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Synthesis and antibacterial activity of amphiphilic lysine-ligated neomycin B conjugates

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ABSTRACT

Amphiphilic lysine-ligated neomycin B building blocks were prepared by reductive amination of a protected C5″-modified neomycin B-based aldehyde and side chain-unprotected lysine or lysine-containing peptides. It was demonstrated that a suitably protected lysine-ligated neomycin B conjugate (NeoK) serves as a building block for peptide synthesis, enabling incorporation of aminoglycoside binding sites into peptides. Antibacterial testing of three amphiphilic lysine-ligated neomycin B conjugates against a representative panel of Gram-positive and Gram-negative strains demonstrates that C5″-modified neomycin-lysine conjugate retains antibacterial activity. However, in most cases the lysine-ligated neomycin B analogs display reduced potency against Gram-positive strains when compared to unmodified neomycin B or unligated peptide. An exception is MRSA where an eightfold enhancement was observed. When compared to unmodified neomycin B, the prepared lysine-neomycin conjugates exhibited a 4–8-fold enhanced Gram-negative activity against *Pseudomonas aeruginosa* and up to 12-fold enhanced activity was observed when compared to unligated reference peptides.

1. Introduction

The rise in antibiotic resistance among pathogenic bacteria and the declining rate of novel antibacterials reaching the market are a major concern in medicine.[1–5] As a result, there is a pressing need for novel classes of antibacterial agents with new or combined mechanisms of action and reduced likelihood to lead to the development of resistance. Cationic antimicrobial peptides and their mimetics are currently investigated as a new source of potential antibacterial agents in preclinical and clinical settings.[6,7] Cationic antimicrobial peptides that contain 10–50 amino acids are amphiphilic, and are rich in lysine or arginine and hydrophobic amino acids.[8–10] However, shorter amphiphilic peptide sequences as short as di- or tri- or hexapeptides with antibacterial activity are known.[11–13] We were especially encouraged by a previous report that has shown that both dipeptides H-D-Lys-Trp-OBn (kW-OBn) and H-L-Lys-Trp-OBn (KW-OBn) display potent antibacterial Gram-positive activity against *Staphylococcus aureus* (*S. aureus*), methicillin resistant *S. aureus* (MRSA), and methicillin resistant *Staphylococcus epidermidis* (MRSE) strains.[11] Interestingly, it was observed that the nature of the *C*-terminus contributes substantially to the antimicrobial activity of these two peptides.[22] For instance, dipeptide kW-OBn exhibits strong *S. aureus* activity while this activity is abolished in peptide kW-NHBzl (see Table 1). The reasons for this peculiar behavior are currently not understood.

Numerous studies with linear, cyclic, and diastereomeric cationic antimicrobial peptides have strongly supported the hypothesis that their physicochemical properties rather than any precise sequence are responsible for their activities.[8–10] It is generally believed that the amphiphilic topology is essential for insertion into and disruption of the cytoplasmic membrane. However, other mode of actions including intracellular targets have also been suggested.[19] In particular, the ability to rapidly kill bacteria and the relative difficulty with which bacteria develop resistance in vitro make cationic antimicrobial peptides attractive targets for drug development.[9] Previously, we and others have shown that aminoglycoside antibiotics-derived amphiphiles (AADAs) form a novel class of potent antibacterial agents.[14–17,31] The physicochemical similarities between AADAs and cationic antimicrobial peptides suggest a membranolytic mode of action. This mechanism is supported by (a) the observed concentration-dependent hemolytic activity of AADAs; (b) the presence of one or more hydrophobe(s) to induce antibacterial activity and (c) the precedence of a plethora of cationic antibacterial amphiphiles with membranolytic mode of actions. In this paper we report on the synthesis of amphiphilic lysine-ligated neomycin B analogs in which the C5″-position of neomycin B is ligated to hydrophobic lysine analogs or lysine-containing hydrophobic peptide sequence

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Table 1
Antibacterial activity (MIC) in μg/mL of various Lys-Trp dipeptides, neomycin B–lysine conjugate **5** and amphiphilic neomycin B–peptide conjugates **7** and **17** and control compounds against various Gram-positive and Gram-negative bacterial strains.

Organisms	Genta-micin	Neo-mycin B	kW-NBn	kW-OBn	Fmoc-kW-OBn	**5** (Fmoc-NeoK-OBn)	**7** (Fmoc-NeoKW-OBn)	**17** (H-WNeoKW-OH)
S. aureus[a]	1	1	512	16	2	16	8	4
MRSA[b]	2	256	512	512	2	128	32	64
S. epidermidis[c]	0.25	0.25	256	2	2	4	4	4
MRSE[d]	32	0.25	512	32	4	4	4	8
S. pneumoniae[e]	32	8	>512	32	64	128	64	32
E. coli[f]	4	2	512	64	128	32	16	32
E. coli (Gent-R)[g]	128	4	512	16	128	32	16	16
P. aeruginosa[h]	4	512	>512	512	128	>256	128	64
P. aeruginosa (Gent-R)[i]	128	512	512	>512	128	>256	64	>128

Representative minimal inhibitory concentrations (MIC) in μg/mL for various bacterial strains: [a] ATCC 29213; [b] Methicillin-resistant *S. aureus* ATCC 33592; [c] *S. epidermidis* ATCC 14990; [d] Methicillin-resistant *S. epidermidis* (CAN-ICU) 61589; [e] ATCC 49619; [f] ATCC 25922; [g] CAN-ICU 61714; [h] ATCC 27853; [i] CAN-ICU 62308.

(Fig. 1). The single primary hydroxymethyl group at the ribose moiety (C5″-position) in neomycin B was chosen as a point of modification due to its expected high reactivity in chemical modifications.[21] Previous studies have indicated that modified C5″-analogs of neomycin B retain high binding affinity to RNA and antimicrobial activities.[18,30–32] Most of the previous work on C5″-modified neomycin B analogs has focused on neomycin B–lipid analogs and neomycin B–fluoroquinolone hybrid antibiotics[34] while very little data exist on amphiphilic neomycin B–amino acid or amphiphilic neomycin B–peptide conjugates that resemble short antibacterial peptides. We were particularly interested to prepare C5″-modified neomycin B conjugates linked to the ε-amino function of lysine thereby producing novel polycationic lysine mimetics, which may find utility in solid phase/solution phase antibacterial peptide synthesis. Moreover, suitably-protected lysine-ligated neomycin B building blocks may find application for incorporation of RNA-binding sites into peptides and in addition may serve as polyfunctional lysine surrogates in antimicrobial peptides.

2. Results and discussion

The chemical manipulation of aminoglycoside antibiotics provides great opportunities for medicinal chemistry due to the unique biological properties of this class of compounds.[27–31,33] However, the polyfunctional nature of aminoglycoside antibiotic scaffold frequently requires multi-synthetic protection–deprotection steps to perform selective chemical modifications. This is often perceived as a serious limitation to drug development making it difficult and expensive to access aminoglycoside analogs. We describe herein, the preparation of lysine-ligated neomycin B analogs by taking advantage of the presence of a single primary hydroxy group at the C5″-position in neomycin B. We were interested to develop a synthetic method that would permit regioselective ligation of the C5″-position of neomycin B to the side chain amino function of lysine. We envisaged that this could be achieved by regioselective oxidation of the primary alcohol into an aldehyde followed by reductive amination with a partially protected lysine building block (Fig. 1). For that purpose, the synthesis of the lysine-ligated neomycin B building block NeoK is outlined in Scheme 1. At first, commercially available neomycin B sulfate **1** was converted into Boc-protected neomycin B according to the literature procedure.[20] The primary hydroxyl group of **2** was selectively oxidized with trichlorocyanuric acid (TCCA) in the presence of catalytic amounts of TEMPO at 0 °C–rt for 3 h, according to Vasella's procedure[21h] to afford aldehyde **3**, which was directly used for the reductive amination reaction. In a model study, aldehyde **3** was ligated to Fmoc-L-Lys-OBn to form intermediate imine in the presence of molecular sieves and catalytic amount of acetic acid in minimum volume of methanol for 4 h at rt. Subsequently, the reaction mixture was diluted with methanol followed by addition of sodium cyanoborohydride and 3% methanol–acetic acid maintaining pH 5–6 and stirring at room temperature for 12 h. The reaction was monitored by TLC using methanol–CH_2Cl_2 (1:10) as solvent and developed in ethanolic ninhydrin solution. The reaction mixture was neutralized with 2% NaOH and then extracted with EtOAc to afford a crude reaction mixture containing both neomycin–lysine conjugate **4** (53%) and alcohol **2** (47%).[20] Separation was achieved by flash column chromatography using methanol–CH_2Cl_2 (1:10) as the eluent. The product was confirmed by mass as well as NMR spectroscopic data. In the ^{1}H NMR spectrum shows the appearance of aromatic protons related to the Fmoc- and benzyl-group between $\delta = 7.80$–6.41 ppm indicating the presence of lysine unit while signals at $\delta = 5.76$–1.40 indicate the neomycin B moiety. Global deprotection of all Boc-groups afforded the desired conjugate **5** as TFA salt (Scheme 1).

Once we had demonstrated that the synthetic strategy is applicable to lysine we then developed an interest to study the reductive amination of neomycin B to the ultrashort lysine-containing antimicrobial dipeptide KW-OBn.[11] For this purpose aldehyde **3** was conjugated to side chain unprotected dipeptide Fmoc-KW-OBn using the same protocol as previously applied to afford

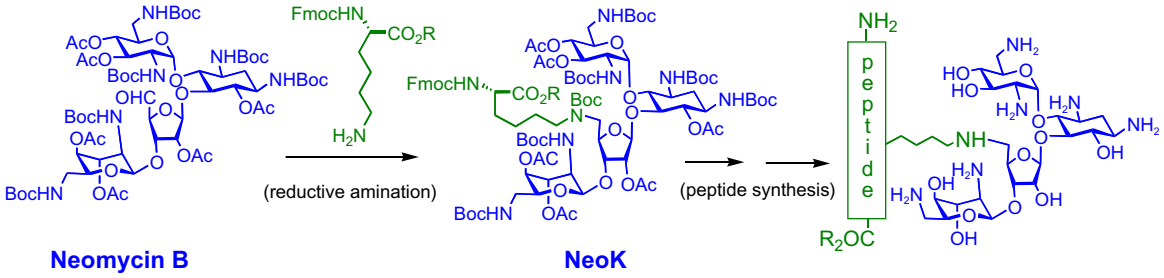
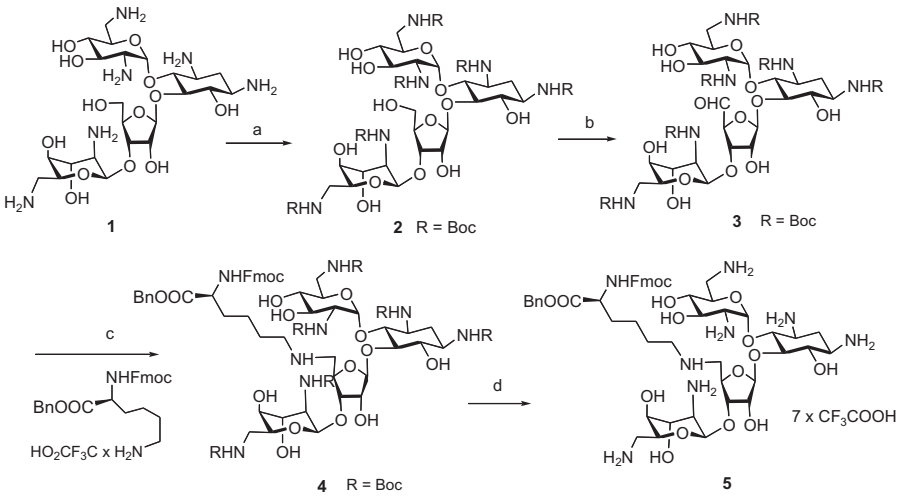


Figure 1. Synthesis of lysine-ligated neomycin B building block (NeoK) and use in peptide synthesis.



Scheme 1. Reagents and conditions: (a) $(Boc)_2O$, Et_3N, MeOH, 55 °C, overnight; (b) TCCA, EtOAc, 0 °C to rt, 3 h; (c) Lysine salt, $NaCNBH_3$, AcOH, MeOH, molecular sieves powder, rt, 10 h; (d) TFA, 0 °C, 3 min.

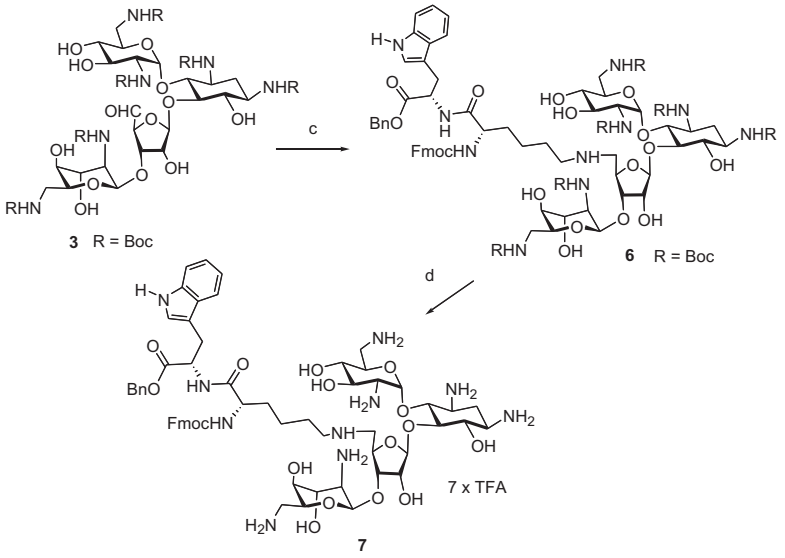
neomycin B–peptide conjugate **6** in 48% isolated yield which upon treatment with TFA produced deblocked **7** as TFA salt (Scheme 2). The structure of neomycin–KW-OBn conjugate was confirmed by spectroscopic analysis.

2.1. Synthesis of AG-AMP using a stepwise approach

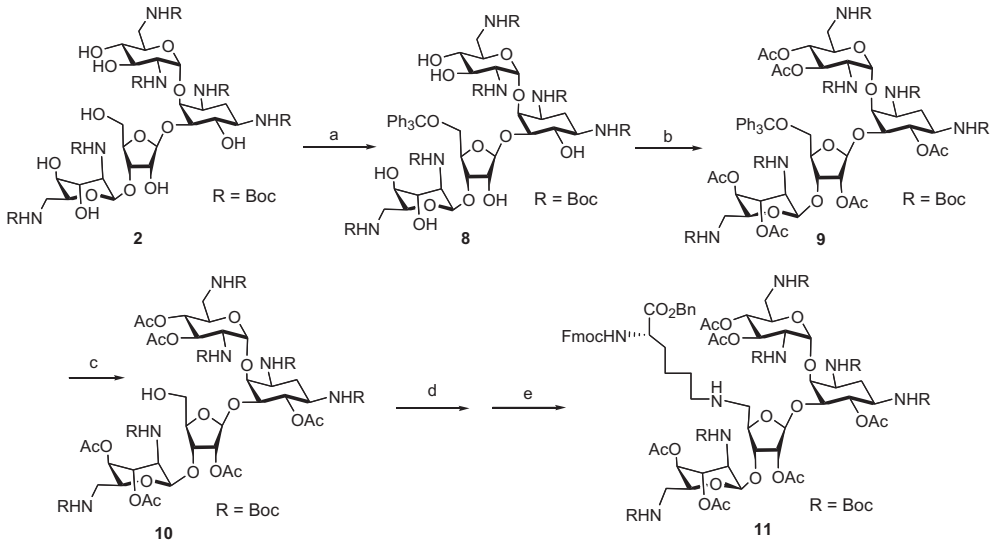
We also explored the synthesis of hydroxyl protected lysine-ligated neomycin B analog **11** using a stepwise approach (Scheme 3). Protected lysine–neomycin B conjugate **11** contains six acetate ester protecting groups which are expected to facilitate peptide elongation by preventing potential side reactions involving the unprotected neomycin B-derived polyol scaffold. Compound **11** was synthesized in a five-step synthesis. At first, the primary hydroxyl group was selectively protected as trityl ether **8** using trityl chloride in pyridine at room temperature in 89% yield. Peracetylation of **8** using acetic anhydride and DMAP in pyridine afforded **9**. The trityl group in **9** was selectively removed with *p*-TSA in methanol to afford alcohol **10**. Subsequently, the primary hydroxyl group in **10** was oxidized with pyridinium chlorochromate (PCC) in the presence of molecular sieves powder to give the corresponding aldehyde which was directly used for ligation to Fmoc-KW-OBn using our previously applied standard protocol to afford conjugate **11** (59%) together with the corresponding primary alcohol **10** (41%). Both compounds could be separated by flash column chromatography (Scheme 3).

2.2. Use of lysine–neomycin B conjugate 13 as building block in peptide chain elongation

In order to use compound **11** as a building block in peptide synthesis, protection of the C5″-amino function is necessary. To facilitate global deprotection using acidic conditions at a later stage, we protected the secondary amine as *tert*-butyl carbamate. This was achieved by exposure of **11** to excess $(Boc)_2O$ and triethylamine in acetonitrile for 6 h to yield fully protected lysine–neomycin B conjugate **12** in 73% yield. To explore peptide chain elongation in solution phase we selected fully protected lysine–neomycin B conjugate **12** for N- and C-terminal elongation. Exposure of **12** to piperidine in DMF produced amine **13** which was coupled to



Scheme 2. Reagents and conditions: (a) TBTU, DIPEA, DMF, rt, 2 h; (b) 50% TFA, CH_2Cl_2, rt, 2 h; (c) $NaCNBH_3$, AcOH, MeOH, rt, 4 h; (d) TFA, 0 °C.

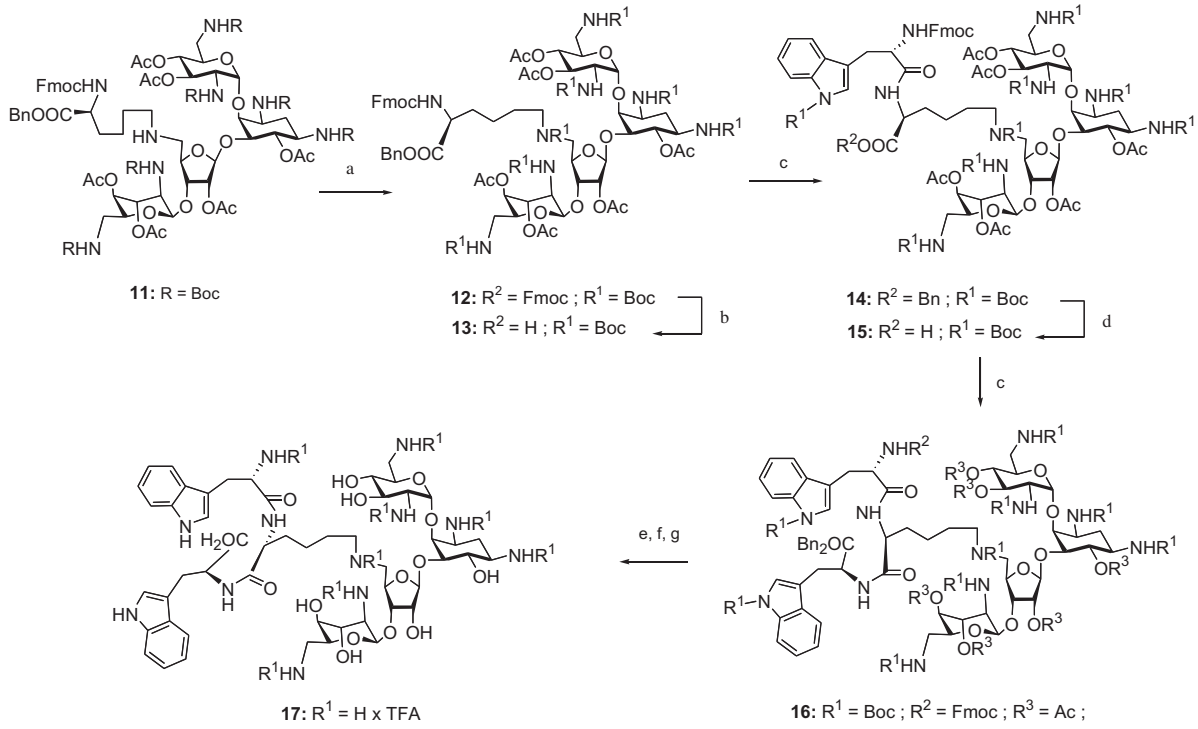


Scheme 3. Reagents and conditions: (a) TrCl, Pyridine, rt; (b) Ac_2O, Py, DMAP, rt, overnight; (c) *p*-TSA, MeOH, rt; (d) PCC, CH_2Cl_2, molecular sieves powder, rt; (e) $NaCNBH_3$, AcOH–MeOH.

Fmoc-L-Trp(Boc)-H using TBTU, DIPEA in DMF to produce neomycin B-ligated dipeptide **14** in quantitative yield. *C*-terminal elongation required deblocking of the benzylester moiety using catalytic hydrogenation with Pearlman's catalyst ($Pd(OH)_2$–C) to produce acid **15**. Coupling of acid **15** to amine H-L-Trp(Boc)-OBn produced the tripeptide-nomycin B conjugate **16** in 58% yield. Global deprotection of **16** using catalytic amounts of sodium methoxide in methanol followed by catalytic hydrogenation and exposure to TFA produced unprotected tripeptide–neomycin B conjugate **17** (Scheme 4).

2.3. Antibacterial properties of lysine-ligated neomycin B conjugates

With amphiphilic lysine–neomycin B conjugates **5**, **7**, and **17** in hand, we determined the antibacterial minimum inhibitory concentration (MIC) in μg/mL of these conjugates against American Type Culture Collection (ATCC) reference strains as well as clinical isolates of Gram-positive strains including *S. aureus*, MRSA, *S. epidermidis*, methicillin-resistant *S. epidermidis* (MRSE), and *Streptococcus pneumoniae* as well as Gram-negative strains



Scheme 4. Reagents and conditions: (a) $(Boc)_2O$, DMAP, Et_3N, acetonitrile; (b) 25% piperidine, DMF, rt; (c) TBTU, DIPEA, DMF; (d) $Pd(OH)_2$, EtOAc, H_2; (e) Na in MeOH, 0 °C to rt; (f) $Pd(OH)_2$, MeOH, H_2; g) TFA, 0 °C.

Escherichia coli, gentamicin-resistant *E. coli*, and *Pseudomonas aeruginosa* (Table 1). We also included three resistant strains obtained from a national surveillance study assessing antimicrobial resistance in Canadian intensive care units CAN-ICU (Table 1).[25] Gentamicin and neomycin B served as positive controls while the dipeptides kW-OBn, kW-NHBn, and Fmoc-kW-OBn served as reference peptides. Our results demonstrate that lysine-ligated neomycin B analog **7** (Fmoc-NeoKW-OBn × 7 TFA, MW = 2038.5) generally exhibits reduced Gram-positive activity (2–8-fold higher MICs) when compared to reference peptide Fmoc-KW-OBn × TFA (MW = 758.3). However, an eightfold reduction in MIC was observed against two Gram-negative *E. coli* strains including a gentamicin-resistant organism. Enhancing the overall amphiphilicity of lysine-ligated neomycin conjugates leads to an increase in antibacterial potency. For instance, amphiphilic dipeptide Fmoc-NeoKW-OBn **7** containing a hydrophobic tryptophan (W) residue shows stronger antibacterial activity when compared to Fmoc-NeoK-OBn **5**. This is consistent with previous findings which have shown that cationic amphiphilicity is necessary for antibacterial activity.[7] A similar trend is seen with dipeptides H-kW-OBn and Fmoc-kW-OBn. The enhanced antibacterial activity of Fmoc-protected cationic peptides has been reported previously and is likely related to strong hydrophobic effect of the Fmoc-protecting group.[12] The similar antibacterial activity of tripeptide–neomycin B conjugate **17** (H-WNeoKW-OH) when compared to **7** demonstrates that the hydrophobicity provided by the Fmoc-group can be substituted by addition of a tryptophan residue. The preference for tryptophan residues in short cationic peptide antibiotics has been previously described.[23,24] Rather surprising is the low activity of amphiphilic neomycin B conjugate **7** against MRSA resulting in a eightfold reduction of antibacterial potency when compared to dipeptide Fmoc-kW-OBn. The loss of activity in **7** is higher than that what would be expected by a 2.5-fold increase in molecular weight. Overall our biological results indicate that there is little to no synergistic effects when neomycin B is conjugated to amphiphilic lysine-containing residues at the C5″-position in neomycin B. In summary, we have developed a synthetic access to suitably-protected polycationic lysine surrogates that serve as building blocks in peptide synthesis. Incorporation of these building blocks permits the incorporation of RNA-binding motifs in peptides. All three lysine–neomycin B conjugates display lower antibacterial activity when compared to unmodified neomycin B. The only exceptions are MRSA and two *P. aeruginosa* strains where modest improvements (up to eightfold lower MICs) were seen. The modest activity of neomycin B conjugates **5**, **7**, and **17** against resistant organisms such as MRSE, gentamicin-resistant *E. coli*, and *P. aeruginosa* strains could be the action of aminoglycoside-modifying enzymes, reduced binding affinity to RNA, reduced membrane permeability or other factors. It is currently not possible to eliminate any of these factors. Based on previous research results obtained with C-5"-modified neomycin B analogs it is unlikely that reduced RNA binding will be the major driving force. Very likely a combination of organism-specific factors will be responsible to explain the observed results. We hypothesize that the introduced amphiphilicity in neomycin B conjugates **5**, **7**, and **17** alters the mode of action(s) in favor of enhanced permeability.

3. Experimental

3.1. General methods—chemistry

NMR spectra were recorded on a Brucker Avance 300 spectrometer (300 MHz for ^{1}H NMR, 75 MHz for ^{13}C) and AMX 500 spectrometer (500 MHz for ^{1}H NMR). Optical rotation was measured at a concentration of g/100 mL, with a Perkin–Elmer polarimeter (accuracy 0.002°). GC–MS analyses were performed on a Perkin–Elmer Turbomass Autosystem XL. Analytical thin-layer chromatography was performed on precoated silica gel plates. Visualization was performed by ultraviolet light and/or by staining with ninhydrin solution in ethanol. Chromatographic separations were performed on a silica gel column by flash chromatography (Kiesel Gel 40, 0.040–0.063 mm; Merck). All the aminoacids used are natural, that is, L-isomer. Yields are given after purification, unless differently stated. When reactions were performed under anhydrous conditions, the mixtures were maintained under nitrogen. Compounds were named following IUPAC rules as applied by Beilstein Institute AutoNom (version 2.1) software for systematic names in organic chemistry.

3.2. 5″-*N*-(Fmoc-Lys-OBn)-1,3,2′,6′,2‴,6‴-hexa-*N*-(*tert*-butoxycarbonyl)-5″-deoxy-neomycin B (4)

A 0.05 M solution of alcohol **2** (1.0 g, 0.823 mmol) in EtOAc at −10 °C was treated with trichloroisocyanuric acid (TCCA; 0.209 g, 0.902 mmol) and a catalytic amount of TEMPO (0.006 g, 0.041 mmol) under N_2 was allowed to warm to 25 °C and stirred for 1 h. After completion the reaction mixture was basified with 1 N NaOH. After separation of the phases, the aq layer was extracted with EtOAc (3 × 50 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to obtain the aldehyde **3** (77%). This crude aldehyde was used for next reaction.

To a solution of **3** (0.08 g, 0.065 mmol) in MeOH (10 mL) was added Fmoc-Lys-OBn (0.060 g, 0.132 mmol) and stirred at room temperature for 1 h and then AcOH (0.36 mL) was added and the mixture was stirred for 30 min. $NaBH_3CN$ (0.045 g, 0.72 mmol) was then added and the mixture was stirred at room temperature for 4 h. The reaction mixture was neutralized with NaOH and extracted with AcOEt (3 × 50 mL). The combined organic layers were washed with satd NaCl (3 × 30 mL), dried over Na_2SO_4, filtered, and the solvents were evaporated in vacuo to give an oily residue. Flash column chromatography afforded the desired product **4** as a white solid. Yield = 53%; R_f 0.46 (CH_2Cl_2–MeOH 11:1); ^{1}H NMR (300 MHz, CD_3OD): δ 7.80 (d, 2H, J = 9.0 Hz), 7.66 (d, 2H, J = 7.6 Hz), 7.40 (t, 2H, J = 7.6 Hz), 7.30 (m, 7H), 5.76 (d, 1H, J = 4.0 Hz), 5.60 (br s, 1H), 5.20–5.10 (m, 4H), 5.09 (br s, 1H), 5.00 (d, 2H, J = 8.3 Hz), 4.79 (m, 1H), 4.61 (m, 1H), 4.49 (d, 1H, J = 5.0 Hz), 4.40–4.30 (m, 4H), 4.20–4.12 (m, 3H), 4.10 (m, 1H), 3.92 (m, 3H), 3.80 (m, 2H), 3.51 (m, 6H), 3.40 (m, 1H), 3.21 (m, 1H), 2.85 (m, 1H), 2.60 (m, 1H), 1.97 (m, 2H), 1.66 (m, 2H), 1.53 (m, 2H), 1.40 (m, 54H); ^{13}C NMR (75 MHz, CD_3OD): δ 173.8 (ester–CO), 159.2, 159.0, 158.7, 158.5, 158.4, 158.3, 157.9, (amide–CO), 145.0–120.7 (aromatic carbons), 111.6, 99.4, 98.1 (anomeric carbons), 80.9 (×2), 80.7 (×2), 80.3 (×4), 79.0, 76.3, 75.2, 74.3, 73.1, 72.9 (×2), 72.8, 72.6, 71.8, 71.4 (×2), 68.4, 68.0 (×2), 67.7, 56.0, 54.2, 53.1, 52.3, 50.6, 42.4, 41.4, 35.8, 32.8, 32.3, 29.0–28.7, 24.7; $[\alpha]_D^{25} = +34.0$ (c 0.2, MeOH); EIMS: calcd for $C_{81}H_{122}N_8NaO_{28}^+$ 1677.84, found: 1678.02 $[M+Na]^+$. Anal. Calcd for $C_{81}H_{122}N_8O_{28}$: C, 58.75; H, 7.43; N, 6.77. Found: C, 59.11; H, 7.67; N, 6.91.

3.3. 1,3,2′,6′,2‴,6‴-Heptaammonium-5″-*N*-(Fmoc-Lys-OBn)-5″-deoxy-neomycin B-hexafluoroacetate (5)

Compound **4** (0.12 g, 0.7255 mmol) was treated with 99% trifluoroacetic acid (2 mL) for 3 min at 0 °C. The volatiles were removed in vacuo. The nonpolar residue was removed by washing with ether–methanol (1%) mixture and decanted the solvent to get the conjugates **5** as TFA salt. Yield = 88%; R_f 0.25 (CH_2Cl_2–MeOH–NH_4OH 7:5:2); ^{1}H NMR (300 MHz, CD_3OD): δ 7.80 (d, 2H, J = 7.3 Hz), 7.66 (t, 2H, J = 7.4 Hz), 7.40 (d, 2H, J = 7.3 Hz), 7.30 (m, 7H), 5.97 (d, 1H, J = 4.3 Hz), 5.82 (d, 1H, J = 1.8 Hz), 5.35 (t, 1H, J = 4.3 Hz), 5.33 (br s, 1H), 5.15 (dd, 2H, J = 6.6, 10.5 Hz), 4.76 (m,

1H), 4.61 (m, 1H), 4.40 (m, 2H), 4.32 (dd, 2H, *J* = 6.6, 10.5 Hz), 4.25–4.12 (m, 3H), 4.10 (m, 1H), 4.05–3.88 (m, 4H), 3.73 (q, 2H, *J* = 9.0 Hz), 3.71 (m, 2H), 3.67–3.58 (m, 2H), 3.49–3.40 (m, 3H), 3.26 (m, 1H), 3.18 (m, 3H), 2.86 (t, 1H, *J* = 7.3 Hz), 2.49 (m, 1H), 2.22 (m, 1H), 1.86 (m, 1H), 1.66 (m, 2H), 1.43 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ 173.8 (ester), 158.7 (Fmoc-CO), 145.3–120.9 (aromatic carbons), 111.2, 99.4, 98.9 (anomeric carbons), 87.8, 77.9, 77.2, 75.0, 74.3, 73.6, 72.0, 71.4, 71.2, 71.0, 70.9, 69.8, 69.4, 69.2, 68.1, 62.1, 61.5, 55.4, 55.2, 52.7, 51.4, 50.4, 50.2, 42.1, 42.0, 40.5, 31.9, 28.1, 24.5; EIMS: calcd for $C_{51}H_{75}N_8O_{16}^+$ 1055.52, found: 1055.60 $[M+H]^+$. Anal. Calcd for $C_{65}H_{81}F_{21}N_8O_{30}$: C, 42.12; H, 4.41; F, 21.53; N, 6.05. Found: C, 42.22; H, 4.39; F, 21.5763; N, 6.11.

3.4. 5″-*N*-(Fmoc-Trp-Lys-OBn)-1,3,2′,6′,2‴,6‴-hexa-*N*-(*tert*-butoxycarbonyl)-5″-deoxy-neomycin B (6)

To a solution of **3** (0.08 g, 0.065 mmol) in MeOH (10 mL) were added Fmoc-Lys-Trp-OBn (0.060 g, 0.132 mmol) and molecular sieves powder and stirred at room temperature for 1 h and then AcOH (0.36 mL) was added and the mixture was stirred for 30 min. $NaBH_3CN$ (0.045 g, 0.72 mmol) was then added and the mixture was stirred at room temperature for 8 h. The reaction mixture was neutralized with NaOH and extracted with AcOEt (3 × 50 mL). The combined organic layers were washed with satd NaCl (3 × 30 mL), dried over Na_2SO_4, filtered, and the solvents were evaporated in vacuo to give an oily residue. Flash column chromatography afforded the desired product **6** as a white solid. Yield = 48%; R_f 0.45 (CH_2Cl_2–MeOH 17:1); ^{1}H NMR (300 MHz, CD_3OD): δ 8.13 (t, 1H, *J* = 8.3 Hz), 7.84 (t, 1H, *J* = 6.3 Hz), 7.82 (d, 2H, *J* = 6.3 Hz), 7.60 (s, 1H), 7.52 (m, 1H), 7.45–7.20 (m, 10H), 7.03 (dd, 1H, *J* = 2.6, 7.6 Hz), 6.98 (d, 1H, *J* = 5.4 Hz), 5.33 (s, 1H), 5.17 (s, 2H), 4.91 (s, 1H), 4.78 (m, 4H), 4.58 (s, 1H), 4.36 (t, 1H, *J* = 7.8 Hz), 4.20 (m, 2H), 3.98 (m, 1H), 3.91 (m, 3H), 3.73 (m, 4H), 3.66 (m, 1H), 3.52 (m, 9H), 3.38 (m, 2H), 3.20 (m, 3H), 2.96 (m, 1H), 2.76 (m, 1H), 2.01 (m, 2H), 1.62 (m, 2H), 1.48 (3 s, 54H), 1.37 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD, HSQC): δ 127.7–119.7 (aromatic carbons), 110.9, 106.1, 99.5 (anomeric carbons), 86.0, 85.7, 85.3, 82.2, 80.9 (×2), 80.7 (×2), 78.2, 77.6, 76.8, 74.0, 73.3, 71.5, 71.1, 70.0, 68.1, 66.5, 65.9, 55.5, 54.6, 53.9, 52.0, 51.6, 50.8, 49.6, 48.3, 46.5, 40.6, 34.3, 34.0, 27.6–28.0, 24.3; $[\alpha]_D^{25} = +43.0$ (*c* 0.35, MeOH); EIMS: calcd for $C_{92}H_{132}N_{10}NaO_{29}^+$ 1863.92, found: 1864.13 $[M+Na]^+$. Anal. Calcd for $C_{92}H_{132}N_{10}O_{29}$: C, 59.99; H, 7.22; N, 7.60. Found: C, 60.11; H, 7.31; N, 7.55.

3.5. 1,3,2′,6′,2‴,6‴-Hexaammonium-5″-*N*-(Fmoc-Trp-Lys-OBn)-5″-deoxy-neomycin B-hexafluoroacetate (7)

Procedure same as above. Yield = 85%; R_f 0.12 (NH_4OH–CH_2Cl_2–MeOH 2:9:4); ^{1}H NMR (300 MHz, CD_3OD): δ 7.82 (dd, 2H, *J* = 3.4, 7.3 Hz), 7.72 (d, 2H, *J* = 7.9 Hz), 7.60 (d, 2H, *J* = 7.3 Hz), 7.41 (t, 2H, *J* = 7.5 Hz), 7.35 (m, 3H), 7.22 (m, 3H), 7.16 (m, 3H), 7.03 (t, 1H, *J* = 7.5 Hz), 5.97 (d, 1H, *J* = 3.4 Hz), 5.85 (d, 1H, *J* = 3.6 Hz), 5.46 (m, 1H), 5.39 (d, 1H, *J* = 3.4 Hz), 5.33 (m, 1H), 5.29 (m, 1H), 5.18 (m, 2H), 4.97 (m, 1H), 4.58 (d, 1H, *J* = 5.5 Hz), 4.45 (d, 1H, *J* = 14.6 Hz), 4.32 (m, 3H), 4.16 (m, 2H), 4.03 (m, 3H), 3.87 (m, 1H), 3.75 (dd, 1H, *J* = 4.8, 10.1 Hz), 3.69 (br d, 1H, *J* = 2.8 Hz), 3.64 (m, 1H), 3.59 (m, 1H), 3.44 (m, 4H), 3.28 (m, 1H), 3.24 (m, 1H), 3.20 (m, 1H), 3.17 (d, 1H, *J* = 4.5 Hz), 3.10 (m, 3H), 2.83 (m, 1H), 2.48 (m, 2H), 2.16 (m, 1H), 2.01 (m, 1H), 1.72 (m, 1H), 1.58 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ 173.6 (ester–CO), 164.0–162.7 (TFA carbons, q with $^1J_{CF}$ ~34.8 Hz), 124.1–112.4 (TFA carbons, q with $^1J_{CF}$ ~292.0 Hz), 158.4 (×2) (amide CO), 145.5–119.7 (aromatic carbons), 110.4, 97.1, 96.6 (anomeric carbons), 86.4, 78.5, 76.9, 75.1, 74.3, 74.0, 73.3, 72.0, 71.8, 71.0, 69.8, 69.4, 69.2, 68.4, 58.9, 55.9, 55.2, 54.9, 52.9, 51.4, 51.2, 50.6, 50.3, 50.2, 44.3, 42.0, 41.6, 31.9, 30.1, 28.9, 23.8; $[\alpha]_D^{25} = +29.0$ (*c* 0.55, MeOH); EIMS: calcd for $C_{62}H_{85}N_{10}O_{17}^+$ 1241.60, found: 1241.64 $[M+H]^+$. Anal. Calcd for $C_{76}H_{91}F_{21}N_{10}O_{31}$: C, 44.76; H, 4.50; F, 19.56; N, 6.87. Found: C, 44.89; H, 4.55; F, 19.87; N, 6.31.

3.6. 5″-*O*-Triphenylmethyl-1,3,2′,6′,2‴,6‴-hexa-*N*-(*tert*-butoxycarbonyl)-neomycin B (8)

A solution of **2** (1.0 g, 0.82 mmol) in dry pyridine (20 mL) was treated with trityl chloride (0.66 g, 2.46 mmol) and stirred at rt for 10 h and then removed the solvent under reduced pressure. The crude reaction mixture was partitioned between H_2O (60 mL) and EtOAc (30 mL). The aqueous layer was separated and extracted with EtOAc (2 × 40 mL). The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. Flash column chromatography afforded **8** as a white solid. Yield = 89%; R_f 0.45 (CH_2Cl_2–MeOH 12:1); ^{1}H NMR (300 MHz, CD_3OD): δ 7.42 (d, 6H, *J* = 7.5 Hz), 7.29 (t, 6H, *J* = 7.2 Hz), 7.21 (t, 3H, *J* = 6.9 Hz), 6.12 (d, 1H, *J* = 8.7 Hz), 5.66 (br s, 1H), 5.32 (s, 1H), 5.11 (s, 1H), 4.90 (m, 2H), 4.74 (br s, 1H), 4.36 (br s, 1H), 4.11 (m, 3H), 3.92 (d, 1H, *J* = 8.7 Hz), 3.70 (m, 3H), 3.60 (m, 5H), 3.25 (m, 5H), 2.66 (br s, 1H), 2.39 (m, 1H), 1.46 (s, 54H); ^{13}C NMR (75 MHz, CD_3OD): 158.9, 158.5, 158.2 (×2), 157.9, 150.1 (Boc-CO), 145.5–128.1 (anomeric carbons), 110.8, 99.6, 98.0 (anomeric carbons), 88.0, 87.7, 82.2, 80.8 (×2), 80.7, 80.5 (×2), 80.0, 78.1, 77.7, 75.6, 75.3, 74.2, 73.5, 73.0, 68.6, 64.2, 56.4, 53.5, 52.4, 42.2, 41.2, 37.1, 36.0, 31.8, 29.3–28.9; $[\alpha]_D^{25} = +22.0$ (*c* 0.55, MeOH); EIMS: calcd for $C_{72}H_{108}N_6NaO_{25}^+$ 1479.74, found: 1480.04 $[M+Na]^+$. Anal. Calcd for $C_{72}H_{108}N_6O_{25}$: C, 59.33; H, 7.47; N, 5.77. Found: 59.11; H, 7.25; N, 5.89.

3.7. 6,3′,4′,2″,5″,4‴-Hexa-*O*-acetyl-1,3,2′,6′,2‴,6‴-hexa-*N*-(*tert*-butoxycarbonyl)- 5″-*O*-triphenylmethyl neomycin B (9)

To a solution of **8** (1.36 g, 10 mmol), in pyridine (10 mL) were added acetic anhydride (1.3 g, 13 mmol) and DMAP (1.5 g, 12 mmol). After stirring at rt for 6 h the reaction mixture was neutralized by adding hydrochloric acid (1.0 N) and aqueous sodium bicarbonate solution and then partitioned between H_2O (100 mL) and EtOAc (100 mL). The organic layer was dried (anhydrous Na_2SO_4), evaporated, and purified by flash column chromatography to afford **9** as a white solid. Yield = 89%; R_f 0.45 (CH_2Cl_2–MeOH 17:1); ^{1}H NMR (300 MHz, CD_3OD): δ 7.50 (d, 3H, *J* = 7.3 Hz), 7.45 (d, 3H, *J* = 7.3 Hz), 7.36 (t, 4H, *J* = 6.6 Hz), 7.26 (m, 2H), 7.12 (d, 3H, *J* = 7.6 Hz), 5.66 (s, 1H), 5.32 (s, 1H), 5.25 (s, 1H), 5.04–4.77 (m, 5H), 4.64 (m, 4H), 3.87 (m, 3H), 3.70 (m, 4H), 3.52 (t, 1H, *J* = 8.8 Hz), 3.32 (m, 1H), 3.22 (m, 4H), 2.91 (m, 1H), 2.28 (m, 1H), 2.22 (s, 3H), 2.18 (s, 3H), 2.11 (s, 3H), 2.01 (s, 3H), 1.93 (s, 3H), 1.66 (s, 3H), 1.46 (s, 54H); ^{13}C NMR (75 MHz, $CDCl_3$): 171.1, 170.3, 169.4, 169.1 (×2), 168.4, 156.6, 155.3 (×2), 154.9 (×2), 154.6, 144.5–127.0 (aromatic carbons), 107.8, 97.6, 96.7 (anomeric carbons), 86.4, 82.9, 80.2, 79.7 (×2), 79.4 (×2), 79.3 (×3), 78.8, 77.3, 75.7, 74.2, 73.2, 71.9, 68.9, 68.6 (×2), 66.6, 61.6, 52.4, 49.4, 49.0, 40.0, 35.8, 28.4–28.0, 21.0–20.5; $[\alpha]_D^{25} = +36.0$ (c 0.55, MeOH); EIMS: calcd for $C_{84}H_{120}N_6NaO_{31}^+$ 1731.80, found: 1731.9 $[M+Na]^+$. Anal. Calcd for $C_{84}H_{120}N_6O_{31}$: C, 59.00; H, 7.07; N, 4.91. Found: C, 59.23; H, 7.38; N, 5.03.

3.8. 6,3′,4′,2″,5″,4‴-Hexa-*O*-acetyl-1,3,2′,6′,2‴,6‴-hexa-*N*-(*tert*-butoxycarbonyl)- neomycin B (10)

To compound **9** (0.76 g, 0.44 mmol) in methanol was added a solution containing *p*-TSA (0.08 g) in MeOH (10 mL) at rt. After 1 h, the reaction mixture was neutralized with 5% $NaHCO_3$ and diluted with CH_2Cl_2 and extracted with CH_2Cl_2 (3 × 50 mL). The organic phase was dried (anhydrous Na_2SO_4), filtered, and the

solvent was removed in vacuo to afford a solid residue. Flash column chromatography afforded **10** as a white solid. Yield = 78%; R_f 0.44 (CH_2Cl_2–MeOH 17:1); ^{1}H NMR (300 MHz, acetone-d_6): δ 6.07 (br s, 1H, NH), 5.92 (d, 1H, J = 8.7 Hz), 5.81 (d, 1H, J = 8.7 Hz), 5.68 (s, 1H), 5.29 (m, 2H), 5.08 (t, 1H, J = 10.3 Hz), 4.74 (br s, 1H), 4.94 (m, 3H), 4.80–4.72 (m, 3H), 4.31 (t, 1H, J = 5.8 Hz), 4.14 (t, 1H, J = 7.7 Hz), 4.08 (m, 2H), 3.97 (dt, 1H, J = 3.6, 9.9 Hz), 3.87 (t, 1H, J = 5.8 Hz), 3.75 (m, 3H), 3.62 (m, 1H), 3.32 (m, 2H), 3.22 (m, 1H), 2.10 (4 s, 12H), 1.97 (s, 3H), 1.90 (s, 3H), 1.75 (t, 1H, J = 12.1 Hz), 1.51 (m, 1H), 1.40 (s, 54H); ^{13}C NMR (75 MHz, CD_3OD): 171.2, 170.7, 170.4, 169.6, 169.2, 168.4 (acetates), 155.9 (×2), 155.7, 155.0 (×3) (Boc-CO), 105.9, 99.1, 98.8 (anomeric carbons), 82.5, 81.9, 80.4, 79.9, 79.5 (×3), 79.4 (×3), 77.3, 76.1, 74.3, 72.9, 71.8, 70.1, 69.1, 68.9, 66.6, 61.1, 53.4, 49.2, 48.9, 41.1, 40.5, 33.9, 28.3–28.2, 21.0–20.2 (acetates); $[\alpha]_D^{25} = +28.0$ (c 0.45, MeOH); EIMS: calcd for $C_{65}H_{106}N_6NaO_{31}^+$ 1489.69, found: 1489.69 $[M+Na]^+$. Anal. Calcd for $C_{65}H_{106}N_6O_{31}$: C, 53.20; H, 7.28; N, 5.73. Found: C, 53.41; H, 7.45; N, 5.41.

3.9. 5″-*N*-(Fmoc-Lys-OBn)-6,3′,4′,2″,5″,4‴-hexa-*O*-acetyl-1,3,2′,6′,-2‴,6‴-hexa-*N*-(*tert*-butoxycarbonyl)-5″-deoxy-neomycin B (11)

To a solution of alcohol **10** (1.0 g, 0.823 mmol) in CH_2Cl_2, pyridinium chlorochromate (0.162 g, 0.74 mmol) and molecular sieves powder were added and stirred at rt for 3 h. After completion the reaction mixture was diluted with CH_2Cl_2 and filtered through pad of Celite. The filtrate was evaporated to obtain the aldehyde. The crude aldehyde was used for next reaction. Coupling was performed with H-Lys-OBn ester as above described procedure to get **11**. Yield = 59%; R_f 0.45 (CH_2Cl_2–MeOH 17:1); ^{1}H NMR (300 MHz, CD_3OD): δ 7.80 (d, 2H, J = 8.1 Hz), 7.65 (d, 2H, J = 7.7 Hz), 7.56 (t, 2H, J = 7.0 Hz), 7.39 (t, 2H, J = 7.2 Hz), 7.35 (m, 3H), 7.29 (m, 2H), 5.66 (d, 1H, J = 4.3 Hz), 5.22–5.13 (m, 5H), 5.08 (t, 1H, J = 9.7 Hz), 4.96 (t, 2H, J = 8.3 Hz), 4.81 (m, 2H), 4.72 (m, 2H), 4.31 (dd, 1H, J = 6.2, 10.2 Hz), 4.18 (t, 2H, J = 6.7 Hz), 4.08 (m, 2H), 3.92 (m, 4H), 3.76–3.52 (m, 5H), 3.44 (m, 2H), 3.25 (m, 2H), 2.93 (m, 2H), 2.17–1.90 (4 s, 18H), 2.08 (m, 2H), 1.87 (m, 1H), 1.71 (m, 2H), 1.61 (m, 2H), 1.55 (m, 1H), 1.50 (3s, 54H); ^{13}C NMR (75 MHz, CD_3OD): 173.7–170.4 (carbonyls), 158.9–157.2 (Boc-CO), 145.3–121.0 (aromatic carbons), 110.3, 98.9, 97.8 (anomeric carbons), 84.8, 81.3, 80.9, 80.7, 80.5 (×2), 80.4 (×2), 78.9, 78.1, 77.4, 75.1, 74.3, 73.3, 70.3, 70.1, 69.7, 68.1 (×2), 68.0 (×2), 67.5, 55.4, 54.3, 52.9, 50.6, 50.1, 48.4, 41.1, 40.5, 34.9, 32.3, 29.0–28.8, 24.2–20.8; $[\alpha]_D^{25} = +29.0$ (c 0.93, MeOH); EIMS: calcd for $C_{93}H_{134}N_8NaO_{34}^+$ 1929.90, found: 1929.90 $[M+Na]^+$. Anal. Calcd for $C_{93}H_{134}N_8O_{34}$: C, 58.54; H, 7.08; N, 5.87. Found: C, 58.81; H, 7.39; N, 5.69.

3.10. 5″-*N* -(Fmoc-Lys-OBn)-6,3′,4′,2″,5″,4‴-hexa-*O*-acetyl-1,3,2′,6′,5″,2‴,6‴-hepta-*N*-(*tert*-butoxycarbonyl)-5″-deoxy-neomycin B (12)

To compound **11** (0.15 g, 0.078 mmol) in acetonitrile (1 mL) were added triethylamine (0.040 mL, 0.314 mmol) and $(Boc)_2O$ (0.685 g, 0.314 mmol) and stirred at rt for 4 h. The reaction mixture was partitioned between EtOAc and H_2O and the organic layer was dried over Na_2SO_4, and filtered, the solvents were evaporated in vacuo to give an oily residue. Flash column chromatography afforded the desired product **12** as a white solid. Yield = 73%; R_f 0.45 (CH_2Cl_2–MeOH 17:1); ^{1}H NMR (300 MHz, CD_3OD): δ 7.81 (d, 2H, J = 7.1 Hz), 7.67 (d, 2H, J = 8.3 Hz), 7.41 (t, 4H, J = 6.2 Hz), 7.31 (t, 5H, J = 6.2 Hz), 5.60 (d, 1H, J = 3.1 Hz), 5.24 (m, 5H), 5.05 (t, 1H, J = 9.7 Hz), 4.96 (m, 2H), 4.83 (m, 2H), 4.70 (m, 2H), 4.37 (t, 1H, J = 6.2 Hz), 4.31 (q, 2H, J = 7.1 Hz), 4.21 (t, 1H, J = 6.2 Hz), 4.01 (m, 3H), 3.99 (m, 2H) 3.85 (t, 1H, J = 7.6 Hz), 3.73 (d, 1H, J = 7.5 Hz), 3.63 (m, 4H), 3.40 (m, 3H), 3.24 (m, 3H), 3.10 (m, 1H), 2.70 (m, 1H), 2.40 (t, 1H, J = 5.8 Hz), 2.28–1.90 (4 s, 18H), 1.60 (m, 2H), 1.50 (s, 9H), 1.40 (3s, 54H), 1.35 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD): 173.6–170.4 (carbonyls), 158.9–157.3 (Boc-CO), 145.3–121.0 (aromatic carbons), 110.3, 99.1, 97.8 (anomeric carbons), 84.8, 81.3, 80.9, 80.7, 80.4, 78.9, 78.1, 77.4, 75.1, 74.3, 73.3, 70.3, 70.1, 69.7, 68.1, 68.0, 67.5, 55.4, 54.3, 52.9, 50.6, 50.1, 41.1, 40.5, 34.9, 32.3, 29.2–28.9, 24.3–20.9; $[\alpha]_D^{25} = +27.0$ (c 0.04, MeOH); EIMS: calcd for $C_{98}H_{142}N_8NaO_{36}^+$ 2030.94, found: 2029.33 $[M+Na]^+$. Anal. Calcd for $C_{98}H_{142}N_8O_{36}$: C, 58.61; H, 7.13; N, 5.58. Found: C, 58.95; H, 7.39; N, 5.31.

3.11. 5″-*N*-(H-Lys-OBn)-6,3′,4′,2″,5″,4‴-hexa-*O*-acetyl-1,3,2′,6′,-5″,2‴,6‴-hepta-*N*-(*tert*-butoxycarbonyl)- 5″-deoxy-neomycin B (13)

To a solution of compound **12** (0.15 g, 0.074 mmol) in DMF (5 mL), 15% piperidine in DMF was added and stirred at rt for 3 h. The solvent was removed under reduced pressure and purified through flash chromatography afforded the desired product as a white solid. Yield = 78%; R_f 0.25 (CH_2Cl_2–MeOH 10:1); ^{1}H NMR (300 MHz, CD_3OD): δ 7.40 (m, 5H), 5.63 (t, 1H, J = 3.1 Hz), 5.27 (m, 1H), 5.21 (m, 2H), 5.17 (m, 2H), 5.05 (t, 1H, J = 9.7 Hz), 4.96 (m, 2H), 4.89 (m, 1H), 4.75 (br s, 1H), 4.64 (m, 1H), 4.12 (m, 3H), 3.96 (m, 3H) 3.88 (m, 2H), 3.74 (m, 2H), 3.67 (m, 2H), 3.63 (t, 2H, J = 7.5 Hz), 3.53 (m, 2H), 3.48 (m, 1H), 3.44 (m, 1H), 3.27 (m, 1H), 3.21 (m, 3H), 2.28 (m, 1H), 2.18–1.90 (4 s, 18H), 1.86 (m, 1H), 1.72–1.63 (m, 2H), 1.52 (s, 9H), 1.48 (3 s, 54H); $[\alpha]_D^{25} = +32.0$ (c 0.65, MeOH); EIMS: calcd for $C_{83}H_{132}N_8NaO_{34}^+$ 1807.88, found: 1808.12 $[M+Na]^+$. Anal. Calcd for $C_{83}H_{132}N_8O_{34}$: C, 55.82; H, 7.45; N, 6.27. Found: C, 55.79; H, 7.38; N, 6.55.

3.12. 5″-*N*-(Fmoc-Trp(Boc)-Lys-OBn)-6,3′,4′,2″,5″,4‴-hexa-*O*-acetyl-1,3,2′,6′,5″,2‴,6‴-hepta-*N*-(*tert*-butoxycarbonyl)-5″-deoxy-neomycin B (14)

To a solution of **13** (1 equiv) in dry DMF, TBTU (2 equiv), Fmoc-Trp(Boc)-OH (1 equiv) and DIPEA (3 equiv) were added and stirred at room temperature for 2 h. The reaction mixture was triturated with H_2O and EtOAc. The EtOAc layer was washed with H_2O, brine, dried over sodium sulfate and concentrated. The crude residue was purified using flash silica gel by eluting with MeOH–CH_2Cl_2 to obtain pure **14** as syrup. Yield = 74%; R_f 0.45 (CH_2Cl_2–MeOH 15:1); ^{1}H NMR (300 MHz, CD_3OD): δ 8.07 (d, 2H, J = 8.3 Hz), 7.75 (d, 2H, J = 8.3 Hz), 7.60 (d, 1H, J = 7.7 Hz), 7.56 (m, 2H), 7.50 (m, 2H), 7.35 (m, 3H), 7.22 (m, 4H), 7.16 (m, 3H), 5.60 (d, 1H, J = 2.9 Hz), 5.26 (m, 2H), 5.20 (m, 2H), 5.08 (t, 1H, J = 9.8 Hz), 4.98 (m, 1H), 4.89 (m, 1H), 4.74 (m, 2H), 4.64 (br s, 1H), 4.57 (m, 2H), 4.57 (m, 1H), 4.36 (m, 1H), 4.10 (m, 4H), 4.00 (dd, 2H, J = 4.5, 10.7 Hz), 3.92 (m, 1H), 3.86 (m, 2H), 3.74 (m, 2H), 3.63 (m, 3H), 3.54 (m, 2H), 3.53 (m, 1H), 3.45 (m, 1H), 3.34 (m, 1H), 3.20 (m, 2H), 3.10 (m, 2H), 2.30–1.90 (m, 20H), 1.67 (m, 2H), 1.56 (s, 9H), 1.49 (s, 9H), 1.40 (s, 54H), 1.30 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ 174.0 (ester–CO), 173.1, 172.9, 172.3, 171.9, 171.7, 170.7 (acetates–CO), 167.4, 164.5 (peptide–amide, NHCO), 158.8, 158.4, 158.1 (×2), 157.9, 157.4 (×2), 157.2 (amide CO), 145.2–118.7 (aromatic carbon), 109.3, 99.7, 98.7 (anomeric carbon), 84.7, 81.7, 81.2 (×2), 81.0, 80.4 (×2), 80.3 (×3), 80.0, 79.4, 78.7, 76.9, 75.3, 74.7, 74.0, 73.3, 70.8, 69.9, 68.4 (×2), 68.1 (×2), 66.5, 55.9, 54.9, 52.9, 50.8, 50.4, 48.3, 41.6, 38.9, 37.6, 37.0, 35.2, 32.4, 31.9, 30.1, 29.9–28.8, 23.8–20.8; $[\alpha]_D^{25} = +36.0$ (c 0.2, MeOH); EIMS: calcd for $C_{114}H_{160}N_{10}NaO_{39}^+$ 2316.08, found: 2316.44 $[M+Na]^+$. Anal. Calcd for $C_{114}H_{160}N_{10}O_{39}$: C, 59.67; H, 7.03; N, 6.10. Found: C, 60.11; H, 7.23; N, 6.16.

3.13. 5″-*N*-(Fmoc-Trp(Boc)-Lys-OH)-6,3′,4′,2″,5″,4‴-hexa-*O*-acetyl-1,3,2′,6′,5″,2‴,6‴-hepta-*N*-(*tert*-butoxycarbonyl)-5″-deoxy-neomycin B (15)

The solution of **14** (130 mg, 0.104 mmol) and 10% $Pd(OH)_2$–C in EtOAc (5 mL) was hydrogenated at normal temperature and pressure for 5 h and then filtered through Celite. The filtrate was concentrated and the residue was purified on flush column chromatography to afford **16** as a white solid. Yield = 87%; R_f 0.16 (CH_2Cl_2–MeOH 9:1); ^{1}H NMR (300 MHz, CD_3OD): δ 8.07 (d, 1H, J = 6.0 Hz), 7.73 (t, 2H, J = 7.3 Hz), 7.70 (br s, 1H), 7.56 (m, 1H), 7.48 (d, 1H, J = 7.1 Hz), 7.41 (d, 1H, J = 7.1 Hz), 7.37 (d, 2H, J = 4.1 Hz), 7.27 (d, 1H, J = 4.2 Hz), 7.23 (s, 1H), 7.25 (t, 2H, J = 7.5 Hz), 5.60 (s, 1H), 5.28 (m, 2H), 5.05 (t, 1H, J = 9.4 Hz), 4.95 (m, 1H), 4.73 (m, 2H), 4.64 (m, 2H), 4.39 (m, 2H), 4.17 (m, 5H), 4.00 (m, 3H), 3.97 (t, 1H, J = 4.4 Hz), 3.92 (t, 1H, J = 4.4 Hz), 3.88 (m, 2H), 3.63 (m, 5H), 3.51 (s, 1H), 3.42 (m, 2H), 3.24–3.10 (m, 5H), 2.30–1.90 (6 s, 18H), 2.10 (m, 2H), 1.89 (m, 1H), 1.68 (m, 1H), 1.57 (m, 2H), 1.50 (2 s, 18H), 1.42 (s, 54H); ^{13}C NMR (75 MHz, CD_3OD from HSQC): δ 127.0–114.5 (aromatic carbons), 106.4, 96.6, 97.8 (anomeric carbons), 80.8, 79.1, 77.8, 76.8, 75.4, 75.1, 73.4, 72.6, 72.4, 71.9, 71.2, 68.5, 68.0, 66.7, 66.0, 65.0, 63.6, 60.3, 55.0, 54.8, 54.4, 53.7, 51.9, 51.2, 48.2, 47.3, 46.8, 39.4, 33.4, 32.8, 28.8–27.3, 22.2–18.9; $[\alpha]_D^{25} = +26.0$ (c 0.2, MeOH); EIMS: calcd for $C_{107}H_{154}N_{10}NaO_{39}^+$ 2226.04, found: 2226.04 $[M+Na]^+$. Anal. Calcd for $C_{107}H_{154}N_{10}O_{39}$: C, 58.30; H, 7.04; N, 6.35. Found: C, 58.77; H, 7.29; N, 6.89.

3.14. 5″-*N*-(Fmoc-Trp(Boc)-Lys-Trp(Boc)-OBn)-1,3,2′,6′,5″,2‴,6‴-hepta-*N*-(*tert*-butoxycarbonyl)-6,3′,4′,2″,5″,4‴-hexa-acetyl-5″-deoxy-neomycin (16)

Coupling was performed as previously described and compound **16** was purified after column chromatography. Yield = 58%; R_f 0.35 (CH_2Cl_2–MeOH 15:1); ^{1}H NMR (300 MHz, CD_3OD): δ 7.93 (d, 1H, J = 8.3 Hz), 7.89 (d, 1H, J = 8.1 Hz), 7.59 (m, 2H), 7.58 (m, 2H), 7.56 (m, 1H), 7.52 (m, 1H), 7.50 (m, 2H), 7.44–7.32 (m, 3H), 7.20 (m, 4H), 7.16–6.98 (m, 6H), 5.42 (s, 1H), 5.05 (m, 2H), 5.00 (s, 1H), 4.97 (s, 1H), 4.88 (m, 2H), 4.74 (m, 2H), 4.54 (s, 1H), 4.43 (m, 1H), 4.22 (m, 2H), 3.90 (m, 5H), 3.76 (m, 4H), 3.53 (m, 1H), 3.43 (m, 4H), 3.40 (t, 2H, J = 2.3 Hz), 3.33 (t, 1H, J = 5.6 Hz), 3.22 (dd, 3H, J = 5.6 Hz), 3.18 (d, 1H, J = 11.1 Hz), 3.03 (m, 4H), 2.88 (m, 1H), 2.80 (m, 3H), 2.07–1.60 (6 s, 18H), 1.67 (m, 2H), 1.48 (m, 1H), 1.43 (m, 2H), 1.37 (s, 3H), 1.30 (s, 15H), 1.22 (s, 63H); ^{13}C NMR (75 MHz, CD_3OD): δ 173.8, 173.2, 172.3–170.5 (ester and acetate–CO), 158.4, 158.1–156.6 (amide CO), 145.1–116.2 (aromatic carbons), 109.3, 99.0, 98.4 (anomeric carbons), 85.2, 84.7, 81.9, 81.2 (×3), 81.0, 80.3 (×3), 79.8, 76.9, 75.8, 75.0, 73.8, 72.3, 71.7, 71.3, 68.4, 67.5 (×3), 69.1, 66.5, 66.1, 60.7, 56.0, 54.5, 53.8, 52.9, 51.7, 50.7, 50.3, 48.6, 46.6, 41.9, 41.2, 34.0, 33.5, 30.5, 29.0–27.1, 22.5–20.7; $[\alpha]_D^{25} = +26.0$ (c 0.26, MeOH); EIMS: calcd for $C_{130}H_{178}N_{12}NaO_{42}^+$ 2579.22, found: 2602.55 $[M+Na]^+$. Anal. Calcd for $C_{130}H_{178}N_{12}O_{42}$ C, 60.50; H, 6.95; N, 6.51. Found: C, 60.73; H, 7.02; N, 6.86.

3.15. 1,3,2′,6′,2‴,6‴-Octaaammonium-5″-*N*-(H-Trp-Lys-Trp-OH)-5″-deoxy-neomycin (17)

A solution of **16** (80 mg, 0.031 mmol) in 0.02 N MeONa in MeOH (2 mL) was stirred at 0 °C to rt under N_2 atmosphere for 12 h, and neutralized with CO_2, filtered, concentrated and the crude residue was debenzylated under hydrogenation condition with $Pd(OH)_2$ in methanol for 5 h. The compound was filtered through a Celite pad and concentrated to get the acid, which was treated with 95% TFA for 3 min at 0 °C. The solvent was removed at reduced pressure and the residue 2% methanol in ether was added and the solvent was decanted to get a solid which was lyophilized to produce **17** as salt. Yield = 33% (in 3 steps); R_f 0.10 (CH_2Cl_2–MeOH–NH_4OH 5:6:1); ^{1}H NMR (300 MHz, CD_3OD): δ 7.62–7.40 (m, 4H), 7.33–7.00 (m, 6H), 5.28 (br s, 2H), 5.11 (br s, 2H), 4.86 (m, 2H), 4.35 (m, 5H), 4.19 (m, 2H), 3.99 (m, 1H), 3.86 (m, 2H), 3.80 (m, 2H), 3.70–3.65 (m, 5H), 3.51 (m, 2H), 3.40 (m, 3H), 3.33 (m, 1H), 3.26 (m, 3H), 3.00 (m, 2H), 2.32 (m, 2H), 2.18 (dt, 2H, J = 3.7, 12.1 Hz), 2.00 (m, 1H), 1.68 (m, 1H), 1.49 (m, 1H), 1.30 (m, 1H); ^{13}C NMR (75 MHz, CD_3OD, HSQC): δ 1214.8–114.4 (aromatic carbons), 109.0, 98.5, 98.3 (anomeric carbons), 84.6, 80.6, 79.4, 78.5, 73.4, 74.1, 71.5, 74.0, 72.9, 71.1, 70.0, 69.4, 66.9, 55.0, 54.6, 54.1, 52.9, 51.9, 51.0, 50.2, 49.6, 49.3, 49.0, 47.4, 41.2, 39.9, 35.1, 34.4, 33.4, 26.9–23.2; $[\alpha]_D^{25} = +21.0$ (c 0.11, MeOH); EIMS: calcd for $C_{51}H_{79}N_{12}O_{16}^+$ 1115.57, found: 1115.99 $[M+H]^+$. Anal. Calcd for $C_{67}H_{86}F_{24}N_{12}O_{32}$: C, 39.69; H, 4.28; F, 22.49; N, 8.29. Found: C, 40.11; H, 4.52; F, 22.76; N, 8.54.

3.16. Determination of the MIC values for aminoglycoside–peptide conjugates 5, 7, and 17

Bacterial isolates were obtained from the American Type Culture Collection (ATCC). Isolates were kept frozen in skim milk at −80 °C until minimum inhibitory concentration (MIC) testing was carried out. Following two subcultures from frozen stock, the in vitro activities of peptides were determined by macrobroth dilution in accordance with the Clinical and Laboratory Standards Institute (CLSI) 2006 guidelines.[26] Stock solutions of peptides were prepared and dilutions were made as described by CLSI. Test tubes contained doubling antimicrobial dilutions of cation adjusted Mueller–Hinton broth and inoculated to achieve a final concentration of approximately 5×10^5 CFU/mL then incubated in ambient air for 24 h prior to reading. Colony counts were performed periodically to confirm inocula. Quality control was performed using ATCC QC organisms.

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