.[12,13] It has been suggested that the antibacterial mode of action of cationic lipids is similar to antibacterial peptides and involves disruption of the bacterial envelope induced by the removal (substitution) of divalent positively charged counter ions by ammonium ions.[12]

In this paper we describe the synthesis and antibacterial properties of a novel class of dicationic lysine- and arginine-related amphiphiles. Previous studies have shown that dicationic, arginine-based cationic lipids prepared by C-terminal amidation of arginine to alkyl amines bearing long alkyl chains (C-10, C-12 and C-14) exhibit broad spectrum antibacterial activity with little to no cytotoxicity.[23] However, dicationic lipids of this type do not

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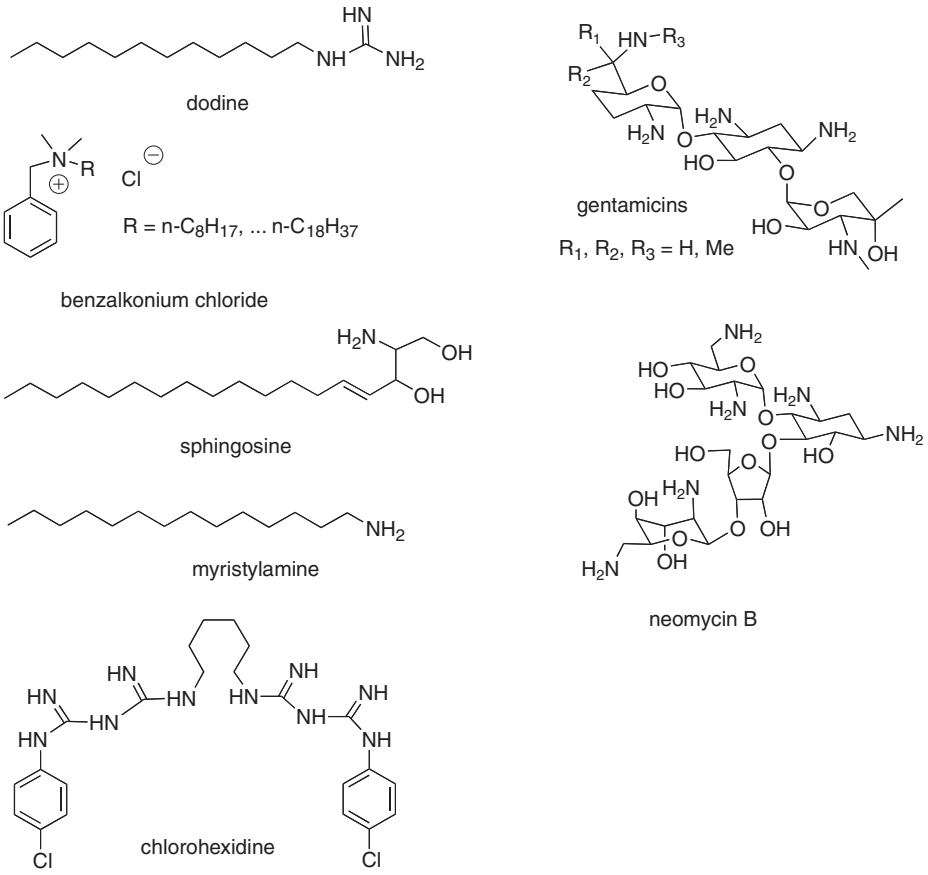


Figure 1. Structures of antibacterial cationic lipids and detergents. Dodine, benzalkonium chlorides, sphingosine, and chlorohexidine are currently used in commercial antiseptics and disinfectants. The structures of the positive controls gentamicin and neomycin B are also provided for general reference.

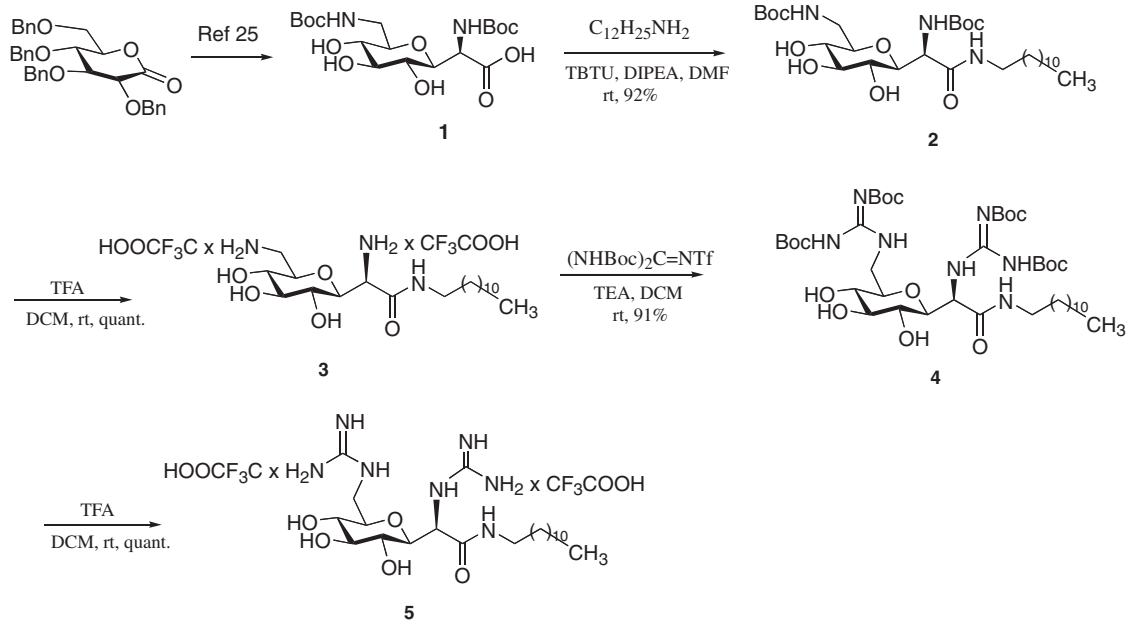
permit additional manipulation as both the dicationic headgroup and the hydrophobic tail form the amphiphilic pharmacophore of antibacterial cationic lipids. We have previously outlined a strategy termed carbohydrate-templated amino acid synthesis (CTAAs) that permits the incorporation of polyfunctional sugar scaffolds into naturally occurring amino acids, such as ornithine,[24] arginine,[24] lysine,[25] proline[26–30], and GABA.[31] The presence of the carbohydrate-scaffold introduces chemical diversity into amino acid side chains and may mimic artificial post-translational modifications, such as hydroxylation and glycosylation. Moreover, chemical derivatization of the auxophoric polyol scaffold can be used to tailor the pharmacodynamic and pharmacokinetic properties of cationic lipids. We have now applied this strategy to prepare novel carbohydrate-templated lysine–lipid conjugates. We were particularly interested to study how incorporation of an auxophoric glucose-based polyol scaffold into the cationic head group of a lysine surfactant influences its antibacterial properties.

2. Results and discussion

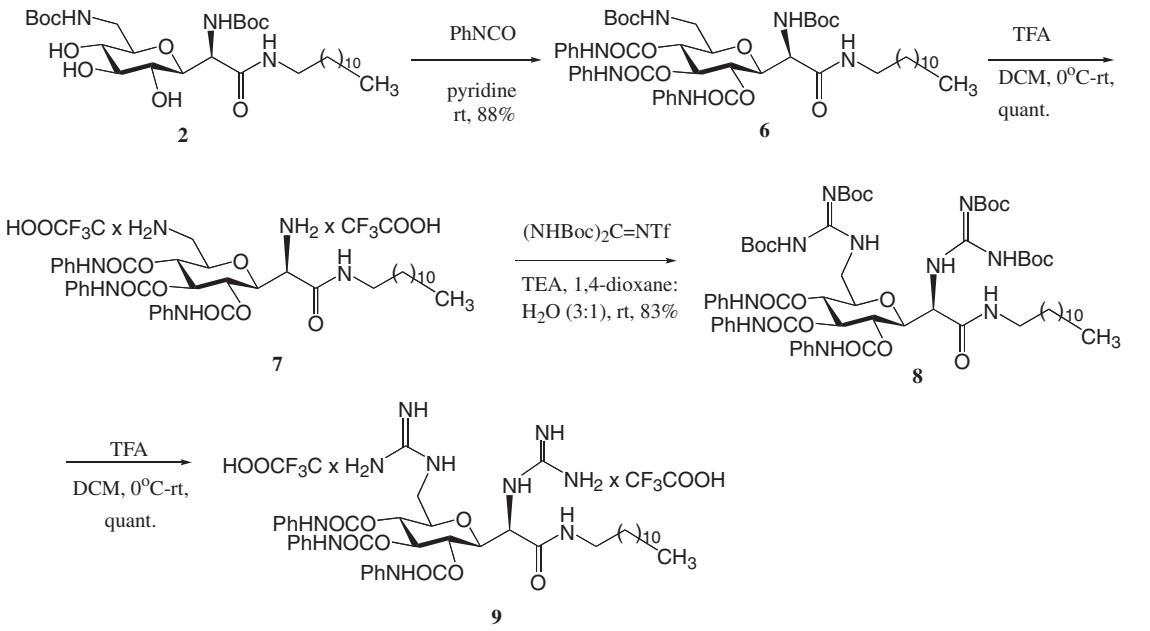
Our synthesis began with partially protected D-*gluco*-templated D-lysine analog **1** previously prepared from commercially available tetra-*O*-benzyl gluconolactone.[25] Acid **1** was coupled to dodecylamine using of 2-(1*H*-benzotriazole-1yl)-1,1,3,3-tetra-methyluronium tetrafluoroborate (TBTU) as coupling reagent and Hünig's base (*N*,*N*-diisopropylethylamine) in *N*,*N*′-dimethylformamide as solvent to afford glycolipid **2** in 92% yield. Exposure of glycolipid **2** to trifluoroacetic acid in dichloromethane at room temperature for 3 h produced deblocked D-lysine-based glycosurfactant **3** as trifluoroacetate salt. We also prepared deblocked homo-D-arginine-derived glycolipid **5** by guanidinylation of the two amino functions in glycosurfactant **3**. This was achieved by reaction of **3** with *N*,*N*′-di-*tert*-butoxycarbonyl-*N*′-triflylguanidine $(NHBoc)_2C{=}NTf$[32] using Et_3N in dichloromethane at ambient temperature for 12 h to generate *tert*-butoxycarbonyl-protected homo-D-arginine-derived glycolipid **4**. Deblocking was achieved by exposure to trifluoroacetic acid in dichloromethane to give unprotected D-*gluco*-templated D-homoarginine-derived glycolipid **5** (Scheme 1). Both glycolipids **3** and **5** were used for antibacterial testing.

To explore how chemical modifications on the D-*gluco*-configured polyol scaffold influence the antibacterial properties we decided to prepare polyol-modified D-lysine-based triphenylcarbamate **7** and polyol-modified homo-D-arginine-based triphenylcarbamte **9** (Scheme 2). Phenylcarbamoylation was selected as a method of derivatization due to the high reactivity of phenyl isocyanate with sugar hydroxyl groups and their antibacterial properties.[34] Compound **7** was prepared by reaction of glycoplipid **2** with phenyl isocyanate in anhydrous pyridine at room temperature for 12 h to afford Boc-protected lysine analog **6** in 88% yield. Exposure of **6** to TFA in dichloromethane produced oligoamphiphilic lysine analog **7** as a TFA salt in quantitative yield. The two deblocked amino functions were guanidinylated with *N*,*N*′-di-*tert*-butoxycarbonyl-*N*′-triflylguanidine $(NHBoc)_2C{=}NTf$[32] to yield protected homoarginine-derived lipid **8** in 83% isolated yield. Deblocking of the Boc-protecting groups in dichloromethane produced polyamphiphilic homoarginine-derived lipid **9** (Scheme 2). We also prepared the D-lysine-based cationic lipid **12** and D-homoarginine-derived lipid **14** from commercially available D-lysine building block **10** as reference compounds (Scheme 3).

With amphiphilic carbohydrate-templated lysine-modified lipids **3**, **5**, **7**, and **9** and lysine-based surfactants **12** and **14** lipids in hand, we determined the antibacterial minimum inhibitory con-



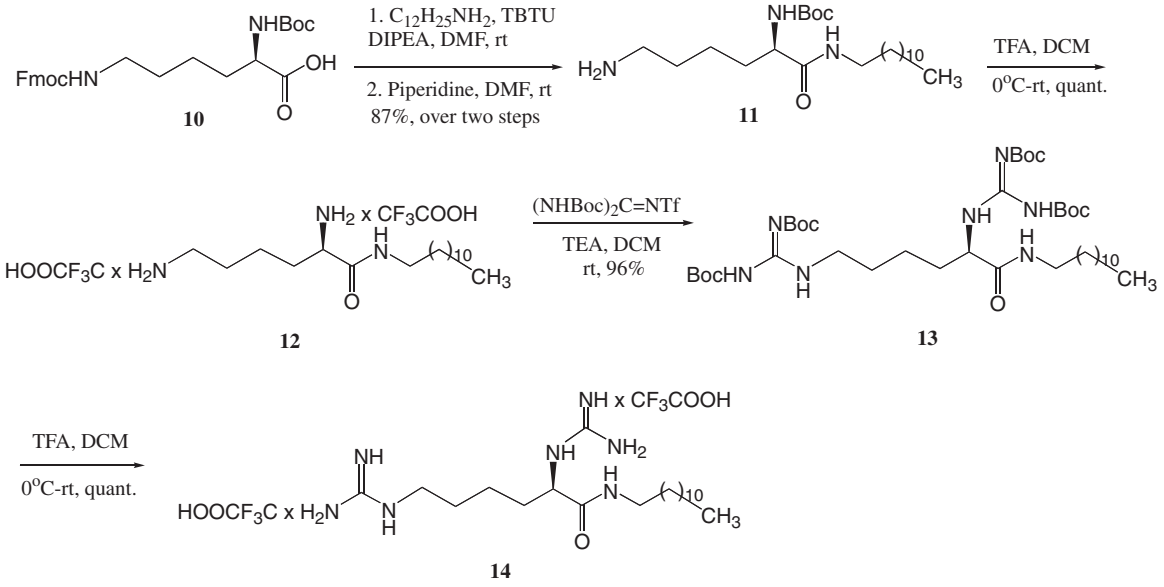
Scheme 1. Synthesis of D-*gluco*-templated D-lysine surfactant **3** and D-*gluco*-templated homo-D-arginine-based surfactant **5**.



Scheme 2. Synthesis of polyol-modified carbohydrate-templated D-lysine- (**7**) and carbohydrate-templated homo-D-arginine-based (**9**) lipids.

centration (MIC) in μg/mL of these analogs against American Type Culture Collection (ATCC) reference strains as well as clinical isolates of Gram-positive strains including *Staphylococcus aureus*, MRSA, *Staphylococcus epidermidis*, methicillin-resistant *S. epidermidis* (MRSE) and *Streptococcus pneumoniae* as well as Gram-negative strains *Escherichia coli*, gentamicin-resistant *E. coli*, and *Pseudomonas aeruginosa* (Table 1). We also included three resistant strains obtained from a national surveillance study assessing antimicrobial resistance in Canadian intensive care units CAN-ICU (Table 1).[33] Gentamicin and neomycin B served as positive controls.

Our results show that substitution of D-lysine in cationic lipid **12** by D-*gluco*-templated D-lysine results in similar antibacterial activity. In most cases, an expected slight increase in MICs for Gram-positive organisms was observed for **3** when compared to **12** because of changes in molecular weight. However, similar potency was observed for MRSA while a fourfold deduction in antibacterial potency was observed against *S. pneumoniae*. A significant fourfold reduction in antibacterial activity of **3** when compared to **12** was found against a Gentamicin-susceptible Gram-negative *E. coli* strain. Interestingly, phenylcarbamoylation of the cationic polyol-based headgroup in compounds **3** and **5** has little to no effect on the antibacterial activity once the changes in molecular weight are considered. This suggests that polyol-based substitutions aimed to enhance the hydrophobicity of the polar headgroup do not provide a tool to enhance the antibacterial properties. Very likely the enhanced hydrophobicity of the polar headgroup in cationic lipids **7** and **9** disturbs the monoamphiphilic nature (single polar head and single hydrophobic tail) of classic cationic deter-



Scheme 3. Synthesis of lysine- (**12**) and guanidinylated lysine-based (**14**) reference lipids.

Table 1
Antibacterial activity (MIC) in μg/mL of carbohydrate-templated lysine-based lipids **3**, **5**, **7** and **9** and reference lipids **12** and **14** against various Gram-positive and Gram-negative bacterial strains

Control organism	Gentamicin	Neomycin B	**3**	**5**	**7**	**9**	**12**	**14**
S. aureus[a]	1	1	64	16	64	32	32	4
MRSA[b]	2	256	64	16	32	32	64	4
S. epidermidis[c]	0.25	0.25	16	8	16	32	8	2
MRSE[d]	32	0.5	32	16	32	64	16	8
S. pneumoniae[e]	4	32	128	64	>128	128	32	32
E. coli[f]	1	4	256	128	>256	64	32	128
E. coli[g]	128	8	256	128	>256	128	128	128
P. aeruginosa[h]	8	512	>256	256	>256	128	128	64
P. aeruginosa[i]	128	512	256	128	>256	128	128	128
MW[j]	[k]	908.9	505.4	589.4	862.7	946.8	415.3	499.4

[a] ATCC 29213.
[b] Methicillin-resistant *S. aureus* ATCC 33592.
[c] *S. epidermidis* ATCC 14990.
[d] Methicillin-resistant *S. epidermidis* (CAN-ICU) 61589.
[e] ATCC 49619.
[f] ATCC 25922.
[g] CAN-ICU 61714.
[h] ATCC 27853.
[i] CAN-ICU 62308.
[j] Molecular weight as TFA salt for compounds **3**, **5**, **7**, **9**, **12** and **14**, trisulfate (Neomycin B).
[k] Mixture of homologues average MW ~ 600.

gents such as **12** and **14** resulting in the formation of polyamphiphilic surfaces. In addition, our results suggest that the enhanced hydrophobicity of amphiphiles **7** and **9** very likely must be compensated by changes in the ionization state or number of cationic charges in the headgroup in order to enhance antibacterial activity. Moreover, guanidinylation of **12**, **3**, and **7** enhances in most cases antibacterial activity. The increase in potency is more obvious for Gram-positive strains in lipids **12** and **3** resulting in a eight to two-fold decrease in MIC with relative little changes for Gram-negative organisms. Overall it appears that carbohydrate-templated lysine–lipid conjugates exhibit higher MICs when compared to their unmodified lysine–lipid conjugates. In particular, cationic lipid **14** exhibits impressive Gram-positive activity when compared to the aminoglycoside antibiotics gentamicin and neomycin B.

In summary, we have synthesized four carbohydrate-templated lysine-derived dicationic lipids as well as two unmodified lysine-derived dicationic reference lipids and explored their antibacterial properties against a panel of clinically relevant Gram-positive and Gram-negative bacterial strains. Our results show that substitution of D-lysine by carbohydrate-templated D-lysine analogs retains, but does not improve, the antibacterial potency of the corresponding cationic lipid.

3. Experimental

3.1. General methods

NMR spectra were recorded on a Brucker Avance 300 spectrometer (300 MHz for ^{1}H NMR, 75 MHz for ^{13}C) and on a AMX 500 spectrometer (500 MHz for ^{1}H NMR). Optical rotation was measured at a concentration of g/100 mL, with a Perkin–Elmer polarimeter (accuracy 0.002°). GC–MS analyses were performed on a Perkin–Elmer Turbomass-Autosystem XL. Analytical thin-layer chromatography was performed on pre-coated silica gel plates.

Visualization was performed by ultraviolet light and/or by staining with ninhydrin solution in ethanol. Chromatographic separations were performed on a silica gel column by flash chromatography (Kiesel gel 40, 0.040–0.063 mm; Merck). Yields are given after purification, unless differently stated. When reactions were performed under anhydrous conditions, the mixtures were maintained under nitrogen. Compounds were named following IUPAC rules as applied by Beilstein-Institute AutoNom (version 2.1) software for systematic names in organic chemistry. The purity of all final compounds used in biological testing was assessed by elemental analysis confirming >95% purity. ^{13}C NMR spectra of compounds in the form of TFA salts usually showed the characteristic carbon peaks for TFA at δ = 164.4 (q) and 116.5 (q) in D_2O when using long acquisition times.

3.2. *N′*-*Dodecyl*-[(2*R*)-2-(*tert*-butoxycarbonylamino)-2-[6-deoxy-6-(*t*-butoxycarbonylamino)-β-D-glucopyranosyl] ethanamide (2)

To a mixture of compound **1** (100 mg, 0.23 mmol) and TBTU (265 mg, 0.83 mmol) in DMF (5.0 mL) were added dodecylamine (51 mg, 0.25 mmol) and *N*,*N*-diisopropylethylamine (200 μL, 1.1 mmol) and stirred for 2 h at room temperature. The solvent was removed in vacuo and the residue was purified by flash column chromatography (MeOH–EtOAc 1:25) to yield **2** (127 mg, 0.21 mmol, 92%) as a thick colorless liquid. R_f = 0.20 (MeOH–EtOAc 1:19); $[\alpha]_D^{25} = +10.0$ (*c* 0.30, MeOH); ^1H NMR (300 MHz, MeOH-d_4): δ 0.91 (t, 3H, *J* = 6.9 Hz), 1.30 (br s, 18H), 1.45–1.55 (m, 20H), 3.01–3.12 (m, 2H), 3.16–3.26 (m, 3H), 3.29 (m, 1H), 3.35–3.40 (m, 2H), 3.55 (d, 1H, *J* = 13.5 Hz), 4.40 (s, 1H); ^{13}C NMR (75 MHz, MeOH-d_4): δ 14.5, 23.7, 28.0, 28.7 (×3), 28.9 (×3), 30.5 (×3), 30.8 (×4), 33.1, 40.4, 42.9, 56.9, 71.9, 72.9, 79.1, 80.3, 80.8, 81.0, 81.2, 157.4, 158.7, 171.9; MS (ES+): *m/z* 626.21 $[M+Na]^+$; Anal. Calcd for $C_{30}H_{57}N_3O_9$: C, 59.68; H, 9.52; N, 6.96. Found: C, 59.43; H, 9.90; N, 7.23.

3.3. *N′*-*Dodecyl*-[[(2*R*)-2-ammonium-2-]-(6-ammonium-6-deoxy)-β-D-glucopyranosyl]ethanamide bistrifluoroacetate (3)

A solution of TFA–CH_2Cl_2 (1:3, 4.0 mL) was added to **2** (127 mg, 0.21 mmol) at room temperature. After 3 h, the solution was diluted with toluene (3 × 10 mL), concentrated in vacuo, to provide **3** (133 mg, quant.) as a trifluoroacetate salt. $[\alpha]_D^{25} = +15.0$ (*c* 0.35, MeOH); ^1H NMR (300 MHz, D_2O): δ 0.84 (t, 3H, *J* = 7.0 Hz), 1.24 (br s, 18H), 1.42–1.55 (m, 2H), 3.04 (m, 1H), 3.12–3.30 (m, 3H), 3.35–3.70 (m, 4H), 3.82 (m, 1H), 4.35 (s, 1H); ^{13}C NMR (75 MHz, D_2O): δ 14.1, 22.9, 27.2, 28.9, 29.4, 29.7, 29.9, 30.0 (×3), 32.2, 40.4, 41.1, 53.7, 70.0, 71.4, 76.4, 76.9, 77.3, 165.3; MS (ES+): *m/z* 404.17 $[M+H]^+$; Anal. Calcd for $C_{24}H_{43}F_6N_3O_9$: C, 45.64; H, 6.86; N, 6.65. Found: C, 45.32; H, 6.53; N, 6.85.

3.4. *N′*-*Dodecyl*-[[(2*R*)-2-*N*,*N′*-di-*tert*-butoxycarbonyl-guanidinyl-2-]-[6-*N*,*N′*-di-*tert*-butoxycarbonyl-guanidinyl-6-deoxy)-β-D-glucopyranosyl]ethanamide (4)

To a solution of diamine salt **3** (0.040 g, 0.006 mmol) in dichloromethane (2.0 mL) was added *N*,*N′*-di-*t*-butoxycarbonyl-*N″*-triflylguanidine (86 mg, 0.22 mmol, 3.5 equiv). After 5 min, NEt_3 (31.0 μL, 0.22 mmol, 3.5 equiv) was added at room temperature. After 12 h, the dichloromethane was removed under reduced pressure. The remaining residue product was purified by flash column chromatography with EtOAc on silica gel to give guanidinylated derivative **4** (52 mg, 0.058 mmol, 91%) as an oil. R_f = 0.20 (EtOAc); $[\alpha]_D^{25} = -55.0$ (*c* 0.30, $CHCl_3$); ^1H NMR (300 MHz, MeOH-d_4): δ 0.91 (t, 3H, *J* = 7.0 Hz), 1.30 (br s, 19H), 1.47 (2s, 18H), 1.56 (m, 19H), 3.16 (dd, 1H, *J* = 8.8, 9.2 Hz), 3.25 (t, 2H, *J* = 7.0 Hz), 3.35–3.45 (m, 2H), 3.48 (dd, 1H, *J* = 6.8, 9.0 Hz), 3.57 (dd, 1H, *J* = 3.3, 9.4 Hz), 3.65 (dd, 1H, *J* = 2.7, 14.4 Hz), 3.80 (dd, 1H, *J* = 4.4, 14.4 Hz),4.83 (d, 1H, *J* = 4.0 Hz); ^{13}C NMR (75 MHz, MeOH-d_4): δ 14.5, 23.7, 28.0, 28.4 (×6), 28.5 (×3), 28.6 (×3), 30.4, 30.5, 30.6, 30.8 (×4), 33.1, 40.6, 42.9, 57.0, 72.0, 72.5, 78.6, 79.8, 80.5, 80.6, 80.9, 84.6, 84.7, 154.0 (×2), 157.1, 158.3, 164.1, 164.3, 170.4; MS (ES+): *m/z* 910.14 $[M+Na]^+$; Anal. Calcd for $C_{42}H_{77}N_7O_{13}$: C, 56.80; H, 8.74; N, 11.04. Found: C, 57.11; H, 9.02; N, 10.83.

3.5. *N′*-*Dodecyl*-[[(2*R*)-2-guanidinyl-2-]-[6-guanidinyl-6-deoxy)-β-D-glucopyranosyl]ethanamide × 2 trifluoroacetic acid (5)

A solution of TFA–CH_2Cl_2 (1:3, 4.0 mL) was added to compound **4** (80 mg, 0.09 mmol) at room temperature. After 3 h, the solution was diluted with toluene (3 × 5 mL), concentrated in vacuo, quantitatively provided guanidine substance **5** (64.0 mg, quant.) as a trifluroacetate salt. $[\alpha]_D^{25} = -15.0$ (*c* 0.60, MeOH); ^1H NMR (300 MHz, D_2O): δ 0.81 (t, 3H, *J* = 6.6 Hz), 1.21 (s, 18H), 1.44 (br s, 2H), 3.01–3.31 (m, 4H), 3.40–3.55 (m, 3H), 3.58 (br d, 1H, *J* = 13.8 Hz), 3.73 (br d, 1H, *J* = 6.9 Hz), 4.50 (s, 1H); ^{13}C NMR (75 MHz, D_2O): δ 14.0, 22.9, 27.1, 29.0, 29.5, 29.7, 29.9, 30.0 (×3), 32.2, 40.2, 42.8, 56.2, 70.3, 70.9, 77.3, 78.4, 79.6, 157.1, 157.8, 168.1; MS (ES+): *m/z* 488.01 $[M+H]^+$; Anal. Calcd for $C_{26}H_{47}F_6N_7O_9$: C, 43.63; H, 6.62; N, 13.70. Found: C, 43.43; H, 6.30; N, 13.33.

3.6. *N′*-*Dodecyl*-[(2*R*)-2-(*t*-butoxycarbonylamino)-2-[6-deoxy-6-(*tert*-butoxycarbonylamino-2,3,4-tri-*O*-phenylcarbamoyl)-β-D-glucopyranosyl]ethanamide (6)

To a solution of *tert*-butoxycarbonyl-protected derivative **2** (1 equiv) in dry pyridine phenyl isocyanate (4.0 equiv) was added and stirred at room temperature for 12 h. Pyridine was removed under reduced pressure and the crude residue was purified by flash column chromatography, eluted by EtOAc–hexane to get pure carbamate derivative **6** as thick liquid in 88% yield. R_f = 0.20 (EtOAc–Hexane 4:1); $[\alpha]_D^{25} = -27.0$ (*c* 1.3, MeOH); ^1H NMR (300 MHz, MeOH-d_4): δ 0.89 (t, 3H, *J* = 7.0 Hz), 1.29 (s, 18H), 1.44 (2s, 18H), 1.50 (m, 2H), 3.04–3.20 (m, 2H), 3.20–3.28 (m, 1H), 3.56 (m, 1H), 3.68 (m, 1H), 4.00 (dd, 1H, *J* = 2.5, 10.0 Hz), 4.45 (m, 1H), 4.89 (m, 1H), 5.09 (dd, 1H, *J* = 9.5, 10.0 Hz), 5.32 (dd, 1H, *J* = 9.5, 10.0 Hz), 6.80 (br s, 1H), 6.92–7.02 (m, 3H), 7.09–7.30 (m, 8H), 7.32–7.44 (m, 4H), 7.93 br s, 1H); ^{13}C NMR (75 MHz, MeOH-d_4): δ 14.5, 23.7, 28.1, 28.7 (×3), 28.8 (×3), 30.3, 30.4, 30.5, 30.8 (×4), 33.1, 40.8, 42.5, 56.8, 71.8, 72.0, 76.3, 78.9, 79.2, 80.4, 81.3, 120.2–139.7 (aromatic carbons), 154.5, 154.9, 155.2, 157.6, 158.2, 170.5; MS (ES+): *m/z* 983.58 $[M+Na]^+$; Anal. Calcd for $C_{51}H_{72}N_6O_{12}$: C, 63.73; H, 7.55; N, 8.74. Found: C, 63.51; H, 7.84; N, 8.67.

3.7. *N′*-*Dodecyl*-[[(2*R*)-2-ammonium-2-]-(6-ammonium-6-deoxy-2,3,4-tri-*O*-phenylcarbamoyl-)-β-D-glucopyranosyl] ethanamide bistrifluoroacetate (7)

Deblocking of the *tert*-butoxycarbonyl groups in derivative **6** (50.0 mg) was performed with 25% trifuoroacetic acid (4.0 mL) in dichloromethane for 3 h at 0 °C to rt. The volatile components were removed in vacuo under reduced pressure and the non-polar residues were removed by washing with ether and decanted the solvent to get the TFA salt **7** in quantitative yield. $[\alpha]_D^{25} = -9.0$ (*c* 1.7, MeOH); ^1H NMR (300 MHz, MeOH-d_4): δ 0.89 (t, 3H, *J* = 7.0 Hz), 1.22–1.48 (m, 18H), 1.58–1.69 (m, 2H), 3.12–3.22 (m, 2H), 3.34–3.41 (m, 2H), 4.06 (m, 1H), 4.27 (m, 1H), 4.35 (br s, 1H), 4.89 (m, 1H), 5.13 (dd, 1H, *J* = 7.9, 8.2 Hz), 5.49 (dd, 1H, *J* = 9.0, 9.5 Hz), 6.96–7.08 (m, 3H), 7.15–7.46 (m, 12H); ^{13}C NMR (75 MHz, MeOH-d_4): δ 14.5, 23.8, 28.2, 30.1, 30.5 (×2), 30.9 (×4), 33.1, 41.2, 41.7, 54.3, 71.1, 71.4, 75.0, 76.2, 77.5, 120.1–139.4 (aro-

matic carbons), 154.3, 154.8, 155.2, 165.5; MS (ES+): *m/z* 761.11 $[M+H]^+$; Anal. Calcd for $C_{45}H_{58}F_6N_6O_{12}$: C, 54.65; H, 5.91; N, 8.50. Found: C, 54.86; H, 6.27; N, 8.59.

3.8. *N′*-*Dodecyl*-[(2*R*)-2-*N*,*N′*-di-*t*-butoxycarbonyl-guanidinyl-2-]-[6-*N*,*N′*-di-*t*-butoxycarbonyl-guanidinyl-6-deoxy-2,3,4-tri-*O*-phenylcarbamoyl)-β-D-glucopyranosyl]ethanamide (8)

To a solution of diamine salt **7** (0.050 g, 0.05 mmol) in the mixture of 1,4-dioxane and water (3:1, 2.0 mL) was added *N*,*N′*-di-*t*-butoxycarbonyl-*N″*-triflylguanidine (69 mg, 0.18 mmol, 3.5 equiv). After 5 min, NEt_3 (25.0 μL, 0.18 mmol, 3.5 equiv) was added at room temperature. After 12 h, the reaction mixture was extracted with dichloromethane (2 × 5 mL) and dried over anhydrous Na_2SO_4. Then the combined organic layer was removed under reduced pressure. The remaining residue product was purified by flash column chromatography with EtOAc on silica gel to give guanidinylated compound **8** (52 mg, 0.042 mmol, 83%) as an oil. R_f = 0.20 (EtOAc–Hexane 2:3); $[\alpha]_D^{25} = -17.0$ (*c* 1.1, $CHCl_3$); ^1H NMR (300 MHz, MeOH-d_4): δ 0.90 (t, 3H, *J* = 6.9 Hz), 1.26–1.31 (m, 15H), 1.33 (br s, 8H), 1.42 (s, 9H), 1.47 (s, 9H), 1.52 (s, 9H), 1.57 (s, 9H), 3.17–3.28 (m, 2H), 3.56–3.67 (m, 1H), 3.86–3.97 (m, 2H), 4.11 (dd, 1H, *J* = 6.0, 10.0 Hz), 4.95–5.05 (m, 2H), 5.19 (dd, 1H, *J* = 9.5, 10.0 Hz), 5.37 (dd, 1H, *J* = 9.5, 10.0 Hz), 6.90–7.04 (m, 3H), 7.09–7.28 (m, 8H), 7.30–7.38 (m, 4H), 8.17 (t, 1H, *J* = 5.5 Hz); ^{13}C NMR (75 MHz, MeOH-d_4): δ 14.5, 23.8, 28.1, 28.2 (×4), 28.4 (×3), 28.5 (×3), 28.6 (×3), 30.2, 30.5, 30.6, 30.8 (×3), 33.1, 40.7, 41.9, 57.0, 70.9, 72.8, 76.3, 77.2, 78.1, 80.4, 80.6, 84.0, 84.6, 120.3–139.8 (aromatic carbons), 153.2, 153.8, 154.2, 154.3, 155.1, 157.1, 158.0, 164.2, 164.4, 169.8; MS (ES+): *m/z* 1267.40 $[M+Na]^+$; Anal. Calcd for $C_{63}H_{92}N_{10}O_{16}$: C, 60.75; H, 7.45; N, 11.25. Found: C, 60.98; H, 7.69; N, 11.63.

3.9. *N′*-*Dodecyl*-[(2*R*)-2-guanidinyl-2-]-[6-guanidinyl-6-deoxy-2,3,4-tri-*O*-phenylcarbamoyl)-β-D-glucopyranosyl]ethanamide × 2 trifluoroacetic acid (9)

Deblocking of the *tert*-butoxycarbonyl groups in derivative **8** (0.050 mmol) was performed with 25% trifluoroacetic acid (4 mL) in dichloromethane for 3 h at 0 °C to rt. The volatile components were removed in vacuo under reduced pressure and the non-polar residues were removed by washing with ether and the solvent was decanted to get the guanidine derivative **9** as TFA salt quantitatively. $[\alpha]_D^{25} = -29.0$ (*c* 1.0, MeOH); ^1H NMR (300 MHz, MeOH-d_4): δ 0.79 (t, 3H, *J* = 6.8 Hz), 1.20 (s, 18H), 1.40–1.50 (m, 2H), 3.04–3.19 (m, 2H), 3.30 (dd, 1H, *J* = 6.0, 15.0 Hz), 3.60 (dd, 1H, *J* = 2.5, 15.0 Hz), 3.83 (m, 1H), 4.17 (dd, 1H, *J* = 2.5, 10.0 Hz), 4.54 (d, 1H, *J* = 2.5 Hz), 4.86 (dd, 1H, *J* = 9.8, 10.0 Hz), 5.10 (dd, 1H, *J* = 9.8, 10.0 Hz), 5.34 (dd, 1H, *J* = 9.8, 10.0 Hz), 6.84–6.98 (m, 3H), 7.02–7.20 (m, 8H), 7.24–7.35 (m, 4H), 8.03 (t, 1H, *J* = 5.4 Hz); ^{13}C NMR (75 MHz, MeOH-d_4): δ 14.4, 23.7, 28.2, 30.3, 30.5, 30.6, 30.7, 30.8, 30.9 (×2), 33.1, 41.1, 43.2, 56.9, 71.1 (×2), 75.8, 78.2, 79.6, 120.3–139.4 (aromatic carbons), 154.6, 154.8, 155.0, 159.0, 159.5, 167.5; MS (ES+): *m/z* 845.13 $[M+H]^+$; Anal. Calcd for $C_{47}H_{62}F_6N_{10}O_{12}$: C, 52.61; H, 5.82; N, 13.05. Found: C, 52.36; H, 6.02; N, 12.87.

3.10. *t*-Butoxycarbonyl-NH-D-Lys-$NHC_{12}H_{25}$ (11)

To the mixture of compound **10** (1 g, 2.1 mmol) and TBTU (2.5 g, 7.8 mmol) in DMF (15.0 mL) were added dodecylamine (540 μL, 2.31 mmol) and *N*,*N*-diisopropylethylamine (1.8 mL, 10.08 mmol) and stirred for 2 h at room temperature. The solvent was removed in vacuo and the residue was stirred with piperidine (5.0 mL) in DMF (20.0 mL) for 1 h at room temperature. The solvent was removed in vacuo and the crude product was purified by flash column chromatography (MeOH–EtOAc 1:20) to afford the **11** (768 mg, 87%). $[\alpha]_D^{25} = -21.0$ (*c* 0.5, MeOH); ^1H NMR (300 MHz, MeOH-d_4): δ 0.91 (t, 3H, *J* = 6.8 Hz), 1.24–1.40 (m, 20H), 1.44 (s, 9H), 1.46–1.60 (m, 4H), 1.60–1.74 (m, 2H), 3.06 (t, 2H, *J* = 6.8 Hz), 3.12–3.30 (m, 3H); ^{13}C NMR (75 MHz, MeOH-d_4): δ 14.6, 23.8, 24.0, 28.1, 28.9 (×3), 30.5 (×2), 30.6, 30.7, 30.8 (×2), 30.9 (×2), 33.1, 36.3, 40.4, 41.2, 56.1, 79.8, 158.5, 177.5; MS (ES+): *m/z* 414.61 $[M+H]^+$, 436.60 $[M+Na]^+$; Anal. Calcd for $C_{23}H_{47}N_3O_3$: C, 66.78; H, 11.45; N, 10.16. Found: C, 66.56; H, 11.82; N, 10.41.

3.11. D-Lys-$NHC_{12}H_{25}$ × 2 trifluoroacetic acid (12)

Deblocking of the *tert*-butoxycarbonyl groups in derivative **11** (100 mg) was performed with 25% trifluoroacetic acid (4 mL) in dichloromethane for 3 h at 0 °C to room temperature. The volatile components were removed in vacuo under reduced pressure and the non-polar residues were removed by washing with ether and the solvent was decanted to get the compound **12** as quantitative as a TFA salt. $[\alpha]_D^{25} = -12.0$ (*c* 1.1, MeOH); ^1H NMR (300 MHz, MeOH-d_4): δ 0.90 (t, 3H, *J* = 7.0 Hz), 1.30 (m, 18H), 1.42–1.63 (m, 4H), 1.63–1.79 (m, 2H), 1.80–2.00 (m, 2H), 2.94 (t, 2H, *J* = 7.5 Hz), 3.23 (t, 2H, *J* = 7.0 Hz), 3.85 (t, 1H, *J* = 6.5 Hz), 7.99 (s, 1H), 8.45 (s, 1H); ^{13}C NMR (75 MHz, MeOH-d_4): δ 14.5, 23.0, 23.7, 28.0 (×2), 30.3, 30.4, 30.5, 30.7, 30.8 (×3), 32.1, 33.1, 40.3, 40.7, 54.3, 169.9; MS (ES+): *m/z* 314.19 $[M+H]^+$; Anal. Calcd for $C_{22}H_{41}F_6N_3O_5$: C, 48.79; H, 7.63; N, 7.76. Found: C, 49.10; H, 8.00; N, 7.38.

3.12. *N*,*N′*-Di-*t*-Butoxycarbonyl-guanidinyl-D-Lys(*N*,*N′*-di-*t*-butoxycarbonyl-guanidinyl)-$NHC_{12}H_{25}$ (13)

To a solution of diamine **12** (95 mg, 0.175 mmol) in dichloromethane (2.0 mL) was added *N*,*N′*-di-*t*-butoxycarbonyl-*N″*-triflylguanidine (206 mg, 0.53 mmol, 3 equiv). After 5 min, NEt_3 (73 μL, 0.53 mmol, 3 equiv) was added at room temperature. After over night, the dichloromethane was removed under reduced pressure. The remaining residue product was purified by flash column chromatography (hexane–EtOAc 1:9) on silica gel to give guanidinylated derivative **13** in 96% yield as an oil. R_f = 0.20 (EtOAc); $[\alpha]_D^{25} = -17.0$ (*c* 0.6, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 0.84 (t, 3H, *J* = 6.8 Hz), 1.16–1.30 (m, 18H), 1.32–1.41 (m, 4H), 1.44 (2s, 36H), 1.53–1.63 (m, 2H), 1.70 (m, 1H), 1.88 (m, 1H), 3.10–3.22 (m, 2H), 3.30–3.40 (m, 2H), 4.42 (q, 1H, *J* = 7.0 Hz), 6.76 (t, 1H, *J* = 5.5 Hz), 8.27 (br t, 1H, *J* = 4.8 Hz), 8.62 (d, 1H, *J* = 7.3 Hz), 11.3 (br s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.1, 22.6, 22.8, 26.8, 28.0 (×6), 28.2 (×6), 28.7, 29.2 (×2), 29.3, 29.5, 29.6 (×3), 31.0, 31.9, 39.5, 40.6, 54.0, 79.3, 79.5, 83.1, 83.6, 152.3, 153.2, 156.0, 156.1, 162.9, 163.5, 170.8; MS (ES+): *m/z* 820.48 $[M+Na]^+$; Anal. Calcd for $C_{40}H_{75}N_7O_9$: C, 60.20; H, 9.47; N, 12.29. Found: C, 60.03; H, 9.19; N, 12.53.

3.13. Guanidinyl-D-Lys(guanidinyl)-$NHC_{12}H_{25}$ (14)

Deblocking of the *tert*-butoxycarbonyl groups in derivative **13** (0.050 mmol) was performed with 25% trifluoroacetic acid (4 mL) in dichloromethane for 3 h at 0 °C to room temperature. The volatile components were removed in vacuo under reduced pressure and the non-polar residues were removed by washing with ether and the solvent was decanted to get guanidine derivative **14** as TFA salt quantitatively. $[\alpha]_D^{25} = -24.0$ (*c* 1.5, MeOH); ^1H NMR (300 MHz, MeOH-d_4): δ 0.90 (t, 3H, *J* = 7.0 Hz), 1.20–1.38 (m, 18H), 1.39–1.58 (m, 4H), 1.59–1.68 (m, 2H), 1.78 (m, 1H), 1.89 (m, 1H), 3.15–3.26 (m, 4H), 4.12 (dd, 1H, *J* = 5.7, 7.5 Hz), 8.22 (t, 1H, *J* = 5.7 Hz); ^{13}C NMR (75 MHz, MeOH-d_4): δ 14.5, 23.6, 23.7, 28.0, 29.5, 30.3, 30.4, 30.5, 30.7 (×2), 30.8 (×2), 33.1, 33.4, 40.8, 42.2, 56.5, 158.6, 158.7, 172.1; MS (ES+): *m/z* 398.46 $[M+H]^+$; Anal.

Calcd for $C_{24}H_{45}F_6N_7O_5$: C, 46.07; H, 7.25; N, 15.67. Found: C, 46.28; H, 7.57; N, 15.33.

3.14. Determination of the MIC values for cationic lipids 3, 5, 7, 9, 12 and 14

Bacterial isolates were obtained from the American Type Culture Collection (ATCC). Isolates were kept frozen in skim milk at −80 °C until minimum inhibitory concentration (MIC) testing was carried out. Following two subcultures from frozen stock, the in vitro activities of peptides were determined by macrobroth dilution in accordance with the Clinical and Laboratory Standards Institute (CLSI) 2006 guidelines.[35] Stock solutions of peptides were prepared and dilutions made as described by CLSI. Test tubes contained doubling antimicrobial dilutions of cation adjusted Mueller–Hinton broth and inoculated to achieve a final concentration of approximately 5×10^5 CFU/mL then incubated in ambient air for 24 h prior to reading. Colony counts were performed periodically to confirm inocula. Quality control was performed using ATCC QC organisms.

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