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(54) **Title:** MEMBRANE-TARGETING AMINOGLYCOSIDE-BASED CATIONIC AMPHIPHILES AND THERAPEUTIC USES

(57) **Abstract:** The present invention relates to amphiphilic aminoglycoside (AG) derivatives, especially derivatives of tobramycin, kanamycin A and paromomycin comprising hydrophobic side chains linked to the AG moiety by various linkers. These novel derivatives are useful as broad spectrum antibacterial agents targeting bacterial cell membranes, while having minimal toxicity due to non-selective targeting of eukaryotic cell membranes. The present invention further relates to methods for preparing the novel derivatives, pharmaceutical compositions including such compounds, and methods of using these compounds and compositions, especially as membrane-targeting antibacterial agents for treating bacterial infections, and for inhibiting bio film growth.



MEMBRANE-TARGETING AMINOGLYCOSIDE-BASED CATIONIC AMPHIPHILES AND THERAPEUTIC USES

FIELD OF THE INVENTION

The present invention relates to bacterial membrane-targeting aminoglycoside (i.e., tobramycin, kanamycin or paromomycin) based cationic amphiphiles having potent antibacterial and reduced hemolytic activity, methods for their preparation, pharmaceutical compositions including such compounds, and methods of using these compounds and compositions, especially as antibacterial agents having broad antibacterial activity against both Gram-positive and Gram-negative bacteria (e.g., for treating soft tissue and skin infections while having reduced hemolytic activity).

BACKGROUND OF THE INVENTION

Membranes and cell walls are essential constituents required for the viability of all bacteria and therefore serve as attractive targets for the development of antibiotics. Amongst cell-wall-targeting antibiotics, several families of peptidoglycan biosynthesis inhibitors, including β -lactams that irreversibly inhibit the activity of the peptidoglycan trans-peptidation biosynthetic step (Chung, 2009; Fisher, 2005); glyco-peptide antibiotics such as vancomycin that competitively inhibit the trans-peptidation step, and the glyco-lipid antibiotic moenomycin A inhibits the peptidoglycan trans-glycosylation step (Healy, 2000; Ge, 1999; Yuan, 2008; Fuse, 2010). To date, disruption of the bacterial membrane bi-layer has been poorly exploited as a strategy for the development of antibiotics.

Bacterial membrane-disrupting-antibiotics offer several advantages over antimicrobial agents that target intracellular bacterial targets. First, membrane disruption is not dependent on the bacterial cell cycle state and is therefore a promising strategy for the eradication of dormant bacteria and treatment of persistent infections (Hurdle, 2011). Second, antimicrobial agents that act in the extracellular bacterial environment evade intracellular resistance mechanisms and are expected to maintain prolonged clinical efficacy. Finally, cell permeability consideration, which is often a significant challenge for drug designers, is not necessary for the design of membrane-targeting antibiotics. Although peptidoglycan exists solely in bacteria, membranes

composed of lipid bi-layers are common to all cells; therefore, avoiding cytotoxicity to eukaryotic cells through non-selective membrane disruption is a major challenge. In contrast to most eukaryotic cell membranes, both Gram-positive and Gram-negative bacteria membranes are highly negatively charged due to a high content of anionic lipids such as cardiolipins and phosphatidylglycerol (Weghuber, 2011; Epan, 2007). Gram-negative bacteria membranes also have the negatively charged core of the lipopolysaccharide (LPS), while negatively charged teichoic acids are major constituents of Gram-positive bacteria cell walls (Silhavy, 2010; Swoboda, 2010).

Hence, both Gram-positive and Gram-negative bacteria membranes attract positively charged organic compounds through ionic interactions. LPS that compose the Gram-negative outer membrane leaflet is unique to bacteria and serves as a target for the polymixin family of antibiotics (Figure 1). Polymixins composed of a cyclic cationic decapeptide with an *N*-terminal hydrophobic residue are highly potent and clinically used antibiotics that bind to the negatively charge LPS core and disrupt the outer membrane of Gram negative bacteria (Tsubery, 2002; Tsubery, 2000). The potency and broad-spectrum activity of polymixins against Gram-negative bacteria demonstrates the potential that lies in the development of membrane-targeting antibiotics and the need to develop such antimicrobials that will target Gram-positive bacteria as well.

Bacterial skin infections, including chronic infections related to diabetes, venous stasis, or arterial insufficiency account for a significant percentage of infectious diseases (Landis, 2008; Lipsky, 2004; Abdulrazak, 2005). Patients with infected wounds are frequently treated with systemic antibiotics and, in addition, topical antibiotic treatments (Schwartz, 2010; Lipsky, 2009). There is a large repertoire of potent antimicrobial agents that are unfit for internal use due to their toxicity but that are tolerated topically (Gelmetti, 2008). 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, polymixins, and aminoglycosides such as neomycin B (Schwartz, 2010, Lipsky, 2009; Gelmetti, 2008; Hancock, 1999). 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 extremely toxic when taken internally (Gelmetti, 2008; Hartmann, 2010, Mogi, 2009). The membrane targeting cyclic lipo-peptides polymixins are

active against several Gram negative pathogens, but polymyxins are not active against Gram positive bacteria and are also highly toxic when used systemically (Mogi, 2009; Zavascki, 2007). The clinical efficacy of the aminoglycoside neomycin B is continuously reduced as an ever increasing number of bacterial strains that acquire resistance to this aminoglycoside antibiotic (Glupczynski, 1999; Houghton, 2010). Topical use of neomycin B may be accompanied by undesired contact dermatitis side effects (Gehrig, 2008). Aminoglycosides are also highly nephrotoxic and ototoxic when used internally (Huth, 2011), Mingeot-Leclercq, 1999). 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 that exhibit minimal toxic side effects.

In recent years, several studies have demonstrated the potential of positively charged aminoglycosides (AGs) as scaffolds for the development of membrane-targeting cationic amphiphilic antimicrobial agents by the attachment of hydrophobic residues to one or more positions on the AG (Ouberaï, 2011; Bera, 2010(a); Bera, 2010 (b); Baussanne, 2010; Hanessian, 2010). It was recently demonstrated that the attachment of aliphatic chains to the 6"-position of the aminoglycoside tobramycin (Figure 1, compound 1) resulted in potent antimicrobial agents and provided evidence for their membrane-disruption activity (Herzog, 2012). Several lines of evidence support the hypothesis that these cationic amphiphiles act by disrupting bacterial membranes (Herzog, 2012, Bera, 2010(a); Bera 2010 (b); Ouberaï, 2011).

WO 2010/004433 discloses hydrophobically enhanced aminoglycosides, such as aminoglycoside-lipid conjugates, and their use as antibacterial agents.

There is an unmet need in the art for new antibacterial agents that are active against both gram-positive and gram-negative bacteria on the one hand, and that have reduced hemolytic activity on the other hand.

SUMMARY OF THE INVENTION

The present invention relates to amphiphilic aminoglycoside (AG) derivatives, especially derivatives of tobramycin, kanamycin A and paromomycin comprising hydrophobic side chains linked to the AG moiety by various linkers as described herein. These novel

derivatives are useful as broad spectrum antibacterial agents by targeting bacterial cell membranes, while having minimal toxicity due to non-selective targeting of eukaryotic cell membranes. The present invention further relates to methods for preparing the novel derivatives, pharmaceutical compositions including such compounds, and methods of using these compounds and compositions, especially as membrane-targeting antibacterial agents, to combat bacteria that cause, e.g., soft tissue and skin infections, and for inhibiting biofilm growth.

The present invention is based on the discovery of novel aminoglycoside-based cationic amphiphiles differing in the chemical bond linking their hydrophobic and hydrophilic parts. In some embodiments, the novel compounds are based on the scaffold of aminoglycosides such as tobramycin and kanamycin A, linked to hydrophobic aliphatic chains via a linker such as a thioether, sulfone, sulfonyl, amide or triazolyl. In other embodiments, the novel compounds are paromomycin-based cationic amphiphiles that are based on the scaffold of paromomycin, linked at the two primary hydroxy sites to hydrophobic aliphatic chains via a linker such as a thioether, sulfone, sulfonyl, amide or triazolyl. Several compounds of the invention demonstrate potent antimicrobial and biofilm growth inhibition properties (e.g., they combat bacteria that cause soft tissue and skin infection), while exhibiting a dramatic reduction in red blood cells hemolysis, thus demonstrating that it is possible to maintain the antimicrobial potency of such molecules while reducing their undesired hemolytic effect.

Compared to mammalian cell membranes, bacterial membranes contain a high percentage of anionic lipids (Weghuber, 2011; Epan, 2007). It was 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. In some embodiments, anchoring of the positively-charged aminoglycoside to the bacterial membrane was enhanced by attaching aliphatic chains on two different positions on the aminoglycoside scaffold.

The amphiphilic aminoglycoside analogues of the present invention include four structural motifs that affect their antibacterial activity as well as their specificity towards bacterial membranes: (1) the sugar backbone; (2) the length of the aliphatic chain (or chains); (3) the type of chemical bond that links the hydrophobic aliphatic chain and the pseudo-oligosaccharide; and (4) the number of positive charges on the molecule.

Tobramycin and Kanamycin A Derivatives:

In some embodiments, the compounds of the present invention are based on tobramycin (1) and kanamycin A (6) (Figure 1), modified with lipophilic linear alkyl chains on the 6'-primary alcohol and/or the 4'-primary alcohol of each aminoglycoside. While kanamycin A and tobramycin share high structural similarity, these two pseudo-oligosaccharides differ in the number of amine groups (four for kanamycin A and five for tobramycin) and therefore, under physiological conditions, the overall positive charge of tobramycin is higher than that of kanamycin A. Tobramycin (1) based cationic amphiphiles are particularly interesting since similar to polymyxins, this AG also contains five primary amines which are positively charged under physiological conditions.

Without wishing to be bound by any particular mechanism or theory, it is contemplated that the selectivity towards bacterial membranes is based on ionic interactions of the positively charged aminoglycoside-based cationic amphiphiles with the negatively charged bacterial membranes. A decrease in the overall positive charge of the cationic amphiphiles by decreasing the number of amines on the aminoglycoside scaffold (e.g., by using kanamycin A analogs vs. tobramycin analogs), may not dramatically affect the affinity to bacterial membranes which are highly negatively charged, yet on the other hand it may significantly reduce the affinity of the kanamycin A amphiphiles towards eukaryotic cell membranes which are far less negatively charged.

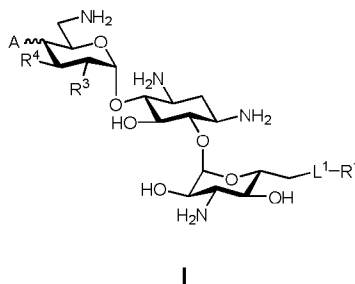
Moreover, selectivity of the amphiphilic aminoglycoside analogues towards bacterial membranes may also be achieved based on the high overall negative charge of bacterial membranes, by significantly enhancing the net positive charge by increasing the number of ammonium groups on these analogues. This may be accomplished by creating amphiphilic dimeric structures of the amphiphilic aminoglycosides, as demonstrated hereinbelow (e.g., compounds XII and 10), therefore doubling the net positive charge of these molecules compared to analogues with a single aminoglycoside scaffold.

Selectivity towards bacterial cells may be demonstrated by comparing the hemolytic activity and eukaryotic cell toxicity of the kanamycin A and tobramycin analogues, as described herein.

The linear alkyl chains typically range from 12 to 16 carbons in length which were found to be the optimal lengths leading to the most potent and broad spectrum antibacterial activity (Herzog, 2012). When two alkyl chains are present, the length of each chain is

preferably between 4 and 8 carbon atoms. The total number of carbon atoms (whether originating from one alkyl or two alkyl chains) is preferably from 12 to 16. The linker can be any functional group that links the aminoglycoside to the lipophilic alkyl group. Non-limiting examples include thioether, sulfone and sulfonyl ester-bond based compounds, amide bond based compounds and triazole analogues, as further described hereinbelow.

Thus, in one embodiment, the present invention relates to a tobramycin or kanamycin A derivative represented by the structure of formula I:



wherein

A is OH or $-L^2-R^2$, wherein:

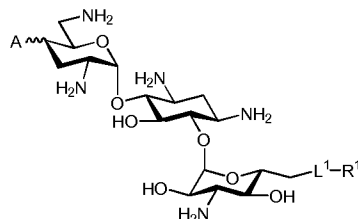
- (i) when A is OH, L^1 is selected from the group consisting of $-\text{OSO}_2-$, triazolyl, and $-\text{NH}-\text{C}(=\text{O})-$; and R^1 is a $\text{C}_{12}-\text{C}_{16}$ linear alkyl;
- (ii) when A is $-L^2-R^2$, each of L^1 and L^2 is selected from the group consisting of $-\text{S}-$, $-\text{SO}_2-$, $-\text{OSO}_2-$, triazolyl and $-\text{NH}-\text{C}(=\text{O})-$; and each of R^1 and R^2 is a linear C_4-C_8 alkyl; and

R^3 and R^4 are each OH; or R^3 is NH_2 and R^4 is H;

including salts, solvates, polymorphs, optical isomers, geometrical isomers, enantiomers, diastereomers, and mixtures thereof.

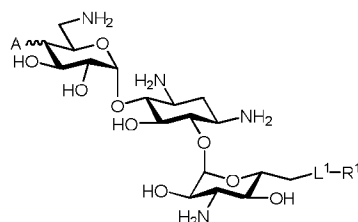
In one embodiment of formula I, R^3 is NH_2 and R^4 is H. In accordance with this embodiment, the compound is a tobramycin derivative, represented by the structure of formula II:

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II

In another embodiment of formula I, R^3 and R^4 are each OH. In accordance with this embodiment, the compound is a kanamycin A derivative represented by the structure of formula III:



III

The compounds of the present invention have, in some embodiments, different functional groups linking their hydrophobic (alkyl) and hydrophilic (aminoglycoside) components.

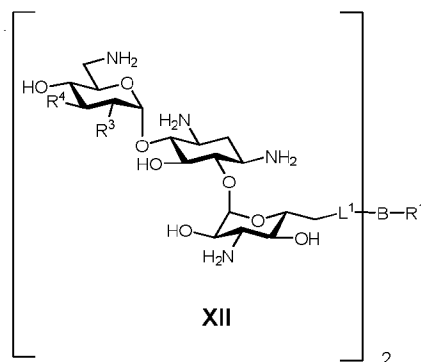
In some embodiments, A in the compounds of formula I-III is OH, in which case the linker L^1 is selected from a sulfonyl ester, a triazolyl and an amide. Preferably, A is in the equatorial position. Non-limiting examples of such compounds are depicted in compounds of Formula IV, V and VI, respectively, with each possibility representing a separate embodiment of the present invention. For each of compounds IV, V and VI, R^3 and R^4 may each be OH (kanamycin A derivative) or R^3 is NH_2 and R^4 is H (tobramycin derivative), with each possibility representing a separate embodiment of the present invention. The structures of compounds IV, V and VI are depicted in the detailed description hereinbelow.

As contemplated herein, it has been discovered that linkage of aminoglycosides of formula I wherein A is OH to lipophilic hydrocarbon chains, and in particular linear alkyl chains of 12 to 16 carbons in length, lead to potent and broad spectrum antibacterial activity. Thus, in some embodiments, R^1 in any of the compounds of formula I-VI is a linear alkyl of 12 to 16 carbons, examples of which include $-(CH_2)_{11}CH_3$, $-(CH_2)_{13}CH_3$ and $-(CH_2)_{15}CH_3$. Each possibility represents a separate embodiment of the present invention.

In some embodiments, A in the compounds of formula I-III is $-L^2-R^2$ wherein R^2 and L^2 are as defined above. In this case, each of the linkers L^1 and L^2 is selected from a thioether, a sulfone, a sulfonyl ester, a triazolyl and an amide. Preferably, A is in the axial position. Non-limiting examples of such compounds are depicted in compounds of Formula VII, VIII, IX, X and XI, respectively, with each possibility representing a separate embodiment of the present invention. For each of compounds VII, VIII, IX, X and XI, R^3 and R^4 may each be OH (kanamycin A derivative) or R^3 is NH_2 and R^4 is H (tobramycin derivative), with each possibility representing a separate embodiment of the present invention. A currently preferred compound is compound (17). The structures of compounds (17), VII, VIII, IX, X and XI, are depicted in the detailed description hereinbelow.

As contemplated herein, it has further been discovered that linkage of aminoglycosides of formula I wherein A is $-L^2-R^2$ to lipophilic hydrocarbon chains, and in particular linear alkyl chains of 4 to 8 carbons in length, lead to potent and broad spectrum antibacterial activity. Thus, in some embodiments, each of R^1 and R^2 in any of the compounds of formula VII-XII is a linear alkyl of 4 to 8 carbons, preferably 6 to 8 carbon atoms. Without wishing to be bound by any particular mechanism or theory, it is contemplated that the sum of the carbons of the two alkyl groups in such compounds is preferably between 12 and 16 carbons, which has been found to be a preferred length for the monoalkylated derivatives described above.

In another embodiment, the present invention relates to a dimeric compound represented by the structure of formula XII:



wherein

R^1 is a C_{12} - C_{16} linear alkyl;

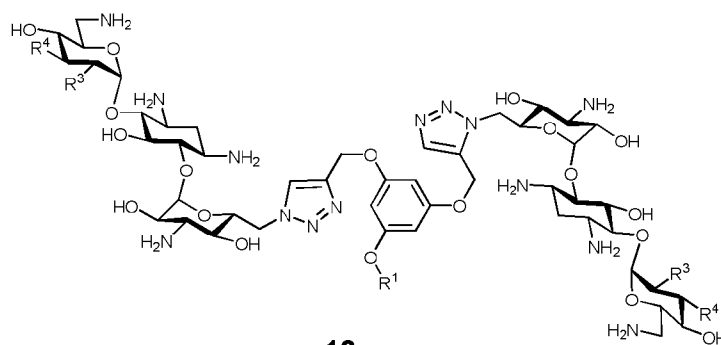
L^1 is selected from the group consisting of $-S-$, $-SO_2-$, $-OSO_2-$, triazolyl and $-NH-C(=O)-$;

R^3 and R^4 are each OH; or R^3 is NH_2 and R^4 is H; and

B is selected from an unsubstituted or substituted alkyl, aryl, aryloxy, alkyloxy and amide, preferably wherein B is phenoxy;

including salts, solvates, polymorphs, optical isomers, geometrical isomers, enantiomers, diastereomers, and mixtures thereof.

An example of such compound is compound **10** (e.g., compounds 10a and 10b).



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10a: $R^3 = NH_2$, $R^4 = H$

10b: $R^3, R^4 = OH$

Paromomycin Derivatives:

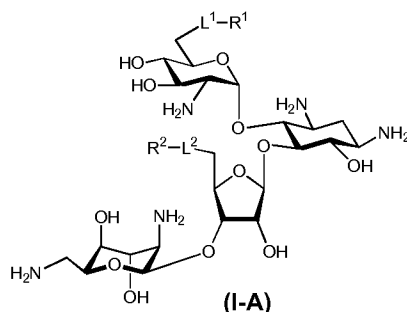
In additional embodiments, the compounds of the present invention are based on dialkylated derivatives of paromomycin. For the preparation of di-alkylated aminoglycosides the pseudo-tetrasaccharide paromomycin (**1A**) was chosen (Figure 1) for two reasons: Like tobramycin (Figure 1), this aminoglycoside scaffold has five amine functionalities that are positively charged under physiological conditions. However, unlike tobramycin, paromomycin 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. As demonstrated herein, 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 substantial 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, C_6 , C_7 , and C_8 alkyl chains were chosen for the preparation of the di-alkylated paromomycin derivatives.

A collection of paromomycin-based di-alkylated cationic amphiphiles differing in the lengths of their aliphatic chain residues were thus designed, synthesized, and evaluated against

14 Gram positive pathogens that are known to cause skin infections. Paromomycin derivatives that were di-alkylated with C₇ and C₈ 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. A comparison between a di-alkylated and a mono-alkylated paromomycin indicated that, for this sugar backbone, 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 novel di-alkylated paromomycin derivatives.

Selectivity towards bacterial cells may be demonstrated by comparing the hemolytic activity and eukariotic cell toxicity of the paromomycin analogues, as described herein.

In one embodiment, the present invention relates to a paromomycin derivative represented by the structure of formula I-A:



wherein

L¹ and L² are each independently selected from the group consisting of -S-, -SO₂, -OSO₂-, triazolyl and -NH-C(=O)-; and

R¹ and R² are each independently a linear C₄ to C₈ alkyl;

including salts, solvates, polymorphs, optical isomers, geometrical isomers, enantiomers, diastereomers, and mixtures thereof.

The paromomycin derivatives of the present invention comprise different functional groups (linkers L¹ and L²) linking their hydrophobic (alkyl) and hydrophilic (aminoglycoside) components. The linkers are selected from thioethers (-S-), sulfonyls (-SO₂), sulfonyl esters (-OSO₂-), triazolyl and amides (-NH-C(=O)-). Non-limiting examples of such compounds are depicted in compounds of Formula II-A, III-A, IV-A, V-A and VI-A, respectively, with each

possibility representing a separate embodiment of the present invention. Currently preferred compounds are represented by formula 22, 23 or 24, with each possibility representing a separate embodiment of the present invention. The structures of compounds II-A, III-A, IV-A, V-A, VI-A, 22, 23 and 24 are depicted in the detailed description hereinbelow.

As contemplated herein, it has been discovered that linkage of aminoglycosides of formula I-A to lipophilic hydrocarbon chains, and in particular linear alkyl chains of 4 to 8 carbons in length, lead to potent and broad spectrum antibacterial activity. Thus, in some embodiments, each of R^1 and R^2 in any of the compounds of formula I-A to VI-A is a linear alkyl of 4 to 8 carbons, preferably 6 to 8 carbon atoms ($\text{CH}_3(\text{CH}_2)_5$ -, $\text{CH}_3(\text{CH}_2)_6$ -, and $\text{CH}_3(\text{CH}_2)_7$ -). Without wishing to be bound by any particular mechanism or theory, it is contemplated that the sum of the carbons of the two alkyl groups in such compounds is preferably between 12 and 16 carbons, which has been found to be a preferred length for conferring potent antibacterial on the one end, and minimal hemolytic potential on the other.

In another aspect, the present invention relates to an anti-bacterial pharmaceutical composition comprising a compound according to any of formulae I to XII, or I-A to VI-A as described herein, or any compound covered by said formulae, and a pharmaceutically acceptable carrier or excipient. The pharmaceutical compositions of the invention may be a form suitable for oral administration, intravenous administration by injection, topical administration, administration by inhalation, or administration via a suppository.

In another aspect, the present invention relates to a method of combating bacteria, or treating bacterial infections, comprising the step of administering to a subject in need thereof a compound of any of formulae I to XII, or I-A to VI-A as described herein, or any compound covered by said formulae, or a pharmaceutical composition comprising such compound. The bacteria may be Gram-positive or Gram-negative, with each possibility representing a separate embodiment of the present invention. In some embodiments, the bacteria cause skin infections. In other embodiments, the bacteria cause soft tissue (e.g., throat) infections.

In another aspect, the present invention relates to a method of combating bacteria, comprising the step of contacting the bacteria with a compound of any of formulae I to XII, or I-A to VI-A as described herein, or any compound covered by said formulae, or a composition comprising such compound.

In another aspect the present invention relates to the use of a compound of any of formulae I to XII, or I-A to VI-A as described herein, or any compound covered by said

formulae, or a pharmaceutical composition comprising such compound, for the manufacture of a medicament for combating bacteria or treating bacterial infections.

In another aspect, the present invention relates to a compound of any of formulae I to XII, or I-A to VI-A as described herein, or any compound covered by said formulae, or to a pharmaceutical composition comprising such compound, for use in combating bacteria or treating bacterial infections.

In another embodiment, the present invention relates to a method of inhibiting biofilm growth, comprising the step of contacting the biofilm or a surface comprising the biofilm with a compound of any of formulae I to XII, or I-A to VI-A as described herein, or any compound covered by said formulae, or a pharmaceutical composition comprising such compound.

Further embodiments and the full scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Structures of the Gram-negative bacteria targeting polymixins and bacterial ribosome targeting aminoglycoside antibiotics tobramycin, kanamycin A, and paromomycin.

Figure 2: Laboratory rat RBC hemolysis assay. Amphiphilic tobramycin analogues were incubated with RBCs isolated from a laboratory rat at concentrations of (A) 32 $\mu\text{g/mL}$, (B) 64 $\mu\text{g/mL}$, and (C) 128 $\mu\text{g/mL}$ for 1 hour at 37°C. All experiments were performed in triplicate, and results are the average from two different sets of experiments using blood samples from two laboratory rats.

Figure 3: Rat RBCs were incubated with tested compounds for 1 hour at 37°C: **22** (X), **23** (▲), **24** (●), **25** (--♦--), 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.

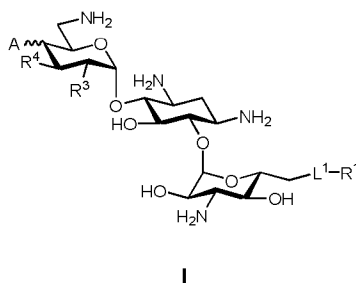
Figure 4: Scanning electron microscopic (SEM) images of *S. epidermidis* ATCC12228 with and without drug: (a) Untreated control bacteria cells. (b) Cells after 1 hour of incubation at 37 °C with 1 μ g/mL paromomycin **1A**. (c) Cells after 1 hour of incubation at 37 °C with 1 μ g/mL of compound **24**.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to amphiphilic aminoglycoside (AG) derivatives, especially derivatives of tobramycin, kanamycin A and paromomycin comprising hydrophobic side chains linked to the AG moiety by various linkers. These novel derivatives are useful as broad spectrum antibacterial agents targeting bacterial cell membranes, while having minimal toxicity due to non-selective targeting of eukaryotic cell membranes. The present invention further relates to methods for preparing the novel derivatives, pharmaceutical compositions including such compounds, and methods of using these compounds and compositions, especially as membrane-targeting antibacterial agents to combat bacteria that cause, e.g., soft tissue and skin infections, and for inhibiting biofilm growth.

Tobramycin and Kanamycin A Derivatives:

In one embodiment, the present invention relates to a tobramycin or kanamycin A derivative represented by the structure of formula I:



wherein

A is OH or $-L^2-R^2$, wherein:

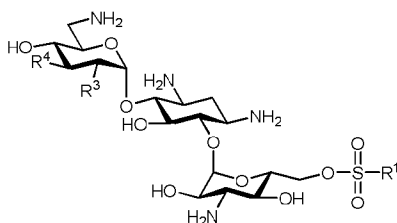
- (i) when A is OH, L^1 is selected from the group consisting of $-\text{OSO}_2-$, triazolyl, and $-\text{NH}-\text{C}(=\text{O})-$; and R^1 is a C_{12} - C_{16} linear alkyl;

(ii) when A is $-L^2-R^2$, each of L^1 and L^2 is selected from the group consisting of $-S-$, $-SO_2-$, $-OSO_2-$, triazolyl and $-NH-C(=O)-$; and each of R^1 and R^2 is a linear C_4-C_8 alkyl; and

R^3 and R^4 are each OH; or R^3 is NH_2 and R^4 is H;

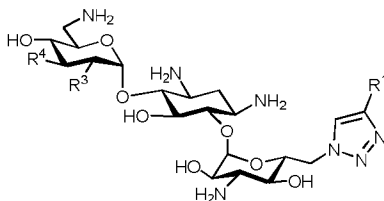
including salts, solvates, polymorphs, optical isomers, geometrical isomers, enantiomers, diastereomers, and mixtures thereof. Several non-limiting embodiments of the compounds of formula I are compounds of formulae II, III, IV, V, VI, VII, VIII, IX, X and XI, or any of the exemplified compounds of formula (4a), (4b), (4c), (5a), (5b), (5c), (6a), (6b), (6c), (14a), (14b), (14c), (15a), (15b), (15c), (16a), (16b), (16c) and (17), which are described hereinbelow. Dimeric structures of any of such compounds, as represented by Formula XII (e.g., compound 10) may also be prepared and are encompassed by the present invention. Each possibility represents a separate embodiment of the present invention.

In some embodiments, A in the compounds of formula I-III is OH, in which case the linker L^1 is selected from a sulfonyl ester, a triazolyl and an amide. Preferably, A is in the equatorial position. Non-limiting examples of such compounds are depicted in compounds of Formula IV, V and VI, respectively, with each possibility representing a separate embodiment of the present invention. For each of compounds IV, V and VI, R^3 and R^4 may each be OH (kanamycin A derivative) or R^3 is NH_2 and R^4 is H (tobramycin derivative), with each possibility representing a separate embodiment of the present invention.



IV

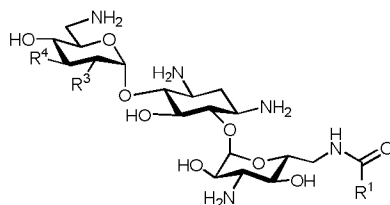
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V

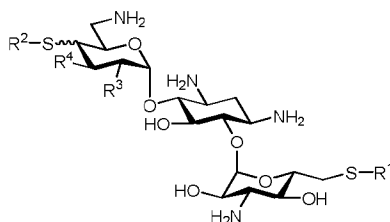
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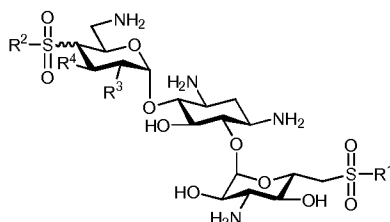
VI

In some embodiments, A in the compounds of formula I-III is $-L^2-R^2$ wherein R^2 and L^2 are as defined above. In this case, each of the linkers L^1 and L^2 is selected from a thioether, a sulfone, a sulfonyl ester, a triazolyl and an amide. Preferably, A is in the axial position. Non-limiting examples of such compounds are depicted in compounds of Formula VII, VIII, IX, X and XI, respectively, with each possibility representing a separate embodiment of the present invention. For each of compounds VII, VIII, IX, X and XI, R^3 and R^4 may each be OH (kanamycin A derivative) or R^3 is NH_2 and R^4 is H (tobramycin derivative), with each possibility representing a separate embodiment of the present invention.



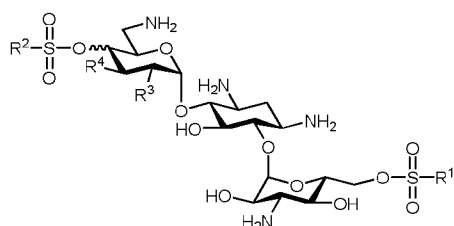
VII

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VIII

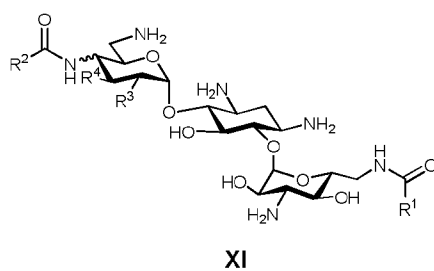
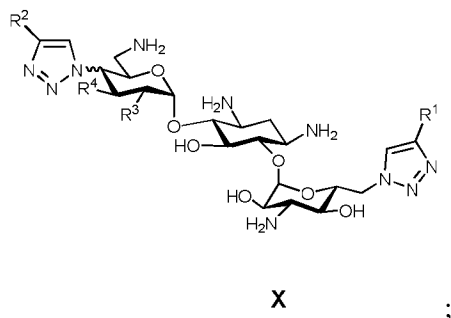
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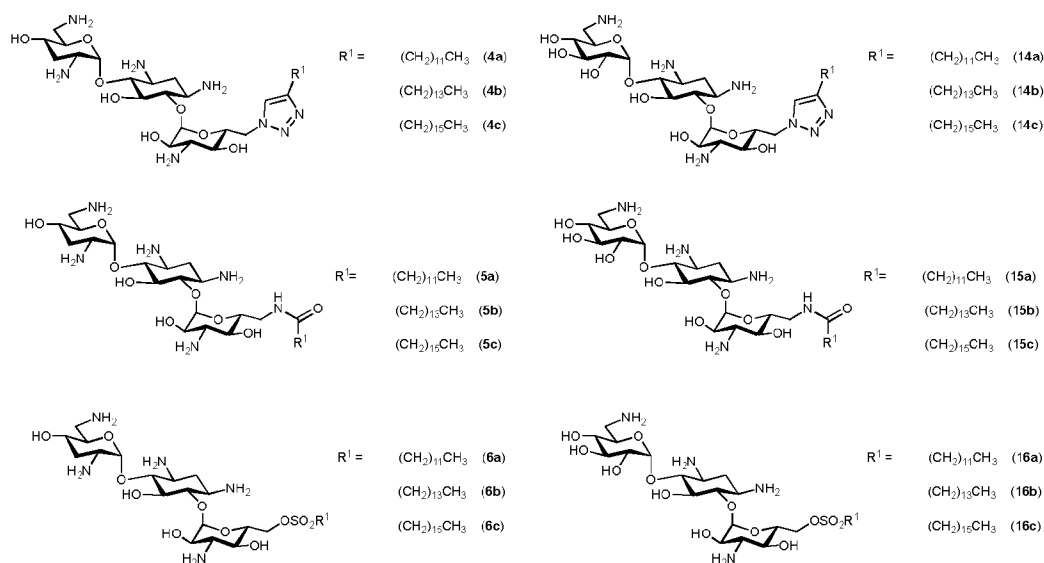
IX

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16

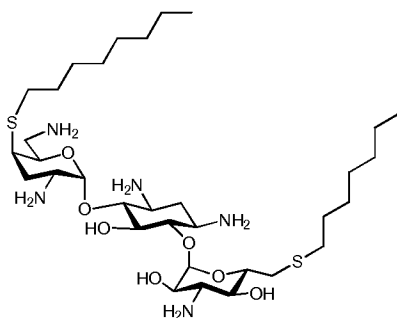


According to some embodiments, the compound is a tobramycin or kanamycin A derivative selected from the group consisting of the following structures, with each possibility representing a separate embodiment of the present invention:



In an additional preferred embodiment, the compound is represented by the structure of formula 17:

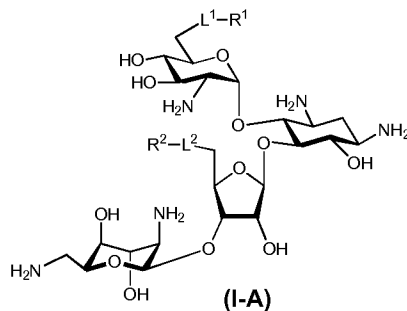
17



(17)

Paromomycin Derivatives:

In one embodiment, the present invention relates to a paromomycin derivative represented by the structure of formula I-A:



wherein

L^1 and L^2 are each independently selected from the group consisting of -S-,
-SO₂-, -OSO₂-, triazolyl and -NH-C(=O)-; and

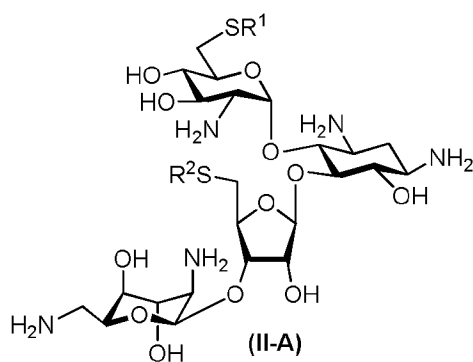
R^1 and R^2 are each independently a linear C₄ to C₈ alkyl;

including salts, solvates, polymorphs, optical isomers, geometrical isomers, enantiomers, diastereomers, and mixtures thereof.

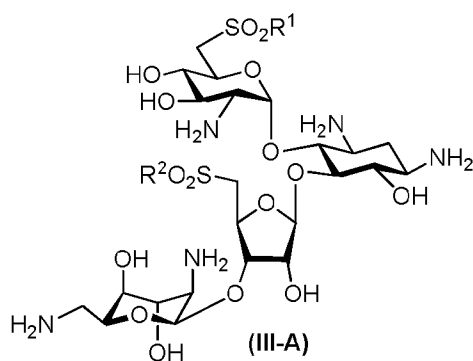
Several non-limiting embodiments of the compounds of formula I are compounds of formulae II-A, III-A, IV-A, V-A and VI-A, or any of the exemplified compounds of formula (22), (23), (24), (26a), (26b), (26c), (27a), (27b), (27c), (28a), (28b), (28c), (29a), (29b) and (29c), which are described hereinbelow. Each possibility represents a separate embodiment of the present invention.

In some embodiments, each of the linkers L^1 and L^2 is selected from a thioether, a sulfone, a sulfonyl ester, a triazolyl and an amide.

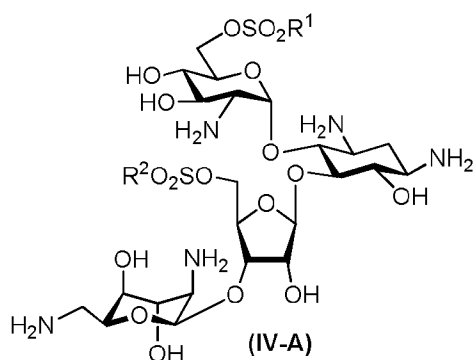
In one particular embodiment, L^1 and L^2 are each a thioether of the formula -S-, and the compound is represented by the structure of formula II-A:



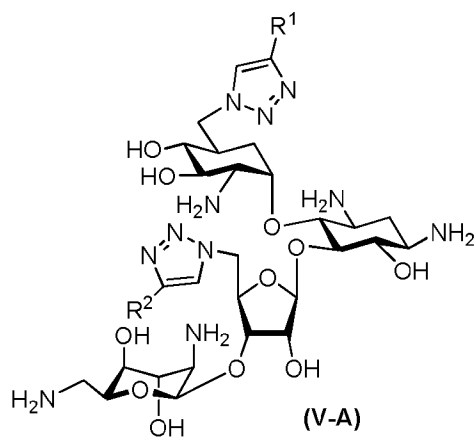
In another particular embodiment, L^1 and L^2 are each a sulfonyl of the formula -SO₂-, and the compound is represented by the structure of formula III-A:



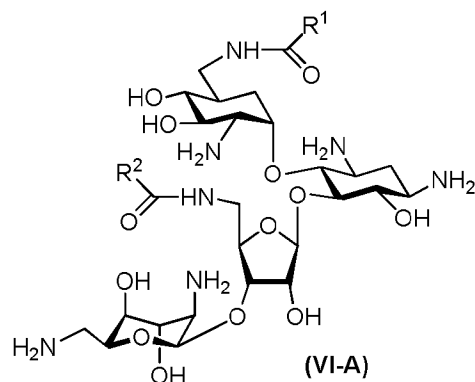
In another particular embodiment, L^1 and L^2 are each a sulfonyl ester of the formula -OSO₂-, and the compound is represented by the structure of formula IV-A:



In another particular embodiment, L^1 and L^2 are each a triazolyl, and the compound is represented by the structure of formula V-A:

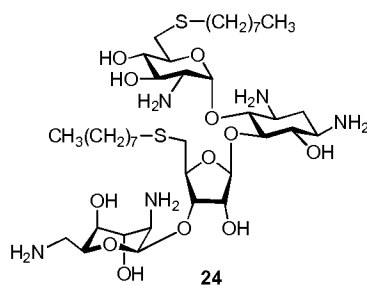
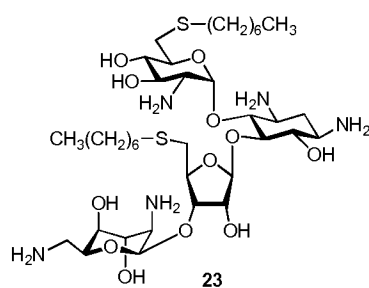
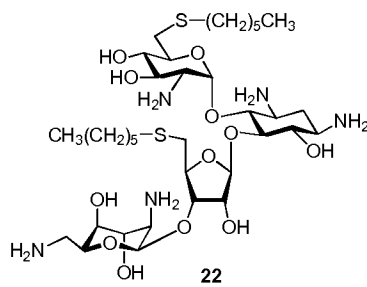


In another particular embodiment, L^1 and L^2 are each an amide of the formula -NH-C(=O)-, and the compound is represented by the structure of formula VI-A:

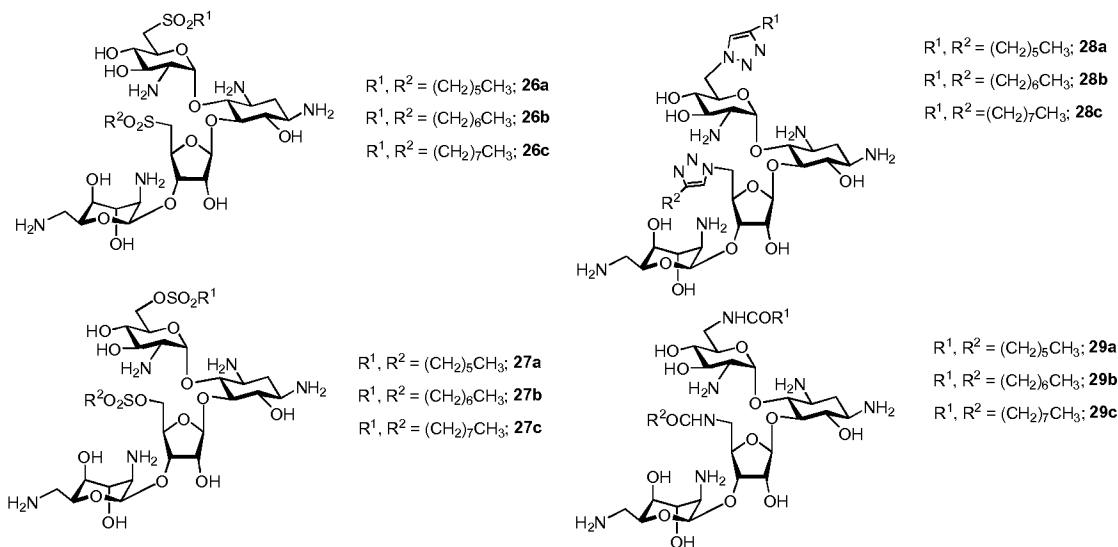


In some preferred embodiments, the compound is represented by the structure of formula 22, 23 or 24, with each possibility representing a separate embodiment of the present invention.

20



According to other embodiments, the compound is a paromomycin derivative selected from the group consisting of the following structures, with each possibility representing a separate embodiment of the present invention:



Chemical Definitions:

The term "alkyl", used herein alone or as part of another group denotes linear and branched, saturated groups having from 1 to 20 carbon atoms. Some preferred alkyl groups for use in the compounds of the present invention include linear alkyl groups having from 12 to 16 carbon atoms. Other preferred alkyl groups for use in the compounds of the present invention include linear alkyl groups having from 4 to 8 carbon atoms. The alkyl group may be unsubstituted or substituted. An alkyloxy is an alkyl that is bonded to an oxygen atom (alkyl-O). In one embodiment, the alkyl is a linear alkyl of 6 carbon atoms ($CH_3(CH_2)_5-$). In one embodiment, the alkyl is a linear alkyl of 7 carbon atoms ($CH_3(CH_2)_6-$). In one embodiment, the alkyl is a linear alkyl of 8 carbon atoms ($CH_3(CH_2)_7-$). In general, for monalkylated derivatives, the length of the alkyl group is preferably from 12 to 16 carbon atoms, and for dialkylated derivatives, the length of the alkyl group is preferably from 6 to 8 carbon atoms.

The term "aryl" used herein alone or as part of another group denotes an aromatic ring system containing from 6-14 ring carbon atoms. The aryl ring can be a monocyclic, bicyclic, tricyclic and the like. Non-limiting examples of aryl groups are phenyl, naphthyl including 1-naphthyl and 2-naphthyl, and the like. The aryl group can be unsubstituted or substituted through available carbon atoms with one or more groups defined hereinabove for alkyl. An aryloxy is an aryl that is bonded to an oxygen atom (aryl-O, e.g., phenoxy).

The term "amino protecting group" as used herein refers to a readily cleavable group bonded to an amino group. The nature of the amino protecting group is not critical so long as

the derivatized amino group is stable. Examples of amino-protecting groups include t-butoxycarbonyl (BOC), benzyloxycarbonyl, acetyl, phenylcarbonyl, or a silyl group, which can be substituted with alkyl (trialkylsilyl), with an aryl (triarylsilyl) or a combination thereof (e.g., dialkylphenylsilyl), e.g., trimethylsilyl (TMS) or t-butyldimethyl silyl (TBDMS). Other suitable amino-protecting agents and amino-protecting groups, as well as methods of protection and deprotection, have been described in, e.g., T. W. Greene and P. G. M. Wuts, *Protective Groups in Organic Synthesis*, 2nd Ed., John Wiley and Sons (1991) and A. J. Pearson and W. R. Roush, *Activating Agents and Protecting Groups*, John Wiley and Sons (1999), each of which is incorporated herein by reference.

All stereoisomers of the above compounds are contemplated, either in admixture or in pure or substantially pure form. The compounds of the present invention can have asymmetric centers at any of the atoms. Consequently, the compounds can exist in enantiomeric or diastereomeric forms or in mixtures thereof. The present invention contemplates the use of any racemates (i.e. mixtures containing equal amounts of each enantiomers), enantiomerically enriched mixtures (i.e., mixtures enriched for one enantiomer), pure enantiomers or diastereomers, or any mixtures thereof. The chiral centers can be designated as R or S or R,S or d,D, l,L or d,l, D,L. The sugar residues include residues of D-sugars, L-sugars, or racemic derivatives of sugars.

One or more of the compounds of the invention, may be present as a salt. The term "salt" encompasses both basic and acid addition salts, including but not limited to salts with amine nitrogens, and include salts formed with the organic and inorganic anions discussed below. Furthermore, the term includes salts that form by standard acid-base reactions with basic groups (such as amino groups) and organic or inorganic acids. Such acids include, but are not limited to, hydrochloric, hydrofluoric, trifluoroacetic, sulfuric, phosphoric, acetic, succinic, citric, lactic, maleic, fumaric, palmitic, cholic, pamoic, mucic, D-glutamic, D-camphoric, glutaric, phthalic, tartaric, lauric, stearic, salicylic, methanesulfonic, benzenesulfonic, sorbic, picric, benzoic, cinnamic, and like acids.

The present invention also includes solvates of the compounds of the present invention and salts thereof. "Solvate" means a physical association of a compound of the invention with one or more solvent molecules. This physical association involves varying degrees of ionic and covalent bonding, including hydrogen bonding. In certain instances the solvate will be capable of isolation. "Solvate" encompasses both solution-phase and isolatable solvates. Non-limiting

examples of suitable solvates include ethanlates, methanlates and the like. "Hydrate" is a solvate wherein the solvent molecule is water.

The present invention also includes polymorphs of the compounds of the present invention and salts thereof. The term "polymorph" refers to a particular crystalline state of a substance, which can be characterized by particular physical properties such as X-ray diffraction, IR spectra, melting point, and the like.

Therapeutic Uses

As contemplated herein, the present invention is based on the finding that tobramycin or kanamycin A derivatives of formula I, or compounds of formula II-XI or any compounds encompassed by such formulae, or dimeric structures according to formula XII as described above, are active as antibacterial agents against both Gram-positive and Gram-negative bacteria, targeting their membranes selectively, while at the same time exhibiting minimal toxicity due to non-selective targeting of eukaryotic cell membranes. In other embodiments, the present invention is based on the finding that paromomycin derivatives of formula I-A, or compounds of formula II-A to VI-A or any compounds encompassed by such formulae as described above, are active as antibacterial agents against both Gram-positive and Gram-negative bacteria, targeting their membranes selectively, while at the same time exhibiting minimal toxicity due to non-selective targeting of eukaryotic cell membranes.

The antibacterial compositions of the invention may be used for medicinal purposes and in such a case the composition is a pharmaceutical composition for the treatment of bacterial infections, e.g., bacteria that cause skin infections or soft tissue (e.g., throat) infections, or for inhibiting of biofilm growth.

Thus, in one, the present invention relates to a method of combating bacteria, or treating bacterial infections, comprising the step of administering to a subject in need thereof (1) a tobramycin or kanamycin A derivative of formula I or a compound of any of formulae II-XI or any compounds encompassed by such formulae; (2) a paromomycin derivative of formula I-A or a compound of any of formulae II-A to VI-A or any compounds encompassed by such formulae; or (3) dimeric structures according to formula XII as described herein, or a pharmaceutical composition comprising any of the aforementioned compounds. Each possibility represents a separate embodiment of the present invention.

In another aspect the present invention relates to the use of (1) a tobramycin or kanamycin A derivative of formula I or compounds of formula II-XI or any compounds encompassed by such formulae; (2) a paromomycin derivative of formula I-A or a compound of any of formulae II-A to VI-A or any compounds encompassed by such formulae; or (3) dimeric structures according to formula XII as described above, or a pharmaceutical composition comprising any of the aforementioned compounds, for the manufacture of a medicament for combating bacteria or treating bacterial infections. Each possibility represents a separate embodiment of the present invention.

In another aspect, the present invention relates to (1) a tobramycin or kanamycin A derivative of formula I or compounds of formula II-XI or any compounds encompassed by such formulae; (2) a paromomycin derivative of formula I-A or a compound of any of formulae II-A to VI-A or any compounds encompassed by such formulae; or (3) dimeric structures according to formula XII as described above, or to a pharmaceutical composition comprising any of the aforementioned compounds, for use in combating bacteria or treating bacterial infections. Each possibility represents a separate embodiment of the present invention.

The anti bacterial composition may also be used for disinfecting purposes for example of surfaces, devices (including medical devices), cultures of eukaryotic cells or tissue, water pipes and water filters, food and agricultural products. The anti bacterial compositions may also be used to inhibit biofilm growth. The present invention further concerns a method for combating bacteria by contacting the bacteria with an effective amount of (1) a tobramycin or kanamycin A derivative of formula I or compounds of formula II-XI or any compounds encompassed by such formulae; (2) a paromomycin derivative of formula I-A or a compound of any of formulae II-A to VI-A or any compounds encompassed by such formulae; or (3) dimeric structures according to formula XII as described above, or to a pharmaceutical composition comprising any of the aforementioned compounds.

The contact may be *ex vivo* on a surface, on a device, in cell/tissue culture dish, in food, water, agricultural product as described above. Alternatively the contact may be in the body of a human or non human subject.

The term "*anti-bacterial*" may refer to one or more of the following effects: killing the bacteria (bactericide), causing halt of growth of bacteria (bacteriostatic), prevention of bacterial infection, prevention of bio-film formation and disintegration of a formed biofilm, and decrease in bacterial virulence.

Examples of bacterial strain that can be treated/disinfected by the composition of the invention (both as a disinfecting composition and as a pharmaceutical composition) are all Gram-negative and Gram-positive bacteria and in particular pathogenic gram negative and gram positive bacteria.

The term "*combating bacteria*" or "*treating bacterial infection*" may refer to one of the following: decrease in the number of bacteria, killing or eliminating the bacteria, inhibition of bacterial growth (stasis), inhibition of bacterial infestation, inhibition of biofilm formation, disintegration of existing biofilm, or decrease in bacterial virulence.

The compounds of the present invention are effective against a wide variety of Gram-positive and Gram-negative cells, non-limiting examples of which include

Gram-positive: *Streptococcus pyogenes* M12 (strain MGAS9429), *Methicillin-resistant Staphylococcus aureus* (MRSA), *Streptococcus mutans* UA 159, *vancomycin-resistant enterococci* (VRE), *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* (Oxford strain ATCC9144), *Staphylococcus epidermidis* ATCC 35984, and *Staphylococcus epidermidis* ATCC 12228, *S. aureus oxford* NCTC6571; *S. aureus* Cowan ATCC12598; **G**, *S. pyogenes* M1T1; **H**, *S. pyogenes* M2; **I**, *S. pyogenes* M3; **J**, *S. pyogenes* M5; **K**, *S. pyogenes* M24; **L**, *S. pyogenes* JRS75; **M**, *S. pyogenes* glossy; and **N**, *S. pyogenes* T5.

Gram-negative: *Pseudomonas aeruginosa* ATCC33347, *Shigella sonnei* clinical isolate 6831(*O*-antigen positive), and *Shigella sonnei* clinical isolate 6831 (*O*-antigen negative).

The methods of the invention both *ex-vivo* and in the body of the subject may further comprise co administration of at least one additional anti-bacterial agent such as state of the art antibiotics.

As used herein, the term "administering" refers to bringing in contact with a compound of the present invention. Administration can be accomplished to cells or tissue cultures, or to living organisms, for example humans. In one embodiment, the present invention encompasses administering the compounds of the present invention to a human subject.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs of pathology for the purpose of diminishing or eliminating those signs. A "therapeutically effective amount" is that amount of compound which is sufficient to provide a beneficial effect to the subject to which the compound is administered.

Pharmaceutical Compositions

Although the compounds of the present invention can be administered alone, it is contemplated that such compounds will be administered in pharmaceutical compositions further containing at least one pharmaceutically acceptable carrier or excipient. Where combination treatments are used, each of the components can be administered in a separate pharmaceutical composition, or the combination can be administered in one pharmaceutical composition.

Thus, in one embodiment, the present invention contemplates pharmaceutical compositions that comprise a compound of formula I, and a pharmaceutically acceptable excipient. In one embodiment, the compound is represented by the structure of formula II. In another embodiment, the compound is represented by the structure of formula III. In another embodiment, the compound is represented by the structure of formula IV. In another embodiment, the compound is represented by the structure of formula V. In another embodiment, the compound is represented by the structure of formula VI. In another embodiment, the compound is represented by the structure of formula VII. In another embodiment, the compound is represented by the structure of formula VIII. In another embodiment, the compound is represented by the structure of formula IX. In another embodiment, the compound is represented by the structure of formula X. In another embodiment, the compound is represented by the structure of formula XI. In another embodiment, the compound is a dimeric structure represented by the structure of formula XII.

In another embodiment, the present invention contemplates pharmaceutical compositions that comprise a compound of formula IA, and a pharmaceutically acceptable excipient. In another embodiment, the compound is represented by the structure of formula II-A. In another embodiment, the compound is represented by the structure of formula III-A. In another embodiment, the compound is represented by the structure of formula IV-A. In another embodiment, the compound is represented by the structure of formula V-A. In another embodiment, the compound is represented by the structure of formula VI-A. Each possibility represents a separate embodiment of the present invention.

The pharmaceutical compositions of the present invention can be formulated for administration by a variety of routes including oral, rectal, transdermal, parenteral (subcutaneous, intraperitoneal, intravenous, intra-arterial, transdermal and intramuscular), topical, intranasal, or via a suppository. Such compositions are prepared in a manner well

known in the pharmaceutical art and comprise as an active ingredient at least one compound of the present invention as described hereinabove, and a pharmaceutically acceptable excipient or a carrier. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals and, more particularly, in humans.

During the preparation of the pharmaceutical compositions according to the present invention the active ingredient is usually mixed with a carrier or excipient, which may be a solid, semi-solid, or liquid material. The compositions can be in the form of tablets, pills, capsules, pellets, granules, powders, lozenges, sachets, cachets, elixirs, suspensions, dispersions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active compound, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders.

The carriers may be any of those conventionally used and are limited only by chemical-physical considerations, such as solubility and lack of reactivity with the compound of the invention, and by the route of administration. The choice of carrier will be determined by the particular method used to administer the pharmaceutical composition. Some examples of suitable carriers include lactose, glucose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water and methylcellulose. The formulations can additionally include lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents, surfactants, emulsifying and suspending agents; preserving agents such as methyl- and propylhydroxybenzoates; sweetening agents; flavoring agents, colorants, buffering agents (e.g., acetates, citrates or phosphates), disintegrating agents, moistening agents, antibacterial agents, antioxidants (e.g., ascorbic acid or sodium bisulfite), chelating agents (e.g., ethylenediaminetetraacetic acid), and agents for the adjustment of tonicity such as sodium chloride. Other pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like, polyethylene glycols, glycerin, propylene glycol or other synthetic solvents. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions.

In one embodiment, in the pharmaceutical composition the active ingredient is dissolved

in any acceptable lipid carrier (e.g., fatty acids, oils to form, for example, a micelle or a liposome).

In some embodiments, nanocarriers are used to effectuate intracellular uptake or transcellular transport of the compounds of the invention. Nanocarriers are miniature devices or particles that can readily interact with biomolecules on cell surfaces and within cells. Pharmaceutical nanocarriers such as viral vectors, polymeric nanoparticles, and liposomes are advantageous for delivering pharmaceutically active agents more selectively to target cells. Nanocarriers also effectively enhance the delivery of poorly-soluble therapeutics and control the release rate of encapsulated compounds. Many nanocarriers are natural or synthetic polymers that have defined physical and chemical characteristics. As a consequence, a person skilled in the art can engineer desired properties, such as target selectivity, biodegradability, biocompatibility, and responsiveness to environmental factors (e.g., pH or temperature changes), into nanocarriers to improve performance. Any nanocarriers can be used in the context of the present invention.

For preparing solid compositions such as tablets, the principal active ingredient(s) is mixed with a pharmaceutical excipient to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention. When referring to these preformulation compositions as homogeneous, it is meant that the active ingredient is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules. This solid preformulation is then subdivided into unit dosage forms of the type described above containing from, for example, from about 0.1 mg to about 2000 mg, from about 0.1 mg to about 500 mg, from about 1 mg to about 100 mg, from about 100 mg to about 250 mg, etc. of the active ingredient(s) of the present invention.

Any method can be used to prepare the pharmaceutical compositions. Solid dosage forms can be prepared by wet granulation, dry granulation, direct compression and the like. The solid dosage forms of the present invention may be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer, which serves to resist disintegration in the stomach and permit the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for

such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol, and cellulose acetate.

The liquid forms in which the compositions of the present invention may be incorporated, for administration orally or by injection, include aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored emulsions with edible oils such as cottonseed oil, sesame oil, coconut oil, or peanut oil, as well as elixirs and similar pharmaceutical vehicles.

Compositions for inhalation include solutions and suspensions in pharmaceutically acceptable aqueous or organic solvents, or mixtures thereof, and powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as described above. Preferably the compositions are administered by the oral or nasal respiratory route for local or systemic effect. Compositions in preferably pharmaceutically acceptable solvents may be nebulized by use of inert gases. Nebulized solutions may be breathed directly from the nebulizing device or the nebulizing device may be attached to a face masks tent, or intermittent positive pressure breathing machine. Solution, suspension, or powder compositions may be administered, preferably orally or nasally, from devices that deliver the formulation in an appropriate manner.

Another formulation employed in the methods of the present invention employs transdermal delivery devices ("patches"). Such transdermal patches may be used to provide continuous or discontinuous infusion of the compounds of the present invention in controlled amounts. The construction and use of transdermal patches for the delivery of pharmaceutical agents is well known in the art.

In yet another embodiment, the composition is prepared for topical administration, e.g. as an ointment, a gel a drop or a cream. For topical administration to body surfaces using, for example, creams, gels, drops, ointments and the like, the compounds of the present invention can be prepared and applied in a physiologically acceptable diluent with or without a pharmaceutical carrier. The present invention may be used topically or transdermally bacterial infections. Adjuvants for topical or gel base forms may include, for example, sodium carboxymethylcellulose, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol and wood wax alcohols.

Alternative formulations include nasal sprays, liposomal formulations, slow-release formulations, pumps delivering the drugs into the body (including mechanical or osmotic

pumps) controlled-release formulations and the like, as are known in the art.

The compositions are preferably formulated in a unit dosage form. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

In preparing a formulation, it may be necessary to mill the active ingredient to provide the appropriate particle size prior to combining with the other ingredients. If the active compound is substantially insoluble, it ordinarily is milled to a particle size of less than 200 mesh. If the active ingredient is substantially water soluble, the particle size is normally adjusted by milling to provide a substantially uniform distribution in the formulation, e.g. about 40 mesh.

It may be desirable to administer the pharmaceutical composition of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, infusion to the liver via feeding blood vessels with or without surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material.

The compounds may also be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.), and may be administered together with other therapeutically active agents. It is preferred that administration is localized, but it may be systemic. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

A compound of the present invention can be delivered in an immediate release or in a controlled release system. In one embodiment, a compound of the invention is administered in combination with a biodegradable, biocompatible polymeric implant, which releases the compound over a controlled period of time at a selected site. Examples of preferred polymeric materials include polyanhydrides, polyorthoesters, polyglycolic acid, polylactic acid, polyethylene vinyl acetate, copolymers and blends thereof (See, Medical applications of

controlled release, Langer and Wise (eds.), 1974, CRC Pres., Boca Raton, Fla.). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, thus requiring only a fraction of the systemic dose.

Furthermore, at times, the pharmaceutical compositions may be formulated for parenteral administration (subcutaneous, intravenous, intraarterial, transdermal, intraperitoneal or intramuscular injection) and may include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Oils such as petroleum, animal, vegetable, or synthetic oils and soaps such as fatty alkali metal, ammonium, and triethanolamine salts, and suitable detergents may also be used for parenteral administration. Further, in order to minimize or eliminate irritation at the site of injection, the compositions may contain one or more nonionic surfactants. Suitable surfactants include polyethylene sorbitan fatty acid esters, such as sorbitan monooleate and the high molecular weight adducts of ethylene oxide with a hydrophobic base, formed by the condensation of propylene oxide with propylene glycol.

The parenteral formulations can be presented in unit-dose or multi-dose sealed containers, such as ampoules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described and known in the art.

Doses and Dosing Schedules

The amount of a compound of the invention that will be effective in the treatment of a particular bacterial infection will depend on the nature of the infection, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. A preferred dosage will be within the range of 0.01-1000 mg/kg of body weight, more preferably, 0.1mg/kg to 100 mg/kg and even more preferably 1 mg/kg to

10mg/kg. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test bioassays or systems.

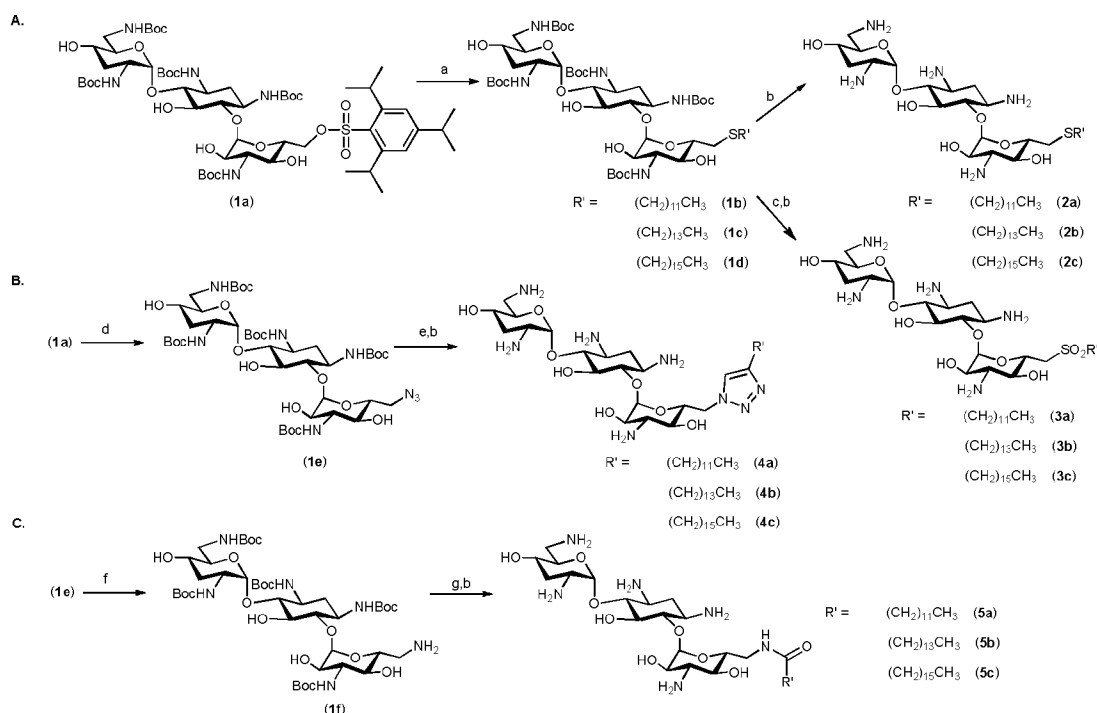
The administration schedule will depend on several factors such as the bacterial infection being treated, the severity and progression, the patient population, age, weight etc. For example, the compositions of the invention can be taken once-daily, twice-daily, thrice daily, etc.

EXAMPLES

The following are non-limiting examples for the preparation of AE-based compounds attached to amino sugars and amino carba-sugars. It is apparent to a person of skill in the art that the present invention is not limited to the currently recited synthetic schemes and that variations in reaction conditions and reagents are possible and are encompassed by the broad scope of the present invention.

Example 1 - Preparation of Amphiphilic Tobramycin and Kanamycin A Aminoglycoside Derivatives

Scheme 1 generally represents various embodiments of the amphiphilic tobramycin and kanamycin A aminoglycoside derivatives of the present invention. Such derivatives may be prepared in accordance with the processes as described herein. Some currently preferred embodiments are described hereinbelow, while it is understood that various modifications in the synthetic scheme such as the choice of protecting groups, reagents and solvents are encompassed within the scope of the present invention.



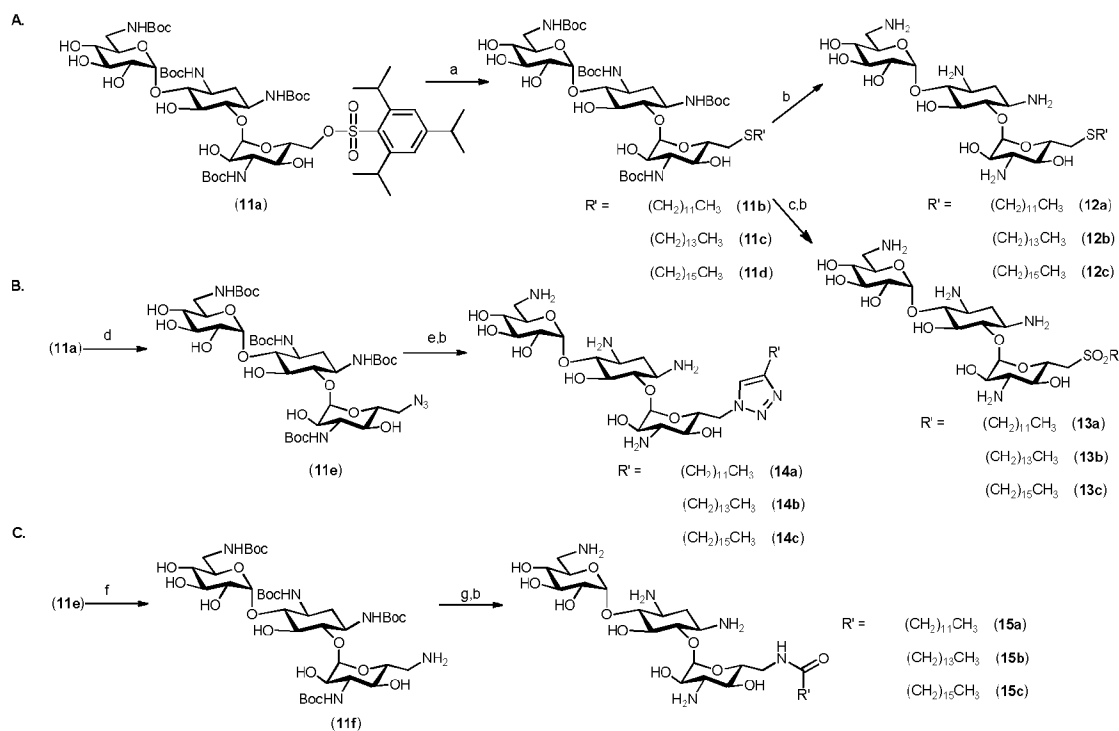
Scheme 1: Synthesis of amphiphilic tobramycin analogues: *Reagents and conditions:* (a) R'SH, Cs₂CO₃, DMF, 25-60°C, 63-92%; (b) neat TFA, rt, 97%-quantitative yields; (c) *m*CPBA (3 equiv), CHCl₃, r.t. 70-99%; (d) NaN₃, DMF, 60°C, 12 h, 91%; (e) R'CCH, CuSO₄·5H₂O (0.1 equiv), sodium ascorbate (0.2 equiv), DMF, microwave irradiation, 87-94%; (f) PMe₃ (1M in THF), aqueous NaOH, rt, 80%; (g) R'COOH, HBTU, DIEA, DMF, 71-86%.

It is noted that other nitrogen protecting groups as defined herein may be used instead of the BOC protecting group described hereinabove, with suitable protection and deprotection methods being utilized as known in the art.

The most potent and broad-spectrum antimicrobial activity was observed for thioether analogues containing C₁₂-, C₁₄-, and C₁₆-linear alkyl chains (Scheme 1A, **2a-c**). The aliphatic chain length affected not only antimicrobial activity but also the level of undesired red blood cell (RBC) hemolysis; the C₁₂ analogue had the least hemolytic activity. It was hypothesized that the aliphatic alkyl chains and the AG scaffold are required for optimal antimicrobial activity but that altering the link between these two segments should not have a dramatic effect on the antimicrobial performance, yet may affect the specificity of these compounds towards different membranes. It was further contemplated that linking the AG scaffold to the aliphatic chain through different chemical groups may affect the binding interactions with specific membrane components, therefore affecting the specificity of these compounds towards bacterial and RBC membranes. To test this using the tobramycin scaffold as an exemplary

scaffold, several types of chemical bonds between the aliphatic chain and tobramycin were evaluated. Thioether-linked analogues (Scheme 1A, **2a-c**) were compared to sulfone-linked analogues (**3a-c**), triazole ring-linked analogues (Scheme 1B, **4a-c**), and amide bond-linked analogues (Scheme 1C, **5a-c**). The thioethers **2a-c** were prepared from penta *NH*-Boc protected-6''-*O*-trisyl tobramycin (Scheme 1A, **1a**) as previously reported.^{18, 19} Oxidation of the protected thioether analogues (**1b-d**) using *m*CPBA (70-99% yield) followed by the removal of the *NH*-Boc protecting groups in neat TFA, gave the sulfone analogues (**3a-c**). The 6''-*O*-trisyl group of **1a** was replaced by an azide to yield compound **1e**,²⁰ which served as a precursor for the preparation of the triazole analogues (Scheme 1B, **4a-c**). Microwave-heated click reaction using **1e** and terminal alkynyl aliphatic chains, followed by the removal of the *NH*-Boc groups, gave the desired triazole analogues **4a-c**. Reduction of the 6''-azido group of **1e** using the Staudinger reaction conditions resulted in superior yields (80%) of the 6''-amino tobramycin analogue **1f** (Scheme 1C) compared to the reduction of the azide under catalytic hydrogenation conditions (H₂, Pd/C, MeOH), although both methods can be used to prepare this compound. Compound **1f** served as the precursor for the preparation of the amide analogues (Scheme 1C); **1f** was coupled to linear aliphatic carboxylic acids using HBTU (71-86% yield), and the *NH*-Boc groups were removed to yield the amide-linked analogues (Scheme 1C, **5a-c**).

It is understood that, although Scheme 1 is described with respect to the synthesis of tobramycin analogs, similar compounds and reactions can be obtained using the kanamycin A scaffold. Scheme 2 generally represents various aminoglycosides of the present invention that are based on the kanamycin A scaffold.



Scheme 2: Synthesis of amphiphilic kannamycin A analogues: *Reagents and conditions:* (a) R'SH, Cs₂CO₃, DMF, 25-60°C; (b) neat TFA, rt; (c) *m*CPBA (3 equiv), CHCl₃, r.t.; (d) NaN₃, DMF, 60°C, 12 h; (e) R'CCH, CuSO₄·5H₂O (0.1 equiv), sodium ascorbate (0.2 equiv), DMF, microwave irradiation; (f) PMe₃ (1M in THF), aqueous NaOH, rt.; (g) R'COOH, HBTU, DIEA, DMF.

Example 2 - Results: Tobramycin Derivatives

The minimum inhibitory concentrations (MICs) of the semi-synthetic tobramycin amphiphiles were determined for 11 Gram-positive and Gram-negative strains (Table 1) (see Example 4 for experimental conditions). Amongst the Gram-positive bacteria were pathogenic strains such as *Streptococcus pyogenes* M12 (strain A), a hospital isolate of methicillin-resistant *Staphylococcus aureus* (MRSA; strain B), and vancomycin-resistant *Enterococcus* (VRE; strain D) with high levels of resistance to tobramycin (MIC = 64 µg/mL for strain A and >128 µg/mL for strains B and D). Amongst the Gram-negative isolates were the pathogenic and highly tobramycin resistant (MIC > 128 µg/mL) *Pseudomonas aeruginosa* (ATCC 33347; strain I) and *Shigella sonnei*, which is responsible for the severe foodborne disease shigellosis. Two types of *S. sonnei* were tested: *O*-antigen positive (strain J), and *O*-antigen negative (strain K) (Shepherd, 2000). In general, analogues with a C₁₄ linear aliphatic chain (2b, 3b, 4b,

and **5b**) exhibited the most potent antimicrobial activity, which was in most cases one to two double dilutions more potent than the activity of the corresponding C₁₂ and C₁₆ linear aliphatic chain analogues. The chemical links between the AG and the aliphatic chain did not have significant effects on MIC values against the tested strains with the exception of the sulfone linked analogues **3a-c**. These analogues were somewhat less potent than the corresponding unoxidized thioether analogues **2a-c**. Some of the amphiphilic tobramycin analogues demonstrated high potency against strains that were highly tobramycin resistant: The MIC of tobramycin against *S. pyogenes* M12 (strain **A**) was 64 µg/mL; the thioether **2b**, triazole **4b**, and the amide analogue **5b** were 32 and 16 times more potent against this strain, respectively. A significant improvement in antimicrobial activity of the semi-synthetic analogues compared to that of tobramycin was also observed in the case of *S. mutans* UA159 and *S. epidermis* ATCC35984 (strains **C** and **G**, respectively). Although most of the synthetic analogues were not active against the tested *P. aeruginosa* (strain **I**), the C₁₂ chain triazole analogue **4a** and amide analogue **5a** demonstrated improved antimicrobial activity against this strain relative to tobramycin (MICs = 64 and 32 µg/mL, respectively, and MIC >128 µg/mL for tobramycin). The antibacterial activity of tobramycin and six out of the twelve synthetic analogues was better against *O*-antigen positive *S. sonnei* (strain **J**) than against the corresponding *O*-antigen negative strain **K**. This difference may be explained by the higher overall negative charge of the membrane of the *O*-antigen positive *S. sonnei*, which contains the negatively charged 2-acetamido-2-deoxy-L-altruronic acid (Liu, 2010).

Table 1: MIC values (µg/mL) of tobramycin and its amphiphilic analogues

AG ^a	MICs (µg/ml) for tested bacterial strains ^b										
	A	B	C	D	E	F	G	H	I	J	K
1	64	>128	128	>128	>128	16	128	<1	>128	16	32
2(a)	8	64	8	64	128	16	8	8	128	32	64
2(b)	2	32	2	16	64	8	4	4	128	16	32
2(c)	4	64	4	64	128	16	8	4	>128	32	64
3(a)	32	>128	16	64	>128	64	64	128	>128	>128	>128
3(b)	4	64	4	32	64	16	16	16	128	32	64

3(c)	8	32	8	32	64	8	16	16	>128	32	128
4(a)	8	128	4	32	128	16	8	16	64	64	64
4(b)	4	64	4	32	128	8	8	4	128	32	32
4(c)	16	32	8	16	128	16	8	4	>128	128	64
5(a)	16	128	4	64	128	16	8	8	32	128	128
5(b)	4	32	4	32	64	8	4	4	128	16	32
5(c)	8	32	4	32	128	8	4	4	128	32	32

^a AG = Aminoglycoside. ^b MIC values were determined against Gram-positive bacterial strains: **A**, *S. pyogenes* serotype M12 (strain MGAS9429); **B**, MRSA; **C**, *S. mutans* UA159; **D**, VRE; **E**, *E. faecalis* ATCC29212; **F**, *S. aureus* ATCC9144; **G**, *S. epidermis* ATCC35984; **H**, *S. epidermis* ATCC12228 and Gram-negative bacterial strains: **I**, *P. aeruginosa* ATCC33347; **J**, *S. sonnei* clinical isolate 6831 (*O*-antigen positive); and **K**, *S. sonnei* clinical isolate 6831 (*O*-antigen negative). All strains were tested by using the double-dilution method (from a starting concentration of 128 µg/mL). All experiments were performed in triplicate, and results were obtained from two different sets of experiments.

It was previously demonstrated that low micromolar concentrations of saturated fatty acids inhibit the formation of biofilms formed by *S. aureus* and *Listeria monocytogenes* strains (Nguyen, 2012). This activity may be explained by the fact that the amphiphilic structures of fatty acids destabilize interactions between protein and polysaccharide components that form biofilm matrices. Interestingly, the most potent inhibitors were C₁₂-C₁₄ aliphatic chain carboxylic acids. The applicants have therefore determined the minimal biofilm inhibition concentration (MBIC) values for each of the C₁₂ and C₁₄ chain tobramycin analogues (Table 2) (see Example 5 for experimental conditions). MBIC tests were performed using *S. mutans* UA 159 and *S. epidermidis* ATCC 35984 grown under biofilm-forming conditions. Compared to tobramycin (MBIC range of 64-128 µg/mL), the tested analogues demonstrated improved biofilm growth inhibition properties (MBIC range of 4-32 µg/mL) against the tested strains (Table 2).

Table 2: Biofilm growth inhibition. MBIC values ($\mu\text{g/mL}$) of the amphiphilic tobramycin analogues and tobramycin (**1**).

Strain ^b	AG ^a								
	1	2a	2b	3a	3b	4a	4b	5a	5b
C	64	4	4	16	4	4	4	8	4
G	128	16	8	32	8	16	16	8	8

^a AG = Aminoglycoside. ^b *S. mutans* UA159; **C**, *S. epidermis* ATCC35984; **G**. All strains were tested by using the double-dilution method (starting form 128 $\mu\text{g/mL}$). *S. mutans* biofilm was grown in BHI+Sucrose 2%, at final dilution 1:100. *S. epidermidis* biofilm was grown in TSB+Glucose 1%, at final dilution 1:100. Biofilms were stained using crystal violet. All experiments were performed in triplicates and results were obtained from two different sets of experiments.

Finally, the hemolytic activity was determined using a hemolysis assay using laboratory rat RBCs (Figure 2A-C) (see Example 6 for experimental details).¹⁸ The MIC and MBIC values were significantly lower than the concentrations required for 100% hemolysis for all analogues (Figure 2). In most cases, the MIC range of analogues with the C₁₄ aliphatic chain was 2-32 $\mu\text{g/mL}$; these analogues caused significant hemolysis (~23-43%) at 32 $\mu\text{g/mL}$. All of the C₁₄ aliphatic chain analogues caused extensive hemolysis (74.4 $\pm$ 5.5%-100%) at a concentration of 64 $\mu\text{g/mL}$. The C₁₆ aliphatic chain analogues also caused high levels of hemolysis at 64 $\mu\text{g/mL}$ 37.9 $\pm$ 5.1%- 81.8 $\pm$ 2.3%).

No direct correlation between the antibacterial potency and the hemolytic activity was detected for the thioether, triazole, or amide analogues. As initially hypothesized, the hemolytic activity of the different tobramycin analogues was affected by the type of bond between the aliphatic chain and the AG scaffold. The biggest effect was observed for the C₁₂ aliphatic chain analogues. At 64 $\mu\text{g/mL}$ the triazole C₁₂ aliphatic chain analogue **4a** demonstrated the highest hemolytic effect (26.3 $\pm$ 2.7%) of the C₁₂ aliphatic chain tobramycin analogues. The C₁₂ aliphatic chain amide analogue **5a** caused almost no hemolysis at the same concentration (0.0 $\pm$ 0.4%). At 128 $\mu\text{g/mL}$, the triazole analogue **4a** caused extensive hemolysis (89.1 $\pm$ 1.6%), the thioether **2a** caused 71.6 $\pm$ 8.3% hemolysis, yet the amide analogue **5a** caused significantly less hemolysis (10.2 $\pm$ 0.8%). The lowest hemolytic activity at all of the tested concentrations was

observed for the C₁₂ sulfone analogue **3a**, however, this compound had poor antimicrobial activity against the tested strains. In contrast, while the C₁₂ amide analogue **5a** was potent against several of the tested bacterial strains, and was the most potent analogue against the tested *P. aeruginosa* (strain I), it caused the lowest levels of hemolysis at a concentration which was 16-32 times higher than the MIC values of this compound against several of the tested strains.

In conclusion, twelve 6"-aliphatic chain tobramycin analogues differing in the chemical linkage between the AG and the hydrophobic chain (thioether, sulfone, sulfonyl, triazole, and amide bonds) and in the length of their hydrophobic linear aliphatic chain (C₁₂, C₁₄, and C₁₆ chains) were synthesized and evaluated for their antimicrobial activity against eleven bacterial strains. Of the three chain lengths tested, the C₁₄ aliphatic chain analogues were the most potent antimicrobial agents, and were in most cases one or two double dilutions more potent than corresponding C₁₂ and C₁₆ chain analogues. In some cases, the antimicrobial activity was at least 32 fold more potent than that of the parent AG tobramycin. MBIC tests indicated that some of the compounds possessed biofilm growth inhibition properties that were significantly more potent than that of tobramycin. Finally, RBC hemolysis tests revealed that there was not a linear correlation between the antimicrobial potency and the hemolytic activity of the amphiphilic tobramycin analogues. Both the aliphatic chain length and the type of chemical linkage between the hydrophilic and hydrophobic parts of the molecule affect the specificity towards bacterial membranes. The C₁₂ linear aliphatic chain 6"-amide analogue **5a** is of particular interest. This analogue was significantly more potent than tobramycin and caused little measurable hemolysis of laboratory rat RBCs at concentrations up to 32 times higher than the MIC values of this compound against some of the tested strains. The results of this study demonstrate that the choice of the hydrophobic segment and of the chemical group that links the hydrophobic region to the AG are important factors in the design of such membrane targeting antibiotics.

Example 3: Synthetic Methods: Tobramycin Derivatives

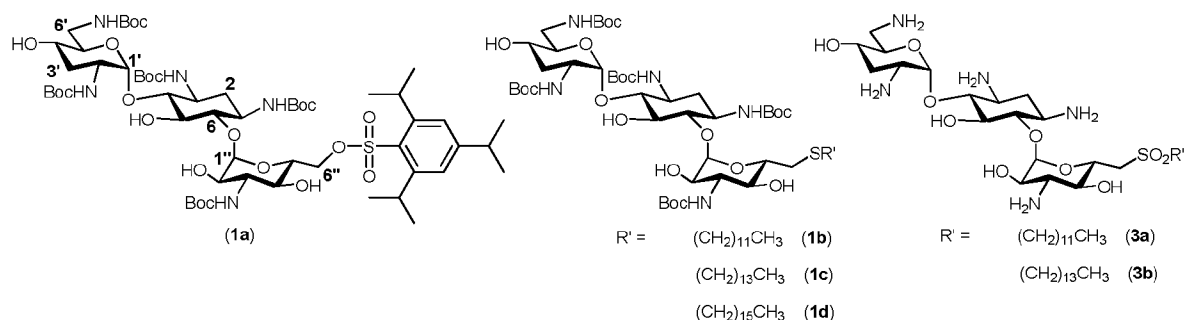
General information.

¹H NMR spectra (including 1D-TOCSY) were recorded on a Bruker Avance™ 400 spectrometer, and chemical shifts reported (in ppm) were calibrated to CD₃OD (δ = 3.31) with CD₃OD as the solvent, and to HOD (δ = 4.63) with D₂O as the solvent. ¹³C NMR spectra were

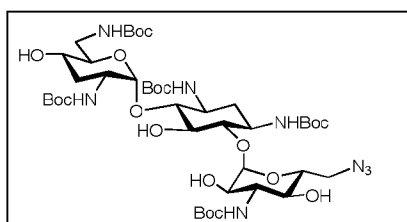
recorded on a Bruker AvanceTM 400 spectrometer at 100.6 MHz. Multiplicities are reported using the following abbreviations: br = broad, s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, t = triplet, dt = doublet of triplets, app. q = appears quartet, m = multiplet, and td = triplet of doublets. 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 F₂₅₄). Visualization was achieved using a cerium-molybdate stain ((NH₄)₂Ce(NO₃)₆ (5 g), (NH₄)₆Mo₇O₂₄•4H₂O (120 g), H₂SO₄ (80 mL), H₂O (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. Tobramycin was bought from Tzamal D-Chem Laboratories Ltd. Compound **1a** was prepared as previously described (Michael, 1999).

Compounds **1b**, **1c**, **1d**, **3a** and **3b** were prepared as previously reported (Herzog, 2012). Compounds were purified flash chromatography (SiO₂, Merck, Kieselgel 60). *S. pyogenes* serotype M12 (strain MGAS9429) (**A**), methicillin-resistant *S. aureus* (MRSA) (**B**), and vancomycin-resistant *Enterococcus* (VRE) (**D**) were a gift from Prof. Itzhak Ofek (Faculty of Medicine, Tel Aviv University). *S. mutans* UA159 (**C**) was a gift from Prof. Doron Steinberg (Faculty of Dental Medicine, The Hebrew University of Jerusalem). *E. faecalis* ATCC29212 (**E**), *S. aureus* ATCC9144 Oxford strain (**F**), *S. epidermidis* ATCC35984 (**G**), *S. epidermidis* ATCC12228 (**H**), and *P. aeruginosa* ATCC33347 (**I**) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *S. sonnei* clinical isolate 6831(O-antigen positive) (**J**), and *S. sonnei* clinical isolate 6831 (O-antigen negative) (**K**) were a gift from Prof. Dani Cohen (School of Public Health, Tel Aviv University).

Scheme 3: Tobramycin-based precursors and numbering system demonstrated for compound **1a**.

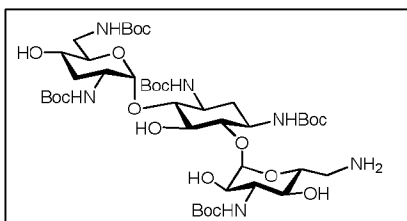


2. Synthetic procedures



Compound 1e: Compound 1e was prepared as previously reported (Disney, 2007) with the following changes: Compound 1a (2.3 gr, 1.9 mmol) in DMF (12 mL) was added sodium azide (197 mg, 3.0 mmol) and the reaction was stirred under argon at 75°C overnight.

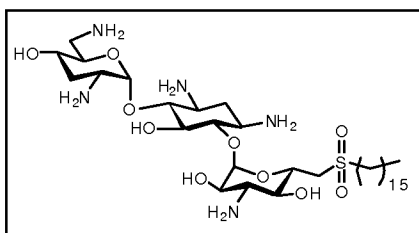
Propagation of the reaction was monitored by TLC (EtOAc/petroleum ether: 7/3). Upon completion, the solvent was removed by evaporation under reduced pressure; the crude was re-dissolved in EtOAc (80 mL), washed twice with brine (2x60 mL), dried over anhydrous MgSO₄ and concentrated under reduced pressure. Purification by flash column chromatography (SiO₂, EtOAc: petroleum ether) afforded compound 1e as a white solid (1.68, 91%); LR-ESI *m/z* calc'd for C₄₃H₇₆N₈O₁₈ 992.53, found 994.07 [M+H]⁺.



Compound 1f: Compound 1e (470 mg, 0.47 mmol) was dissolved in THF (2 mL), NaOH 0.01M (100 μL) and stirred at ambient temperature for 10 minutes after which PMe₃ (1M solution in THF, 0.5 mL, 0.50 mmol) was added to the reaction mixture. Propagation of the reaction

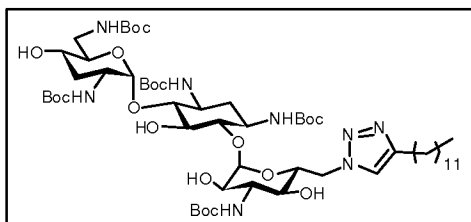
was monitored by TLC (EtOAc/petroleum ether: 7/3). Upon completion, the solvent was removed by evaporation under reduced pressure, and the crude was purified by flash column chromatography (SiO₂, MeOH: CH₂Cl₂) to afford compound 1f (366 mg, 80%) as a white solid: ¹H NMR (400 MHz, D₂O) (Fig. S1) δ 5.10 (br s, 2H, H-1', H-1''), 3.95 (ddd, *J*₁ = 9.8 Hz, *J*₂ = 7.0 Hz, *J*₃ = 3.0 Hz, 1H, H-5''), 3.69 (t, *J* = 10.2 Hz, 1H, H-3''), 3.65-3.35 (m, 11H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6'(2H), H-2''), 3.18 (t, *J* = 9.8 Hz, 1H, H-4''), 3.02 (br

d, $J = 13.0$ Hz, 1H, H-6''), 2.70 (dd, $J_1 = 13.5$ Hz, $J_2 = 6.9$ Hz, 1H, H-6''), 2.10 (br d, $J = 10.6$ Hz, 1H, H-2eq), 2.01 (m, 1H, H-3'eq), 1.65 (app. q, $J_1 = J_2 = J_3 = 12.1$ Hz, 1H, H-3'ax), 1.52-1.40 (m, 46H, H-2ax, 5xCO₂C(CH₃)₃); ¹³C NMR (100 MHz, CD₃OD) (Fig. S2) δ 159.5, 159.3, 157.9, 157.8, 99.4 (anomeric C), 99.3 (anomeric C), 82.8, 80.7, 80.5, 80.4, 80.2, 76.9, 74.1, 73.5, 72.1, 71.5, 66.4, 57.2, 51.6, 51.1, 51.0, 43.5, 42.0, 35.9, 34.3, 28.9, 28.8; LR-ESI m/z calc'd for C₄₃H₇₈N₆O₁₈ 966.54, found 967.94 [M+H]⁺

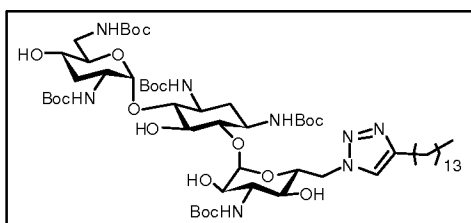


Compound 3c: Compound **1d** (100 mg, 0.08 mmol) in CHCl₃ (3 mL) was treated with *m*-chloroperbenzoic acid (70-75%) (54 mg, ~0.31 mmol), and stirred at ambient temperature under argon atmosphere. Propagation of the reaction was monitored by ESI-MS by following the

disappearance of the starting material ([M+H]⁺, m/z 1209.43) and the formation of the corresponding sulfone ([M+H]⁺, m/z 1241.15). Upon completion, the reaction mixture was diluted with CHCl₃ (15 mL), washed twice with a 1M water solution of KOH (5 mL), concentrated under reduced pressure, and treated with 99% TFA (1.0 mL) for 3 min after which reaction mixture was evaporated under reduced pressure. The product was dissolved in a minimal volume of H₂O and freeze-dried to afford the TFA salt of compound **3c** as a white foam (89 mg, 82%). ¹H NMR (400 MHz, D₂O) (Fig. S3) δ 5.57 (d, $J = 3.4$ Hz, 1H, H-1'), 4.97 (d, $J = 3.2$ Hz, 1H, H-1''), 4.28 (t, $J = 9.2$ Hz, 1H, H-5''), 3.91-3.76 (m, 4H, H-4, H-5, H-5', H-2''), 3.67 (t, $J = 9.0$ Hz, 1H, H-6), 3.60-3.35 (m, 7H, H-1, H-3, H-2', H-4', H-3'', H-4'', H-6''), 3.33-3.23 (m, 2H, H-6', H-6''), 3.08 (m, 2H, SO₂CH₂(CH₂)₁₄CH₃), 3.02 (dd, $J_1 = 13.5$ Hz, $J_2 = 7.8$ Hz, 1H, H-6'), 2.43 (m, 1H, H-2eq), 2.16 (m, 1H, H-3'eq), 1.86 (m, 2H, H-2ax, H-3'ax), 1.62 (m, 2H, SO₂CH₂(CH₂)₁₄CH₃), 1.26 (m, 2H, SO₂CH₂(CH₂)₁₄CH₃), 1.22-1.07 (m, 24H, SO₂CH₂(CH₂)₁₄CH₃), 0.74 (t, 3H, $J = 6.9$ Hz, SO₂CH₂(CH₂)₁₄CH₃); ¹³C NMR (100 MHz, D₂O) (Fig. S4) δ 162.8 (q, $J = 35$ Hz, CF₃CO₂H), 116.4 (q, $J = 290$ Hz, CF₃CO₂H), 100.6 (anomeric C), 94.8 (anomeric C), 84.4, 77.6, 74.6, 70.2, 67.9, 67.7, 64.9, 54.52, 54.46, 52.4, 49.3, 48.2, 48.0, 40.2, 31.7, 29.9, 29.52, 29.49, 29.3, 29.2, 29.1, 28.7, 28.5, 27.9, 27.8, 22.5, 21.7, 13.7; HRESI-MS m/z calc'd for C₃₄H₆₉N₅O₁₀SNa 762.4663, found 762.4659 [M+Na]⁺.

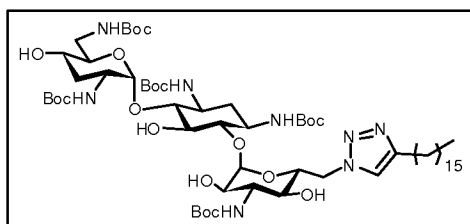


NHBoc-protected 4a: Compound **1e** (151 mg, 0.15 mmol), in DMF (1 mL), was added $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (3.6 mg, 0.01 mmol), sodium ascorbate (5.0 mg, 0.03 mmol) and 1-tetradecyne 90% (75 μL , 0.30 mmol). The reaction mixture was irradiated by a microwave for 4 min. Propagation of the reaction was monitored by TLC (EtOAc/petroleum ether: 7/3) and upon completion, reaction mixture was diluted with EtOAc (10 mL) and the organic layer was washed twice with brine (2x20 mL). The aqueous layer was extracted again with EtOAc (10 mL) and the combined organic layers were dried over anhydrous MgSO_4 and concentrated under reduced pressure. Purification by flash column chromatography (SiO_2 , EtOAc: petroleum ether) afforded the corresponding **NHBoc-protected 4a** as a white solid (171 mg, 95%). ^1H NMR (400 MHz, CD_3OD) δ 7.75 (s, 1H, triazole ring), 5.11 (d, $J = 3.3$ Hz, 1H, H-1"), 5.06 (br s, 1H, H-1'), 4.68 (br d, $J = 14.0$ Hz, 1H, H-6"), 4.55 (m, 1H, H-6"), 4.42 (m, 1H, H-5"), 3.74 (t, $J = 10.0$ Hz, H-3"), 3.64-3.27 (m, 11H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2"), 2.99 (t, $J = 9.8$ Hz, 1H, H-4"), 2.68 (t, $J = 7.7$ Hz, 2H, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$), 2.04 (m, 2H, H-2eq, H-3'eq), 1.66 (m, 3H, H-3'ax, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$), 1.47-1.28 (m, 64H, H-2ax, $5 \times \text{CO}_2\text{C}(\text{CH}_3)_3$, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$), 0.90 (t, $J = 7.0$ Hz, 3H, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 159.4, 159.2, 157.9, 157.7, 149.0 (triazole ring), 124.6 (triazole ring), 99.8 (anomeric C), 98.8 (anomeric C), 83.2, 81.7, 80.7, 80.5, 80.4, 80.2, 76.5, 73.6, 71.8, 71.7, 70.4, 66.5, 57.1, 51.7, 51.3, 42.1, 36.1, 34.3, 33.1, 31.8, 30.8, 30.6, 30.5, 30.4, 28.9, 28.84, 28.80, 26.4, 23.7, 14.4; LR-ESI m/z calc'd for $\text{C}_{57}\text{H}_{102}\text{N}_8\text{O}_{18}$ 1186.73, found 1188.27 $[\text{M}+\text{H}]^+$.



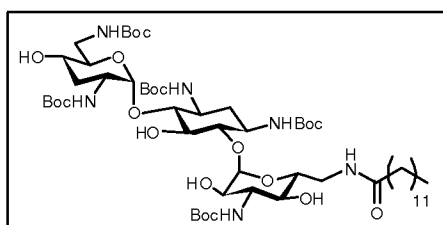
NHBoc-protected 4b: Compound **1e** (200 mg, 0.20 mmol), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (5 mg, 0.02 mmol), sodium ascorbate (9 mg, 0.05 mmol), DMF (1.5 mL), 1-hexadecyne 90% (120 μL , 0.43 mmol). Purification by flash column chromatography (SiO_2 , EtOAc:petroleum ether) afforded the corresponding **NHBoc-protected 4b** as a white solid (212 mg, 87%). ^1H NMR (400 MHz, CD_3OD) δ 7.75 (s, 1H, triazole ring), 5.11 (d, $J = 3.3$ Hz, 1H, H-1"), 5.06 (br s, 1H, H-1'), 4.69 (br d, $J = 13.9$ Hz, 1H, H-6"), 4.55 (m, 1H, H-6"), 4.42 (m, 1H, H-5"), 3.74 (t, $J = 9.9$ Hz, H-3"), 3.64-3.27 (m, 11H, H-1, H-3, H-4, H-5, H-6, H-2', H-4',

H-5', H-6' (2H), H-2''), 2.99 (t, $J = 9.8$ Hz, 1H, H-4''), 2.68 (t, $J = 7.8$ Hz, 2H, $C_2HN_3CH_2(CH_2)_{12}CH_3$), 2.04 (m, 2H, H-2eq, H-3'eq), 1.66 (m, 3H, H-3'ax, $C_2HN_3CH_2(CH_2)_{12}CH_3$), 1.47-1.28 (m, 68H, H-2ax, $5 \times CO_2C(CH_3)_3$, $C_2HN_3CH_2(CH_2)_{12}CH_3$), 0.90 (t, $J = 7.0$ Hz, 3H, $C_2HN_3CH_2(CH_2)_{12}CH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 159.4, 159.2, 157.8, 157.7, 149.0 (triazole ring), 124.6 (triazole ring), 99.8 (anomeric C), 98.8 (anomeric C), 83.1, 81.8, 80.7, 80.4, 80.3, 80.2, 76.5, 73.6, 71.8, 71.6, 70.4, 66.5, 57.0, 51.7, 51.2, 42.1, 36.0, 34.3, 33.0, 31.8, 30.8, 30.6, 30.5, 30.4, 30.3, 28.9, 28.84, 28.80, 26.4, 23.7, 14.5; LR-ESI m/z calc'd for $C_{59}H_{106}N_8O_{18}$ 1214.76, found 1216.27 $[M+H]^+$.



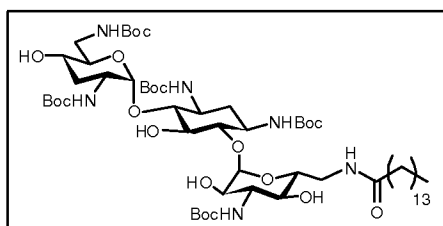
NHBoc-protected 4c: Compound **1e** (150 mg, 0.15 mmol), $CuSO_4 \cdot 5H_2O$ (2 mg, 0.01 mmol), sodium ascorbate (3 mg, 0.02 mmol), DMF (1 mL), 1-hexadecyne >95% (100 μ L, 0.32 mmol). Purification by flash column chromatography (SiO_2 ,

EtOAc:petroleum ether) afforded the corresponding NHBoc-protected **4c** as a white solid (170 mg, 90%). 1H NMR (400 MHz, CD_3OD) δ 7.75 (s, 1H, triazole ring), 5.11 (d, $J = 3.3$ Hz, 1H, H-1''), 5.06 (br s, 1H, H-1'), 4.69 (br d, $J = 13.9$ Hz, 1H, H-6''), 4.55 (m, 1H, H-6''), 4.42 (m, 1H, H-5''), 3.74 (t, $J = 10.0$ Hz, H-3''), 3.66-3.26 (m, 11H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2''), 2.99 (t, $J = 9.9$ Hz, 1H, H-4''), 2.68 (t, $J = 7.8$ Hz, 2H, $C_2HN_3CH_2(CH_2)_{14}CH_3$), 2.04 (m, 2H, H-2eq, H-3'eq), 1.66 (m, 3H, H-3'ax, $C_2HN_3CH_2(CH_2)_{14}CH_3$), 1.48-1.26 (m, 72H, H-2ax, $5 \times CO_2C(CH_3)_3$, $C_2HN_3CH_2(CH_2)_{14}CH_3$), 0.90 (t, $J = 7.0$ Hz, 3H, $C_2HN_3CH_2(CH_2)_{14}CH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 159.4, 159.2, 157.8, 157.7, 148.9 (triazole ring), 124.6 (triazole ring), 99.8 (anomeric C), 98.8 (anomeric C), 83.1, 81.8, 80.7, 80.4, 80.3, 80.2, 76.5, 73.6, 71.8, 71.6, 70.4, 66.5, 57.0, 51.7, 51.2, 42.0, 36.0, 34.3, 33.0, 31.8, 30.8, 30.6, 30.5, 30.4, 30.3, 28.9, 28.84, 28.80, 26.4, 23.7, 14.5; LR-ESI m/z calc'd for $C_{61}H_{110}N_8O_{18}$ 1242.79, found 1243.89 $[M+H]^+$.



NHBoc-protected 5a: Tridecanoic acid 98% (36 mg, 0.17 mmol) and *N,N*-diisopropylethylamine (DIEA) (83 μ L, 0.50 mmol) in dry DMF (2 mL), was added HBTU (77 mg, 0.20 mmol) and stirred at ambient

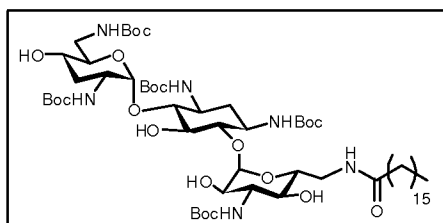
temperature for 15 min under argon atmosphere. The mixture was then cooled in an ice-bath, added with compound **1f** (81 mg, 0.08 mmol) and allowed to reach ambient temperature. Propagation of the reaction was monitored by TLC (EtOAc: petroleum ether/7:3), and upon completion, the reaction mixture was diluted with EtOAc (50 mL) and the organic layer was washed three times with brine (3x40 mL). The aqueous layer was extracted again with EtOAc (50 mL) and the combined organic layers were dried over anhydrous MgSO_4 and concentrated under reduced pressure. Purification by flash column chromatography (SiO_2 , EtOAc:petroleum ether) gave the corresponding *NHBoc*-protected **5a** (84 mg, 86%) as a white solid.: ^1H NMR (400 MHz, CD_3OD) δ 5.13 (br s, 1H, H-1'), 5.04 (br d, $J = 3.4$ Hz, 1H, H-1''), 4.04 (m, 1H, H-5''), 3.69-3.31 (m, 14H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2'', H-3'', H-6'' (2H)), 3.16 (t, $J = 9.9$ Hz, 1H, H-4''), 2.21 (t, $J = 7.7$ Hz, 2H, $\text{NHCOCH}_2(\text{CH}_2)_{10}\text{CH}_3$), 1.98-2.12 (m, 2H, H-2eq, H-3'eq), 1.65 (app. q, $J = 12.1$ Hz, 1H, H-3'ax), 1.62-1.25 (m, 66H, H-2ax, $5\times\text{CO}_2\text{C}(\text{CH}_3)_3$, $\text{NHCOCH}_2(\text{CH}_2)_{10}\text{CH}_3$), 0.90 (t, $J = 7.0$ Hz, 3H, $\text{NHCOCH}_2(\text{CH}_2)_{10}\text{CH}_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 177.0 (NHCO), 159.3, 159.2, 158.0, 157.8, 157.6, 99.8 (anomeric C), 99.4 (anomeric C), 83.2, 82.6, 80.7, 80.5, 80.3, 80.1, 77.0, 73.5, 72.6, 72.1, 70.7, 66.5, 57.0, 51.7, 51.0, 42.1, 41.5, 37.1, 34.3, 33.1, 31.8, 30.8, 30.7, 30.5, 28.8, 27.0, 23.7, 14.5; LR-ESI m/z calc'd for $\text{C}_{56}\text{H}_{102}\text{N}_6\text{O}_{19}$ 1162.72, found 1163.76 $[\text{M}+\text{H}]^+$.



***NHBoc*-protected 5b:** Pentadecanoic acid 99% (40 mg, 0.17 mmol), DIEA (82 μL , 0.49 mmol), dry DMF (2 mL), HBTU (75 mg, 0.20 mmol), **1f** (80 mg, 0.08 mmol). Purification by flash column chromatography (SiO_2 , EtOAc:petroleum ether) gave the corresponding

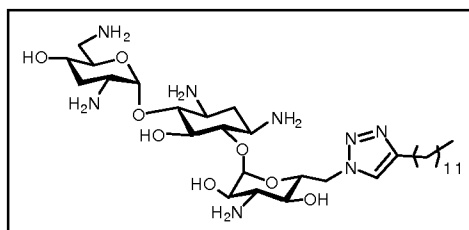
NHBoc-protected **5b** as a white solid (77 mg, 78%). ^1H NMR (400 MHz, CD_3OD) δ 5.13 (br s, 1H, H-1'), 5.04 (br d, $J = 3.6$ Hz, 1H, H-1''), 4.03 (m, 1H, H-5''), 3.67-3.30 (m, 14H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2'', H-3'', H-6'' (2H)), 3.16 (t, $J = 9.9$ Hz, 1H, H-4''), 2.21 (t, $J = 7.8$ Hz, 2H, $\text{NHCOCH}_2(\text{CH}_2)_{12}\text{CH}_3$), 2.13-1.98 (m, 2H, H-2eq, H-3'eq), 1.66 (app. q, $J = 12.2$ Hz, 1H, H-3'ax), 1.62-1.28 (m, 70H, H-2ax, $5\times\text{CO}_2\text{C}(\text{CH}_3)_3$, $\text{NHCOCH}_2(\text{CH}_2)_{12}\text{CH}_3$), 0.90 (t, $J = 7.0$ Hz, 3H, $\text{NHCOCH}_2(\text{CH}_2)_{12}\text{CH}_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 177.0 (NHCO), 159.3, 159.2, 158.0, 157.8, 157.6, 99.9 (anomeric C), 99.4 (anomeric C), 83.2, 82.7, 80.7, 80.5, 80.4, 80.2, 77.0, 73.5, 72.7, 72.1, 70.7, 66.6, 57.0, 51.8,

51.1, 42.0, 41.6, 37.1, 35.9, 34.3, 33.1, 31.8, 30.8, 30.7, 30.5, 28.9, 28.83, 28.81, 27.0, 23.7, 14.4; LR-ESI m/z calc'd for $C_{58}H_{106}N_6O_{19}$ 1190.75, found 1191.83 $[M+H]^+$.



NHBoc-protected 5c. Heptadecanoic acid >98% (45 mg, 0.17 mmol), DIEA (83 μ L, 0.50 mmol), dry DMF (2 mL), HBTU (75 mg, 0.20 mmol), **1f** (80 mg, 0.08 mmol). Purification by flash column chromatography (SiO_2 , EtOAc:petroleum ether) gave the corresponding

NHBoc-protected 5c as a white solid (72 mg, 71%). 1H NMR (400 MHz, CD_3OD) δ 5.13 (br s, 1H, H-1'), 5.04 (br d, $J = 3.6$ Hz, 1H, H-1''), 4.03 (m, 1H, H-5''), 3.69-3.30 (m, 14H, H-1, H-3, H-4, H-5, H-6, H-2', H-4', H-5', H-6' (2H), H-2'', H-3'', H-6'' (2H)), 3.16 (t, $J = 9.9$ Hz, 1H, H-4''), 2.21 (t, $J = 7.8$ Hz, 2H, $NHCOCH_2(CH_2)_{14}CH_3$), 2.13-1.98 (m, 2H, H-2eq, H-3'eq), 1.66 (app. q, $J = 12.2$ Hz, 1H, H-3'ax), 1.62-1.27 (m, 74H, H-2ax, $5 \times CO_2C(CH_3)_3$, $NHCOCH_2(CH_2)_{14}CH_3$), 0.90 (t, $J = 7.0$ Hz, 3H, $NHCOCH_2(CH_2)_{14}CH_3$); ^{13}C NMR (100 MHz, CD_3OD) δ 177.0 ($NHCO$), 159.3, 159.2, 158.0, 157.8, 157.6 99.9 (anomeric C), 99.4 (anomeric C), 83.2, 82.7, 80.7, 80.5, 80.4, 80.1, 77.0, 73.5, 72.7, 72.1, 70.7, 66.6, 57.0, 51.8, 51.1, 42.0, 41.6, 37.1, 34.3, 33.1, 31.8, 30.8, 30.5, 28.9, 28.8, 27.0, 23.7, 14.4; LR-ESI m/z calc'd for $C_{60}H_{110}N_6O_{19}$ 1218.78, found 1219.71 $[M+H]^+$.

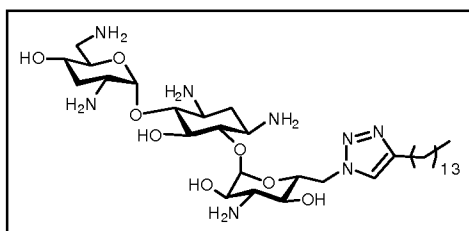


Compound 4a: **NHBoc-protected 4a** (42 mg, 0.04 mmol) was treated with 99% TFA (2mL) at ambient temperature for 3 min. The TFA was then removed under reduced pressure, the product was re-dissolved in a minimal volume of H_2O and freeze-dried to

afford **4a** as a white foam (42 mg, 94%). 1H NMR (400 MHz, D_2O) (Fig. S5) δ 7.60 (s, 1H, triazole ring), 5.69 (d, $J = 2.9$ Hz, 1H, H-1'), 4.91 (d, $J = 3.5$ Hz, 1H, H-1''), 4.62 (m, 1H, H-6''), 4.46 (dd, $J_1 = 13.8$ Hz, $J_2 = 7.0$ Hz, 1H, H-6''), 4.21 (ddd, $J_1 = 9.1$ Hz, $J_2 = 7.0$ Hz, $J_3 = 2.1$ Hz, 1H, H-5''), 3.84 (t, $J = 9.8$ Hz, 1H, H-4), 3.79-3.72 (m, 2H, H-5', H-2''), 3.65 (t, $J = 8.9$ Hz, 1H, H-5), 3.63-3.52 (m, 3H, H-6, H-2', H-4'), 3.47-3.28 (m, 5H, H-1, H-3, H-6', H-3'', H-4''), 3.08 (m, 1H, H-6'), 2.53 (t, $J = 6.3$ Hz, 1H, $C_2HN_3CH_2(CH_2)_{10}CH_3$), 2.40 (dt, $J_1 = 12.6$ Hz, $J_2 = J_3 = 4.1$ Hz, 1H, H-2eq), 2.20 (dt, $J_1 = 12.1$ Hz, $J_2 = J_3 = 4.4$ Hz, 1H, H-3'eq), 1.88 (app. q, $J_1 = J_2 = J_3 = 11.6$ Hz, 1H, H-3'ax), 1.78 (app. q, $J_1 = J_2 = J_3 = 12.6$ Hz, 1H, H-2ax), 1.43 (m, 2H,

$\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$), 1.18-1.06 (m, 18H, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$), 0.65 (t, 3H, $J = 6.8$ Hz, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{10}\text{CH}_3$); ^{13}C NMR (100 MHz, D_2O) (Fig. S6) δ 162.9 (q, $J = 35$ Hz, $\text{CF}_3\text{CO}_2\text{H}$), 148.6 (triazole ring), 124.9 (triazole ring), 116.4 (q, $J = 290$ Hz, $\text{CF}_3\text{CO}_2\text{H}$), 100.9 (anomeric C), 93.8 (anomeric C), 84.0, 76.7, 74.4, 70.9, 70.2, 67.9, 66.6, 64.7, 54.8, 50.3, 49.6, 48.4, 47.8, 40.1, 31.3, 29.9, 29.6, 29.4, 28.9, 28.7, 28.6, 28.3, 27.7, 24.5, 22.1, 13.5; HRESI-MS m/z calc'd for $\text{C}_{32}\text{H}_{62}\text{N}_8\text{O}_8\text{Na}$ 709.4588, found 709.4583 $[\text{M}+\text{Na}]^+$.

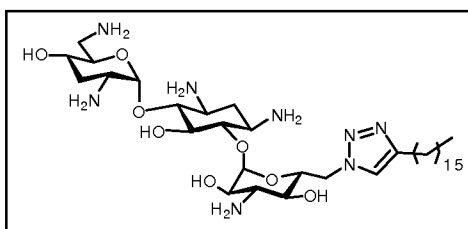
Compound 4b: *NHBoc*-protected **4b** (59 mg, 0.05 mmol) was treated with 99% TFA (2mL) at



ambient temperature for 3 min. The TFA was removed under reduced pressure, the product was re-dissolved in a minimal volume of H_2O and freeze-dried to afford **4b** as a white foam (58 mg, 93%). ^1H NMR (400 MHz, D_2O) (Fig. S7) δ 7.47 (s, 1H,

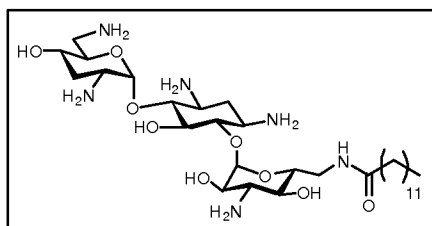
triazole ring), 5.72 (d, $J = 3.2$ Hz, 1H, H-1'), 4.89 (d, $J = 3.1$ Hz, 1H, H-1''), 4.58(m, 1H, H-6''), 4.40 (dd, $J_1 = 14.5$ Hz, $J_2 = 5.8$ Hz, 1H, H-6''), 4.21 (m, 1H, H-5''), 3.87 (t, $J = 9.6$ Hz, 1H, H-4), 3.77 (td, $J_1 = J_2 = 8.6$ Hz, $J_3 = 3.4$ Hz, 1H, H-5'), 3.71 (dd, $J_1 = 10.7$ Hz, $J_2 = 3.1$ Hz, 1H, H-2''), 3.68-3.56 (m, 2H, H-5, H-6), 3.55-3.24 (m, 7H, H-1, H-3, H-2', H-4', H-6', H-3'', H-4''), 3.01 (dd, $J_1 = 13.4$ Hz, $J_2 = 7.8$ Hz, 1H, H-6'), 2.48 (t, $J = 7.4$ Hz, 2H, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{12}\text{CH}_3$), 2.40 (dd, $J_1 = 12.2$ Hz, $J_2 = 3.6$ Hz, 1H, H-2eq), 2.19 (dd, $J_1 = 7.3$ Hz, $J_2 = 4.5$ Hz, 1H, H-3'eq), 1.91 (app. q, $J_1 = J_2 = J_3 = 11.4$ Hz, 1H, H-3'ax), 1.83 (app. q, $J_1 = J_2 = J_3 = 12.6$ Hz, 1H, H-2ax), 1.44 (m, 2H, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{12}\text{CH}_3$), 1.56-1.04 (m, 22H, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{12}\text{CH}_3$), 0.68 (t, 3H, $J = 6.8$ Hz, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{12}\text{CH}_3$); ^{13}C NMR (100 MHz, D_2O) (Fig. S8) δ 162.7 (q, $J = 35$ Hz, $\text{CF}_3\text{CO}_2\text{H}$), 148.5 (triazole ring), 124.4 (triazole ring), 116.4 (q, $J = 290$ Hz, $\text{CF}_3\text{CO}_2\text{H}$), 100.9 (anomeric C), 93.7 (anomeric C), 84.1, 76.7, 74.4, 70.7, 70.2, 67.9, 66.5, 64.9, 54.8, 50.2, 49.7, 48.4, 47.9, 40.3, 31.7, 30.7, 29.8, 29.6, 29.4, 29.3, 29.1, 29.0, 28.8, 27.7, 24.7, 22.4, 13.6; HRESI-MS m/z calc'd for $\text{C}_{34}\text{H}_{67}\text{N}_8\text{O}_8$ 715.5082, found 715.5089 $[\text{M}+\text{H}]^+$.

Compound 4c: *NHBoc*-protected **4c** (46 mg, 0.04 mmol) was treated with 99% TFA (2mL) at



ambient temperature for 3 min. The TFA was removed under reduced pressure, the product was re-dissolved in a minimal volume of H_2O and freeze-

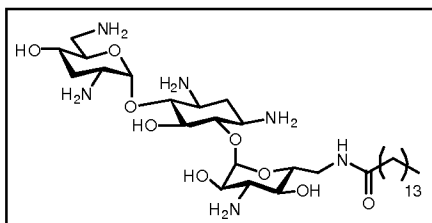
dried to afford **4c** as a white foam (48 mg, 99%). ^1H NMR (400 MHz, D_2O) (Fig. S9) δ 7.43 (s, 1H, triazole ring), 5.73 (d, $J = 3.2$ Hz, 1H, H-1'), 4.88 (d, $J = 3.0$ Hz, 1H, H-1''), 4.57 (m, 1H, H-6''), 4.38 (dd, $J_1 = 14.2$ Hz, $J_2 = 5.4$ Hz, 1H, H-6''), 4.21 (m, 1H, H-5''), 3.88 (t, $J = 9.6$ Hz, 1H, H-4), 3.77 (td, $J_1 = J_2 = 8.5$ Hz, $J_3 = 3.4$ Hz, 1H, H-5'), 3.71 (dd, $J_1 = 10.7$ Hz, $J_2 = 3.1$ Hz, 1H, H-2''), 3.68-3.56 (m, 2H, H-5, H-6), 3.54-3.28 (m, 6H, H-1, H-3, H-2', H-4', H-6', H-3''), 3.25 (t, $J = 9.9$ Hz, 1H, H-4''), 2.99 (dd, $J_1 = 13.3$ Hz, $J_2 = 7.9$ Hz, 1H, H-6'), 2.47 (t, $J = 7.2$ Hz, 2H, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{14}\text{CH}_3$), 2.40 (dd, $J_1 = 8.2$ Hz, $J_2 = 3.6$ Hz, 1H, H-2eq), 2.19 (dd, $J_1 = 7.4$ Hz, $J_2 = 4.4$ Hz, 1H, H-3'eq), 1.92 (app. q, $J_1 = J_2 = J_3 = 11.5$ Hz, 1H, H-3'ax), 1.84 (app. q, $J_1 = J_2 = J_3 = 12.6$ Hz, 1H, H-2ax), 1.43 (m, 2H, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{14}\text{CH}_3$), 1.19-1.03 (m, 26H, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{14}\text{CH}_3$), 0.69 (t, 3H, $J = 6.9$ Hz, $\text{C}_2\text{HN}_3\text{CH}_2(\text{CH}_2)_{14}\text{CH}_3$); ^{13}C NMR (100 MHz, D_2O) (Fig. S10) δ 162.7 (q, $J = 35$ Hz, $\text{CF}_3\text{CO}_2\text{H}$), 148.5 (triazole ring), 124.2 (triazole ring), 116.4 (q, $J = 290$ Hz, $\text{CF}_3\text{CO}_2\text{H}$), 100.9 (anomeric C), 93.7 (anomeric C), 84.1, 76.7, 74.4, 70.7, 70.2, 67.9, 66.5, 65.0, 54.9, 50.2, 49.7, 48.4, 47.9, 40.3, 31.8, 30.8, 29.8, 29.6, 29.4, 29.3, 29.2, 29.0, 28.9, 27.7, 24.8, 22.5, 13.7; HRESI-MS m/z calc'd for $\text{C}_{36}\text{H}_{70}\text{N}_8\text{O}_8\text{Na}$ 765.5214, found 765.5216 $[\text{M}+\text{Na}]^+$.



Compound 5a: *NHBoc*-protected **5a** (23.8 mg, 0.02 mmol) was treated with 99% TFA (1mL) at ambient temperature for 3 min. The TFA was then removed under reduced pressure, the residue was re-dissolved in a minimal volume of H_2O and freeze-dried to afford **5a**

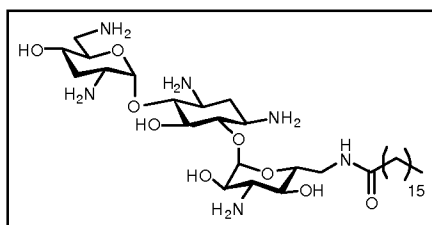
as a white foam (21.5 mg, 85%). ^1H NMR (400 MHz, D_2O) (Fig. S11) δ 5.67 (d, $J = 3.5$ Hz, 1H, H-1'), 4.91 (d, $J = 3.5$ Hz, 1H, H-1''), 3.88-3.73 (m, 4H, H-4, H-5', H-2'', H-5''), 3.70 (t, $J = 9.0$ Hz, 1H, H-5), 3.65-3.26 (m, 10H, H-1, H-3, H-6, H-2', H-4', H-6', H-3'', H-4'', H-6''(2H)), 3.12 (dd, $J_1 = 13.6$ Hz, $J_2 = 7.0$ Hz, 1H, H-6'), 2.40 (dt, $J_1 = 12.5$ Hz, $J_2 = J_3 = 4.2$ Hz, 1H, H-2eq), 2.17 (m, 1H, H-3'eq), 2.11 (t, $J = 7.5$ Hz, 2H, $\text{NHCOCH}_2(\text{CH}_2)_{10}\text{CH}_3$), 1.89 (app. q, $J_1 = J_2 = J_3 = 11.0$ Hz, 1H, H-3'ax), 1.79 (app. q, $J_1 = J_2 = J_3 = 12.6$ Hz, 1H, H-2ax), 1.43 (m, 2H, $\text{NHCOCH}_2(\text{CH}_2)_{10}\text{CH}_3$), 1.18-1.06 (m, 18H, $\text{NHCOCH}_2(\text{CH}_2)_{10}\text{CH}_3$), 0.70 (t, 3H, $J = 7.0$ Hz, $\text{NHCOCH}_2(\text{CH}_2)_{10}\text{CH}_3$); ^{13}C NMR (100 MHz, D_2O) (Fig. S12) δ 178.2 (NHCO), 162.9 (q, $J = 35$ Hz, $\text{CF}_3\text{CO}_2\text{H}$), 116.4 (q, $J = 290$ Hz, $\text{CF}_3\text{CO}_2\text{H}$), 100.9 (anomeric C), 94.1 (anomeric C), 83.6, 77.3, 74.2, 71.0, 70.4, 68.1, 66.6, 64.4, 54.7, 49.8, 48.4, 47.8, 39.8, 38.9,

35.8, 31.2, 29.3, 28.8, 28.7, 28.6, 28.5, 28.4, 28.3, 27.8, 25.4, 22.0, 13.4; HRESI-MS m/z calc'd for $C_{31}H_{63}N_6O_9$ 663.4657, found 663.4648 $[M+H]^+$.



Compound 5b: *NHBoc*-protected **5b** (24.0 mg, 0.02 mmol) was treated with 99% TFA (1mL) at ambient temperature for 3 min. The TFA was removed under reduced pressure, the residue was dissolved in a minimal volume of H_2O and freeze-dried to afford **5b** as

a white foam (24.9 mg, 98%). 1H NMR (400 MHz, D_2O) (Fig. S13) δ 5.66 (d, $J = 3.5$ Hz, 1H, H-1'), 4.92 (d, $J = 3.6$ Hz, 1H, H-1''), 3.86-3.73 (m, 4H, H-4, H-5', H-2'', H-5''), 3.69 (t, $J = 9.0$ Hz, 1H, H-5), 3.65-3.26 (m, 10H, H-1, H-3, H-6, H-2', H-4', H-6', H-3'', H-4'', H-6''(2H)), 3.11 (dd, $J_1 = 13.6$ Hz, $J_2 = 7.1$ Hz, 1H, H-6'), 2.39 (dt, $J_1 = 12.5$ Hz, $J_2 = J_3 = 4.2$ Hz, 1H, H-2eq), 2.19-2.09 (m, 3H, H-3'eq, $NHCOCH_2(CH_2)_{12}CH_3$), 1.89 (app. q, $J_1 = J_2 = J_3 = 12.0$ Hz, 1H, H-3'ax), 1.78 (app. q, $J_1 = J_2 = J_3 = 12.6$ Hz, 1H, H-2ax), 1.43 (m, 2H, $NHCOCH_2(CH_2)_{12}CH_3$), 1.17-1.08 (m, 22H, $NHCOCH_2(CH_2)_{12}CH_3$), 0.71 (t, 3H, $J = 7.0$ Hz, $NHCOCH_2(CH_2)_{12}CH_3$); ^{13}C NMR (100 MHz, D_2O) (Fig. S14) δ 178.1 ($NHCO$), 162.9 (q, $J = 35$ Hz, CF_3CO_2H), 116.4 (q, $J = 290$ Hz, CF_3CO_2H), 100.9 (anomeric C), 94.0 (anomeric C), 83.7, 77.6, 74.2, 71.0, 70.3, 68.1, 66.6, 64.5, 54.7, 49.8, 48.4, 47.8, 39.9, 38.9, 35.8, 31.3, 29.8, 29.4, 28.9, 28.7, 28.6, 28.5, 28.4, 28.0, 25.4, 22.1, 13.5; HRESI-MS m/z calc'd for $C_{33}H_{67}N_6O_9$ 691.4970, found 691.4963 $[M+H]^+$.



Compound 5c: *NHBoc*-protected **5c** (33.2 mg, 0.03 mmol) was treated with 99% TFA (1.3mL) at ambient temperature for 3 min. The TFA was removed under reduced pressure, the product was re-dissolved in a minimal volume of H_2O and freeze-dried to afford **5c** as

a white foam (33.8 mg, 96%). 1H NMR (400 MHz, D_2O) (Fig. S15) δ 5.67 (d, $J = 3.1$ Hz, 1H, H-1'), 4.90 (d, $J = 2.8$ Hz, 1H, H-1''), 3.86 (t, $J = 9.3$ Hz, 1H, H-4), 3.82-3.75 (m, 3H, H-5', H-2'', H-5''), 3.68 (t, $J = 8.8$ Hz, 1H, H-5), 3.62 (t, $J = 9.1$ Hz, 1H, H-6), 3.57-3.20 (m, 9H, H-1, H-3, H-2', H-4', H-6', H-3'', H-4'', H-6''(2H)), 3.04 (dd, $J_1 = 13.4$ Hz, $J_2 = 7.7$ Hz, 1H, H-6'), 2.38 (m, 1H, H-2eq), 2.18-2.04 (m, 3H, H-3'eq, $NHCOCH_2(CH_2)_{14}CH_3$), 1.89 (app. q, $J_1 = J_2 = J_3 = 11.9$ Hz, 1H, H-3'ax), 1.81 (app. q, $J_1 = J_2 = J_3 = 12.4$ Hz, 1H, H-2ax), 1.40 (m, 2H,

NHCOCH₂(CH₂)₁₄CH₃), 1.18-1.06 (m, 26H, NHCOCH₂(CH₂)₁₄CH₃), 0.71 (t, 3H, *J* = 6.8 Hz, NHCOCH₂(CH₂)₁₄CH₃); ¹³C NMR (100 MHz, D₂O) (Fig. S16) δ 177.2 (NHCO), 162.5 (q, *J* = 35 Hz, CF₃CO₂H), 116.0 (q, *J* = 290 Hz, CF₃CO₂H), 100.5 (anomeric C), 93.5 (anomeric C), 83.3, 77.0, 73.8, 70.8, 70.3, 67.8, 66.1, 64.4, 54.2, 49.5, 48.0, 47.5, 39.7, 38.5, 35.5, 31.3, 29.4, 29.1, 28.9, 28.8, 28.6, 28.5, 27.5, 25.1, 22.1, 13.3; HRESI-MS *m/z* calc'd for C₃₅H₇₀N₆O₉Na 741.5102 found 741.5101 [M+Na]⁺.

Example 4: Minimal Inhibitory Concentration (MIC) test.

Starter cultures were incubated for 24 hours (37 °C, 5% CO₂, aerobic conditions), and diluted in fresh broth medium to obtain an optical density of 0.008 (OD₆₅₅). All strains were tested using a double-dilution method starting at 128 µg/mL in 96-well plates (Sarstedt, Newton, NC). After 24 hours of incubation, MTT reagent (50 µL of a 1 mg/mL solution in H₂O) was added to each well followed by additional incubation at 37°C for 30 min. 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. Tested strains: **Gram-positive:** *Streptococcus pyogenes* M12 (strain MGAS9429) (A), *Methicillin-resistant Staphylococcus aureus* (MRSA) (B), *Streptococcus mutans* UA 159 (C), *vancomycin-resistant enterococci* (VRE) (D), *Enterococcus faecalis* ATCC 29212 (E), *Staphylococcus aureus* (Oxford strain ATCC9144) (F), *Staphylococcus epidermidis* ATCC 35984, and *Staphylococcus epidermidis* ATCC 12228. **Gram-negative:** *Pseudomonas aeruginosa* ATCC33347 (I), *Shigella sonnei* clinical isolate 6831 (*O*-antigen positive) (J), and *Shigella sonnei* clinical isolate 6831 (*O*-antigen negative) (K). All strains were grown in Brain Heart Infusion broth (BHI) (BBL Microbiology Systems, Cockeysville, MD) with the exception of *Shigella sonnei* that was grown in Trypticase Soy Broth (TSB).

Example 5: Minimal Biofilm Inhibitory Concentration (MBIC) test

S. epidermis ATCC35984 (G) and *S. mutans* UA 159 (C) were grown in biofilms in Trypticase Soy Broth (TSB) (BBL Microbiology Systems, Cockeysville, MD) supplemented with glucose 1%, and in BHI supplemented with sucrose 2%, respectively with presence of the tested analogues. Strains were tested using a double-dilution starting at 128 µg/mL, aerobically at 37 °C, 5% CO₂, 96-well plates. After 24 hours of growth, the plates were vigorously washed

three times with phosphate-buffered saline (PBS) to remove any unattached bacteria and then dried for 1 hour at 60 °C. The air-dried wells were stained with 0.1% crystal violet (200 µL) for 30 min, and the plate was rinsed with PBS. The air-dried plates were added with 200 µL of acetic acid 30%, and the OD at 570 nm was measured by microtiter plate reader (Tecan). Experiments were performed in triplicate.

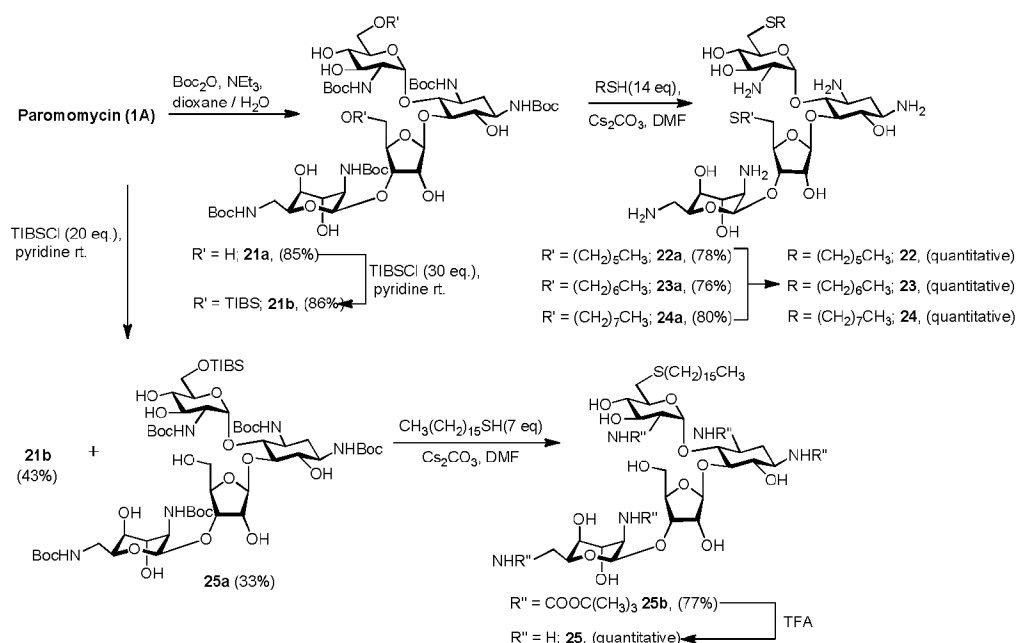
Example 6: Red blood cells (RBCs) hemolysis assay

Rat RBC solution (2% w/w) was incubated with 6"-tobramycin analogues using the double dilution method starting at concentration of 256 µg/mL for 1 h at 37 °C, 5% CO₂. Negative control was PBS and positive control was 1% w/v solution of Triton X100 (100% lysis). Following centrifugation (2,000 rpm, 10 min, ambient temperature), the supernatant was drawn off and its absorbance measured at 550 nm using a microplate reader (Genios, TECAN). The results were expressed as percentage of hemoglobin released relative to the positive control (Triton X100).

This experiment was performed in triplicate, and the results are an average of two different sets of blood.

Example 7 - Design and Synthesis of Di-Alkylated Paromomycin-Based Cationic amphiphiles

Scheme 4 generally represents various embodiments of the amphiphilic paromomycin-based aminoglycoside derivatives of the present invention. Such derivatives may be prepared in accordance with the processes as described herein. Some currently preferred embodiments are described hereinbelow, while it is understood that various modifications in the synthetic scheme such as the choice of protecting groups, reagents and solvents are encompassed within the scope of the present invention.



Scheme 4: Synthesis of mono- and di-alkylated paromomycin-based cationic amphiphiles.

The five amine groups of commercially available paromomycin were protected with Boc groups to afford the penta-*NHBoc* protected paromomycin derivative **21a** in 85% yield (Scheme 4) (Michael, 1999; Pathak, 2005). Selective conversion of the two primary alcohols at positions C-6' and C-5'' of **21a** to the corresponding *O*-trisyl leaving groups using 30 equivalents of 2,4,6-triisopropylbenzene-sulfonyl chloride gave compound **21b** in 86%. Compound **21b** was reacted with 1-*n*-hexanethiol, 1-*n*-heptanethiol, or 1-*n*-octanethiol resulting in the *NHBoc*-protected di-alkylated paromomycin derivatives **22a**, **23a**, and **24a** in yields ranging from 76 to 80%. Removal of the *NHBoc* protecting groups in neat TFA gave the penta TFA salts of the 6', 5'' dithioether paromomycin derivatives **22**, **23**, and **24** with no need for further purification. When the penta-*NHBoc* paromomycin **21a** was reacted with 20 equivalents of 2,4,6-triisopropylbenzene-sulfonyl chloride, a mixture of the di-trisylated product **21b** and of the mono-trisylated product **25a** was obtained (43% and 33% isolated yields, respectively, Scheme 4). NMR characterization of **25a** 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 **25a** after the removal of the *NHBoc* groups). The 6'-*O*-trisyl of **25a** was displaced by 1-*n*-hexadecanethiol resulting in compound **25b**; **25b** was treated with TFA to yield the C₁₆ aliphatic chain thioether **25**. Compound **25** was prepared as the single aliphatic chain anchor analogue of the di-C₈ aliphatic chain paromomycin analogue **24**.

It is noted that other nitrogen protecting groups as defined herein may be used instead of the BOC protecting group described hereinabove, with suitable protection and deprotection methods being utilized as known in the art.

Example 8 - Results: Paromomycin-based cationic amphiphiles

A. Antibacterial Activity Tests Against Skin Injection Causing Bacteria

Compounds **22-25** 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 3).

Antibiotics	Bacterial strains ^[a]													
	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
1A	>64	>64	2	>64	16	>64	64	64	32	32	64	64	64	16
22	32	>64	8	16	64	8	16	8	4	8	8	8	8	4
23	8	16	4	4	16	4	4	2	2	4	4	4	4	4
24	4	8	2	2	4	4	2	2	2	2	2	2	2	2
25	64	64	32	64	64	32	>64	16	32	64	32	8	32	8

Table 3. Antibacterial activity: MIC values ($\mu\text{g/mL}$) of mono- and di-alkylated amphiphiles (**22-25**), the parent drug paromomycin (**1A**), and the membrane targeting antibiotic gramicidin D. ^[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* MIT1; **H**, *S. pyogenes* M2; **I**, *S. pyogenes* M3; **J**, *S. pyogenes* M5; **K**, *S. pyogenes* M24; **L**, *S. pyogenes* JRS75; **M**, *S. pyogenes* glossy; and **N**, *S. pyogenes* T5.

These studies 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**) (Bearden, 2008), *Staphylococcus aureus* (Cowan, Strain **E**) (Löffler, 2010), which causes skin infections in patients with compromised immune systems such as HIV carriers, and two strains of *Staphylococcus epidermidis* (strains **C** and **D**) (Otto, 2009) that were once regarded as harmless human skin colonizing bacteria but are now recognized as major opportunistic pathogens. Also tested were 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 (Johansson, 2010).

Of the tested di-alkylated paromomycin derivatives, the C₆ alkyl chain derivative **22** was the least active against all of the tested strains. Compound **22** was less potent than the

parent paromomycin **1A** against the five tested *Staphylococci* strains **A-E**. The di-C₆ alkylated derivative **22** had superior activity compared to paromomycin **1A** against all nine *Streptococcus pyogenes* strains **F-N** (MIC range from 4 to 16 $\mu\text{g/mL}$ for compound **22**, and 16 to >64 $\mu\text{g/mL}$ for paromomycin **1A**). The di-C₇ alkyl chain derivative **23** was superior to compound **22** against most of the 14 tested strains (MIC range from 2 to 16 $\mu\text{g/mL}$). The most potent antimicrobial activity was observed for the di-C₈ alkyl chain derivative **24** with MICs ranging from 2 to 8 $\mu\text{g/mL}$ against all tested strains. The 6'-C₁₆ linear aliphatic chain paromomycin derivative **25**, designed as the mono-alkyl chain anchor analogue of the di-C₈ alkyl chain paromomycin derivative **24**, had poor antimicrobial activity against all of the tested bacterial strains (MIC range from 8 to >64 $\mu\text{g/mL}$). In addition to comparing the antimicrobial activity of the alkylated paromomycins to that of the ribosome targeting parent antibiotic paromomycin **1A**, their activity was evaluated against that of the membrane targeting oligopeptide mixture gramicidin D (Koo, 2001). With the exceptions of the two *Staphylococcus epidermidis* strains (**C** and **D**), gramicidin D was ineffective against the tested *Staphylococci* strains (Table 3). Compounds **23** and **24** 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 $\mu\text{g/mL}$, and for strain **N**, MIC = 2 $\mu\text{g/mL}$). Although more potent than compounds **22**, **23**, and **24** 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 $\mu\text{g/mL}$, and MIC = 16 $\mu\text{g/mL}$, respectively); compounds **22-24** were more potent than gramicidin D against these *Streptococci* strains. The antimicrobial activity tests demonstrated that the di-alkylated paromomycin amphiphiles have a broader spectrum of antimicrobial activity than the parent aminoglycoside **1A** or the membrane targeting antibiotic gramicidin D. The significantly more potent antimicrobial activity of the di-C₈ alkyl chain paromomycin derivative **24** compared to that of the mono C₁₆ linear aliphatic chain paromomycin derivative **25** demonstrates the favorable effect that may result from the attachment of more than one linear aliphatic chain to the aminoglycoside scaffold.

B. Red blood cell hemolysis test

The selectivity of all of the amphiphilic paromomycin derivatives **22-25** 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 one hour of incubation with increasing concentrations of the tested compounds (up to 256 $\mu\text{g/mL}$) at 37 °C. The membrane targeting gramicidin D caused hemolysis at low concentrations close to the MIC range of this antimicrobial agent ($2.4 \pm 1.4\%$ at 2 $\mu\text{g/mL}$), and a steep concentration-dependent elevation in the percentage of hemolysis was observed for gramicidin D (Figure 3). In contrast, even at concentrations of 128 $\mu\text{g/mL}$, compounds **22**, **23**, and **24** were significantly less hemolytic than gramicidin D (Figure 3). The aliphatic chain length affected the percentage of hemolysis with the lowest hemolysis caused by the di- C_6 alkyl chain paromomycin derivative **22** ($3.4 \pm 1.2\%$ hemolysis at a concentration of 256 $\mu\text{g/mL}$) and the highest by the di- C_8 alkyl chains paromomycin derivative **24** ($62.5 \pm 7.9\%$ at 256 $\mu\text{g/mL}$).

Whereas the mono C_{16} linear aliphatic chain paromomycin derivative **25** 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 $\mu\text{g/mL}$, compound **25** already caused $33.8 \pm 5.2\%$ hemolysis of rat RBCs; at the same concentration compounds **22**, **23**, and **24** caused almost no measurable hemolysis (0% for **22** and **23**, and $0.8 \pm 0.3\%$ for **24**). Moreover, at a concentration of 32 $\mu\text{g/mL}$ compound **25** caused almost a 100% hemolysis; the di-alkylated paromomycin derivative **24** and the highly hemolytic gramicidin D caused no more than 60% hemolysis even at a concentration of 256 $\mu\text{g/mL}$. Hemolysis experiments demonstrated that compound **25** 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 $\mu\text{g/mL}$, the di- C_6 alkyl chain paromomycin derivative **22** 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 **23** against the 14 tested bacterial strains was 16 $\mu\text{g/mL}$; for 11 of the tested strains the MIC of compound **23** was not higher than 4 $\mu\text{g/mL}$. However, at 32 $\mu\text{g/mL}$ which is two to eight times higher than the MICs, this compound caused almost no measurable hemolysis ($3.6 \pm 1.9\%$).

The di-C₈ alkyl chain paromomycin derivative **24** demonstrated the most potent antimicrobial activity against all of the tested bacterial strains (MIC range from 2 to 8 $\mu\text{g/mL}$). The paromomycin derivative with the di-C₇ alkyl chain, **23**, was either as active or one double dilution less active than compound **24** against the tested bacterial strains and caused significantly less RBC hemolysis than compound **24**. At 256 $\mu\text{g/mL}$, compound **23** caused $17.6 \pm 4.3\%$ hemolysis; compound **24** caused $62.5 \pm 7.9\%$ hemolysis at the same concentration. The high level of hemolysis caused by compound **24** at 256 $\mu\text{g/mL}$ was similar to that caused by gramicidin D. Hence, of the three di-alkylated paromomycins, the di-C₇ alkyl chain derivative **23** that demonstrated potent antimicrobial activities against all 14 bacterial strains and was dramatically less hemolytic than both Gramicidin D and di-C₈ alkyl chain paromomycin derivative **24**. Therefore, in terms of the ratio of hemolysis to antimicrobial activity, compound **23** is the most potent of the di-alkylated paromomycins that were studied.

C. 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 (**1A**) and to the most potent of the di-alkylated paromomycins, compound **24** at a concentration of 1 $\mu\text{g/mL}$; 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 (Figure 4a). After 1 hour of incubation with paromomycin **1A**, moderate wrinkling of the bacterial cell surface was observed (Figure 4b). As paromomycin **1A** 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-C₈ alkyl chain paromomycin derivative **24** were severely damaged (Figure 4c). These cells had severe surface wrinkling and roughening that resulted from irregularly-shaped cell walls and damaged membranes. At higher concentrations of compound **24**, 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 (Wallin, 1998). Unlike the minor cell surface damage caused by paromomycin **1A** that presumably results from impaired membrane protein synthesis, the severe cell surface damage

caused by compound **24** can be rationalized by direct and rapid membrane disrupting effects of this compound.

D. Conclusions

In conclusion, three 5",6'-di-alkylated paromomycin-based cationic amphiphiles differing in the length of the linear hydrophobic chain (C₆, C₇, and C₈ 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-C₈ aliphatic chain paromomycin derivative **24** 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 **1A** against most of the tested *Staphylococci* strains and some of the tested *Streptococci* strains.

RBC hemolysis tests indicated that both the aliphatic chain length and number of chains affected the level of undesired hemolysis of red blood cells by the reported paromomycin-based cationic amphiphiles. The C₆ chain-based paromomycin derivative **22** was the least hemolytic; however, this compound was also the least potent antimicrobial agent of the three di-alkylated paromomycins tested. The di-C₇ aliphatic chain paromomycin analogue **23** is of particular interest. This compound was either as effective or one double dilution less potent than the di-C₈ aliphatic chain paromomycin analogue **24** against all of the tested bacteria, yet this compound caused considerably less hemolysis of laboratory rat RBCs compared to both compound **24** and the clinically used gramicidin D. Moreover, the mono C₁₆ linear aliphatic chain paromomycin derivative **25**, which was synthesized as the mono aliphatic chain analogue of the di-C₈ chain paromomycin analogue **24**, 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-C₈ aliphatic chain paromomycin analogue **24** had extensive cell surface damage compared to that caused by the parent ribosome-targeting aminoglycoside **1A**. This extensive damage caused by analogue **24** is attributed to the disruption of the membranes of the treated bacteria and 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-*n*-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-*n*-alkylation approach may be more favorable than the mono-*n*-alkylation approach in designing aminoglycoside-based cationic amphiphiles both in terms of enhancing the antimicrobial activity and in reducing the undesired hemolytic effect.

Example 9: Synthetic Methods: Paromomycin Derivatives

A. General methods and instrumentation

Methods and instrumentation for these experiments are as described above in Example 3.

All chemicals, unless otherwise stated, were obtained from commercial sources. Compounds were purified flash chromatography (SiO₂, Merck, Kieselgel 60).

B. Synthetic procedures

Compound 21a. 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 Boc₂O (2.6 g, 12 mmol) was added, and the mixture was stirred at ambient temperature for 12 hours. Reaction progress was monitored by TLC (9% methanol in dichloromethane). Upon completion, the reaction mixture was concentrated, diluted with ethyl acetate (150mL), washed with brine, dried over MgSO₄, and concentrated under reduced pressure. The crude was purified by flash column chromatography over silica gel (7.5% methanol/dichloromethane) to afford compound **21a** as a white solid (1.15 g, 85%).

Compound 21b. A solution of **21a** (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 MgSO₄, and concentrated under reduced pressure. The concentrated crude was purified by flash column chromatography over silica gel (2.5% methanol in dichloromethane) to afford the desired product **21b** as a white solid (1.9 g, 86%): ¹H NMR (500 MHz, methanol-d₄) δ 7.32 (s, 2H, SO₃C₆H₂(CH)₃(CH₃)₆), 7.31 (s, 2H, SO₃C₆H₂(CH)₃(CH₃)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''(2H), 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''', $\text{SO}_3\text{C}_6\text{H}_2(\text{CH})_3(\text{CH}_3)_6(4\text{H})$), 3.20 (m, 1H, H-6'''), 2.99 (m, 3H, $\text{SO}_3\text{C}_6\text{H}_2(\text{CH})_3(\text{CH}_3)_6(2\text{H})$, H-6'''), 1.97 (m, 1H, H-2eq), 1.47 - 1.43 (m, 12H, 46H, $5\text{XCO}_2\text{C}(\text{CH}_3)_3$, H-2ax), 1.33 - 1.23 (m, 36H, $\text{SO}_3\text{C}_6\text{H}_2(\text{CH})_3(\text{CH}_3)_6$). ^{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 (anomeric), 98.8 (anomeric), 97.3 (anomeric), 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 calc'd for $\text{C}_{78}\text{H}_{128}\text{N}_5\text{O}_{28}\text{S}_2$ 1646.83, found 1646.01 $[\text{M}-\text{H}]^-$.

Compound 22a. To a solution of compound **21b** (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 over silica gel (4% methanol/dichloromethane) to afford compound **22a** (159 mg, 80%) as a white solid: ^1H 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''(2H)), 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, $2\text{xSCH}_2\text{CH}_2(\text{CH}_2)_3\text{CH}_3$), 2.05 - 1.94 (m, 1H, H-2eq), 1.67 - 1.53 (m, 4H, $2\text{xSCH}_2\text{CH}_2(\text{CH}_2)_3\text{CH}_3$), 1.53 - 1.37 (m, 58H, $5\text{xCO}_2\text{C}(\text{CH}_3)_3$, $2\text{xSCH}_2\text{CH}_2(\text{CH}_2)_3\text{CH}_3$, H-2eq), 0.99 - 0.82 (m, 6H, $2\text{xSCH}_2\text{CH}_2(\text{CH}_2)_3\text{CH}_3$). ^{13}C NMR (125 MHz, methanol- d_4) δ 157.4, 157.0, 156.8, 156.7, 156.3, 109.9 (anomeric), 98.9 (anomeric), 97.5 (anomeric), 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 calc'd for $\text{C}_{60}\text{H}_{110}\text{N}_5\text{O}_{22}\text{S}_2$ 1316.70, found 1316.58 $[\text{M}+\text{H}]^+$.

Compound 23a. To a solution of compound **21b** (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 over silica gel (4% methanol/dichloromethane) to afford compound **23a** (155 mg, 76%) as a white solid: ^1H NMR (500 MHz, methanol- d_4) δ 5.47 (bs, 1H, H-1'), 5.15 (d, $J = 3.6$ Hz, 4H, 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'''(2H)), 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, $2\times\text{SCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_3$), 2.02 (dd, $J = 13.5, 4.8$ Hz, 1H, H-2eq), 1.66-1.59 (m, 4H, $2\times\text{SCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_3$), 1.57 – 1.15 (m, 62H, $5\times\text{CO}_2\text{C}(\text{CH}_3)_3$, $2\times\text{SCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_3$, H-2ax), 0.94- 0.91 (m, 6H, $2\times\text{SCH}_2\text{CH}_2(\text{CH}_2)_5\text{CH}_3$). ^{13}C NMR (125 MHz, methanol- d_4) δ 157.4, 157.1, 156.7, 156.3, 109.9 (anomeric), 98.8 (anomeric), 97.5 (anomeric), 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 calc'd for $\text{C}_{62}\text{H}_{114}\text{N}_5\text{O}_{22}\text{S}_2$ 1344.73, found 1344.67 $[\text{M}+\text{H}]^+$.

Compound 24a. To a solution of compound **21b** (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 **24a** (162 mg, 78%) as a white solid: ^1H 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'''(2H)), 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, $2\times\text{SCH}_2\text{CH}_2(\text{CH}_2)_5\text{CH}_3$), 2.02 (m, 1H, H-2eq), 1.62 (m, 4H, $2\times\text{SCH}_2\text{CH}_2(\text{CH}_2)_5\text{CH}_3$), 1.53 – 1.41 (m, 66H, $5\times\text{CO}_2\text{C}(\text{CH}_3)_3$,

2xSCH₂CH₂(CH₂)₅CH₃, H-2ax), 0.94 – 0.91 (m, 6H, 2xSCH₂CH₂(CH₂)₅CH₃). ¹³C NMR (125 MHz, methanol-d₄) δ 157.4 , 157.0 , 156.8 , 156.7 , 156.3 , 109.9 (anomeric), 98.9 (anomeric) , 97.5 (anomeric), 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* calc'd for C₆₄H₁₁₈N₅O₂₂S₂ 1372.76, found 1372.54 [M+H]⁺.

Compound 22. Compound **22a** (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 H₂O and freeze-dried to afford compound **22** (21 mg, quantitative yield) as a white foam: ¹H NMR (400 MHz, methanol-d₄) 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, 2xSCH₂CH₂(CH₂)₃CH₃), 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, 2xSCH₂CH₂(CH₂)₃CH₃), 1.50-1.24 (m, 12H, 2xSCH₂CH₂(CH₂)₃CH₃), 0.90 (m, 6H, 2xSCH₂CH₂(CH₂)₃CH₃). ¹³C NMR (100 MHz, methanol-d₄) δ 161.7 (q, *J* = 34.6 Hz, CF₃CO₂H), 116.7 (q, *J* = 292.8 Hz, CF₃CO₂H), 110.7 (anomeric) , 96.2 (anomeric) , 95.5 (anomeric) , 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* calc'd for C₃₅H₇₀N₅O₁₂S₂ 816.4462 found 816.4460 [M+H]⁺.

Compound 23. Compound **23a** (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 H₂O and freeze-dried to afford compound **23** (15.6 mg, quantitative yield) as a white foam: ¹H NMR (400 MHz, methanol-d₄) 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, 2xSCH₂CH₂(CH₂)₄CH₃), 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, 2xSCH₂CH₂(CH₂)₄CH₃), 1.50 – 1.19 (m, 16H, 2xSCH₂CH₂(CH₂)₄CH₃), 0.98 – 0.78 (m, 6H, 2xSCH₂CH₂(CH₂)₃CH₃). ¹³C NMR (125 MHz, methanol-d₄) δ 161.7 (q, $J = 34.6$ Hz, CF₃CO₂H), 116.7 (q, $J = 292.7$ Hz, CF₃CO₂H), 110.7 (anomeric), 96.2 (anomeric), 95.5 (anomeric), 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 calc'd for C₃₇H₇₄N₅O₁₂S₂ 844.4775 found 844.4770 [M+H]⁺.

Compound 24. Compound **24a** (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 H₂O and freeze-dried to afford compound **24** (31.5 mg, quantitative yield) as a white foam: ¹H NMR (400 MHz, methanol-d₄) 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, 2xSCH₂CH₂(CH₂)₅CH₃), 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, 2xSCH₂CH₂(CH₂)₅CH₃), 1.48 – 1.17 (m, 20H, 2xSCH₂CH₂(CH₂)₅CH₃), 0.97 – 0.67 (m, 6H, 2xSCH₂CH₂(CH₂)₅CH₃). ¹³C NMR (100 MHz, methanol-d₄) δ 161.7 (q, $J = 34.7$ Hz, CF₃CO₂H), 116.7 (q, $J = 292.7$ Hz, CF₃CO₂H), 110.7 (anomeric), 96.1 (anomeric), 95.5 (anomeric), 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 calc'd for C₃₉H₇₈N₅O₁₂S₂ 872.5088 found 872.5087 [M+H]⁺.

Compound 25a. A solution of **21a** (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 (100mL), washed with brine, dried over MgSO₄, and concentrated under reduced pressure. The concentrated crude was purified by flash column chromatography over silica gel (3% methanol in dichloromethane) to afford **21b** (0.31, 43%) and the desired product **25a** as a white solid (0.2 g, 33%): ¹H NMR (400 MHz, methanol-d₄) δ 7.29 (s, 2H, SO₃C₆H₂(CH)₃(CH₃)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'(2H), 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'''(2H), SO₃C₆H₂(CH)₃(CH₃)₆ (2H)), 2.95 (hep, *J* = 6.9 Hz, 1H, SO₃C₆H₂(CH)₃(CH₃)₆), 1.93 (dt, *J* = 12.1, 4.3 Hz, 1H, H-2eq), 1.51-1.38 (m, 46H, 5xCO₂C(CH₃)₃, H-2ax), 1.32-1.22 (m, 18H, SO₃C₆H₂(CH)₃(CH₃)₆). ¹³C NMR (100 MHz, methanol-d₄) δ 159.7, 159.4, 159.0, 159.3, 158.7, 156.2, 153.0, 131.6, 125.8, 111.9 (anomeric), 100.9 (anomeric), 100.0 (anomeric), 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* calc'd for C₆₃H₁₀₆N₅O₂₆S 1380.69, found 1380.58 [M-H]⁻.

Compound 25b. To a solution of compound **25a** (80 mg, 0.057 mmol) and Cs₂CO₃ (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 MgSO₄, and concentrated under reduced pressure. The concentrated crude was purified by flash column chromatography over silica gel (4% methanol / dichloromethane) to afford compound **25b** (60 mg, 77%) as a white solid: ¹H NMR (400 MHz, methanol-d₄) 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'''(2H)), 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, SCH₂CH₂(CH₂)₁₂CH₃), 1.98 (dt, *J* = 13.0, 4.0 Hz, 1H, H-2eq), 1.59 (m, 2H,

SCH₂CH₂(CH₂)₁₂CH₃), 1.53 – 1.39 (m, 46H, 5xCO₂C(CH₃)₃, H-2ax), 1.30- 1.29 (m, 24H, SCH₂CH₂(CH₂)₁₂CH₃), 0.97 – 0.70 (m, 3H, SCH₂CH₂(CH₂)₁₂CH₃). ¹³C NMR (100 MHz, methanol-d₄) δ 157.5 , 157.1 , 156.8 , 156.3 , 109.6 (anomeric) , 98.9 (anomeric), 97.8 (anomeric), 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* calc'd for C₆₄H₁₁₈N₅O₂₃S 1356.79, found 1356.34 [M+H]⁺.

Compound 25. Compound **25b** (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 H₂O and freeze-dried to afford compound **25** (21 mg, quantitative yield) as a white foam: ¹H NMR (400 MHz, methanol-d₄) 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''(2H)), 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''(2H)), 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, SCH₂CH₂(CH₂)₁₂CH₃), 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, SCH₂CH₂(CH₂)₁₂CH₃), 1.41-1.25 (m, 24H, SCH₂CH₂(CH₂)₁₂CH₃), 0.90-0.85 (m, 3H, SCH₂CH₂(CH₂)₁₂CH₃). ¹³C NMR (100 MHz, methanol-d₄) δ 161.7 (q, *J* = 34.8 Hz, CF₃CO₂H), 116.7 (q, *J* = 292.4, CF₃CO₂H), 110.3 (anomeric), 97.3 (anomeric), 95.6 (anomeric), 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* calc'd for C₃₉H₇₈N₅O₁₃S 856.5317 found 856.5318 [M+H]⁺.

C. Biological assays

1. Minimal inhibitory concentration protocol

Starter cultures were incubated for 24 hours (37 °C, 5% CO₂, aerobic conditions) and then diluted in fresh medium to obtain an optical density of 0.004 (OD₆₀₀). All strains were tested using the double-dilution method starting at 128 μg/mL in 96-well plates (Sarstedt). After 24 hours of incubation, MTT (50 μL of a 1 mg/mL solution in H₂O) was added to each well

followed by additional incubation at 37°C for 2 hours. MIC values ($\mu\text{g/mL}$) 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.

2. Red blood cell hemolysis protocol

A sample of rat RBCs (2% w/w) were incubated with each of the tested compounds for 1 hour at 37 °C, 5% CO₂ using the double dilution method starting at concentration of 256 $\mu\text{g/mL}$. Negative control was PBS and positive control was 1% w/v solution of Triton X100 (100% hemolysis). Following centrifugation (2,000rpm, 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.

3. Scanning electron microscopy protocol

Cultures of *S. epidermidis* ATCC12228 were incubated for 4 hours at 37 °C under aerobic conditions to obtain an optical density of 0.2 (OD₆₀₀) and then harvested by centrifugation at 4,000 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 (OD₆₀₀). The bacterial suspension was diluted two fold after treatment with the tested compound at a concentration of 1 $\mu\text{g/mL}$ at 37 °C for 1 hour. The cells were then spun down at 6,000 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% to 100%), and dried in vacuum desiccation. Finally, the samples were coated with palladium-gold and viewed via scanning electron microscopy (Quanta 200FEG ESEM).

Abbreviations

BHI, brain heart infusion; BOC, *tert*-butoxycarbonyl; 1-D-TOCSY, Total Correlation Spectroscopy; DIEA, *N,N*-Diisopropylethylamine; DMF, dimethylformamide; EtOAc, ethyl acetate; HBTU, *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; HR-ESI- High resolution electron spray ionization; LR-ESI, Low resolution electron spray

ionization MIC, minimum inhibitory concentration; MBIC, minimal biofilm inhibitory concentration; MTT, thiazolyl blue tetrazolium bromide; PBS, phosphate buffered saline; RBC, red blood cells; rt, room temperature; SEM, Scanning electron microscopic; TIBSCL, 2,4,6-triisopropylbenzenesulfonyl chloride; TFA, trifluoroacetic acid; TLC, thin layer chromatography; TSB, trypticase soy broth.

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The contents of each of the references cited are incorporated by reference herein in their entirety as if fully set forth herein.

It will be appreciated by persons skilled in the art that the present invention is not limited by what has been particularly shown and described herein above. Rather the scope of the invention is defined by the claims that follow.

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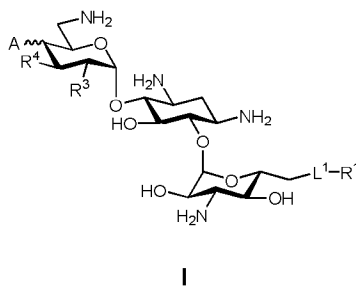
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CLAIMS

1. A tobramycin or kanamycin A derivative represented by the structure of formula I, or a paromomycin derivative represented by the structure of formula I-A:

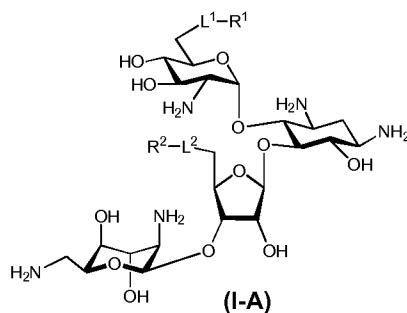


wherein, in formula I:

A is OH or -L²-R², wherein:

- (i) when A is OH, L^1 is selected from the group consisting of $-OSO_2-$, triazolyl, and $-NH-C(=O)-$; and R^1 is a C_{12} - C_{16} linear alkyl;
- (ii) when A is $-L^2-R^2$, each of L^1 and L^2 is selected from the group consisting of $-S-$, $-SO_2-$, $-OSO_2-$, triazolyl and $-NH-C(=O)-$; and each of R^1 and R^2 is a linear C_4 - C_8 alkyl; and

R³ and R⁴ are each OH; or R³ is NH₂ and R⁴ is H;



wherein, in formula I-A:

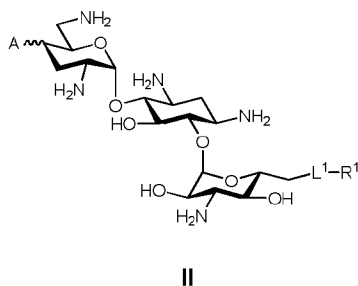
L¹ and L² are each independently selected from the group consisting of -S-, -SO₂-, -OSO₂-, triazolyl and -NH-C(=O)-; and

R¹ and R² are each independently a linear C₄ to C₈ alkyl;

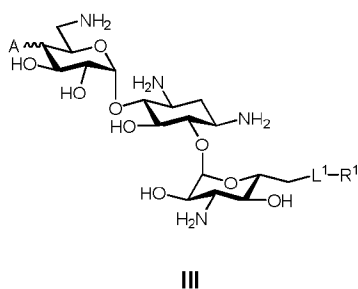
including salts, solvates, polymorphs, optical isomers, geometrical isomers,

enantiomers, diastereomers, and mixtures thereof.

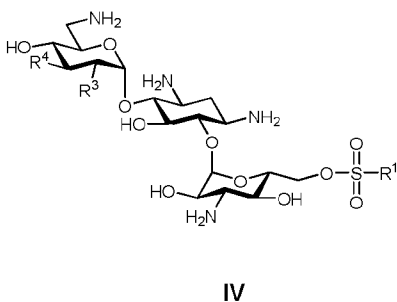
2. A tobramycin or kanamycin A derivative represented by the structure of formula I according to claim 1.
3. The compound according to claim 2, wherein R^3 is NH_2 and R^4 is H, and the compound is a tobramycin derivative, represented by the structure of formula II:



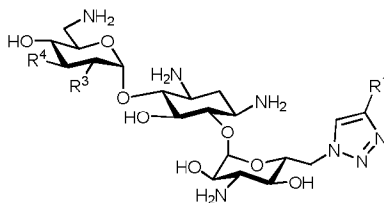
4. The compound according to claim 2, wherein R^3 and R^4 are each OH, and the compound is a kanamycin A derivative represented by the structure of formula III:



5. The compound according to any of claims 2 to 4, wherein A is OH in the equatorial position.
6. The compound according to claim 5, wherein L^1 is a sulfonyl ester of the formula $-OSO_2-$, and the compound is represented by the structure of formula IV:

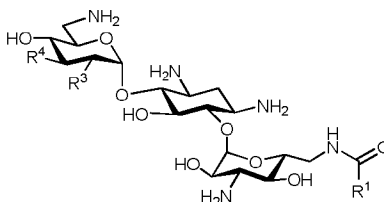


7. The compound according to claim 5, wherein L^1 is a triazolyl, and the compound is represented by the structure of formula V:



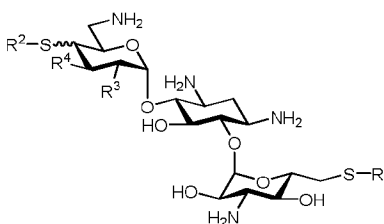
V

8. The compound according to claim 5, wherein L^1 is an amide of the formula $-NH-C(=O)-$, and the compound is represented by the structure of formula VI:



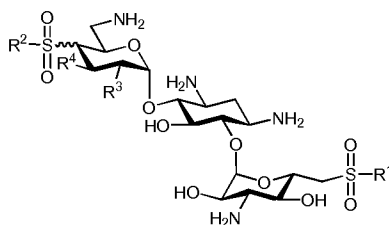
VI

9. The compound according to any of claims 2 to 8, wherein R^1 is selected from the group consisting of $-(CH_2)_{11}CH_3$, $-(CH_2)_{13}CH_3$ and $-(CH_2)_{15}CH_3$.
10. The compound according to claim 5, which is selected from the group consisting of compound (4a), (4b), (4c), (5a), (5b), (5c), (6a), (6b), (6c), (14a), (14b), (14c), (15a), (15b), (15c), (16a), (16b) and (16c).
11. The compound according to any of claims 2 to 4, wherein A is $-L^2-R^2$.
12. The compound according to claim 11, wherein A is in the axial position.
13. The compound according to claim 11, wherein L^1 and L^2 are each a thioether of the formula $-S-$, and the compound is represented by the structure of formula VII:



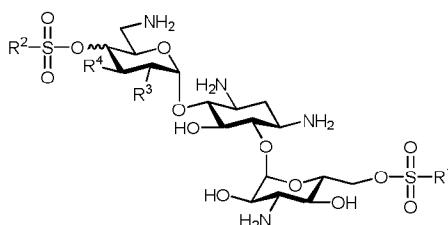
VII

14. The compound according to claim 11, wherein L^1 and L^2 are each a sulfonyl of the formula $-\text{SO}_2-$, and the compound is represented by the structure of formula VIII:



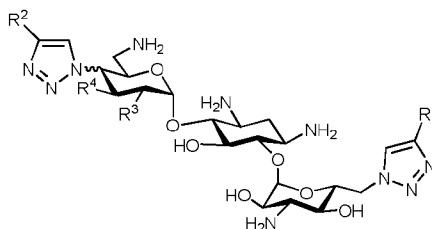
VIII

15. The compound according to claim 11, wherein L^1 and L^2 are each a sulfonyl ester of the formula $-\text{OSO}_2-$, and the compound is represented by the structure of formula IX:



IX

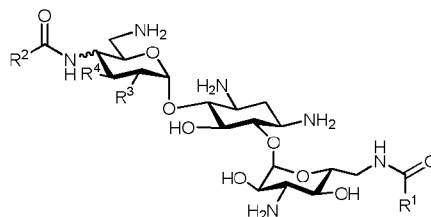
16. The compound according to claim 11, wherein L^1 and L^2 are each a triazolyl, and the compound is represented by the structure of formula X:



X

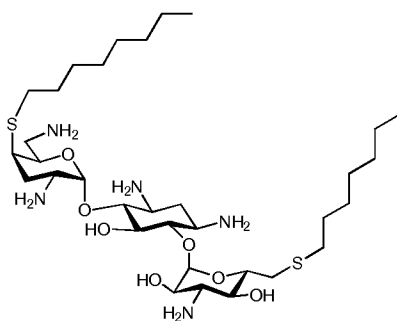
17. The compound according to claim 11, wherein L^1 and L^2 are each an amide, and the compound is represented by the structure of formula XI:

74



XI

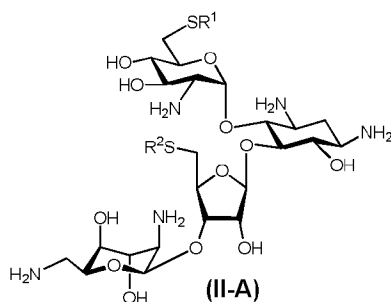
18. The compound according to any of claims 11 to 17, wherein R^1 and R^2 are each a C_6 - C_8 linear alkyl.
19. The compound according to any of claims 11 to 13, which is represented by the structure of formula 17:



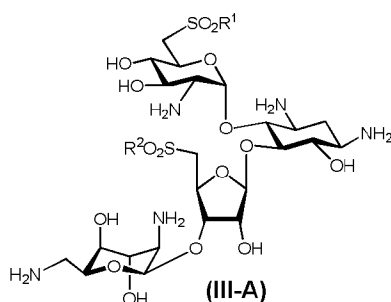
17

20. A paromomycin derivative represented by the structure of formula I-A according to claim 1.
21. The compound according to claim 20, wherein each of R^1 and R^2 is independently selected from the group consisting of $-(CH_2)_5CH_3$, $-(CH_2)_6CH_3$ and $-(CH_2)_7CH_3$.
22. The compound according to claim 20, wherein L^1 and L^2 are each a thioether of the formula -S-, and the compound is represented by the structure of formula II-A:

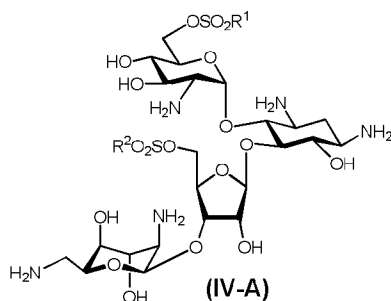
75



23. The compound according to claim 20, wherein L^1 and L^2 are each a sulfonyl of the formula $-\text{SO}_2-$, and the compound is represented by the structure of formula III-A:

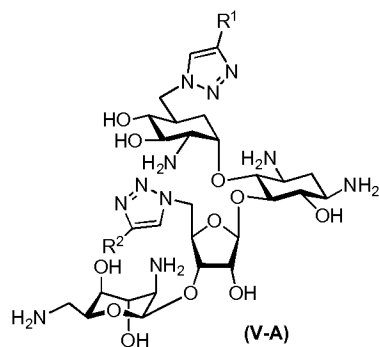


24. The compound according to claim 20, wherein L^1 and L^2 are each a sulfonyl ester of the formula $-\text{OSO}_2-$, and the compound is represented by the structure of formula IV-A:

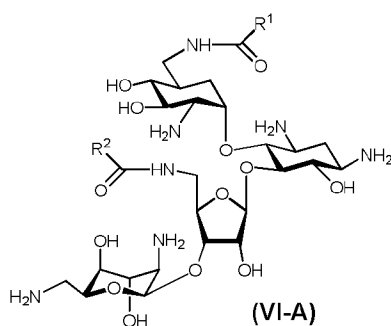


25. The compound according to claim 20, wherein L^1 and L^2 are each a triazolyl, and the compound is represented by the structure of formula V-A:

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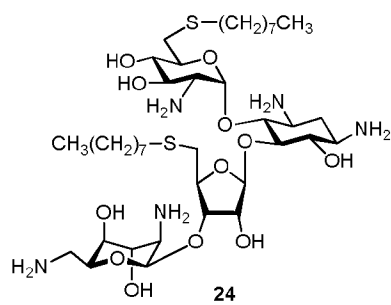
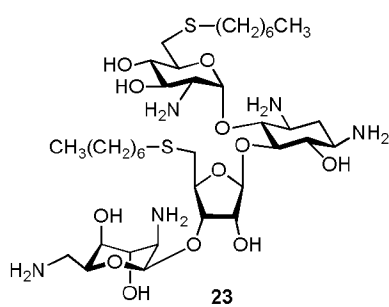
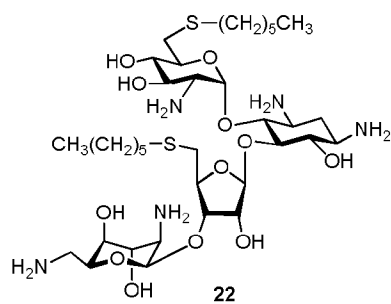


26. The compound according to claim 20, wherein L¹ and L² are each an amide of the formula -NH-C(=O)-, and the compound is represented by the structure of formula VI-A:



27. The compound according to claim 20, which is represented by the structure of formula 22, 23 or 24:

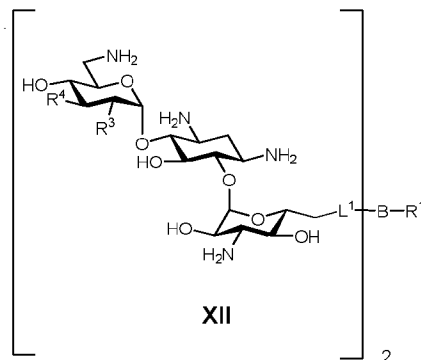
77



28. The compound according to claim 20, which is selected from the group consisting of formula (26a), (26b), (26c), (27a), (27b), (27c) (28a), (28b), (28c), (29a), (29b) and (29c).

29. A dimeric compound represented by the structure of formula XII:

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wherein

R^1 is a C_{12} - C_{16} linear alkyl;

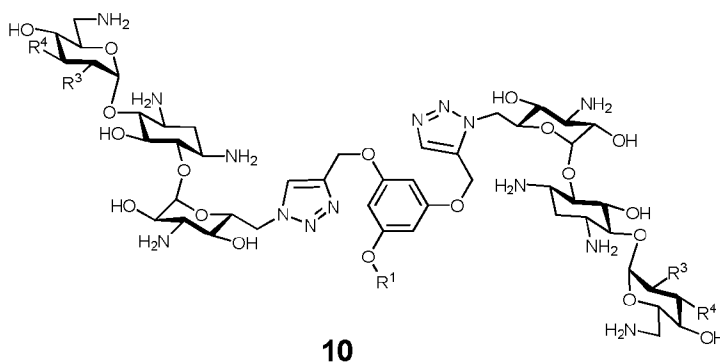
L^1 is selected from the group consisting of -S-, -SO₂-, -OSO₂-, triazolyl and -NH-C(=O)-;

R^3 and R^4 are each OH; or R^3 is NH₂ and R^4 is H; and

B is selected from an unsubstituted or substituted alkyl, aryl, aryloxy, alkyloxy and amide;

including salts, solvates, polymorphs, optical isomers, geometrical isomers, enantiomers, diastereomers, and mixtures thereof.

30. The compound according to claim 29, which is represented by the structure of formula 10:



31. An anti-bacterial pharmaceutical composition comprising a compound according to any of claims 1 to 30, and a pharmaceutically acceptable carrier or excipient.

32. The pharmaceutical composition of claim 31, wherein the composition is in a form suitable for oral administration, intravenous administration by injection,

topical administration, administration by inhalation, or administration via a suppository.

33. A method of combating bacteria, or treating a bacterial infection, comprising the step of administering to a subject in need thereof a compound according to any one of claims 1-30, or a pharmaceutical composition according to claim 31 or 32.
34. Use of a compound according to any one of claims 1-30 or a pharmaceutical composition according to claim 31 or 32, for the manufacture of a medicament for combating bacteria or treating a bacterial infection.
35. A compound according to any one of claims 1-30 or a pharmaceutical composition according to claim 31 or 32, for use in combating bacteria or treating a bacterial infection.
36. A method for combating bacteria or treating a bacterial infection, comprising the step of contacting the bacteria with a compound according to any one of claims 1-30, or a pharmaceutical composition according to claim 31 or 32.
37. The method, use or compound according to any one of claims 33 to 36, wherein the bacteria is a Gram-positive bacteria.
38. The method, use or compound according to any one of claims 33 to 36, wherein the bacteria is a Gram-negative bacteria.
39. The method, use or compound according to any one of claims 33 to 36, wherein the bacteria causes skin infections or soft tissue infections.
40. A method of inhibiting biofilm growth, comprising the step of contacting the biofilm or a surface comprising the biofilm with a compound according to any one of claims 1-30, or a pharmaceutical composition according to claim 31 or 32.

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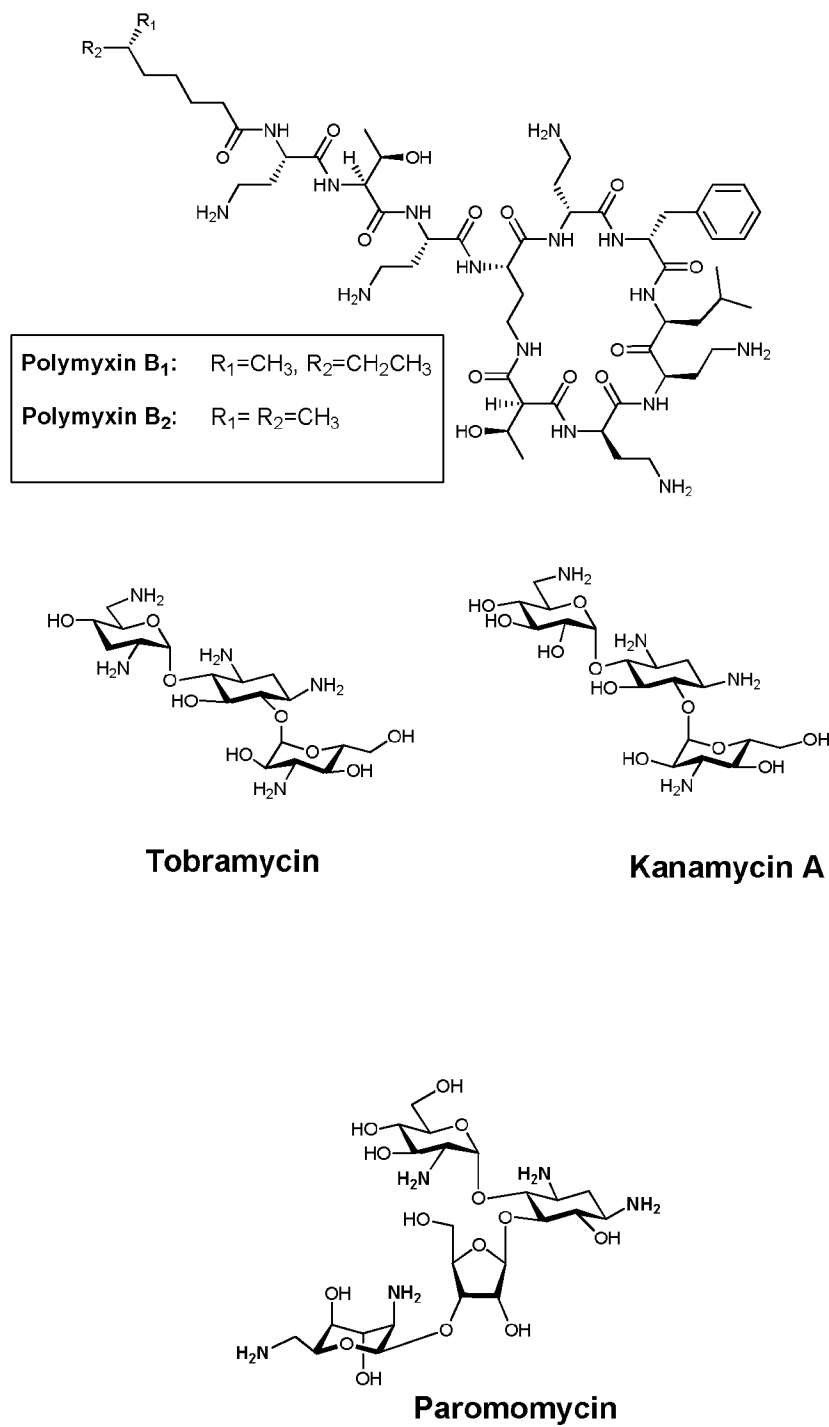


Figure 1

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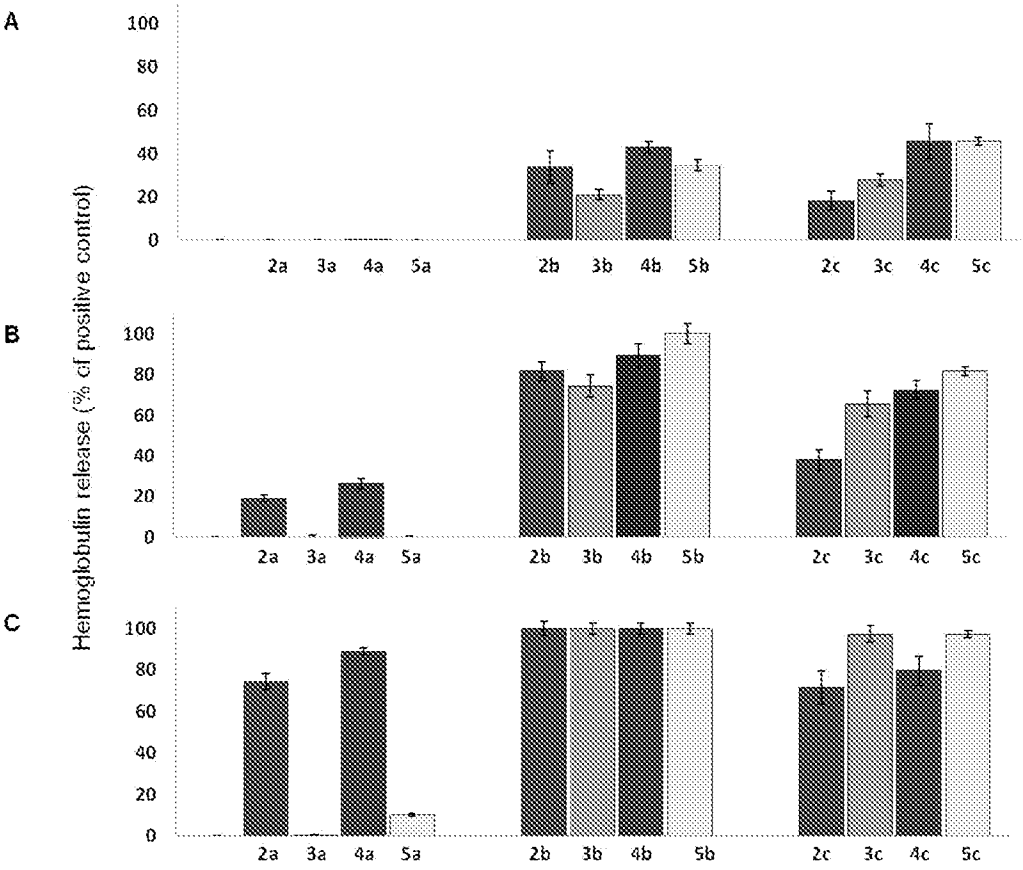


Figure 2

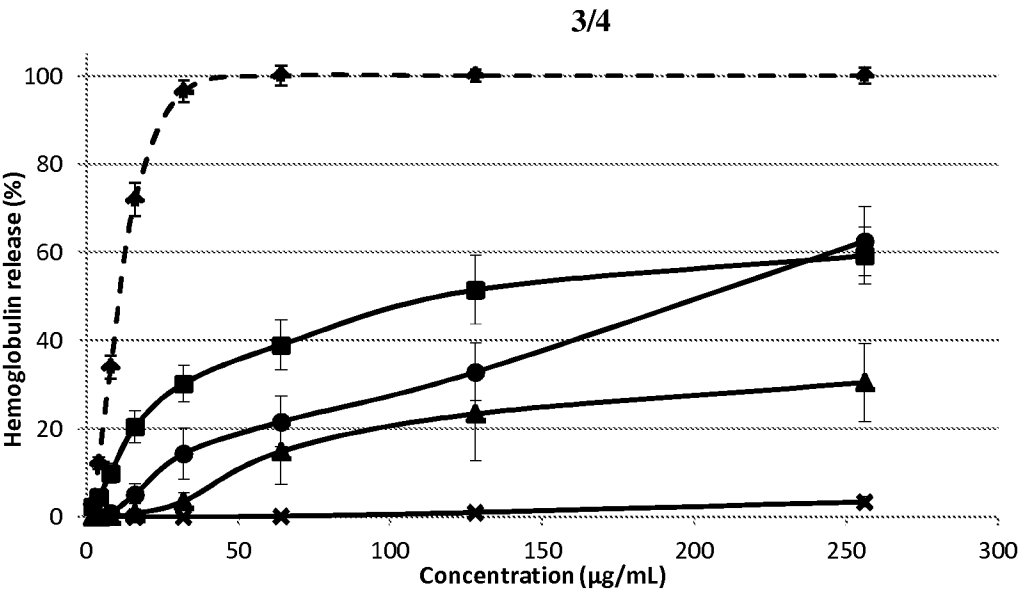


Figure 3

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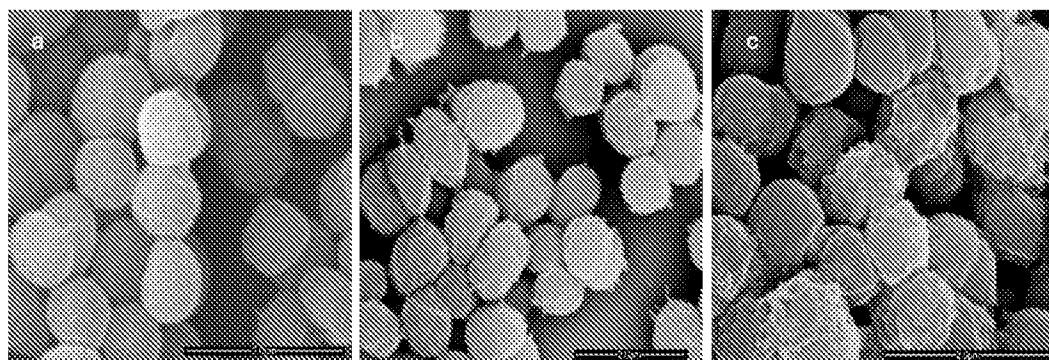


Figure 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2013/050612

A. CLASSIFICATION OF SUBJECT MATTER IPC (2013.01) C07H 15/22, C07H 11/00, C07H 13/06 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC (2013.01) C07H 15/22, C07H 11/00, C07H 13/06 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Databases consulted: Google Patents, CAPLUS, REGISTRY Search terms used: Tobramycin, kanamycin, paromomycin, linear alkyl, hydrophobic, aliphatic.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010004433 A2 UNIV MANITOBA [CA]; BERA SMRITILEHHA [CA]; ZHANEL GEORGE G [CA]; SCHWEIZER FRANK [CA] 14 Jan 2010 (2010/01/14) page 5 and claims . (cited in the application)	1,2,4,5,8,10,31-40
Y	claims (cited in the application)	1-5,7-10,29,31-40
X	WO 2012082650 A2 UNIV UTAH STATE [US]; CHANG CHENG-WEI TOM [US]; TAKEMOTO JON Y [US] 21 Jun 2012 (2012/06/21) claims, table 2	1-6,29,31-40
Y	" Antibacterial activity of amphiphilic tobramycin" Department of Chemistry, University of Manitoba, Winnipeg, Manitoba, Canada. J Antibiot (Tokyo). 11 Jul 2012 (2012/07/11) abstract	1-5,7,29,31-40
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 13 Oct 2013		Date of mailing of the international search report 16 Oct 2013
Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616		Authorized officer VALKOV Karina Telephone No. 972-2-5651777

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2013/050612

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	6"-Thioether tobramycin analogues: Towards selective targeting of bacterial membranes" Angew Chem Int Ed Engl. : Ido M. Herzog, Keith D. Green, Yifat Berkov-Zrihen, Mark Feldman, Roe R. Vidavski, Anat Eldar-Boock, Ronit Satchi-Fainaro, Avigdor Eldar, Sylvie Garneau-Tsodikova, and Micha Fridman 04 Jun 2012 (2012/06/04) all document	1-5,8-10,29,31-40
A	Antibacterial activity of guanidinylated neomycin B- and kanamycin A-derived amphiphilic lipid conjugates Bera S, Zhanel GG, Schweizer F 23 Mar 2010 (2010/03/23) abstract	1-40
P,X	Design of membrane targeting tobramycin-based cationic amphiphiles with reduced hemolytic activity : Ido M. Herzog,a Mark Feldman,a Anat Eldar-Boock,b Ronit Satchi-Fainarob and Micha Fridman 13 Aug 2012 (2012/08/13) abstract	1-40

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/IL2013/050612

Patent document cited search report	Publication date	Patent family member(s)	Publication Date
WO 2010004433 A2	14 Jan 2010	CA 2729852 A1	14 Jan 2010
		EP 2318422 A2	11 May 2011
		US 2011178037 A1	21 Jul 2011
		WO 2010004433 A2	14 Jan 2010
		WO 2010004433 A3	08 Apr 2010
WO 2012082650 A2	21 Jun 2012	US 2012316125 A1	13 Dec 2012
		WO 2012082650 A2	21 Jun 2012
		WO 2012082650 A3	01 Nov 2012