Bioorganic & Medicinal Chemistry 21 (2013) 3624–3631

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Di-alkylated paromomycin derivatives: Targeting the membranes of Gram positive pathogens that cause skin infections

Yifat Berkov-Zrihen, Ido M. Herzog, Mark Feldman, Adar Sonn-Segev, Yael Roichman, Micha Fridman *

School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel

ARTICLE INFO

Article history:
Available online 2 April 2013

Keywords:
Amphiphilic aminoglycosides
Cationic amphiphiles
Membrane-targeting antibiotics
Skin infection causing bacteria
Hemolysis

ABSTRACT

A collection of paromomycin-based di-alkylated cationic amphiphiles differing in the lengths of their aliphatic chain residues were designed, synthesized, and evaluated against 14 Gram positive pathogens that are known to cause skin infections. Paromomycin derivatives that were di-alkylated with C_7 and C_8 linear aliphatic chains had improved antimicrobial activities relative to the parent aminoglycoside as well as to the clinically used membrane-targeting antibiotic gramicidin D; several novel derivatives were at least 16-fold more potent than the parent aminoglycoside paromomycin. Comparison between a di-alkylated and a mono-alkylated paromomycin indicated that the di-alkylation strategy leads to both an improvement in antimicrobial activity and to a dramatic reduction in undesired red blood cell hemolysis caused by many aminoglycoside-based cationic amphiphiles. Scanning electron microscopy provided evidence for cell surface damage by the reported di-alkylated paromomycins.

1. Introduction

Bacterial skin infections, including chronic infections related to diabetes, venous stasis, or arterial insufficiency account for a significant percentage of infectious diseases.[1–3] Patients with infected wounds are frequently treated with systemic antibiotics and, in addition, topical antibiotic treatments.[4,5] There is a large repertoire of potent antimicrobial agents that are unfit for internal use due to their toxicity but that are tolerated topically.[6] These can be used in treatment of skin infections caused by multidrug-resistant organisms that are unaffected by systemic antibiotic treatment.

Amongst the frequently used topical antimicrobial agents are gramicidins, polymyxins, and aminoglycosides such as neomycin B.[4–7] The heterogeneous oligo-peptide mixture of gramicidins is effective mainly against Gram positive bacteria but not against most Gram positive bacilli. Gramicidins are also highly hemolytic, making them too toxic for internal use.[6,8,9] The membrane targeting cyclic lipopeptide antibiotics polymyxins are active against several Gram negative pathogens, but polymyxins are not active against Gram positive bacteria and are also highly toxic when used internally.[9,10] The clinical efficacy of the aminoglycoside neomycin B is continuously reduced as an ever increasing number of bacterial strains acquire resistance to this aminoglycoside antibiotic.[11,12] Topical use of neomycin B may be accompanied by undesired contact dermatitis side effects.[13] Aminoglycosides are also highly nephrotoxic and ototoxic when used internally.[14,15] Although topical antibiotic treatment is tolerated with gramicidins, polymyxins and neomycin B, side effect occur when these antibiotics are used internally, or if high doses of these toxic antimicrobial agents can make their way into the blood system through open wounds or highly damaged external tissue. Therefore, there is a constant need for topical antibiotics that are effective against a wide spectrum of bacteria and exhibit minimal toxic side effects.

In recent years, we and others have demonstrated that the attachment of hydrophobic residues to aminoglycoside antibiotics results in cationic amphiphiles that possess potent antimicrobial activities.[16–21] Several lines of evidence support the hypothesis that these cationic amphiphiles act by disrupting bacterial membranes.[16,20] We previously developed and studied a library of tobramycin-based cationic amphiphiles generated by the alkylation of the 6″ position of this penta-amino aminoglycoside with series of aliphatic chains (Fig. 1). Of the various hydrophobic residues that were tested, tobramycin-based cationic amphiphiles with C_{12}, C_{14}, and C_{16} linear aliphatic chains demonstrated the most potent antimicrobial activity against a wide selection of both Gram positive and Gram negative bacteria.[16,17] The selectivity of the most potent tobramycin-based cationic amphiphiles for bacterial membranes was tested by measuring the damage that these

Abbreviations: BHI, brain heart infusion; BOC, *tert*-butoxycarbonyl; 1-D-TOCSY, total correlation spectroscopy; DMF, dimethylformamide; EtOAc, ethyl acetate; HR-ESI, high resolution electron spray ionization; LR-ESI, low resolution electron spray ionization; MIC, minimum inhibitory concentration; MTT, thiazolyl blue tetrazolium bromide; PBS, phosphate buffered saline; RBC, red blood cells; rt, room temperature; SEM, scanning electron microscope; TIBSCl, 2,4,6-triisopropylbenzenesulfonyl chloride; TFA, trifluoroacetic acid; TLC, thin layer chromatography.

* Corresponding author. Tel.: +972 3 640 8687.
E-mail address: mfridman@post.tau.ac.il (M. Fridman).

0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.03.046

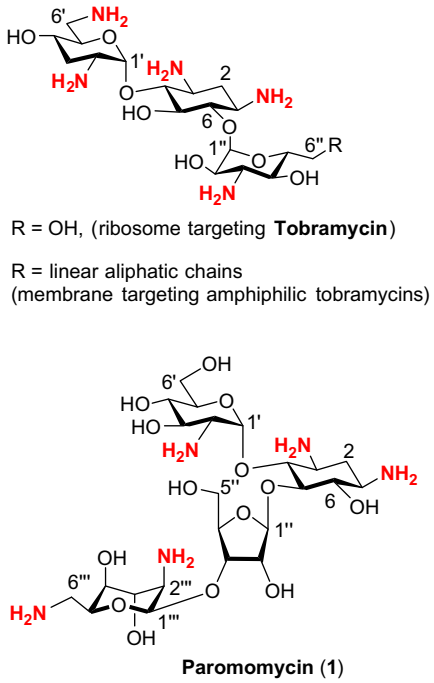


Figure 1. Structures of tobramycin-based cationic amphiphiles and of the aminoglycoside paromomycin (**1**).

compounds inflict on red blood cell (RBC) membranes. Hemolysis tests revealed that there was not a linear correlation between the antimicrobial potency and undesired hemolytic activity of these tobramycin-based cationic amphiphiles and that the minimal inhibitory concentrations (MICs) of the most potent compounds were in some cases well over an order of magnitude lower than the concentrations in which these compounds caused detectible hemolysis.

Although the MIC and hemolysis experiments revealed that tobramycin-based cationic amphiphiles have a degree of selectivity for membranes of some bacterial strains over red blood cells, the therapeutic window of these compounds is still not wide enough to be considered for the development of membrane-targeting antibiotics for internal use.[5,22] We were therefore interested in exploring strategies to increase the selectivity of aminoglycoside-based cationic amphiphiles for bacterial membranes and to widen their potential therapeutic window.

Compared to mammalian cell membranes, bacterial membranes contain a high percentage of anionic lipids.[23,24] We therefore hypothesized that optimization of the interactions between the positively charged amines on the aminoglycoside segment of the cationic amphiphile and the negatively charged bacterial membrane surface may improve the antimicrobial activity of these compounds and their selectivity for bacterial membranes. Our strategy was to enhance anchoring of the positively-charged aminoglycoside to the bacterial membrane by attaching aliphatic chains on two different positions on the aminoglycoside scaffold.

2. Results and discussion

2.1. Design and synthesis of di-alkylated paromomycin-based cationic amphiphiles

For the preparation of di-alkylated aminoglycosides we chose the pseudo-tetrasaccharide paromomycin (**1**, Fig. 1) for two reasons: first, like tobramycin (Fig. 1), this aminoglycoside scaffold has five amine functionalities that are positively charged under physiological conditions. Second, unlike tobramycin, paromomycin (**1**) has two primary alcohols therefore making it possible to readily and chemo-selectively alkylate these two alcohols in the presence of the six secondary alcohols of this aminoglycoside. In our previous studies with tobramycin-based cationic amphiphiles, the optimal antimicrobial activity was obtained by attaching C_{12}, C_{14}, and C_{16} alkyl chains to the aminoglycoside. A dramatic drop in antimicrobial activity was observed for tobramycin-based amphiphiles with shorter or longer alkyl chains. To maintain the hydrophobicity/hydrophilicity ratio that was optimal in the case of the potent tobramycin cationic amphiphiles, we used C_6, C_7, and C_8 alkyl chains for the preparation of the di-alkylated paromomycins.

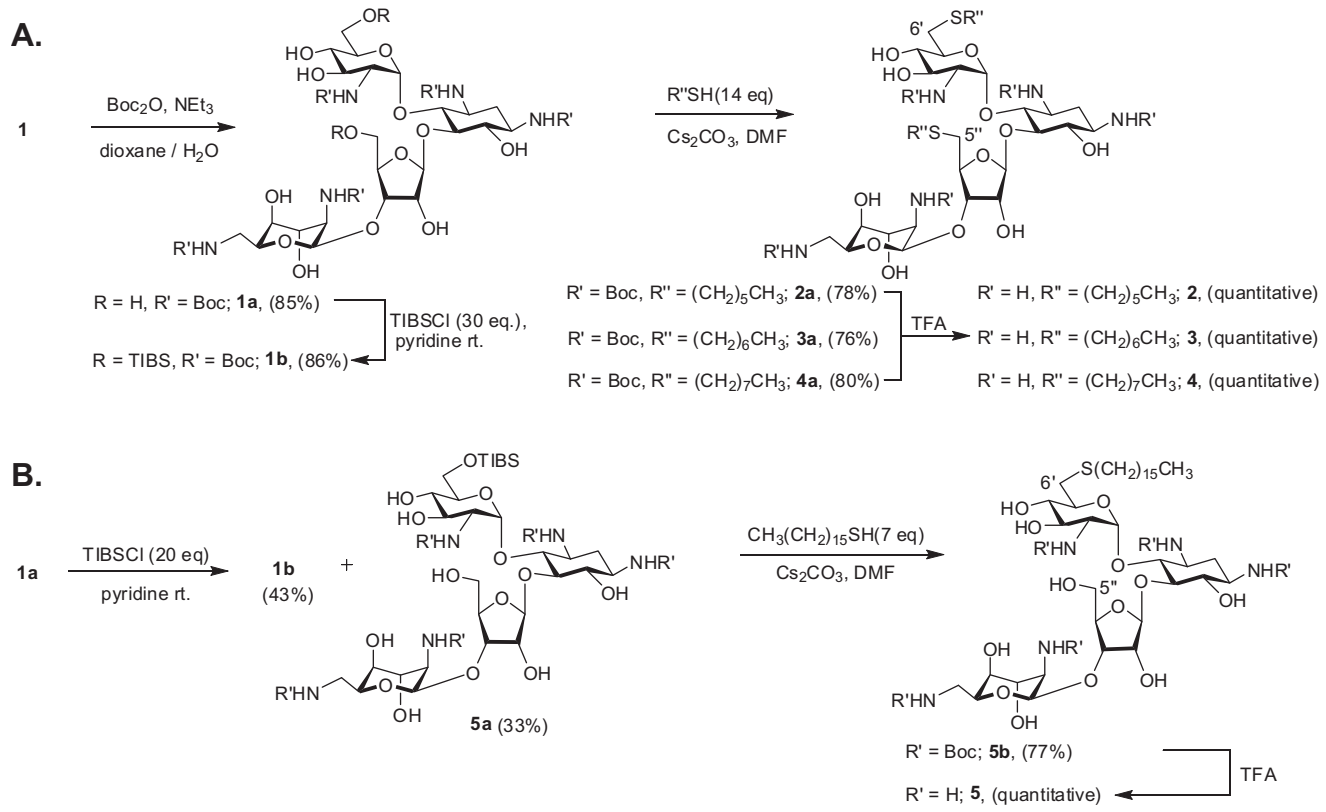
The five amine groups of commercially available paromomycin (**1**) were protected with Boc groups to afford the penta-*NH*Boc protected paromomycin derivative **1a** in 85% yield (Scheme 1A).[25,26] Selective conversion of the two primary alcohols at positions C-6′ and C-5″ of **1a** to the corresponding *O*-trisyl leaving groups using 30 equiv of 2,4,6-triisopropylbenzene-sulfonyl chloride gave compound **1b** in 86%. Compound **1b** was reacted with 1-*n*-hexanethiol, 1-*n*-heptanethiol, or 1-*n*-octanethiol resulting in the *NH*Boc-protected di-alkylated paromomycin derivatives **2a**, **3a**, and **4a** in yields ranging from 76% to 80%. Removal of the *NH*Boc protecting groups in neat TFA gave the penta TFA salts of the 6′, 5″ dithioether paromomycin derivatives **2**, **3**, and **4** with no need for further purification. When the penta-*NH*Boc paromomycin **1a** was reacted with 20 equiv of 2,4,6-triisopropylbenzene-sulfonyl chloride, a mixture of the di-trisylated product **1b** and of the mono-trisylated product **5a** was obtained (43% and 33% isolated yields, respectively, Scheme 1B). NMR characterization of **5a** confirmed that the O-trisylation took place on the C-6′ primary alcohol of paromomycin (confirmed by 1D-TOCSY NMR performed on a sample of **5a** after the removal of the *NH*Boc groups). The 6′-*O*-trisyl of **5a** was displaced by 1-*n*-hexadecanethiol resulting in compound **5b**; **5b** was treated with TFA to yield the C_{16} aliphatic chain thioether **5**. Compound **5** was prepared as the single aliphatic chain anchor analogue of the di-C_8 aliphatic chain paromomycin analogue **4**.

2.2. Antimicrobial activity tests against skin infection causing bacteria

Compounds **2**–**5** were screened against 14 bacterial strains known to cause skin infections, and their minimum inhibitory concentrations (MICs) were determined using the double dilution protocol (Table 1).

We focused on strains belonging to two major families of Gram positive bacteria: *Staphylococci* and *Streptococci*. Of the strains belonging to the *Staphylococci* genus were pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA, strain **B**)[27], *Staphylococcus aureus* (Cowan, Strain **E**),[28] which causes skin infections in patients with compromised immune systems such as HIV carriers, and two strains of *Staphylococcus epidermidis* (strains **C** and **D**)[29] that were once regarded as harmless human skin colonizing bacteria but are now recognized as major opportunistic pathogens. We also tested nine strains of *Streptococcus pyogenes.* These bacteria colonize mainly the throat and skin of humans and are often the cause of skin and soft-tissue infections.[30]

Of the tested di-alkylated paromomycin derivatives, the C_6 di-alkyl chain derivative **2** was the least active against all of the tested strains. However, compound **2** had superior activity compared to paromomycin **1** against all nine *Streptococcus pyogenes* strains **F**–**N** (MIC range from 4 to 16 μg/mL for compound **2**, and 16 to >64 μg/mL for paromomycin **1**). The di-C_7 alkyl chain derivative **3** was superior to compound **2** against most of the 14 tested strains (MIC range from 2 to 16 μg/mL). The most potent antimicrobial activity was observed for the di-C_8 alkyl chain derivative **4** with MICs ranging from 2 to 8 μg/mL against all



Scheme 1. Synthesis of mono- and di-alkylated paromomycin-based cationic amphiphiles.

Table 1
Antibacterial activity: MIC values (μg/mL) of mono- and di-alkylated amphiphiles (**2**–**5**), the parent drug paromomycin (**1**), and the membrane targeting antibiotic gramicidin D

	Bacterial strains[a]													
Antibiotics	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Gramicidin D	32	64	8	2	>64	>64	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	16	2
1	>64	>64	2	>64	16	>64	64	64	32	32	64	64	64	16
2	32	>64	8	16	64	8	16	8	4	8	8	8	8	4
3	8	16	4	4	16	4	4	2	2	4	4	4	4	4
4	4	8	2	2	4	4	2	2	2	2	2	2	2	2
5	64	64	32	64	64	32	>64	16	32	64	32	8	32	8

[a] MIC values were determined against: **A**, *S. aureus oxford* NCTC6571; **B**, MRSA; **C**, *S. epidermidis* ATCC12228 (biofilm negative); **D**, *S. epidermidis* ATCC35984/RP62A (biofilm positive); **E**, *S. aureus* Cowan ATCC12598; **F**, *S. pyogenes* serotype M12 (strain MGAS9429); **G**, *S. pyogenes* serotype M1T1; **H**, *S. pyogenes* serotype M2; **I**, *S. pyogenes* serotype M3; **J**, *S. pyogenes* serotype M5; **K**, *S. pyogenes* serotype M24; **L**, *S. pyogenes* JRS75; **M**, *S. pyogenes* glossy; and **N**, *S. pyogenes* serotype T5.

tested strains. The 6′-C_{16} linear aliphatic chain paromomycin derivative **5**, designed as the mono-alkyl chain anchor analogue of the di-C_8 alkyl chain paromomycin derivative **4**, had poor antimicrobial activity against all of the tested bacterial strains. In addition to comparing the antimicrobial activity of the alkylated paromomycins to that of the ribosome targeting parent antibiotic paromomycin **1**, we evaluated their activity against that of the membrane targeting oligopeptide mixture gramicidin D.[31] With the exceptions of the two *Staphylococcus epidermidis* strains (**C** and **D**), gramicidin D was ineffective against the tested *Staphylococci* strains (Table 1). Compounds **3** and **4** were significantly more potent than gramicidin D against the tested *Staphylococci* strains **A**, **B**, and **E** with MIC values comparable to or better than those of gramicidin D against strains **C** and **D**. In contrast to the lack of activity against *Staphylococci* strains, gramicidin D demonstrated very good antimicrobial activity against seven of the nine tested *Streptococci* strains (for strains **G**–**L**, MICs <0.5 μg/mL, and for strain **N**, MIC = 2 μg/mL). Although more potent than compounds **2**, **3**, and **4** against seven of the tested *Streptococci* strains, gramicidin D demonstrated very poor activity against *S. pyogenes* serotype M12 (strain **F**) and only moderate activity against *S. pyogenes* glossy (strain **M**; MIC >64 μg/mL, and MIC = 16 μg/mL, respectively); compounds **2**–**4** were more potent than gramicidin D against these *Streptococci* strains. However, gramicidin D was at least eight times more potent than compounds **3** and **4** against 6 of the tested streptococci strains (Table 1). The antimicrobial activity tests demonstrated that the di-alkylated paromomycin amphiphiles have a broader spectrum of antimicrobial activity than the parent aminoglycoside **1** or the membrane targeting antibiotic gramicidin D. The significantly more potent antimicrobial activity of the di-C_8 alkyl chain paromomycin derivative **4** compared to that of the mono C_{16} linear aliphatic chain paromomycin derivative **5** demonstrates the favorable effect that may result from the attachment of more than one linear aliphatic chain to the aminoglycoside scaffold.

2.3. Red blood cells hemolysis test

The selectivity of all of the amphiphilic paromomycin derivatives **2**–**5** for bacterial membranes was studied by testing the hemolytic activity of these compounds on red blood cells (RBCs) isolated from laboratory rats. The percentage of hemolysis was determined after 1 h of incubation with increasing concentrations of the tested compounds (up to 256 μg/mL) at 37 °C. The membrane targeting gramicidin D caused hemolysis even at low concentrations close to the MIC range of this antimicrobial agent (2.4 ± 1.4% at 2 μg/mL, Fig. 2). In contrast, even at concentrations of 128 μg/mL, compounds **2**, **3**, and **4** were significantly less hemolytic then gramicidin D (Fig. 2). The aliphatic chain length affected the percentage of hemolysis with the lowest hemolysis caused by the di-C_6 alkyl chain paromomycin derivative **2** (3.4 ± 1.2% hemolysis at a concentration of 256 μg/mL) and the highest by the di-C_8 alkyl chains paromomycin derivative **4** (62.5 ± 7.9% at 256 μg/mL).

Interestingly, whereas the mono C_{16} linear aliphatic chain paromomycin derivative **5** demonstrated poor antimicrobial activity against the tested bacterial strains, this compound caused drastic RBC hemolysis at concentrations that were lower than its MIC range. At a concentration of 16 μg/mL, compound **5** already caused 33.8 ± 5.2% hemolysis of rat RBCs; at the same concentration compounds **2**, **3**, and **4** caused almost no measurable hemolysis (0% for **2** and **3**, and 0.8 ± 0.3% for **4**). Moreover, at a concentration of 32 μg/mL compound **5** caused almost a 100% hemolysis; the di-alkylayed paromomycin derivative **4** and the highly hemolytic gramicidin D caused no more than 60% hemolysis even at a concentration of 256 μg/mL. Hemolysis experiments demonstrated that compound **5** had no selectivity for bacterial membranes and acted in a manner similar to that of non-specific membrane disrupting detergents.

At a concentration of 128 μg/mL, the di-C_6 alkyl chain paromomycin derivative **2** did not cause any measurable hemolysis; this compound was also the least potent antimicrobial agent against the 14 tested bacterial strains. The high end of the MIC range of derivative **3** against the 14 tested bacterial strains was 16 μg/mL; for 11 of the tested strains the MIC of compound **3** was not higher than 4 μg/mL. However, at 32 μg/mL which is two to eight times higher than the MICs, this compound caused almost no measurable hemolysis (3.6 ± 1.9%).

The di-C_8 alkyl chain paromomycin derivative **4** demonstrated the most potent antimicrobial activity against all of the tested bacterial strains (MIC range from 2 to 8 μg/mL). The paromomycin derivative with the di-C_7 alkyl chain, **3**, was either as active or one double dilution less active than compound **4** against the tested bacterial strains and caused significantly less RBCs hemolysis than compound **4**. At 256 μg/mL, compound **3** caused 30.4 ± 8.9% hemolysis; compound **4** caused 62.5 ± 7.9% hemolysis at the same concentration. The high level of hemolysis caused by compound **4** at 256 μg/mL was similar to that caused by gramicidin D. Hence, of the three di-alkylated paromomycins, the di-C_7 alkyl chain derivative **3** demonstrated potent antimicrobial activities against all 14 bacterial strains and was dramatically less hemolytic then both gramicidin D and di-C_8 alkyl chain paromomycin derivative **4**. Up to a concentration of 32 μg/mL compound **3** caused considerably less hemolysis than gramicidin D and as of a concentration of 64 μg/mL and up to 256 μg/mL this amphiphilic paromomycin derivative was approximately twofold less hemolytic than gramicidin D. Therefore, in terms of the ratio of hemolysis to antimicrobial activity, compound **3** is the most potent of the di-alkylated paromomycins that were studied.

2.4. Scanning electron microscopy (SEM) evidence for bacterial cell damage

In order to visualize bacterial cell morphological changes that result from exposure to the synthetic di-alkylated paromomycin-based cationic amphiphiles, we incubated a culture of *S. epidermidis* ATCC12228, which was susceptible to both the parent aminoglycoside paromomycin (**1**) and to the most potent of the di-alkylated paromomycins, compound **4** at a concentration of 1 μg/mL; this concentration is half of the MIC of the two selected antimicrobial agents against this bacterial strain.

Under these experimental conditions, untreated bacterial cells had a smooth surface morphology (Fig. 3a). After 1 h of incubation with paromomycin **1**, moderate wrinkling of the bacterial cell surface was observed (Fig. 3b). As paromomycin **1** is a protein synthesis inhibitor, the accumulation of damaged and non-functional membrane proteins may explain the observed wrinkled cell surfaces. Bacterial cells that were treated with the di-C_8 alkyl chain paromomycin derivative **4** were severely damaged (Fig. 3c). These cells had severe surface wrinkling and roughening that resulted from irregularly-shaped cell walls and damaged membranes. At higher concentrations of compound **4**, mainly severely damaged cells and cellular debris were observed. The percentage of genes in the bacterial genome that encode bacterial membrane proteins averages between 20% and 30% depending on the strain.[32] Unlike the minor cell surface damage caused by paromomycin **1** that

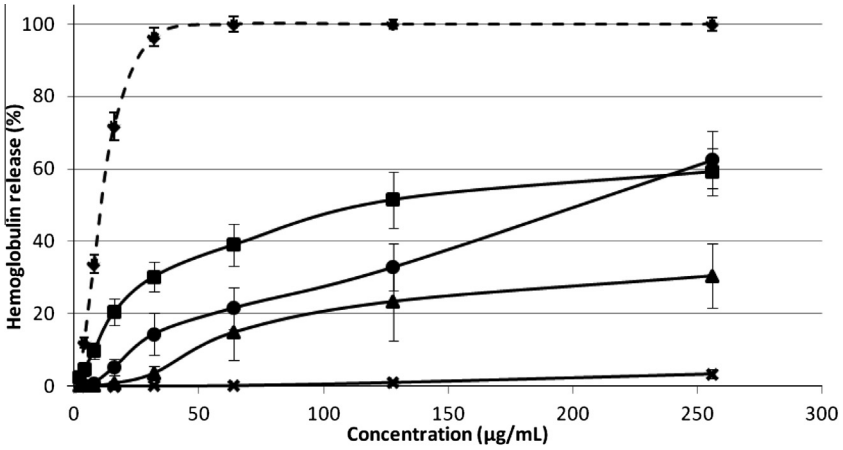


Figure 2. Rat RBCs were incubated with tested compounds for 1 h at 37 °C: **2** (**X**), **3** (▲), **4** (●), **5** (–♦–), gramicidin D (■). All experiments were performed in triplicate, and results are the average of at least two different sets of experiments using blood samples from different laboratory rats.

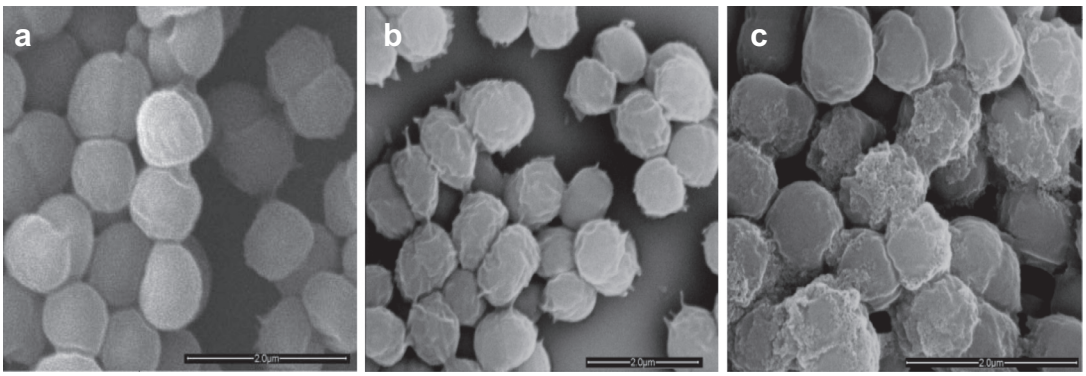


Figure 3. Scanning electron microscopic (SEM) images of *S. epidermidis* ATCC12228 with and without drug: (a) Untreated control bacteria cells. (b) Cells after 1 h of incubation at 37 °C with 1 μg/mL paromomycin **1**. (c) Cells after 1 h of incubation at 37 °C with 1 μg/mL of compound **4**.

presumably results from impaired membrane protein synthesis, the severe cell surface damage caused by compound **4** can be rationalized by direct and rapid membrane disrupting effects of this compound.

3. Summary and conclusions

In conclusion, three 5″,6′-di-alkylated paromomycin-based cationic amphiphiles differing in the length of the linear hydrophobic chain (C_6, C_7, and C_8 chains) were synthesized and evaluated for their antimicrobial activity against 14 bacterial pathogens known to cause skin infections.

Of the three chain lengths tested, the di-C_8 aliphatic chain paromomycin derivative **4** was the most potent antimicrobial agent against all of the tested strains and was at least 16 times more potent than the membrane targeting antibiotic gramicidin D and the parent ribosome-targeting aminoglycoside paromomycin **1** against most of the tested *Staphylococci* strains and some of the tested *Streptococci* strains.

However, the di-C_7 aliphatic chain paromomycin analogue **3** is of particular interest. This compound was either as effective or one double dilution less potent than the di-C_8 aliphatic chain paromomycin analogue **4** against all of the tested bacteria, yet this compound caused considerably less hemolysis of laboratory rat RBCs compared to both compound **4** and the clinically used gramicidin D. Moreover, the mono C_{16} linear aliphatic chain paromomycin derivative **5**, which was synthesized as the mono aliphatic chain analogue of the di-C_8 chain paromomycin analogue **4**, demonstrated poor antimicrobial activity against all tested bacterial strains and caused almost 100% hemolysis of RBCs even at a concentrations close to the MIC.

Scanning electron microscopy (SEM) experiments revealed that bacterial cells that were incubated with the di-C_8 aliphatic chain paromomycin analogue **4** had extensive cell surface damage compared to that caused by the parent ribosome-targeting aminoglycoside **1**. This observed extensive damage caused by analogue **4** further supports the suggestion that these aminoglycoside-based cationic amphiphiles exert their biological activity through disruption of the bacterial cell membrane.

The results of this study demonstrate that di-alkylation of paromomycin results in potent antimicrobial agents that are effective against a broad spectrum of Gram positive pathogens. The newly synthesized compounds caused significantly less hemolysis compared to membrane targeting antibiotics such as gramicidin D. This study also demonstrates that the di-alkylation approach may be more favorable than the mono-alkylation approach in designing aminoglycoside-based cationic amphiphiles both in terms of enhancing the antimicrobial activity and in reducing the undesired hemolytic effect.

4. Experimental

4.1. General methods and instrumentation

^{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 (δ = 3.31) when CD_3OD was the solvent. ^{13}C NMR spectra were recorded on Bruker Avance™ 400 or spectrometers at 100.6 or 125 MHz. Multiplicities are reported using the following abbreviations: b = broad, s = singlet, d = doublet, t = triplet, hep = heptet, m = multiplet. Coupling constants (J) are given in Hertz. Low-resolution electron spray ionization (LR-ESI) mass spectra were measured on a Waters 3100 mass detector. High-resolution electron spray ionization (HR-ESI) mass spectra were measured on a Waters Synapt instrument. Chemical reactions were monitored by TLC (Merck, Silica gel 60 F_{254}). Visualization was achieved 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), H_2O (720 mL)). All reactions were carried out under an argon atmosphere with anhydrous solvents, unless otherwise noted. All chemicals, unless otherwise stated, were obtained from commercial sources. Compounds were purified flash chromatography (SiO_2, Merck, Kieselgel 60).

4.2. Synthetic procedures

4.2.1. Compound 1a

Paromomycin sulfate (1.0 g, 1.2 mmol) was dissolved in a mixture of dioxane/water (2:1, 15 ml), and triethylamine (1.7 ml, 12 mmol) was added. The solution was sonicated for 10 min and then Boc_2O (2.6 g, 12 mmol) was added, and the mixture was stirred at ambient temperature for 12 h. Reaction progress was monitored by TLC (9% methanol in dichloromethane). Upon completion, the reaction mixture was concentrated, diluted with ethyl acetate (150 mL), washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude was purified by flash column chromatography over silica gel (7.5% methanol/dichloromethane) to afford compound **1a** as a white solid (1.15 g, 85%).

4.2.2. Compound 1b

A solution of **1a** (1.5 g, 1.34 mmol) in pyridine (20 mL) was treated with 2,4,6-triisopropylbenzenesulfonyl chloride (12.22 g, 40.35 mmol, 30.0 equiv). The reaction mixture was stirred at 23 °C for 12 h. Reaction progress was monitored by TLC (3% methanol in

dichloromethane). Upon completion, the reaction mixture was concentrated, diluted with ethyl acetate (100 mL), washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The concentrated crude was purified by flash column chromatography (2.5% methanol in dichloromethane) to afford the desired product **1b** as a white solid (1.9 g, 86%): ^{1}H NMR (500 MHz, methanol-d_4) δ 7.32 (s, 2H, $SO_3C_6H_2(CH)_3(CH_3)_6$), 7.31 (s, 2H, $SO_3C_6H_2(CH)_3(CH_3)_6$), 5.47 (s, 1H, H-1′), 5.19 (s, 1H, H-1″), 4.85 (m, 1H, H-1‴), 4.40 (m, 1H, H-6′), 4.38–4.30 (m, 2H, H-2″, H-6′), 4.25-4.14 (m, 4H, H-3″, H-5″, H-4″), 4.01 (m, 1H, H-5′), 3.91 (t, J = 3.1 Hz, 1H, H-4‴), 3.81 (t, J = 7.2 Hz, 1H, H-5‴), 3.77 (m, 1H, H-3‴), 3.62 (dd, J = 10.3, 4.0 Hz, 1H, H-4′), 3.56–3.26 (m, 12H, H-1, H-3, H-4, H-5, H-6, H-2′, H-3′, H-2‴, $SO_3C_6H_2(CH)_3(CH_3)_6$), 3.20 (m, 1H, H-6‴), 2.99 (m, 4H, $SO_3C_6H_2(CH)_3(CH_3)_6$, H-6‴), 1.97 (m, 1H, H-2eq), 1.47–1.43 (m, 46H, $5\times CO_2C(CH_3)_3$, H-2ax), 1.33–1.23 (m, 36H, $SO_3C_6H_2(CH)_3(CH_3)_6$). ^{13}C NMR (100 MHz, methanol-d_4) δ 157.5, 157.1, 156.8, 156.7, 156.3, 154.0, 150.8, 149.4, 148.0, 129.3, 129.1, 123.6, 122.0, 109.2, 98.8, 97.3, 85.0, 79.5, 79.2, 79.1, 78.8, 77.9, 77.1, 74.0, 73.2, 73.0, 72.5, 70.0, 69.9, 68.5, 67.1, 54.6, 52.0, 50.9, 49.5, 39.8, 34.1, 34.0, 33.9, 29.4, 29.4, 29.3, 29.0, 27.5, 27.4, 27.3, 24.0, 23.8, 22.8, 22.5. LR-ESI m/z calcd for $C_{78}H_{128}N_5O_{28}S_2$ 1646.83, found 1646.01 $[M-H]^-$.

4.2.3. Compound 2a

To a solution of compound **1b** (250 mg, 0.15 mmol) and Cs_2CO_3 (198 mg, 0.6 mmol) in dry DMF (4 mL) was added 1-hexanethiol (0.30 mL, 2.12 mmol), and the mixture was stirred at 23 °C overnight. Reaction progress was monitored by TLC (5% methanol in dichloromethane). Upon completion, the reaction mixture was concentrated, diluted with ethyl acetate (100 mL), washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The concentrated crude was purified by flash column chromatography (4% methanol/dichloromethane) to afford compound **2a** (159 mg, 80%) as a white solid: ^{1}H NMR (400 MHz, methanol-d_4) δ 5.44 (bs, 1H, H-1′), 5.13 (d, J = 2.7 Hz, 1H, H-1″), 4.92 (d, J = 1.8 Hz, H-1‴), 4.25 (m, 2H, H-2″, H-4″), 4.09 (m, 1H, H-3″), 3.91 (m, 3H, H-5′, H-3‴, H-5‴), 3.76 (m, 1H, H-4‴), 3.66–3.42 (m, 6H, H-4, H-5, H-6, H-2′, H-3′, H-2‴), 3.33-3.30 (m, 5H, H-1, H-3, H-4′, H-6‴), 2.99 (dd, 1H, J = 13.8, 2.7 Hz, H-6′), 2.85 (m, 2H, H-5″), 2.71(m, 1H, H-6′), 2.69–2.57 (m, 4H, $2\times SCH_2CH_2(CH_2)_3CH_3$), 2.05–1.94 (m, 1H, H-2eq), 1.67–1.53 (m, 4H, $2\times SCH_2CH_2(CH_2)_3CH_3$), 1.53–1.37 (m, 58H, $5\times CO_2C(CH_3)_3$, $2\times SCH_2CH_2(CH_2)_3CH_3$, H-2eq), 0.99–0.82 (m, 6H, $2\times SCH_2CH_2(CH_2)_3CH_3$). ^{13}C NMR (125 MHz, methanol-d_4) δ 157.4, 157.0, 156.8, 156.7, 156.3, 109.9, 98.9, 97.5, 85.9, 81.2, 79.7, 79.4, 79.2, 79.1, 78.8, 74.5, 73.9, 73.0, 72.8, 72.3, 72.2, 70.1, 67.4, 55.2, 52.1, 40.3, 34.2, 33.5, 33.3, 32.3, 31.3, 31.2, 29.6, 29.4, 28.3, 28.2, 27.6, 22.3, 22.3, 13.1, 13.0. LR-ESI m/z calcd for $C_{60}H_{110}N_5O_{22}S_2$ 1316.70, found 1316.58 $[M+H]^+$.

4.2.4. Compound 3a

To a solution of compound **1b** (250 mg, 0.15 mmol) and Cs_2CO_3 (198 mg, 0.6 mmol) in dry DMF (4 mL) was added 1-heptanethiol (0.33 mL, 2.12 mmol), and the reaction was stirred at 23 °C overnight. Reaction progress was monitored by TLC (5% methanol in dichloromethane). Upon completion, the reaction mixture was concentrated, diluted with ethyl acetate (100 mL), washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The concentrated crude was purified by flash column chromatography (4% methanol/dichloromethane) to afford compound **3a** (155 mg, 76%) as a white solid: ^{1}H NMR (500 MHz, methanol-d_4) δ 5.47 (bs, 1H, H-1′), 5.15 (d, J = 3.6 Hz, 1H, H-1″), 4.93 (bs, 1H, H-1‴), 4.39–4.22 (m, 2H, H-4″, H-2″), 4.10 (q, J = 5.2 Hz, 1H, H-3″), 4.02–3.87 (m, 3H, H-5′, H-3‴, H-5‴), 3.79 (bs, 1H, H-4‴), 3.66–3.42 (m, 6H, H-4, H-5, H-6, H-2′, H-3′, H-2‴), 3.38–3.27 (m, 5H, H-1, H-3, H-4′, H-6‴), 3.09–2.96 (m, 1H, H-6′), 2.98–2.84 (m, 2H, H-5″), 2.73 (m, 1H, H-6′), 2.70-2.62 (m, 4H, $2\times SCH_2CH_2(CH_2)_4CH_3$), 2.02 (dd, J = 13.5, 4.8 Hz, 1H, H-2eq), 1.66-1.59 (m, 4H, $2\times SCH_2CH_2(CH_2)_4CH_3$), 1.57–1.15 (m, 62H, $5\times CO_2C(CH_3)_3$, $2\times SCH_2CH_2(CH_2)_4CH_3$, H-2ax), 0.94- 0.91(m, 6H, $2\times SCH_2CH_2(CH_2)_5CH_3$). ^{13}C NMR (125 MHz, methanol-d_4) δ 157.4, 157.1, 156.7, 156.3, 109.9, 98.8, 97.5, 86.0, 81.2, 79.6, 79.4, 79.2, 79.1, 78.8, 74.5, 73.9, 73.0, 72.9, 72.3, 70.2, 67.4, 55.3, 52.1, 50.9, 50.1, 48.4, 40.4, 34.2, 33.6, 33.3, 32.3, 31.6, 31.6, 29.6, 29.5, 28.8, 28.7, 28.6, 28.5, 27.6, 27.4, 27.4, 22.3, 22.3, 13.1. LR-ESI m/z calcd for $C_{62}H_{114}N_5O_{22}S_2$ 1344.73, found 1344.67 $[M+H]^+$.

4.2.5. Compound 4a

To a solution of compound **1b** (250 mg, 0.15 mmol) and Cs_2CO_3 (198 mg, 0.6 mmol) in dry DMF (4 mL) was added 1-octanethiol (0.36 mL, 2.12 mmol), and the mixture was stirred at 23 °C overnight. Reaction progress was monitored by TLC (5% methanol in dichloromethane). Upon completion, the reaction mixture was concentrated, diluted with ethyl acetate (100 mL), washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The concentrated crude was purified by flash column chromatography over silica gel (4% methanol/dichloromethane) to afford compound **4a** (162 mg, 78%) as a white solid: ^{1}H NMR (500 MHz, methanol-d_4) δ 5.48 (bs, 1H, H-1′), 5.15 (d, J = 2.8 Hz, 1H, H-1″), 4.94 (bs, 1H, H-1‴), 4.46–4.22 (m, 2H, H-2″, H-4″), 4.11 (q, J = 5.6 Hz, 1H, H-3″), 3.93 (m, 3H, H-5′, H-3‴, H-5‴), 3.79 (m, 1H, H-4‴), 3.70–3.57 (m, 6H, H-4, H-5, H-6, H-2′, H-3′, H-2‴), 3.39–3.25 (m, 5H, H-1, H-3, H-4′, H-6‴), 3.01 (dd, J = 13.9, 2.7 Hz, 1H, H-6′), 2.87 (m, 2H, H-5″), 2.72 (dd, J = 13.8, 7.3 Hz, 1H, H-6′), 2.70–2.61 (m, 4H, $2\times SCH_2CH_2(CH_2)_5CH_3$), 2.02 (m, 1H, H-2eq), 1.62 (m, 4H, $2\times SCH_2CH_2(CH_2)_5CH_3$), 1.53–1.41 (m, 66H, $5\times CO_2C(CH_3)_3$, $2\times SCH_2CH_2(CH_2)_5CH_3$, H-2ax), 0.94–0.91 (m, 6H, $2\times SCH_2CH_2(CH_2)_5CH_3$). ^{13}C NMR (125 MHz, methanol-d_4) δ 157.4, 157.0, 156.8, 156.7, 156.3, 109.9, 98.9, 97.5, 86.0, 81.2, 79.7, 79.4, 79.2, 79.1, 78.8, 74.5, 73.9, 73.0, 72.9, 72.3, 70.1, 67.4, 55.2, 52.1, 50.9, 50.1, 48.4, 40.3, 34.4, 34.2, 33.6, 33.3, 32.3, 31.7, 31.6, 29.6, 29.5, 29.1, 29.1, 29.0, 28.7, 28.6, 27.6, 27.4, 27.3, 22.3, 13.11. LR-ESI m/z calcd for $C_{64}H_{118}N_5O_{22}S_2$ 1372.76, found 1372.54 $[M+H]^+$.

4.2.6. Compound 2

Compound **2a** (20 mg, 0.014 mmol) was treated with 95% TFA (0.7 mL) at ambient temperature 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 compound **2** (21 mg, quantitative yield) as a white foam: ^{1}H NMR (400 MHz, methanol-d_4) δ 5.73 (d, J = 3.9 Hz, 1H, H-1′), 5.36 (d, J = 3.7 Hz, 1H, H-1″), 5.34 (d, J = 1.7 Hz, 1H, H-1‴), 4.45 (t, J = 4.7 Hz, 1H, H-3″), 4.40 (m, 1H, H-2″), 4.33 (dt, J = 8.4, 4.2 Hz, 1H, H-4″), 4.29 (ddd, J = 7.2, 3.9, 1.5 Hz, 1H, H-5‴), 4.15 (t, J = 3.2 Hz, 1H, H-3‴), 3.99 (t, J = 9.6 Hz, 1H, H-4), 3.96–3.85 (m, 2H, H-3′, H-5′), 3.81 (t, J = 9.0 Hz, 1H, H-5), 3.69 (m, 1H, H-4‴), 3.63 (m, 1H, H-6), 3.57–3.33 (m, 5H, H-3, H-2′, H-4′, H-2‴, H-6‴), 3.29-3.22 (m, 2H, H-1, H-6‴), 3.10-3.04 (m, 2H, H-5″, H-6′), 2.83 (dd, J = 13.0, 8.0 Hz, 1H, H-5″), 2.75 (dd, J = 14.3, 6.7 Hz, 1H, H-6′), 2.66–2.56 (m, 4H, $2\times SCH_2CH_2(CH_2)_3CH_3$), 2.48 (dt, J = 12.4, 4.2 Hz, 1H, H-2eq), 1.92 (q, J = 12.6 Hz, 1H, H-2ax), 1.67–1.54 (m, 4H, $2\times SCH_2CH_2(CH_2)_3CH_3$),1.50–1.24 (m, 12H, $2\times SCH_2CH_2(CH_2)_3CH_3$), 0.90 (m, 6H, $2\times SCH_2CH_2(CH_2)_3CH_3$). ^{13}C NMR (100 MHz, methanol-d_4) δ 161.7 (q, J = 34.6 Hz, CF_3CO_2H), 116.7 (q, J = 292.8 Hz, CF_3CO_2H), 110.7, 96.2, 95.5, 85.2, 80.7, 78.4, 78.0, 74.3, 73.9, 72.4, 71.82, 70.6, 68.8, 67.7, 54.3, 51.5, 49.6, 49.2, 40.2, 35.1, 32.8, 32.6, 32.2, 31.2, 31.1, 29.4, 29.3, 28.3, 28.1, 22.2, 22.2, 13.0, 12.9. HRESI-MS m/z calcd for $C_{35}H_{70}N_5O_{12}S_2$ 816.4462, found 816.4460 $[M+H]^+$.

4.2.7. Compound 3

Compound **3a** (15 mg, 0.011 mmol) was treated with 95% TFA (0.7 mL) at ambient temperature 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 compound **3** (15.6 mg, quantitative yield) as a white foam: ^{1}H NMR (400 MHz, methanol-d_4) δ 5.70 (d, J = 3.9 Hz, 1H, H-1′), 5.35 (d, J = 3.8 Hz, 1H, H-1″), 5.34 (d, J = 2.0 Hz, 1H, H-1‴), 4.45 (t, J = 4.6 Hz, 1H, H-3″), 4.41 (t, J = 4.2 Hz, 1H, H-2″), 4.33 (m, 1H, H-4″), 4.29 (ddd, J = 7.1, 3.8, 1.5 Hz, 1H, H-5‴), 4.14 (t, J = 3.2 Hz, 1H, H-3‴), 3.98–3.84 (m, 3H, H-4, H-3′, H-5′), 3.80 (t, J = 9.0 Hz, 1H, H-5), 3.70 (m, 1H, H-4‴), 3.61 (t, J = 9.7 Hz, 1H, H-6), 3.50–3.34 (m, 5H, H-3, H-2′, H-4′, H-2″, H-6‴), 3.29–3.19 (m, 2H, H-6‴, H-1), 3.07 (m, 2H, H-5″, H-6′), 2.83 (dd, J = 13.0, 8.1 Hz, 1H, H-5″), 2.75 (dd, J = 14.2, 6.9 Hz, 1H, H-6′), 2.69–2.55 (m, 4H, 2×S$CH_2$$CH_2(CH_2)_4CH_3$), 2.44 (dt, J = 12.5, 4.2 Hz, 1H, H-2eq), 1.87 (q, J = 12.6 Hz, 1H, H-2ax), 1.62 (m, 4H, 2×$SCH_2CH_2(CH_2)_4CH_3$), 1.50–1.19 (m, 16H, 2×$SCH_2CH_2(CH_2)_4CH_3$), 0.98–0.78 (m, 6H, 2×$SCH_2CH_2(CH_2)_3CH_3$). ^{13}C NMR (125 MHz, methanol-d_4) δ 161.7 (q, J = 34.6 Hz, CF_3CO_2H), 116.7 (q, J = 292.7 Hz, CF_3CO_2H), 110.7, 96.2, 95.5, 85.4, 80.7, 78.4, 74.3, 73.9, 72.5, 71.8, 70.7, 68.8, 67.8, 67.7, 54.4, 51.5, 49.7, 49.2, 40.2, 35.1, 32.8, 32.7, 32.2, 31.58, 31.5, 29.5, 29.4, 28.7, 28.6, 28.4, 22.3, 22.2, 13.0. HRESI-MS m/z calcd for $C_{37}H_{74}N_5O_{12}S_2$ 844.4775, found 844.4770 $[M+H]^+$.

4.2.8. Compound 4

Compound **4a** (30 mg, 0.021 mmol) was treated with 95% TFA (0.7 mL) at ambient temperature 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 compound **4** (31.5 mg, quantitative yield) as a white foam: ^{1}H NMR (400 MHz, methanol-d_4) δ 5.73 (d, J = 3.9 Hz, 1H, H-1′), 5.36 (d, J = 3.7 Hz, 1H, H-1″), 5.34 (d, J = 1.7 Hz, 1H, H-1‴), 4.45 (t, J = 4.7 Hz, 1H, H-3″), 4.40 (m, 1H, H-2″), 4.33 (dt, J = 8.4, 4.2 Hz, 1H, H-4″), 4.29 (ddd, J = 7.2, 3.9, 1.5 Hz, 1H, H-5‴), 4.15 (t, J = 3.2 Hz, 1H, H-3‴), 3.99 (t, J = 9.5 Hz, 1H, H-4), 3.88 (m, 2H, H-3′, H-5′), 3.81 (t, J = 9.0 Hz, 1H, H-5), 3.69 (m, 1H, H-4‴), 3.63 (dd, J = 10.4, 9.0 Hz, 1H, H-6), 3.56–3.33 (m, 5H, H-3, H-2′, H-4′, H-2″, H-6‴), 3.30–3.19 (m, 2H, H-1, H-6‴), 3.07 (m, 2H, H-6′, H-5″), 2.83 (dd, J = 13.1, 8.0 Hz, 1H, H-5″), 2.76 (dd, J = 14.3, 6.7 Hz, 1H, H-6′), 2.67–2.56 (m, 4H, 2×S$CH_2CH_2(CH_2)5CH_3$), 2.47 (dt, J = 12.5, 4.3 Hz, 1H, H-2eq), 1.92 (q, J = 12.5 Hz, 1H, H-2ax), 1.72–1.51 (m, 4H, 2×$SCH_2CH_2(CH_2)5CH_3$), 1.48–1.17 (m, 20H, 2×$SCH_2CH_2(CH_2)_5CH_3$), 0.97–0.67 (m, 6H, 2×$SCH_2CH_2(CH_2)_5CH_3$). ^{13}C NMR (100 MHz, methanol-d_4) δ 161.7 (q, J = 34.7 Hz, CF_3CO_2H), 116.7 (q, J = 292.7 Hz, CF_3CO_2H), 110.7, 96.1, 95.5, 85.3, 80.7, 78.4, 78.0, 74.4, 73.9, 72.4, 71.7, 70.6, 68.8, 67.8, 54.3, 51.5, 49.6, 49.2, 40.2, 35.7, 32.8, 32.6, 32.2, 31.6, 31.5, 29.5, 29.4, 28.7, 28.4, 28.1, 22.3, 13.0. HRESI-MS m/z calcd for $C_{39}H_{78}N_5O_{12}S_2$ 872.5088, found 872.5087 $[M+H]^+$.

4.2.9. Compound 5a

A solution of **1a** (0.5 g, 0.44 mmol) in pyridine (10 mL) was treated with 2,4,6-triisopropylbenzenesulfonyl chloride (2.71 g, 8.96 mmol, 20.0 equiv). The reaction mixture was stirred at 23 °C for 12 h. Reaction progress was monitored by TLC (3% methanol in dichloromethane). Upon completion, the reaction mixture was concentrated, diluted with ethyl acetate (100 mL), washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The concentrated crude was purified by flash column chromatography over silica gel (3% methanol in dichloromethane) to afford **1b** (0.31, 43%) and the desired product **5a** as a white solid (0.2 g, 33%): ^{1}H NMR (400 MHz, methanol-d_4) δ 7.29 (s, 2H, $SO_3C_6H_2(CH)_3(CH_3)_6$), 5.43 (bs, 1H, H-1′), 5.12 (d, 1H, J = 2.2 Hz, H-1″), 4.89 (bs, 1H, H-1‴), 4.35 (m, 3H, H-6′, H-5′), 4.18 (m, 2H, H-2″, H-3″), 4.01–3.86 (m, 4H, H-4″, H-4′, H-3‴, H-5‴), 3.83–3.73 (m, 2H, H-2‴, H-5″), 3.64 (dd, J = 12.2, 6.7 Hz, 1H, H-5″), 3.61–3.21 (m, 12H, H-1, H-3, H-4, H-5, H-6, H-2′, H-3′, H-4‴, H-6‴, $SO_3C_6H_2(CH)_3(CH_3)_6$), 2.95 (hep, J = 6.9 Hz, 1H, $SO_3C_6H_2(CH)_3(CH_3)_6$), 1.93 (dt, J = 12.1, 4.3 Hz, 1H, H-2eq), 1.51-1.38 (m, 46H, 5×$CO_2C(CH_3)_3$, H-2ax), 1.32-1.22 (m, 18H, $SO_3C_6H_2(CH)_3(CH_3)_6$). ^{13}C NMR (100 MHz, methanol-d_4) δ 159.7, 159.4, 159.0, 159.3, 158.7, 156.2, 153.0, 131.6, 125.8, 111.9, 100.9, 100.0, 88.9, 84.1, 81.6, 81.5, 81.3, 81.0, 79.7, 79.3, 76.8, 76.4, 75.22, 73.9, 72.4, 72.3, 71.9, 70.3, 69.6, 65.1, 57.4, 54.4, 53.3, 52.21, 42.5, 36.4, 31.7, 29.8, 29.7, 29.6, 26.0, 24.8. LR-ESI m/z calcd for $C_{63}H_{106}N_5O_{26}S$ 1380.69, found 1380.58 $[M-H]^-$.

4.2.10. Compound 5b

To a solution of compound **5a** (80 mg, 0.057 mmol) and Cs_2CO_3 (mg, 0.115 mmol) in dry DMF (1 mL) was added 1-hexadecanethiol (0.12 mL, 0.4 mmol), and the mixture was stirred at 23 °C overnight. Reaction progress was monitored by TLC (5% methanol in dichloromethane). Upon completion, the reaction mixture was concentrated, diluted with ethyl acetate (100 mL), washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The concentrated crude was purified by flash column chromatography over silica gel (4% methanol/dichloromethane) to afford compound **5b** (60 mg, 77%) as a white solid: ^{1}H NMR (400 MHz, methanol-d_4) δ 5.32 (bs, 1H, H-1′), 5.14 (d, J = 2.6 Hz, 1H, H-1″), 4.89 (s, 1H, H-1‴), 4.22 (m, 1H, H-2″), 4.17 (m,1H, H-3″), 3.97–3.79 (m, 5H, H-5′, H-4″, H-5″, H-3‴, H-5‴), 3.76 (m, 1H, H-4‴), 3.68 (dd, J = 12.2, 6.4 Hz, 1H, H-5″), 3.63–3.19 (m, 11H, H-1, H-3, H-4, H-5, H-6, H-2′, H-3′, H-4′, H-2‴, H-6‴), 2.99 (dd, J = 13.8, 2.7 Hz, 1H, H-6′), 2.72 (dd, J = 13.8, 7.0 Hz, 1H, H-6′), 2.64 (t, J = 7.2 Hz, 2H, S$CH_2CH_2(CH_2)_{12}CH_3$), 1.98 (dt, J = 13.0, 4.0 Hz, 1H, H-2eq), 1.59 (m, 2H, $SCH_2CH_2(CH_2)_{12}CH_3$), 1.53–1.39 (m, 46H, 5×$CO_2C(CH_3)_3$, H-2ax), 1.30- 1.29 (m, 24H, $SCH_2CH_2(CH_2)_{12}CH_3$), 0.97–0.70 (m, 3H, $SCH_2CH_2(CH_2)_{12}CH_3$). ^{13}C NMR (100 MHz, methanol-d_4) δ 157.5, 157.1, 156.8, 156.3, 109.6, 98.9, 97.8, 86.4, 82.0, 79.3, 79.2, 79.0, 76.9, 74.6, 74.1, 73.0, 72.6, 72.3, 71.6, 70.2, 67.5, 62.4, 55.5, 52.1, 40.4, 34.2, 33.7, 33.2, 31.6, 29.6, 29.3, 29.0, 29.0, 28.5, 27.5, 27.4, 27.3, 22.3, 13.0. LR-ESI m/z calcd for $C_{64}H_{118}N_5O_{23}S$ 1356.79, found 1356.34 $[M+H]^+$.

4.2.11. Compound 5

Compound **5b** (20 mg, 0.014 mmol) was treated with 95% TFA (0.7 mL) at ambient temperature 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 compound **5** (21 mg, quantitative yield) as a white foam: ^{1}H NMR (400 MHz, methanol-d_4) δ 5.57 (d, J = 3.8 Hz, 1H, H-1′), 5.35 (d, J = 3.1 Hz, 1H, H-1″), 5.30 (d, J = 1.7 Hz, 1H, H-1‴), 4.52 (t, J = 5.5 Hz, 1H, H-3″), 4.36–4.24 (m, 2H, H-5‴, H-2″), 4.17 (m, 1H, H-4″), 4.14 (m, 1H, H-3‴), 3.95 (t, J = 9.8 Hz, 1H, H-4), 3.92–3.73 (m, 5H, H-5, H-3′, H-5′, H-5″), 3.68 (m, 1H, H-4‴), 3.61 (m, 1H, H-6), 3.54 (td, J = 12.6, 10.2, 4.3 Hz, 1H, H-3), 3.44 (m, 1H, H-2‴), 3.42–3.21 (m, 5H, H-1, H-2′, H-4′, H-6‴), 3.09 (dd, J = 14.1, 2.2 Hz, 1H, H-6′), 2.68 (dd, J = 14.3, 8.0 Hz, 1H, H-6′), 2.59 (m, 2H, S$CH_2CH_2(CH_2)_{12}CH_3$), 2.48 (dt, J = 12.6, 4.5 Hz, 1H, H-2eq), 1.90 (q, J = 12.6 Hz, 1H, H-2ax), 1.71–1.52 (m, 2H, $SCH_2CH_2(CH_2)_{12}CH_3$), 1.41-1.25 (m, 24H, $SCH_2CH_2(CH_2)_{12}CH_3$), 0.90-0.85 (m, 3H, $SCH_2CH_2(CH_2)_{12}CH_3$). ^{13}C NMR (100 MHz, methanol-d_4) δ 161.7 (q, J = 34.8 Hz, CF_3CO_2H), 116.7 (q, J = 292.4, CF_3CO_2H), 110.3, 97.3, 95.6, 84.2, 82.0, 79.6, 75.5, 74.6, 73.9, 72.5, 72.2, 70.6, 68.9, 67.8, 67.7, 59.6, 54.2, 51.5, 49.7, 49.4, 40.2, 32.7, 32.6, 31.6, 29.4, 29.3, 29.0, 28.9, 22.3, 13.01. HRESI-MS m/z calcd for $C_{39}H_{78}N_5O_{13}S$ 856.5317, found 856.5318 $[M+H]^+$.

4.3. Biological assays

4.3.1. Minimal inhibitory concentration protocol

Starter cultures were incubated for 24 h (37 °C, 5% CO_2, aerobic conditions) and then diluted in fresh medium to obtain an optical density of 0.004 (OD_{600}). All strains were tested using the double-dilution method starting at 64 μg/mL in 96-well plates (Sarstedt). After 24 h of incubation, MTT (50 μL of a 1 mg/mL solution in

H_2O) was added to each well followed by additional incubation at 37 °C for 2 h. MIC values (μg/mL) were determined as the lowest concentration at which no bacterial growth was observed. Results were obtained from two independent experiments, and each experiment was done in triplicate.

4.3.2. Red blood cell hemolysis protocol

A sample of rat RBCs (2% w/w) were incubated with each of the tested compounds for 1 h at 37 °C, 5% CO_2 using the double dilution method starting at concentration of 256 μg/mL. Negative control was PBS and positive control was 1% w/v solution of Triton X100 (100% hemolysis). Following centrifugation (2000 rpm, 10 min, ambient temperature), the supernatant was removed and its absorbance measured at 550 nm using a microplate reader (Genios, TECAN). The results are expressed as percentage of hemoglobin released relative to the positive control (Triton X100). Experiments were performed in triplicate, and the results are an average of experiments in blood samples taken from at least two rats.

4.3.3. Scanning electron microscopy protocol

Cultures of *S. epidermidis* ATCC12228 were incubated for 4 h at 37 °C under aerobic conditions to obtain an optical density of 0.2 (OD_{600}) and then harvested by centrifugation at 4000 rpm for 10 min at 4 °C. The bacterial pellet was washed twice with PBS (pH 7.4) and resuspended in PBS (pH 7.4) to obtain an optical density of 1.0 (OD_{600}). The bacterial suspension was diluted twofold after treatment with the tested compound at a concentration of 1 μg/mL at 37 °C for 1 h. The cells were then spun down at 6000 rpm for 4 min at 4 °C, washed with PBS (pH 7.4) three times, and fixed with 2.5% glutaraldehyde/PBS buffer overnight at 4 °C. The cells were then washed three times in 0.1 M PB (pH 7.4), dehydrated in series of graded ethanol solutions (30–100%), and dried in vacuum desiccation. Finally, the samples were coated with palladium-gold and viewed via scanning electron microscopy (Quanta 200FEG ESEM).

Acknowledgments

This work was supported by the FP7-PEOPLE-2009-RG Marie Curie Action: Reintegration Grants (Grant 246673). We thank Professors Itzhak Ofek, Dani Cohen (Tel Aviv University), and Doron Steinberg (The Hebrew University of Jerusalem) for the gift of bacterial strains. We thank Anat Eldar-Boock from the group of Professor Ronit Satchi-Fainaro (Tel Aviv University) for her help with the hemolysis assays.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.03.046.

References and notes

1. Landis, S. J. *Adv. Skin Wound Care* **2008**, *21*, 531.
2. Lipsky, B. A.; Berendt, A. R.; Deery, H. G.; Embil, J. M.; Joseph, W. S.; Karchmer, A. W., et al *Clin. Infect. Dis.* **2004**, *39*, 885.
3. Abdulrazak, A.; Bitar, Z. I.; Al-Shamali, A. A.; Mobasher, L. A. *J. Diabet. Complications* **2005**, *19*, 138.
4. Schwartz, R. A.; Al-mutairi, N. *Gulf J. Dermatol. Venereol.* **2010**, *17*, 1.
5. Lipsky, B. a.; Hoey, C. *Clin. Infect. Dis.* **2009**, *49*, 1541.
6. Gelmetti, C. *Dermatol. Ther.* **2008**, *21*, 187.
7. Hancock, R. E. W.; Chapple, D. S. *Antimicrob. Agents Chemother.* **1999**, *43*, 1317.
8. Hartmann, M.; Berditsch, M.; Hawecker, J.; Ardakani, M. F.; Gerthsen, D.; Ulrich, A. S. *Antimicrob. Agents Chemother.* **2010**, *54*, 3132.
9. Mogi, T.; Kita, K. *Cell. Mol. Life Sci.* **2009**, *66*, 3821.
10. Zavascki, A. P.; Goldani, L. Z.; Li, J.; Nation, R. L. *Antimicrob. Agents Chemother.* **2007**, *60*, 1206.
11. Glupczynski, Y. *Antimicrob. Agents Chemother.* **1999**, *43*, 727.
12. Houghton, J. L.; Green, K. D.; Chen, W.; Garneau-Tsodikova, S. *ChemBioChem* **2010**, *11*, 880.
13. Gehrig, K. a.; Warshaw, E. M. *J. Am. Acad. Dermatol.* **2008**, *58*, 1.
14. Huth, M. E.; Ricci, A. J.; Cheng, A. G. *Int. J. Otolaryngol.* **2011**, 1.
15. Mingeot-Leclercq, M. P.; Tulkens, P. M. *Antimicrob. Agents Chemother.* **1999**, *43*, 1003.
16. Herzog, I. M.; Green, K. D.; Berkov-Zrihen, Y.; Feldman, M.; Vidavski, R. R.; Eldar-Boock, A.; Satchi-Fainaro, R.; Eldar, A.; Garneau-Tsodikova, S.; Fridman, M. *Angew. Chem., Int. Ed.* **2012**, *51*, 5652.
17. Herzog, I. M.; Feldman, M.; Eldar-Boock, A.; Satchi-Fainaro, R.; Fridman, M. *MedChemCommun* **2013**, *4*, 120.
18. Bera, S.; Zhanel, G. G.; Schweizer, F. *Antimicrob. Agents Chemother.* **2010**, *65*, 1224.
19. Bera, S.; Zhanel, G. G.; Schweizer, F. *J. Med. Chem.* **2010**, *53*, 3626.
20. Ouberai, M.; El Garch, F.; Bussiere, A.; Riou, M.; Alsteens, D.; Lins, L.; Baussanne, I.; Dufrêne, Y. F.; Brasseur, R.; Decout, J.-L.; Mingeot-Leclercq, M.-P. *Biochim. Biophys. Acta* **1808**, *2011*, 1716.
21. Baussanne, I.; Bussière, A.; Halder, S.; Ganem-Elbaz, C.; Ouberai, M.; Riou, M.; Paris, J.-M.; Ennifar, E.; Mingeot-Leclercq, M.-P.; Décout, J.-L. *J. Med. Chem.* **2010**, *53*, 119.
22. Howell-Jones, R. S.; Wilson, M. J.; Hill, K. E.; Howard, A. J.; Price, P. E.; Thomas, D. W. *Antimicrob. Agents Chemother.* **2005**, *55*, 143.
23. Weghuber, J.; Aichinger, M. C.; Brameshuber, M.; Wieser, S.; Ruprecht, V.; Plochberger, B.; Madl, J.; Horner, A.; Reipert, S.; Lohner, K.; Henics, T.; Schütz, G. J. *Biochim. Biophys. Acta* **1808**, *2011*, 2581.
24. Epand, R. F.; Savage, P. B.; Epand, R. M. *Biochim. Biophys. Acta* **2007**, *1768*, 2500.
25. Michael, K.; Wang, H.; Tor, Y. *Bioorg. Med. Chem.* **1999**, *7*, 1361.
26. Pathak, R.; Böttger, E. C.; Vasella, A. *Helv. Chim. Acta* **2005**, *88*, 2967.
27. Bearden, D. T.; Allen, G. P.; Christensen, J. M. *Antimicrob. Agents Chemother.* **2008**, *62*, 769.
28. Löffler, B.; Hussain, M.; Grundmeier, M.; Brück, M.; Holzinger, D.; Varga, G.; Roth, J.; Kahl, B. C.; Proctor, R. a.; Peters, G. *PLoS Pathog.* **2010**, *6*, 1.
29. Otto, M. *Nat. Rev. Microbiol.* **2009**, *7*, 555.
30. Johansson, L.; Thulin, P.; Low, D. E.; Norrby-Teglund, A. *Clin. Infect. Dis.* **2010**, *51*, 58.
31. Koo, S.; Bayer, A. S.; Yeaman, M. R. *Infect. Immun.* **2001**, *69*, 4916.
32. Wallin, E.; Von Heijne, G. *Protein Sci.* **1998**, *7*, 1029.