Design, Synthesis, and Antibacterial Activities of Neomycin−Lipid Conjugates: Polycationic Lipids with Potent Gram-Positive Activity

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Aminoglycoside antibiotics and cationic detergents constitute two classes of clinically important drugs and antiseptics. Their bacteriological and clinical efficacy, however, has decreased recently due to antibiotic resistance. We have synthesized aminoglycoside−lipid conjugates in which the aminoglycoside neomycin forms the cationic headgroup of a polycationic detergent. Our results show that neomycin−C_{16} and neomycin−C_{20} conjugates exhibit strong Gram-positive activity but reduced Gram-negative activity. The MIC of neomycin−C_{16} (C_{20}) conjugates against methicillin-resistant *Staphylococcus aureus* (MRSA) is comparable to clinically used antiseptics.

Introduction

The explosive growth of multidrug resistant (MDR[a]) bacteria in hospitals and the community have led to an emerging crisis where an increasing number of antibiotics cease to be of clinical usefulness.[1] Despite this growing concern, only one new class of antibiotics, the oxazolidinones, has entered the clinic during the past two decades.[2] As a result, there is a pressing need for novel classes of antibacterial agents with new mechanisms of action and reduced likelihood to lead to the development of resistance.

Aminoglycoside antibiotics (AAs) constitute a large family of clinically important drugs used in the treatment of bacterial infections.[3] They effect their antibacterial activity by interfering with ribosomal function (via binding to rRNA), which ultimately results in the disruption of protein biosynthesis.[4,5] AAs demonstrate broad spectrum activity and are effective against most Gram-negative bacteria and certain Gram-positive bacteria. Many of them, e.g., amikacin, gentamicin, kanamycin, neomycin, netilmicin, streptomycin, and tobramycin, have been used clinically for decades as potent antimicrobial agents.[6] Other analogues, e.g., hygromycin A and spectinomycin, are used primarily as animal medicines in veterinary and agricultural applications.[7−9] Although AAs exhibit potent bactericidal activity, their widespread use has been compromised by dose-related nephrotoxicity and ototoxicity.[10,11] The rapid emergence of AA-resistant strains[12−14] has instigated research efforts to develop novel AAs or modified AA-analogues that can delay or avoid acquired resistance by pathogenic bacteria. However, progress has been slow due to the fact that novel and purely synthetic AAs are difficult to synthesize and structural modifications on naturally occurring AAs usually require complex multistep organic synthesis. Moreover, with the exception of neomycin and kanamycin, many commercially available AAs are expensive starting materials that limit their industrial use as synthetic scaffold for chemical modifications.

To exhibit their antibacterial activity AAs must bind to the RNA receptor located within the cell. This requires uptake of the AA by the bacterial cell. However, AA resistance may be manifested by reduced drug uptake as a result of activation of drug efflux pumps, modified membrane potential, changes in membrane composition, and other factors.[15] In an attempt to enhance the uptake of AAs into the bacterial cell, we became interested in the synthesis of AA−lipid conjugates. AA−lipid conjugates are cationic lipids in which a multiple charged cationic headgroup (the aminoglycoside) is linked to a hydrophobic lipid moiety. Over the years, cationic lipids[16,17] have been developed to facilitate transport through membranes. In particular, cationic lipids formulated as cationic liposomes have found applications as drug delivery systems against diseases[18] and gene transfection.[19] In addition, cationic lipids, including dodine,[20] benzalkonium chlorides,[21] sphingosine[22] and fatty amines,[23] chlorohexidine,[24] cationic polymers,[25] and other cationic amphiphiles,[26,27] are known to exhibit broad spectrum antibacterial activities (Figure 1). Several mechanisms have been proposed,[28] including disruption of the bacterial envelope induced by removal (substitution) of divalent positively charged counterions by ammonium ions.[25] Moreover, coadministration of AAs with lipid bilayer permeabilizing agents including ionic lipids has been shown to induce synergistic effects resulting in enhanced antimicrobial activity.[29] Taken together, these features make AA−lipid conjugates attractive targets for antimicrobial drug discovery, development of novel antiseptics, and prevention of biofilms.

Results

In this paper, we report on the synthesis and antibacterial activities of neomycin−lipid conjugates and other hydrophobically enhanced neomycin analogues. Neomycin B was selected as AA due to its commercial availability in multigram quantities and its low price. The low biological potency of neomycin toward several MDR bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) was an additional incentive to develop novel neomycin analogues with reduced resistance and increased activity. The single primary hydroxymethyl group at the ribose moiety (5″ position) in neomycin was chosen as a point of modification due to its expected high reactivity in chemical modifications (Figure 2). In addition, previous studies

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[a] Abbreviations: AA, aminoglycoside antibiotics; MDR, multidrug resistant.

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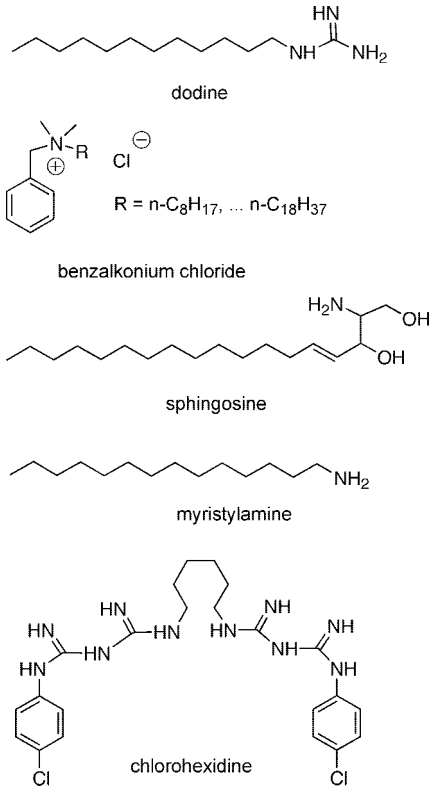


Figure 1. Structures of highly potent cationic lipids and detergents. Dodine, benzalkonium chlorides, sphingosine, and chlorohexidine are currently used in commercial antiseptics and disinfectants.

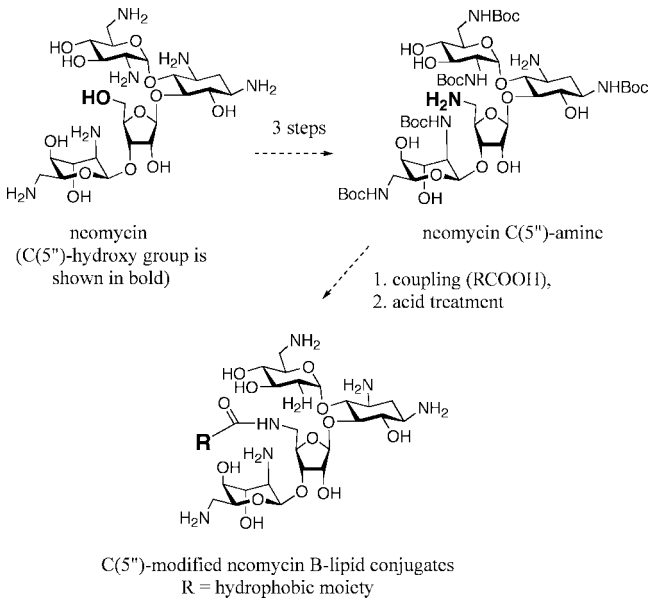
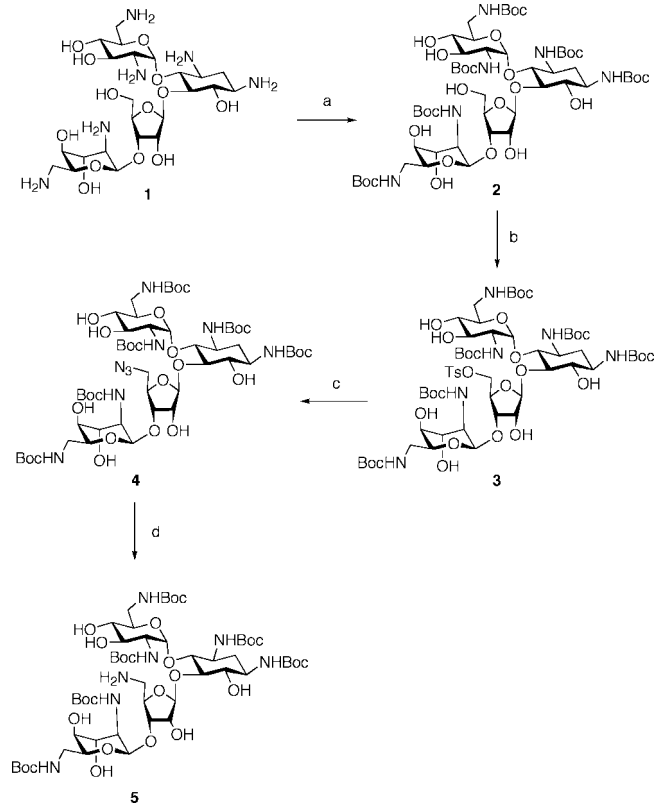


Figure 2. Synthetic strategy for the synthesis of C(5″) neomycin B−lipid conjugates.

have indicated that modified C5″ analogues of neomycin retain high binding affinity to RNA[30] and antimicrobial activities.[31,32]

To investigate how lipid conjugation of neomycin effects the antibacterial activities, we were interested in a short and modular approach in which an easily accessible, suitably protected, and functionalized neomycin analogue could be conjugated to a large array of lipids. Along these lines, we envisioned that neomycin (C5″)−amine could serve as an intermediate for condensation to various activated lipophilic acids (Figure 2). To the best of our knowledge, the antimicrobial activities of aminoglycoside−

Scheme 1[a]



[a] Reagents and conditions: (a) $(Boc)_2O$, Et_3N, DMF-H_2O, 80 °C, 6 h, 74%; (b) TsCl, Py, rt, 10 h, 81%; (c) NaN_3, DMF, 60 °C, 8 h, 53%; (d) $Pd(OH)_2/C$, H_2, rt, 5 h, 85%.

lipid conjugates have not been reported previously. However, kanamycin A−lipid conjugates were previously prepared and used for gene transfection studies.[19,33]

The readily available neomycin sulfate was converted into salt-free neomycin **1** via ionic exchange. The amino functions were protected as carbamate **2** by treatment with $(BOC)_2O$ in DMF−water mixture as previously described.[34a,b] The primary hydroxyl group at the C5″ position was selectively tosylated by treatment of **2** with *p*-toluenesulfonyl chloride in neat pyridine affording monotosylated compound **3** in 81% yield (Scheme 1). Nucleophilic displacement of tosyl-derivative **3** using sodium azide in DMF at 100 °C produced azide **4**, which was subjected to catalytic hydrogenation using Pearlman's catalyst ($Pd(OH)_2/H_2/C$) to provide partially protected 5″-NH_2 neomycin analogue **5** (Scheme 1). Amine **5** served as a precursor for conjugation to lipophilic acids. We selected a variety of acids including saturated C_6-, C_{12}-, C_{16}-, and C_{20}-, a double-unsaturated C_{18}-acid, and other acids containing pyrene and cholic acid in order to study how chain length, saturation, and hybridization of the lipophilic moiety affects the antimicrobial activity. The lipophilic acids were coupled to neomycin-based amine **5** by exposure to 2-(1*H*-benzotriazole-1yl)-1,1,3,3-tetra-methyluronium tetrafluoroborate (TBTU) as coupling reagent and Hünigs base in DMF to yield the lipid conjugates **6**−**12** (Scheme 2). Exposure of the conjugates **6**−**12** to 95% TFA provided the deblocked neomycin−lipid conjugates **13**−**19** as TFA salts, which were used in the bacterial assays against Gram-positive and Gram-negative organisms.

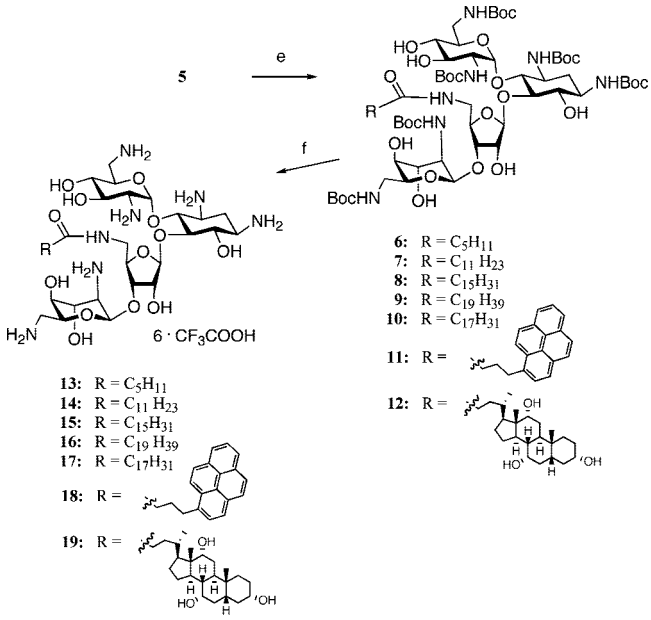
All compounds were tested against American Type Culture Collection (ATCC) reference strains as well as clinical isolates of Gram-positive strains including *S. aureus*, MRSA, *Staphylococcus epidermidis*, methicillin-resistant *S. epidermidis* (MRSE),

Table 1. Representative Minimal Inhibitory Concentrations (MIC) in μg/mL for Various Bacterial Strains

	MIC (μg/mL)							
compd	*S. aureus*[a]	MRSA[b]	*S. epidermidis*[c]	MRSE[d]	*S. pneumoniae*[e]	*E. coli*[f]	*E. coli*[g]	*P. aeruginosa*[h]
gentamicin	1	2	0.25	32	4	1	128	4
neo mycin B	2	256	1	0.5	64	8	4	512
13	16	>512	2	32	64	16	16	>512
14	32	>512	4	8	128	64	64	256
15	4	8	2	2	64	32	64	128
16	8	8	4	4	64	128	128	64
17	16	32	4	4	128	128	64	>512
18	16	256	2	64	>512	32	64	256
19	16	128	8	16	>256	32	64	>256

[a] ATCC 29213. [b] Methicillin-resistant *S. aureus* (ATCC 33592). [c] ATCC 1490. [d] Methicillin-resistant *S. epidermidis* (ATCC 14990). [e] ATCC 49619. [f] ATCC 25922. [g] ATCC 6174 (gentamicin resistant). [h] ATCC 27853.

Scheme 2[a]



[a] Reagents and conditions: (e) TBTU, DMF, DIPEA, rt, 2 h, 85−92%, lipophilic acid, (RCOOH, R = hexanoic acid, dodecanoic acid, palmitic acid, arachidic acid, linoleic acid, pyrene butyric acid, cholic acid); (f) TFA, CH_2Cl_2, 0 °C, 3 min, 90%.

and *Streptococcus pneumoniae* as well as Gram-negative strains *Escherichia coli,* gentamicin resistant *E. coli*, and *Pseudomonas aeruginosa*. The minimum inhibitory concentrations (MIC) in μg/mL were determined using established methods[35,36] and are shown in Table 1. Gentamicin and Neomycin served as positive controls. Our results show that the nature of the lipid moiety in compounds **13**−**19** has a significant influence on the antibacterial activity against Gram-positive organisms. It appears that saturated, aliphatic, and long fatty acid chains induce optimal Gram-positive activity while hydrophobically enhanced neomycin analogues bearing pyrene or cholesterol appendages do result in significantly reduced antibacterial activities. A remarkable 32-fold enhancement against MRSA is observed for compounds **15** and **16** (MIC = 8 μg/mL) when compared to neomycin (MIC = 256 μg/mL). This demonstrates that conjugation of neomycin with C_{16}- or C_{20}-lipids induces optimal antibacterial activity against MRSA while shorter lipids (C_6- or C_{12}-) result in reduced antibacterial activity relative to neomycin. This is in contrast to the antibacterial activity against the Gram-negative *E. coli*. For instance, compound **13** bearing a C_6-lipid chain displays stronger activity against *E. coli* and gentamicin-resistant *E. coli* than the C_{16}- or C_{20}-lipid conjugates. In the case of *P. aeruginosa*, we observed optimal antimicrobial activity for the C_{20}-lipid conjugate **16**. None of the compounds showed antifungal activity against *Candida albicans* in growth inhibition assays with MIC ≥ 512 μg/mL.

Discussion

In this paper we explored the antibacterial activities of seven neomycin−lipid conjugates containing various hydrophobic spacers derived from various lipophilic fatty acids, cholesterol, and pyrene. We envisioned that conjugation of neomycin to lipophilic moieties will enhance penetration in the phospholipid bilayer of bacteria resulting in either enhanced uptake of the AA−lipid conjugate or destabilization of the lipid membrane as proposed for other cationic lipids, peptide antibiotics, and cationic detergents. We observed that neomycin−lipid conjugates display higher antimicrobial potency against Gram-positive than Gram-negative bacteria. Very likely the outer membrane of Gram-negative bacteria provides an entry barrier against polycationic lipids but the cell wall of Gram-positive bacteria may adsorb and transport cationic lipids into the inner membrane. Our results also demonstrate that the nature of the lipid moiety influences the antimicrobial activity of polycationic lipids. A strong dependence is observed on the antimicrobial activity against MRSA, while a much weaker dependence is seen against *S. aureus*, *S. epidermidis*, and MRSE. Optimal activity against *S. aureus* and MRSA is achieved by conjugation of neomycin to a saturated C_{16} or C_{20} fatty acid. Shorter chain length or use of aromatic or cholesterol-based lipophilic acids usually results in weaker Gram-positive potencies. By comparison, no optimized lipid spacer is evident for Gram-negative organisms. Our results are consistent with previous findings using fatty amines.[23] In this case, the relative potency against MRSA followed the order $C_{14} > C_{16} > C_{12} > C_{18} \gg C_{10} > C_8$. By comparison, fatty acids exhibit significantly reduced antimicrobial activities. For instance, the most active fatty acid against MRSA and methicillin-susceptible *S. aureus* (MSSA) is lauric acid (C_{12}, MIC = 400 μg/mL).

Without any doubt a very intriguing finding of our study is the 32-fold enhanced activity of compounds **15** and **16** against MRSA when compared to unmodified neomycin. Our results may be interpreted that optimal lipid conjugation provides a tool to reinstall antimicrobial activity in neomycin-resistant MRSA. Several explanations could account for our observations including improved uptake, changes in mode of action, and enhanced affinity to RNA. Lipids such as free fatty acids, monoglycerides, fatty alcohols, fatty amines, and sphingosines are potent antimicrobial agents that kill Gram-positive and Gram-negative bacteria, fungi, and enveloped viruses on contact.[22,36] Cationic lipids, including sphingosines, phytosphingosines, and fatty amines, generally exhibit higher antimicrobial potency and more extended broad-spectrum antimicrobial activities when compared to neutral or anionic lipids. For

instance, sphingosine exhibits potent antimicrobial activity against *S. aureus* (MIC = 2 μg/mL), MRSA (MIC = 4−5 μg/mL), *E. coli* (MIC = 42 μg/mL), and *C. albicans* (MIC = 6−18 μg/mL).[22] Phytosphingosine, a marketed personal care product isolated from plants and fungi, shows potent broad-spectrum antimicrobial activity and is effective against acne and skin inflammation. Sphingosines are amino alcohols in which a lipophilic (usually C_{18}) carbon chain contains a polar headgroup consisting of two 1,2-hydroxyamine functions (Figure 1). Aminoglycosides are functionally related to sphingosines and contain multiple copies of the 1,2- or 1,3 hydroxyamine motif presented on a cyclic scaffold. Moreover, cationic surface-active detergents such as benzalkonium chloride are clinically used as strong disinfectants being effective on both Gram-positive and Gram-negative organisms. Several modes of action of benzalkonium chlorides have been proposed, including disruption of cell membrane, inactivation of enzymes, and denaturation of cell proteins. The MIC of optimized benzalkonium chlorides is 6.25 μg/mL on MRSA,[23] very similar to the neomycin−lipid conjugates **15** and **16**. This suggests that polycationic aminoglycoside−lipid conjugates may find use as potential antiseptics and antimicrobial disinfectants. Moreover, our results are consistent with previous studies on glycopeptide antibiotics such as teicoplanin and LY264826 that have shown that attachment of hydrophobic moieties can enhance antimicrobial efficacy by affecting the dimerization ratio or incorporation into the bacterial cell membrane.[37,38]

Conclusion

In the present study, we have established that the antibacterial activity of neomycin−lipid conjugates depends on the length and nature of the lipid moiety. Optimal Gram-positive activity is achieved by conjugation to saturated C_{16}- or C_{20}-lipids. Shorter aliphatic chains or aromatic chains result in reduced activity. Neomycin−C16 or neomycin-C20 conjugates are particularly active against MDR strains including MRSA and MRSE. Very importantly, C16− or C20−lipid conjugation in neomycin installs potent antimicrobial activity against clinically relevant, neomycin-resistant MRSA. Our results suggest that aminoglycoside-based polycationic lipids form a new class of antimicrobials with high potency against Gram-positive MDR bacteria. The high activity of C16− or C20−lipid conjugates against MDR Gram-positive bacteria suggests that these compounds may find use as potential antiseptics and antimicrobial disinfectants or in topical infections.

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Supporting Information Available: Synthetic procedures, spectral and analytical data for new compounds and antimicrobial testing protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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