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Journal:	*Medicinal Chemistry Communications*
Manuscript ID:	MD-REV-01-2014-000012.R1
Article Type:	Review Article
Date Submitted by the Author:	23-Feb-2014
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MedChemComm

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REVIEW

Design and synthesis of membrane-targeting antibiotics: From peptides- to aminosugar-based antimicrobial cationic amphiphiles

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Cite this: DOI: 10.1039/x0xx00000x

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

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Infections caused by drug resistant and/or slow-growing bacteria are increasingly becoming a one of the greatest challenges of health organizations worldwide. The decrease in the efficacy of a large percentage of the current repertoire of clinically used antibiotics against these types of infections emphasizes the need for the development of novel antimicrobial agents that will effectively eradicate a broad spectrum of bacteria regardless of the bacterial cell cycle stage. In this Review, we present recent years' progress in the development of cationic amphiphiles that target the bacterial membrane bilayer as a strategy for the development of antibiotics. Synthesis, antimicrobial activity, membrane selectivity and modes of action aspects are discussed.

Antibiotics were once considered miracle drugs, a fact that in 1970, led the Surgeon General of the United States to declare that it was time to "close the book on infectious disease as a major health threat".[1] Decades of use have resulted in enhanced evolution of antibiotic resistance, and bacterial infections have again become a major health problem worldwide.[2] Indeed, a significant percentage of patients admitted to hospitals carry or will develop a serious bacterial infection, and a high percentage of the bacteria that cause these infections are resistant to at least one of the clinically used antibiotics. Since the fate of all clinically used antibiotics is to inevitably evoke the emergence of bacterial resistance, there is an ever growing need for novel approaches that will lead to the discovery of new and broad-spectrum antimicrobials that will ensure the availability of treatments for infectious diseases.[2,3]

Membranes of bacteria as drug targets

Membranes and cell walls are essential for the viability of all bacteria, including bacteria that are dormant, and therefore serve as attractive targets for the development of antibiotics. To date, several families of synthetic as well as natural antibiotics that inhibit different steps of the biosynthesis of the bacterial cell wall peptidoglycan have been discovered. Amongst these antibiotics are β-lactams that irreversibly inhibit the activity of the peptidoglycan trans-peptidation biosynthetic step,[4,5] glyco-peptides such as vancomycin that competitively inhibit the trans-peptidation step, and the glyco-lipid antibiotic moenomycin A that inhibits the peptidoglycan trans-glycosylation biosynthetic step.[6–9]

In contrast to the cell wall, bacterial membranes have only been minimally explored as a target for antibiotics. There are several benefits to the development of such antibiotics: First, targeting bacterial membranes is an effective strategy for combating infections caused by either slow-growing or dormant bacteria that are extremely challenging to treat using the current repertoire of clinically used antibiotics.[10] Second, since bacterial membranes have relatively conserved structures and since membrane-disrupting antibiotics are likely to exert a rapid bactericidal activity,[11] it will be challenging for bacteria to evolve resistance to these antibacterial agents. Hence, membrane targeting antibiotics are likely to maintain prolonged clinical efficacy. Third, the development of antibiotics that target the bacterial membrane does not require bacterial cell permeability considerations that frequently pose an obstacle to the development of antibiotics that target intracellular targets.[12]

However, to be effective and safe for systemic use, membrane-targeting antibiotics must selectively target bacterial, and not mammalian, cell membranes. Avoiding cytotoxicity to eukaryotic cells through non-selective membrane damage is a major challenge in designing membrane-targeting antibiotics. Several fundamental differences between the composition of bacterial membranes and cell surfaces and those of mammalian cells suggest that development of membrane selective antibiotics may in fact be possible: **A.** Membranes of Gram-negative bacteria have a negatively charged core of lipopolysaccharides (LPS), whereas negatively charged teichoic acids are major constituents of Gram-positive bacteria cell walls.[13,14] Neither LPS nor teichoic acids exist in mammalian cells. **B.** Compared to bacterial cell membranes, the plasma membranes of mammalian cells are less negatively charged due to a relatively low percentage of

negatively charged lipids such as cardiolipins (which exist solely in mitochondrial and bacterial membranes), phosphatidylserine, and phosphatidylinositol.[15–18] **C.** The outer leaflet of the membranes of mammalian cells consists mainly of neutral lipids such as phosphatidylcholine and sphingomyelin;[15] negatively charged lipids such as phosphatidylserine and phosphatidylinositol are localized to the inner leaflet of the plasma membrane.[19,20] Since ionic interactions are distance dependent, electrostatic interactions between the negatively charged lipids of the mammalian cell membrane inner leaflet and positively charged molecules arriving from the extracellular environment are reduced compared with the same interactions taking place on the surface of bacterial cell membranes. These differences between the lipid compositions and structures of bacterial and mammalian cell membranes offer the rational basis for the development of several types of positively charged, membrane-targeting antibiotics that will exhibit charge-based selectivity toward bacterial membranes.

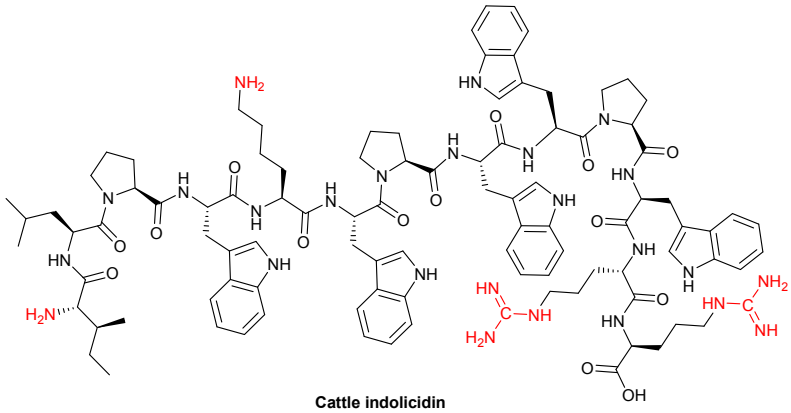
Natural antimicrobial cationic amphiphiles

Cationic antimicrobial peptides

Cationic antimicrobial peptides are produced by the innate immune system of a diverse range of organisms, including mammals, birds, amphibians, crustaceans, fish, and insects, and act by increasing the permeability of bacterial membranes.[21,22] In higher organisms, these short peptides are produced at sites of infection and inflammation and usually possess broad spectrum antibacterial activity. Cationic antimicrobial peptides are not only produced by higher organisms, and can also be found in plants, microbes, and fungi.[23] These natural antimicrobial agents typically consist of 12 to 50 amino acids with a net positive charge ranging between +2 to +7 resulting from the presence of basic amino acids that are positively charged under physiological conditions.[21] An example is indolicidin, which is produced by cattle (Figure 1). Cationic antimicrobial peptides are also amphiphilic, and in most cases over 50% of their amino acids contain hydrophobic side residues. The amphiphilic nature of cationic antimicrobial peptides is key to their interactions with bacterial membranes.[24]

To date, hundreds of antimicrobial peptides have been isolated and characterized from various organisms.[25] All are positively charged cationic amphiphiles, but these peptides do not share high sequence homology.[22,26] In the majority of the cases, antimicrobial cationic peptides form α-helical or β-sheet structures. For example, histatins (Figure 1), which are part of the human repertoire of cationic antimicrobial peptides, adopt an α-helical form when in the presence of organic solvents or lipid bilayers.[27] In these α-helical structures, the hydrophobic amino acid residues face the membrane lipids and the positively charged groups face the aqueous environment. In the case of some β-sheet forming cationic antimicrobial peptides like human β-defencins (Figure 1) disulfide linkages stabilize secondary structures.[28] In others, the β-sheets form upon interaction with membrane lipid bilayers. Natural antimicrobial cationic peptides have provided the inspiration and incentive for the development of several synthetic and semi-synthetic families of small molecules that target bacterial membranes.

Five mechanisms that confer resistance to cationic antimicrobial peptides have been identified so far.[29] The first mechanism involves the degradation of linear cationic antimicrobial peptides by bacterial proteases.[30] The second mechanism involves secretion of bacterial proteins that bind to cationic antimicrobial peptides and prevent their interaction with the bacterial membranes.[31] The third mechanism is based on the incorporation of positively charged chemical groups to various components of the bacterial cell envelope that lead to a reduction in the affinity of the positively charged cationic antimicrobial amphiphiles to the bacterial cell membrane.[32] The fourth mechanism results from the formation of bacterial biofilms which are significantly less permeable to small molecules.[33] Finally, resistance to several cationic antimicrobial peptides has been associated with the action of bacterial efflux pumps.[34] With the exception of bacterial proteases, the mentioned resistance mechanisms may also be relevant to other families of non-peptide-based antimicrobial cationic amphiphiles. However, compared antibiotics that act on intracellular bacterial targets, membrane targeting antibiotics are likely to maintain prolonged clinical efficacy.



Cattle indolicidin

Human β-defensins

β-defencin-1: DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCK

β-defencin-4: LDRICGYGTARCRKKCRSQEYRIGRCPNTYACC LRKPWDESLLNRTK

Histatins

Histatin-1: DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN

Histatin-5: DSHAKRHHGYKRKFHEKHHSHRGY

Fig. 1. Examples of cationic antimicrobial peptides: Amino acids that are positively charged under physiological conditions are marked in red; amino acids with hydrophobic side chains are marked in blue; cysteine is marked in green.

Cationic lipopeptides

This family of natural antimicrobial cationic amphiphiles includes five major compounds (polymyxins A-E, Figure 2) that were discovered during the mid-20th century.[35] Two members of the polymyxin group of antimicrobial agents are in clinical use: polymyxin B and polymyxin E, which is also termed colistin[36]. Isolated from *Bacillus polymyxa*,[37–39] polymyxin B consists of a hepta-peptide ring and a tripeptide side chain with a fatty acid tail. Both polymyxin B and colistin are mixtures of several structurally related compounds that differ from each other in the chemical structure of their fatty acid.[40] In the case of the mixture of polymyxin B, polymyxin B_1 and B_2 (Figure 2) are the major components. There is only one amino acid difference between polymyxin B and colistin, a phenylalanine that is part of the cyclic hepta-peptide of polymyxin B is replaced with a leucine in colistin (Figure 2). In the clinic, the polymyxin B mixture is used as the sulfate salt form, and the colistin mixture is administered as the sodium salt of colistin methanesulfonate, which serves as a prodrug that is activated through hydrolysis.[41] All polymyxins contain five primary amine groups, resulting from the amine side chains of diaminobutyric acid (DAB) that are charged under physiological conditions. Polymyxin B binds to LPS at least three orders of magnitude more tightly than do the natural calcium and magnesium cations that maintain the structure of the Gram-negative bacteria outer membrane intact.[42]

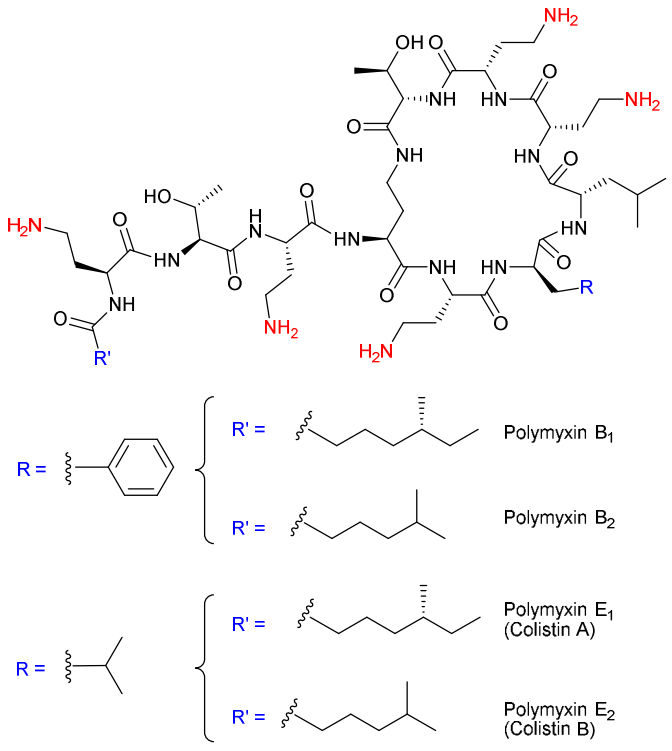


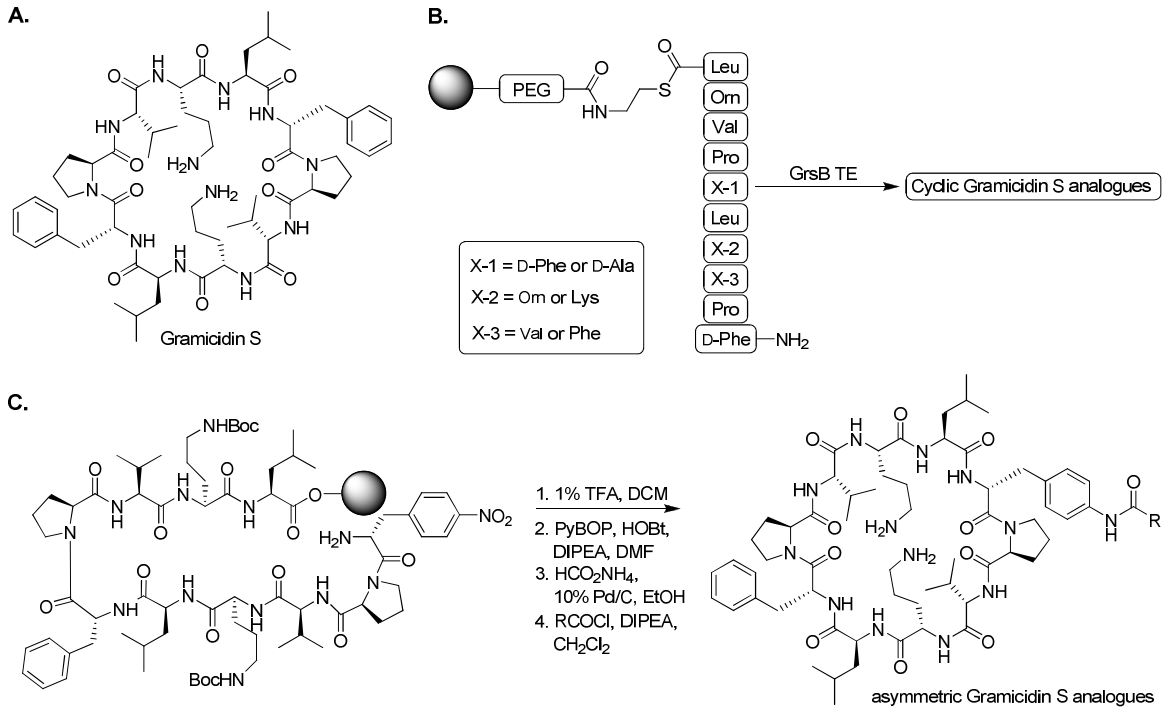
Fig. 2. Structures of cationic lipopeptide antibiotics: Polymyxins.

By displacing calcium and magnesium ions, polymyxin B disrupts the Gram-negative bacteria outer membrane structure. The fatty acid side chain of polymyxin B interacts with the lipid region of the LPS to anchor this antibiotic to the outer membrane.[40] Toxic side effects stand in the way of broader and more frequent clinical use of polymyxin B,[43] but potency against many Gram-negative bacteria, which are challenging to eradicate, make it clinically valuable. It is used as a last resort treatment for life threatening severe systemic infections caused by Gram-negative bacteria. Polymyxins are also widely used for the treatment of topical infections.[36]

Gramicidins

This family of antimicrobial agents includes both linear and cyclic peptides that are extracted from the fermentation broth of *bacillus brevis*. Two fractions that were first isolated in 1939 contained the mixture of linear peptides termed gramicidin A-D and the cyclic peptides termed tyrocidine A-C.[44–46]

Extracts from *B. brevis* found in Russian garden soil were found to contain one main cyclic antimicrobial peptide which was termed gramicidin S (Scheme 1A).[47] This cyclic peptide effectively combats Gram-positive and Gram-negative infections.[48] A major obstacle that prevents the systemic clinical use of gramicidin S and other members of this family is the fact that their therapeutic window is extremely narrow; these peptides cause red blood cell hemolysis at concentrations roughly equivalent to their minimal inhibitory concentrations (MICs) against many strains of pathogenic bacteria. This limitation led chemists to attempt to develop gramicidin derivatives with improved clinical properties. Several groups have reported chemical and chemoenzymatic synthetic routes for the preparation of analogues of the deca-peptide gramicidin S (Scheme 1B).[49] In nature, gramicidin S is synthesized by a thioester intermediate-based mechanism catalyzed by the modular nonribosomal peptide synthases (NRPS) GrsA and GrsB.[50] The C-terminal functional domain of GrsB is predicted to serve as a thioesterase (GrsB TE). GrsB TE is responsible for both dimerizing the linear penta-peptide gramicidin S precursors generated by the NRPS to a symmetric deca-peptide and the head-to-tail cyclization step that results in gramicidin S.[51] The activity of the overexpressed and purified GrsB TE was tested using linear substrates that were generated on solid support for the chemoenzymatic generation of gramicidin S analogues (Scheme 1B).[52] The 34-kD GrsB TE with a histidine tag at the C-terminus was purified from an *E. coli* and was used to catalyze the cyclization of fully deprotected synthetic decapeptide precursors of gramicidin S that were prepared using TentaGel-OH resin as solid support. GrsB TE converted the solid-support-attached precursors into head-to-tail cyclic products with high selectivity similar to the activity of this enzyme during the *in vivo* gramicidin S biosynthetic process. Only a fraction of the linear precursors served as GrsB TE substrates, demonstrating that this thioesterase has relatively high substrate specificity. Therefore, this enzyme is a poor tool for a chemoenzymatic synthesis of gramicidin S analogues.



Scheme 1. The structure of gramicidin S and the preparation of synthetic analogues.

On the other hand, the synthetic linear decapeptide precursors undergo spontaneous head-to-tail cyclization to form corresponding gramicidin S analogues in an ammonia solution with high selectivity and yields, offering a more reliable chemical approach for the preparation of gramicidin S analogues.[52] The importance of the symmetry of gramicidin S was studied by the generation of derivatives modified at the D-phenylalanine of gramicidin S.[53] This was done by the solid-support-based synthesis in which modified D-phenylalanine residues were incorporated to form a collection of asymmetric gramicidin S derivatives (Scheme 1C) that maintained the same β-hairpin secondary structure of the parent antibiotic in solution as demonstrated by NMR experiments and X-ray crystallography.[53] Of the synthesized, substituted D-phenylalanine derivatives of gramicidin S the most potent antimicrobials were also the most hemolytic. However, some of the tested asymmetric gramicidin S analogues demonstrated a significant reduction in the undesired hemolytic activity and had antimicrobial activity similar to that of the parent gramicidin S against *Streptococcus mitis* strains.

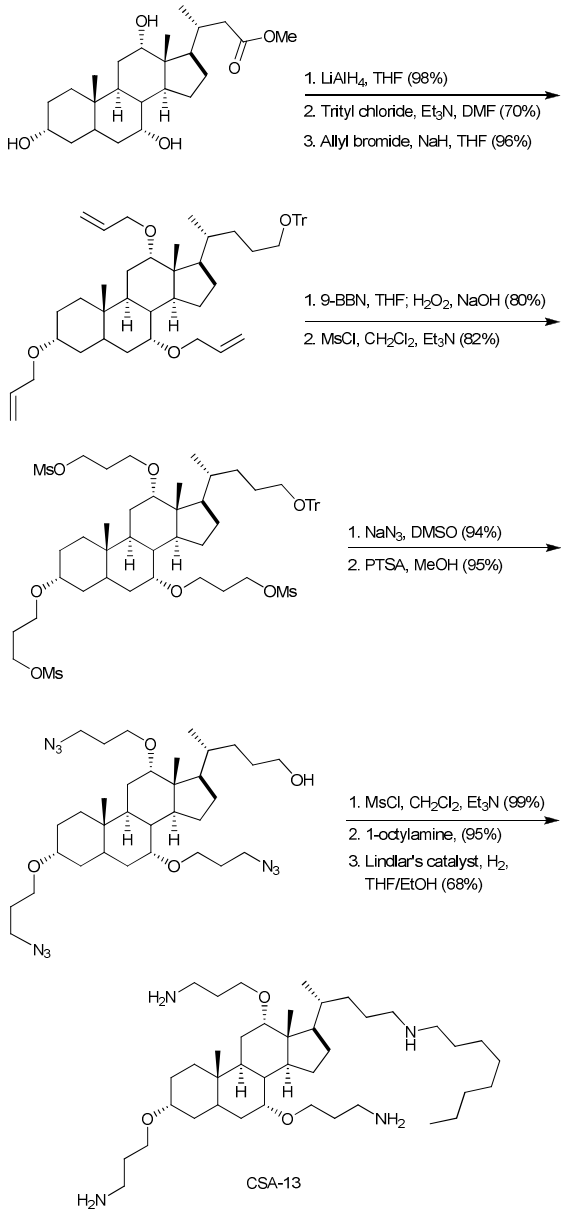
Synthetic antimicrobial cationic amphiphiles

Quaternary ammonium compounds (QACs) are synthetic antimicrobial cationic amphiphiles that are used as antiseptics worldwide.[54] While all of the rest of the antimicrobial cationic amphiphiles in this review contain free amine groups that are charged under physiological conditions, the quaternary ammonium groups of QACs are permanently positively charged. QACs act by binding to phospholipids with high affinity and increase membrane permeability.[55] Unfortunately, the high chemical stability of QACs poses an environmental problem and their toxicity to mammalian cells stands in the way of considering these compounds for the development of antibiotics which will be safe for the treatment of systemic infections.[56]

Ceragenins

Ceragenins are semi-synthetic antimicrobial cationic amphiphiles derived from bile acid, which serves as their hydrophobic backbone.[57] Like antimicrobial cationic peptides, the bile acid segments of ceragenins have been modified to yield an amphiphilic cationic morphology through the installation of amine functionalities that are positively charged under physiological conditions.[58,59] Like the secondary structure of cationic antimicrobial peptides, ceragenins possess a secondary structure in which their ammonium groups can orientate on one face of the molecule and their steroidal segment on the opposite side.[60] Several ceragenin analogues demonstrated impressive levels antimicrobial activity with MIC values in the single μg/mL range when tested against a broad spectrum of both Gram-positive and Gram-negative bacteria.[61] In addition, several ceragenins exhibited high levels of selectivity for bacterial relative to mammalian cell membranes. Membrane selectivity is usually tested on red blood cells (RBCs). Some of the ceragenin analogues did not cause any observable RBC hemolysis at a concentration of 200 μg/mL.[60] Ceragenins were shown to efficiently and rapidly depolarize bacterial membranes.[61] Electron microscopy images of bacterial

cells that were treated with ceragenins resemble images obtained after exposure of bacterial cells to membrane-targeting antimicrobial cationic peptides.[62] Ceragenin analogue CSA-13 was prepared in 10 synthetic steps from methyl cholate as described in Scheme 2 and was recently moved into pre-clinical development by Ceragenix.[59] Most of the synthetic steps were accomplished in high yields, and the total yield of the synthesis was ~25%. CSA-13 is proposed to accumulate at the bacterial cell membrane through electrostatic interactions and upon reaching a critical concentration, lead to the rupture of membrane patches, a mode of action similar to that of some families of natural antimicrobial peptides. CSA-13 acts as a bactericidal against a large panel of bacterial strains including a variety of oral pathogens.

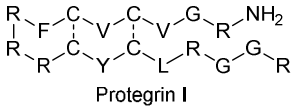


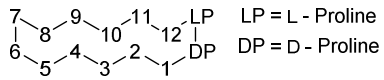
Scheme 2. Synthesis of ceragenin CSA-13.[59]

CSA-13 has MIC ranges of 1–8 μg/mL against *Streptococcus mutans* strains, 2–16 μg/mL against protease-positive *Porphyromonas gingivalis* and *P. cangingivalis*, and 1–2 μg/mL against protease-negative *P. circumdentaria*.[63] In addition to its impressive antimicrobial activity spectrum, ceragenin CSA-13 eliminates established biofilms of both Gram-negative and Gram-positive bacteria and has potent activity against several antibiotic-resistant bacteria.[64] Hence, ceragenins like CSA-13 offer a promising opportunity for the development of clinically useful and novel antimicrobial agents that are likely to be safe for treatment of a variety of topical infections and for the prevention of biofilm formation on medical equipment and devices.

PEPTIDOMIMETIC-BASED ANTIMICROBIAL CATIONIC AMPHIPHILES THAT DO NOT ACT BY DIRECT DISRUPTION OF THE BACTERIAL MEMBRANE LIPID BILAYER

Protegrin I (Figure 3) is a natural cationic antimicrobial disulfide-bridged β-hairpin–shaped peptide composed of 18 amino acids that is produced by the innate immune system and induces bacterial membrane lysis.[65] Inspired by protegrin I, a collection of β-hairpin–shaped peptidomimetics was designed and synthesized by solid-state peptide synthesis.[66] These compounds are composed of loop forming sequences of 14 amino acids with a D-proline and an L-proline on both ends of the sequences to stabilize a β-hairpin structure. Of these synthetic protegrin I analogues, a sequence termed L8-1 (Figure 3), demonstrated broad-spectrum antimicrobial activity equivalent to that of the parent protegrin I.[67] However, compared to protegrin I, L8-1 caused less hemolysis of human red blood cells. An attempt to further optimize the performance of L8-1 through a structure-activity relationship search resulted in another protegrin I analogue termed L27-11 (Figure 3).[67] This tetradeca-peptide has MIC values in the nanomolar range against a large collection of *Pseudomonas aeruginosa* and *Pseudomonas spp.* strains, yet this compound was relatively inactive against Gram-positive strains and most interestingly against other Gram-negative bacteria.





General structure of protegrin I analogues

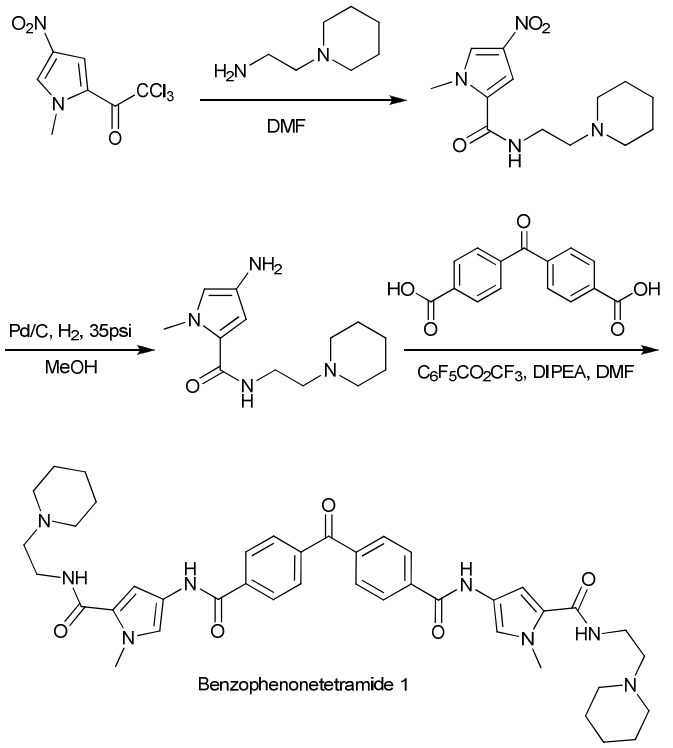
Compd. #	Amino acid # 1	2	3	4	5	6	7	8	9	10	11	12
L 1 - 8	L	R	L	K	K	R	R	W	K	Y	Y	V
L 19 - 45	R	W	L	K	K	R	R	W	K	Y	Y	V
L 26 - 19	T	W	L	K	K	R	R	W	K	K	V	K
L 27 - 11	T	W	L	K	K	R	R	W	K	K	A	K

Fig. 3 The structure of protegrin I and synthetic analogues.

Even though protegrin I and L27-11 share high structural similarities, the fact that the former displays broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria in the low micromolar range suggests that these two compounds do not share a similar mode of action. Interestingly, the enantiomeric analogue of L27-11 is inactive against *Pseudomonas aeruginosa*, whereas the enantiomeric structures of protegrin I have potent antimicrobial activity.[68,69] This observation suggests that the target of the L27-11 is likely to contain chiral structural motifs as do all proteins. Indeed, several lines of evidence suggested that the mechanism of action of the L27-11 is likely to involve the perturbation of LPS transport by the membrane protein LptD.[67] LptD is an essential outer-membrane protein found in Gram-negative bacteria that plays a key role in the assembly of LPS in the outer leaflet of the outer membrane.[70,71] The N-terminal domain of LptD varies among Gram-negative strains and may explain the specificity of L27-11 for *Pseudomonas aeruginosa*.[67] Photoaffinity labeling experiments followed by protease digestion/liquid chromatography–electrospray ionization–tandem mass spectrometry analysis, as well as immunoblotting with polyclonal antibodies raised against a synthetic C-terminal peptide, strongly supported the hypothesis that that LptD is the target of L27-11.[67] These analogues of protegrin I offer a very promising direction for the development of effective antibiotics that will selectively target infections caused by the pathogen *P. aeruginosa* without harming the natural flora of the host. Furthermore, this study demonstrates that even though many antimicrobial cationic amphiphiles efficiently disrupt bacterial membranes, not all of them act by directly disrupting the lipid bilayers; a portion may target other or additional determinants on the bacterial cell surface.

BENZOPHENONE-BASED ANTIMICROBIAL CATIONIC AMPHIPHILES

In 2009, Firestein et al. reported a three-step synthetic route for the preparation of a collection of symmetrically substituted benzophenone derivatives such as benzophenonetetramide 1 (Scheme 3) which proved to be a potent antimicrobial cationic amphiphile.[72] Certain of the substituted benzophenones had activity against both Gram-positive and Gram-negative bacteria with MIC values in the range of 0.5-1.0 mg/L; some analogues inhibited growth of Gram-positive antibiotic-resistant pathogens including methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* strains.[73] Pyrrole-imidazole polyamides bind to double-stranded DNA with high affinities and sequence specificity.[74,75] When tested for DNA binding properties, the benzophenone-based cationic amphiphiles were found to interact with DNA, yet these compounds did not inhibit macromolecular, lipid, or bacterial cell wall biosynthesis.[72] The most potent of the antimicrobial benzophenone analogues caused rapid bacterial membrane depolarization, suggesting that these antimicrobial cationic amphiphiles may target the membrane directly. Interestingly, unlike other antimicrobial cationic amphiphiles, the reported antimicrobial benzophenonetetramides were not toxic to mammalian cells; these compounds caused no red blood cells hemolysis at a concentration two orders of magnitude higher than the potent MIC range of these compounds. Thus, these compounds demonstrated promising selectivity to bacterial membranes and have potential as leads for the development of membrane targeting antimicrobial cationic amphiphiles that will be safe for systemic use.



Scheme 3. Three-step synthesis of benzophenone-derived antimicrobial cationic amphiphiles (demonstrated for benzophenonetetramide 1).[72]

DCAP

An abbreviation of 2-((3-(3,6-dichloro-9H-carbazol-9-yl)-2-hydroxypropyl)amino)-2-(hydroxymethyl)propane - 1, 3 - diol (Figure 4), DCAP was discovered during high-throughput screening in search for inhibitors of the ATPase that regulates division site placement in *Caulobacter crescentus*.[76] This compound targets the membranes of both Gram-positive and Gram-negative bacteria. Although it initially seemed that the antimicrobial activity of DCAP resulted from its ability to decrease the transmembrane potential in bacteria, this compound actually interferes with the integrity of the bacterial membrane by causing mis-localization of membrane-associated proteins.

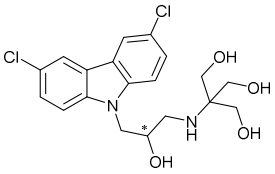


Fig. 4. The structure of DCAP.

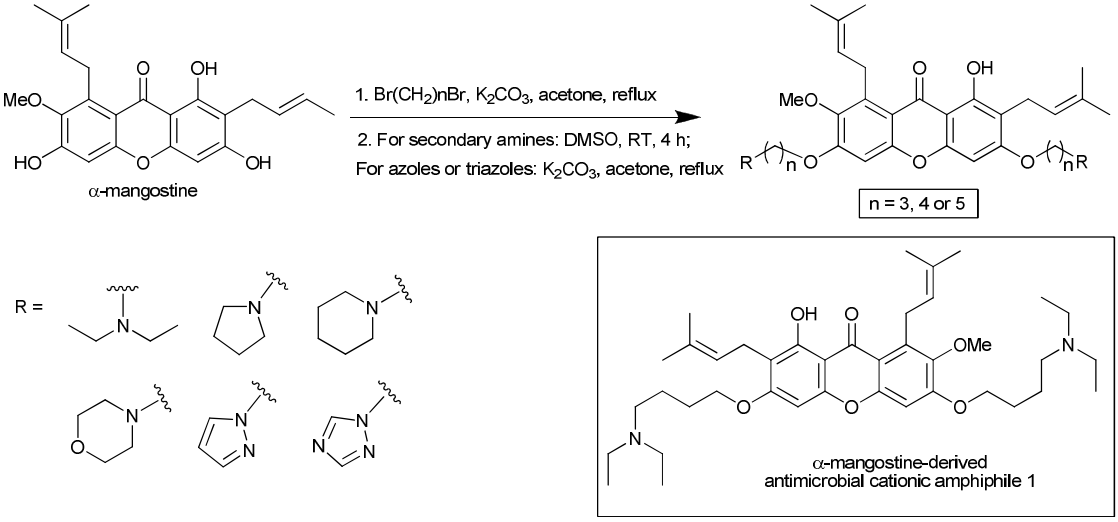
Fluorescent experiments indicated that DCAP associates with bacterial membrane lipids and interferes with the localization of MinD, a membrane ATPase essential for cell division in *Bacillus subtilis*. In addition, DCAP affects the natural distribution of FtsA, a peripheral membrane protein that regulates the recruitment of downstream division proteins in *Caulobacter crescentus*. This compound effectively eradicates stationary-phase bacterial cells as well as actively growing bacteria and biofilm-associated cells. DCAP does not damage red blood cell membranes at concentrations at which it has potent antimicrobial activity; however it reduces mitochondrial transmembrane potential and is toxic to kidney epithelial cells and is therefore more likely to serve as a lead for the development of topical antimicrobial agents.

XANTHONE-BASED ANTIMICROBIAL CATIONIC AMPHIPHILES

Exploration of natural products from a Southeast Asian fruit *Garcinia mangostana* resulted in the isolation of α-mangostin (Scheme 4), which disrupts Gram-positive bacteria membranes including that of the clinical pathogen MRSA.[77] However, α-mangostin failed to exhibit high levels of selectivity for bacterial relative to mammalian cell membranes. The lack of membrane selectivity was rationalized by the hydrophobically substituted xanthone of α-mangostin that is likely to enhance the disruption of membrane lipid bilayers of both eukaryotic and prokaryotic cells. In an attempt to resolve the membrane selectivity problem, a collection of semi-synthetic α-mangostin analogues was designed and synthesized (Scheme 4).[78] In these α-mangostin analogues, the xanthone scaffold of the parent compound was modified by the installation of substituted amine groups with different pKa values to alter the log P value of the parent compound.[78] Of these tested cationic amphiphiles, α-mangostin-derived antimicrobial cationic amphiphile 1 (Scheme 4) demonstrated impressive antimicrobial activity with an MIC range of 0.095 – 1.56 μg/mL against *Staphylococci* including MRSA and vancomycin-resistant *S. aureus* strains yet was not active against Gram-negative bacteria. The membrane selectivity of these compounds was tested by measuring the concentration at which 50% RBC hemolysis (HC-50) was observed. The measured HC-50 values for the α-mangostin analogues that demonstrated potent antimicrobial activities ranged between 16-26 μg/mL and were higher than the HC-50 caused by the parent α-mangostin (9 μg/mL). The ratio of the HC-50 to the MIC values of the most potent of α-mangostin-derived antimicrobial cationic amphiphile 1 was between 12.6 and 206.3 depending on the bacterial strain;[78] this proved that the installation of amine functionalities on the α-mangostin scaffold could result in a significant improvement of both the antimicrobial potency and membrane selectivity of the resultant compounds. The membrane-targeting activity of α-mangostin-derived antimicrobial cationic amphiphile 1 was supported by several experiments:[78] First, similar to other known cationic antimicrobial peptides, this compound inflicted rapid bacterial cell death (within minutes after exposure to 4 fold of the MIC). Second, several membrane depolarization assays indicated that this compound induced rapid depolarization. Third, scanning electron micrograph (SEM) images of bacteria that were exposed to α-mangostin-derived antimicrobial cationic amphiphile 1 revealed that this compound induced significant membrane damage to these cells. Therefore, future screens for amphiphilic xanthone-derived membrane-targeting antibiotics may very well lead to the discovery of novel and clinically relevant antimicrobial agents.

AMINOGLYCOSIDE-DERIVED ANTIMICROBIAL CATIONIC AMPHIPHILES

Aminoglycosides (AGs) are broad-spectrum antibiotics commonly used for the treatment of external bacterial infections and as a last resort drug for the treatment of serious systemic infections. These antibiotics bind to the decoding A-site of the 16S ribosomal RNA (rRNA) and interfering with the codon-anticodon recognition step during protein synthesis.[79]



Scheme 4. Synthesis of α-mangostin-derived antimicrobial cationic amphiphiles.[78]

Widespread and prolonged use of AGs has led to the emergence of bacterial resistance to this family of antibiotics.[80,81] When used systemically, these antimicrobial agents may cause nephrotoxic[82] and ototoxic[83] side effects, and this has reduced the clinical value of this family of antibiotics.

In recent years, several research groups, including ours, reported that the pseudo-oligosaccharide structures of AGs, which are positively charged under physiological conditions, can be used for the of preparation membrane-disrupting antimicrobial agents by the installation of one or more hydrophobic residues on the alcohols of AGs or their fragments. Chang et al. described the synthesis of modified neomycin B derivatives with potent antimicrobial activity compared to that of the parent AG through what seemed to be a mode of action that differed from that of neomycin B.[84] In these neomycin B derivatives, the single primary alcohol was displaced by a hydrophobic residue.

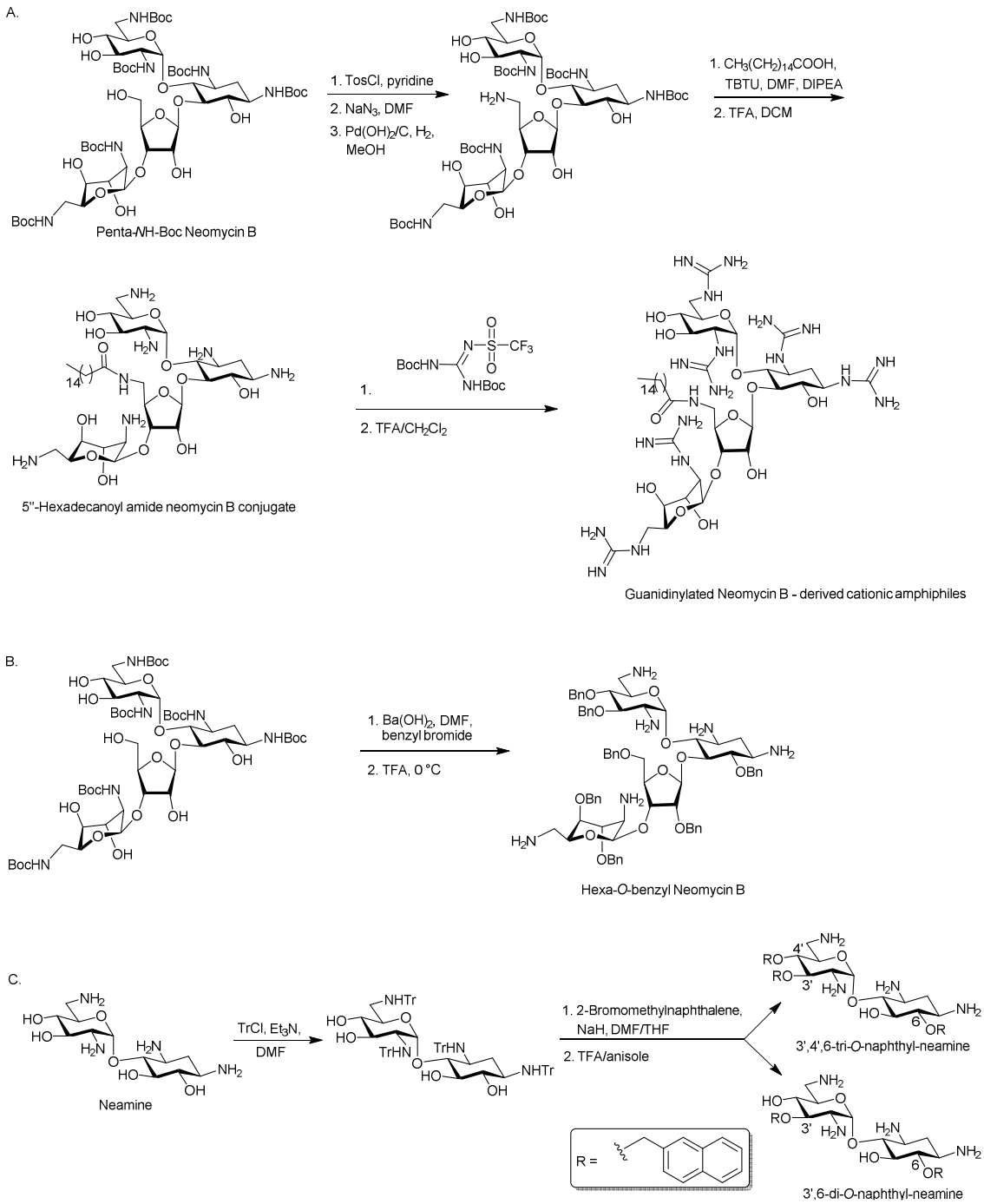
Schweizer et al. reported the design and synthesis of several families of AG-derived antimicrobial cationic amphiphiles[85–91] including neomycin B-based lipid conjugates as shown in Scheme 5A.[89] The incentive for the preparation of these AG-lipid conjugates was to improve their incorporation into the phospholipid bilayers of the bacteria membranes therefore resulting in either enhanced uptake of the AG-lipid conjugate or destabilization of the lipid membrane as was proposed for other antimicrobial cationic amphiphiles. Optimal antimicrobial activity against Gram-positive strains, particularly against several multi-drug resistant strains including MRSA and methicillin-resistant *Staphylococcus epidermidis* (MIC = 8 μg/mL and 2 μg/mL, respectively), was achieved by conjugation of saturated lipids such palmitic acid through an amide bond to yield the 5"-hexadecanoylamide neomycin B derivative (Scheme 5A).[89] This compound was 32 times more potent than the parent neomycin B against MRSA. However, exposure of red blood cells to a concentration of 100 μg/mL of 5"-hexadecanoylamide neomycin B causes 56% of red blood cell hemolysis indicating that this compound had limited membrane selectivity. A significant improvement in the membrane selectivity was achieved by the conversion of the free amines of the AG-lipid conjugates to the corresponding guanidines.[90] At a concentration of 100 μg/mL penta-guanidinyl-5"-hexadecanoylamide neomycin B (Scheme 5A) causes only 13% hemolysis and has antimicrobial activity comparable to that of 5"-hexadecanoylamide neomycin B. In another type of AG-derived antimicrobial cationic amphiphile reported by Schweizer et al. all of the AG alcohol groups were converted into the hydrophobic ether groups.[91] For example, hepta-*O*-benzyl-neomycin B (Scheme 5B) has higher antimicrobial activity against broad-spectrum Gram-positive bacteria than the parent AG and modest activity against some pathogenic Gram-negative strains.

Marked improvement in the activity of AG-derived antimicrobial cationic amphiphiles against Gram negatives bacteria was observed in the case of compounds derived from the pseudo-disaccharide neamine as reported by Décout et al.[92–94] These neamine-derived cationic amphiphiles were generated in three synthetic steps (Scheme 5C).[93] Initially, the four free amine groups of neamine were converted to the corresponding *N*H-trityl groups followed by Williamson's etherification with a collection of aryl bromides and acidic removal of the trityl protecting groups to yield mixtures of 3′,6-di- and 3′,4′,6-tri-*O*-alkylated neamine derivatives as shown for the preparation of di- and tri-*O*-2-naphthyl neamine analogues in Scheme 5C. The 3′,4′,6-tri-*O*-2-naphthyl neamine demonstrated potent antimicrobial activity against pathogenic Gram-negative bacteria including *A. lwoffii*, *E. coli*, *P. aeruginosa*, and *K. pneumonia*, with MIC values between 2–16 μg/mL. The potency of some of the neamine derived antimicrobial cationic amphiphiles against Gram-negative strains was associated with the affinity that these compounds demonstrated to LPS.[92] The 3′,4′,6-tri-*O*-2-naphthyl neamine demonstrated *in-vitro* LPS affinity which was comparable to that of polymyxine B.

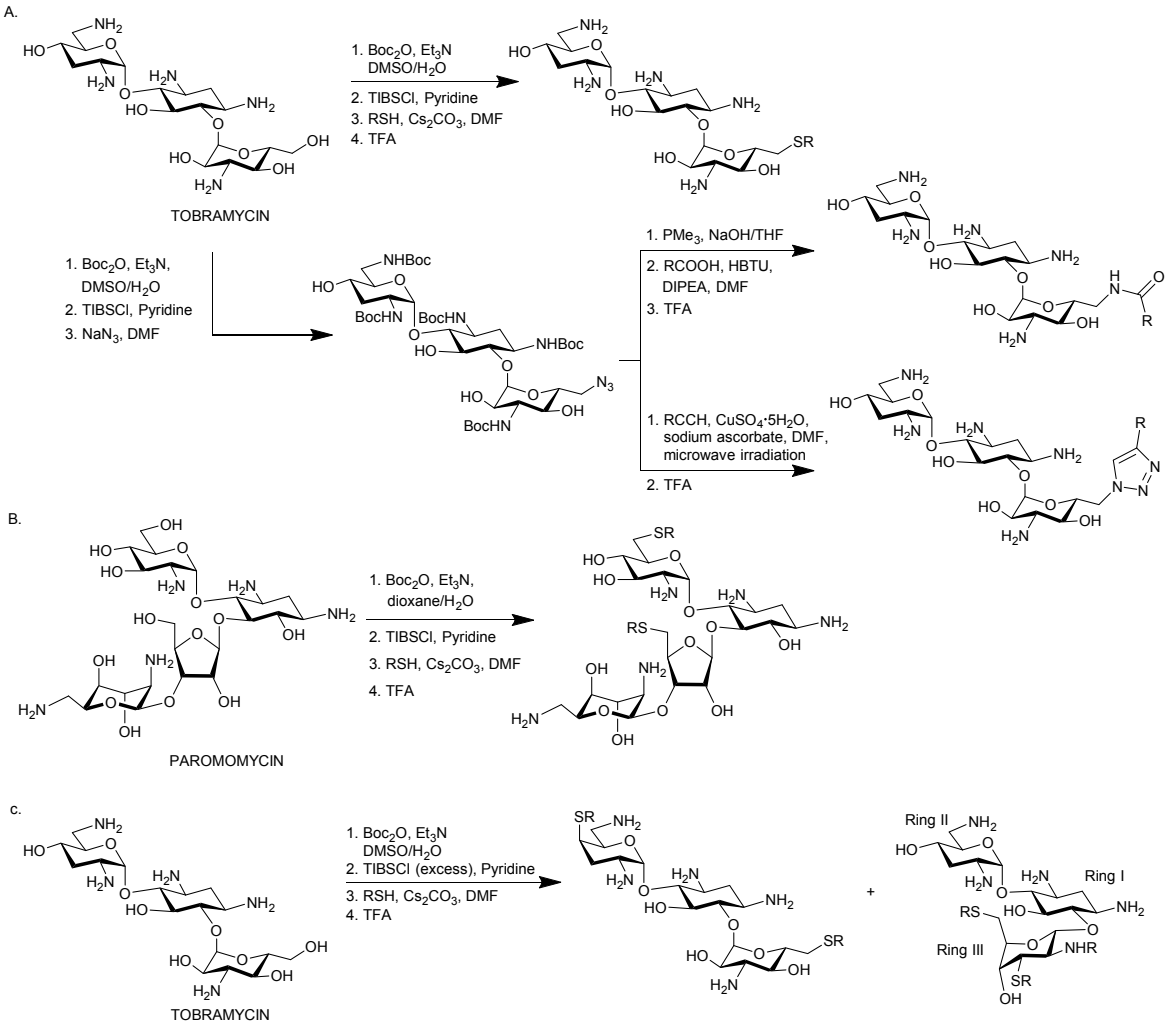
In an attempt to identify the structural parameters that affect both the antimicrobial activity and the membrane selectivity of AG-derived antimicrobial cationic amphiphiles our group performed several structure-activity relationship and mechanistic studies. We identified several structural parameters that govern both the antimicrobial activity potency as well as the level of selectivity of AG-derived antimicrobial cationic amphiphiles to bacterial membranes.[95–98] We demonstrated that in designing AG-derived antimicrobial cationic amphiphiles from the AG tobramycin (Scheme 6A), linear aliphatic chain residues are favored over aryl-based hydrophobic residues.[95] We also found that the length of the aliphatic chain, the chemical bond through which it is connected to the AG, the number of aliphatic chains, and their positions on the AG are all important parameters that affect antimicrobial activity and the membrane selectivity of these antimicrobial agents.[96] By altering these parameters, we were able to develop several antimicrobial cationic amphiphiles with potent antimicrobial activity against a broad spectrum of bacteria with MIC values ranging from 1 to 8 μg/mL. The attachment of a single C_{12}, C_{14}, or C_{16} aliphatic chain to the C-6" position of tobramycin following the synthetic routes outlined in Scheme 6A resulted in the most potent of the tested tobramycin-derived antimicrobial cationic amphiphiles. The tobramycin-derived antimicrobial cationic amphiphiles in which the aliphatic chain was linked to the AG through a triazole ring were highly hemolytic. On the other hand, tobramycin-derived antimicrobial cationic amphiphiles in which the aliphatic chain was attached to the AG through an amide bond were both potent antimicrobials and caused significantly less hemolysis.

To test whether anchoring the AG to the bacterial membrane through more than one aliphatic chain anchor would optimize the interactions between AG-derive antimicrobial cationic amphiphile and the bacterial membrane, we prepared a collection of paromomycin-based di-alkylated cationic amphiphiles (Scheme 6B).[97] We chose paromomycin since, like tobramycin, this aminoglycoside scaffold has five amine functionalities that are positively charged under physiological conditions. To maintain the hydrophobicity/hydrophilicity ratio that was optimized in the case of tobramycin-derived

antimicrobial cationic amphiphiles, we used C_6, C_7, and C_8 aliphatic chains for the preparation of the di-alkylated paromomycins. The di-C_7 aliphatic chain thioether paromomycin analogue demonstrated potent antimicrobial activity against a collection of Gram-positive bacteria that are associated with skin infections with an MIC range of 2-16 μg/mL. At 32 μg/mL, which is 2-16 times higher than the measured MICs, this compound caused almost no measurable hemolysis (3.6±1.9%) and therefore proved more selective for bacterial membranes. We recently reported a method for site-selective nucleophilic displacement of secondary alcohols of tobramycin for the generation of di-*n*-alkylated tobramycin-derived antimicrobial cationic amphiphiles.[98] Site selectivity was affected by the amine protecting groups used and facilitated the generation of homo- and hetero-dithioether tobramycin-derived cationic amphiphiles.



Scheme 5. Synthesis of AG-derived antimicrobial cationic amphiphiles.[89–91,93]



Scheme 6. Synthesis of mono-and di-*n*-alkylated AG-based antimicrobial cationic amphiphiles.[95–98]

In these compounds the thioether-linked aliphatic chains were either at the 3'' and 6'' positions or at the 4' and 6'' positions (Scheme 6C). Antimicrobial activity tests revealed that there were bacterial strains that were susceptible to the 3'', 6''-dithioether tobramycin derivatives and not to those alkylated at the 4', 6''-positions, therefore demonstrating that the positioning of the aliphatic chains on the AG affects the antimicrobial activity of these AG-derived cationic amphiphiles. As was observed in the case of the paromomycin-derived antimicrobial cationic amphiphiles, compared to mono-*n*-alkylated tobramycin-derived antimicrobial cationic amphiphiles, the di-*n*-alkylated were considerably less hemolytic. There was not a linear correlation between the antimicrobial potency of AG-based antimicrobial cationic amphiphiles and the undesired hemolysis that these compounds cause. The MICs of the most potent AG-derived antimicrobial cationic amphiphiles in our studies were in some cases well over an order of magnitude lower than the concentrations at which these compounds caused detectible hemolysis. Several of the AG-derived antimicrobial cationic amphiphiles developed by our group as well as by other groups are far more membrane selective and possess a broader spectrum of potent antimicrobial activity than clinically used membrane-targeting antibiotics such as gramicidins; these molecules may therefore lead to clinically useful antimicrobials. Additional optimization of AG-based antimicrobial cationic amphiphiles based on the structural parameters that were found to affect the antimicrobial activity/selectivity of these compounds is likely to lead to such antimicrobial agents.

Mechanistic studies of antimicrobial cationic amphiphiles

The fact that the majority of clinically used drugs target specific and well defined cellular processes by binding to specific proteins made it possible to gain a detailed understanding of the mode of action of these drugs on an atomic level through numerous experimental approaches and techniques such as X-ray crystallography, biochemical characterization of isolated

target proteins, and directed mutagenesis. The dimensions, chemical characteristics, variability and complexity of membranes make it challenging to provide experimental evidence for the action of membrane disrupting agents at a molecular level. Most of what is currently known about the action of antimicrobial cationic amphiphiles is derived from the study of the mode of action of antimicrobial cationic peptides, and only very few of these have been well characterized mechanistically. Experimental tools that have been applied for the mode of action studies of several families antimicrobial cationic peptides include neutron diffraction, fluorescence spectroscopy, solid-state NMR, oriented CD, electron paramagnetic resonance (EPR), and Fourier transform infrared (FTIR) spectroscopy.[99–104] These studies relied on the use of model phospholipid membranes in the form of monolayers, bilayers, or liposomes.[105] Three main models have been suggested to explain the activity of antimicrobial cationic peptides: the "carpet" model, the "barrel-stave" model, and the toroidal model.[106–108] Linear α-helical antimicrobial peptides are thought to act via the "barrel-stave" model (Figure 5A). In this model, α-helical antimicrobial peptides form transmembrane pores that disrupt membrane function. This model is supported by the fact that such peptides can form channels through membranes lipid bilayers. [99,109–111] To form transmembrane pores, antimicrobial peptides must possess a hydrophobic face that forms hydrophobic interactions with the membrane lipid bilayer. Moreover, these antimicrobial peptides are capable of self-associating to form transmembrane channel structures that vary in diameter due to differences in the number of monomers composing the specific channel.[107,112] In the "carpet" model,[106] antimicrobial cationic peptides interact with negatively charged components of the bacterial membrane through ionic interactions. Once a critical local concentration is reached, the hydrophobic segments of the oligopeptides are driven out from the water and interact hydrophobically with the membrane lipids. The permeation of the membrane takes place when a critical local concentration, which is different for every antimicrobial peptide, is reached. Formation of ruptures and decomposition of the lipid bilayer structure leads to membrane depolarization and dysfunction and decomposition (Figure 5B). Finally, according to the toroidal model which is also termed the "wormhole" model, to relieve the curvature strain caused by the antimicrobial peptide binding, layers of phospholipids bend continuously from one membrane leaflet to the other (Figure 5c). Peptide chains that were lying on the membrane surface already submerged at the hydrophilic/hydrophobic interface get pulled together with the lipid molecules, resulting in a pore structure in which peptide chains and lipid headgroups form the wall of the pore. Unlike most of the antimicrobial cationic peptides, most of the other known families of natural and synthetic antimicrobial cationic amphiphiles have a small molecular weight and are incapable of forming defined α-helical or β-sheet structures. Hence it is likely that most of the small-molecule-based antimicrobial cationic amphiphiles disrupt membrane function through the less ordered "carpet" type model. The complexity of the experiments and the large

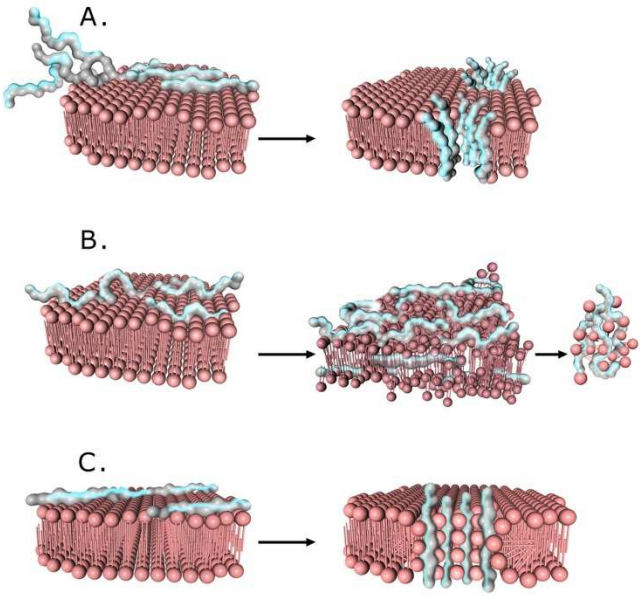


Fig. 5. Proposed models for the mode of action of antimicrobial cationic amphiphiles: (A) barrel - stave model, (B) carpet model, and (C) toroidal model.

structural diversity amongst these antimicrobial agents pose great challenges to mode of action studies. Hence, there is a great need for the development of tools and experiments that will enable study of these important antimicrobial agents interacting with the variety of bacterial cell wall components at the molecular level of the intact organisms.

Conclusion

The interest in the development of antimicrobial cationic amphiphile-based antibiotics has become more relevant than ever due to the worldwide increase in cases of antibiotics resistance. A successful development of clinically useful antibiotics of this type is likely to result in broad spectrum antimicrobial agents with little tendency to induce antimicrobial resistance and with the ability offer an effective solution to infections caused by dormant bacteria or slow dividing bacteria. However, developers of antimicrobial cationic amphiphiles still face one major challenge: Systemic toxicity is caused by all of the currently known families of antimicrobial cationic peptides; this makes them unsafe for internal use and limits their clinical utility. However, the development of several families of antimicrobial cationic amphiphiles in the last decade clearly demonstrated that it is possible to significantly increase the selectivity of antimicrobial cationic amphiphiles for bacterial relative to eukaryotic cell membranes. Amongst the reported antimicrobial cationic amphiphiles are compounds with broad spectrum activity against highly drug resistant pathogens that cause little damage to the membranes of several types of mammalian cells. The examples presented in this review suggest that synthetic antimicrobial cationic amphiphiles may eventually be highly effective and safe for treatment of both

topical and systemic infections yet additional studies are required to reach this goal.

Acknowledgements

This work was supported by the FP7-PEOPLE-2009-RG Marie Curie Action: Reintegration Grants (Grant 246673).

Notes and references

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