

ORIGINAL ARTICLE

# Synthesis and combinational antibacterial study of 5''-modified neomycin

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A library of 5''-modified neomycin derivatives were synthesized for an antibacterial structure–activity optimization strategy. Two leads exhibited prominent activity against both methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Antibacterial activities were measured when combined with other clinically used antibiotics. Significant synergistic activities were observed, which may lead to the development of novel therapeutic practices in the battle against infectious bacteria.

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**Keywords:** aminoglycoside; antibiotic; MRSA; synergism; VRE

## INTRODUCTION

Aminoglycosides are an important class of antibiotics used against infectious diseases. Their usefulness, however, has been significantly compromised by the emergence of resistant bacteria, especially those equipped with aminoglycoside-modifying enzymes (AMEs).<sup>1–3</sup> Structural modification of aminoglycosides remains an effective approach for reviving their antibacterial activities against resistant bacteria. The typical goal is to provide novel aminoglycosides with structural motifs that cannot be accommodated by AMEs but can still bind to the targeted site on rRNA.<sup>4,5</sup> Most aminoglycosides exert their antibacterial activity by binding selectively to the A-site decoding region of the 16S rRNA and disrupting the functions that are vital to bacteria. Although such modification strategies may lead to the development of novel broad-spectrum antibiotics, X-ray structural and enzymatic studies suggest that higher concentrations of AMEs as compared with that of rRNA, and the substrate promiscuity of AMEs, could limit the success of this approach.<sup>6</sup>

Recently, we discovered that structural modifications of aminoglycosides may alter the traditional mode of action of aminoglycosides and may lead to revived activities against resistant bacteria.<sup>7</sup> Encouraged by these findings and prompted by an article that reported a similar discovery,<sup>8</sup> we conducted further syntheses of derivatives on the basis of the leads to reveal structure–activity relationships (SAR). In an effort to gain more insight into possible modes of action, we also investigated the use of these new aminoglycosides in combination with other clinically used antibiotics with known modes of action.

## RESULTS

### Chemistry

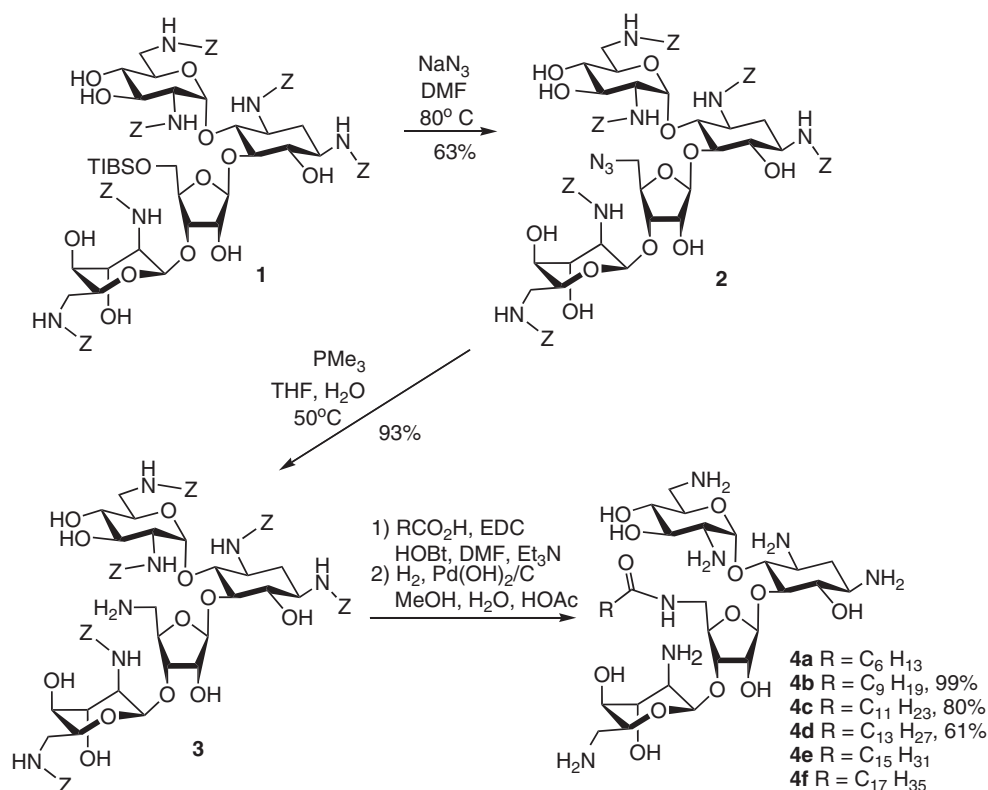
We have demonstrated that neomycin derivatives bearing linear acyl groups at the 5'' position display unusual antibacterial activities. Three additional derivatives with acyl groups of various chain lengths (C7, C16 and C18) were synthesized (Scheme 1). Although the derivative with a heptanoyl group (**4a**)<sup>7</sup> maintains its traditional antibacterial profile, derivatives with a hexadecanoyl group from palmitic acid (C16, **4e**)<sup>7</sup> and an octadecanoyl group from stearic acid (C18, **4f**)<sup>7</sup> manifest unexpected activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). MRSA strains harbor genes that encode APH(3'), ANT(4') and AAC(6')/APH(2''), which render the bacteria resistant to various aminoglycosides, such as gentamicin and tobramycin.<sup>9</sup> VRE contains *vanB*-, *ant(6)-I*- and *aac(6')-aph(2'')*-resistance genes with high levels of resistance to aminoglycosides and vancomycin.<sup>10,11</sup> The *vanB* gene and other genes encode for proteins that produce abnormal D-Ala-D-Lac terminal ends of peptidoglycan precursors.<sup>12</sup> Vancomycin binds to D-Ala-D-Lac with a much lower affinity than does the normal D-Ala-D-Ala, allowing the bacteria to become resistant to vancomycin. As both MRSA and VRE are known to possess high levels of resistance against traditional aminoglycosides,<sup>10,9,13</sup> we decided to further explore SAR with regard to the linear acyl groups by synthesizing **4b**, **4c** and **4d** with a C10 (decanoyl), C12 (dodecanoyl) and C14 (tetradecanoyl) chain length, respectively. The synthesis of these 5''-acylated neomycin derivatives proceeded through the reported method using neomycin B as the starting material (Scheme 1).<sup>7</sup>

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Scheme 1 Synthesis of neomycin derivatives.

Table 1 MIC of the 5'-modified neomycin derivatives<sup>a</sup>

Compounds	Antibiotic-susceptible strains				Antibiotic-resistant strains			
	<i>E. coli</i> ATCC 25922	<i>S. aureus</i> ATCC 25923	<i>K. pneumoniae</i> ATCC13883	<i>S. aureus</i> ATCC 33591 (MRSA)	<i>K. pneumoniae</i> ATCC 700603	<i>P. aeruginosa</i> ATCC 27853	<i>E. faecalis</i> ATCC 29212	<i>E. faecalis</i> ATCC51299 (VRE)
Neomycin B	4	1	4	125	16–32	64	64–125	≥250
Amikacin	1	1	1	8–16	0.5	0.5–1	32–64	≥250
Vancomycin	125–250	0.5–1	≥250	1	ND	≥250	1–2	125
4a	16	2–4	16–32	125	32–64	≥250	32–64	≥250
4b	32	16	64–125	125–250	125	16–32	32–64	64–125
4c	16–32	8–16	64–125	16–32	64–125	16	8–16	64–125
4d	8–16	2–4	64	4–8	32–64	8	2–4	8–16
4e	4–8	1–2	8–16	2–4	16–32	4	2–4	4
4f	4–8	2–4	8–16	2–4	32	8–16	4–8	8–16

<sup>a</sup>Unit:  $\mu\text{g ml}^{-1}$ , ND, not determined.

After the protection of amino groups of neomycin B with a carbobenzyloxy (Cbz or Z) group, 5'-OH was selectively substituted by compound **1**<sup>14</sup> with azide, forming compound **2**. After the Staudinger reduction of azide, compound **3**<sup>7</sup> with 5'-NH<sub>2</sub> was used and coupled with desired carboxylic acids, leading to the preparation of desired neomycin derivatives.

#### Biological testing

The synthesized aminoglycosides were assayed against both Gram-positive (G+) and Gram-negative (G-) susceptible and resistant bacterial strains using neomycin B, amikacin and vancomycin as

controls. Aminoglycoside-susceptible *Escherichia coli* (G-, ATCC 25922) and *S. aureus* (G+, ATCC 25923) were used as standard reference strains. Also used were *Klebsiella pneumoniae* (G-, ATCC 13883) resistant to ampicillin but susceptible to aminoglycosides, *Pseudomonas aeruginosa* (G-, ATCC 27853), which expresses APH(3')-IIb and manifests a modest resistance toward aminoglycosides,<sup>15</sup> MRSA (ATCC 33591), *Enterococcus faecalis* (G+, ATCC51299, VRE) and an *E. faecalis* strain (ATCC 29212), which is susceptible to vancomycin but moderately resistant to aminoglycosides. The minimum inhibitory concentrations (MICs) are summarized in Table 1.

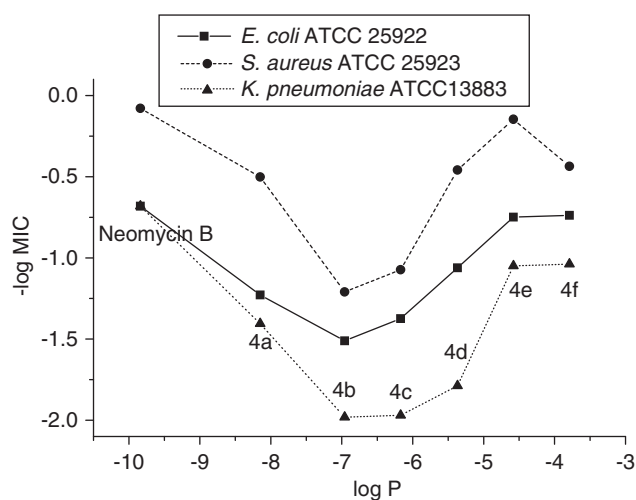
From the MIC values, the 5''-acylated neomycin derivatives are found to be generally more active against G+ bacteria than against G- bacteria. When comparing the antibacterial activity of each compound, compound **4a** showed a similar MIC profile as neomycin, although it was four- to eightfold less active than neomycin against aminoglycoside-susceptible strains, although inactive against aminoglycoside-resistant strains. Increasing the acyl chain length from heptanoyl (**4a**) to decanoyl (**4b**) led to a decrease in antibacterial activity: 8- to 16-fold less active than neomycin against aminoglycoside-susceptible strains and inactive against aminoglycoside-resistant strains. However, the MIC profile of **4b** remained similar to that of **4a** and neomycin. As the acyl chain length was extended to C14, C16 and C18 (designated **4d**, **4e** and **4f**, respectively), the activities against aminoglycoside-resistant strains increased, which implicates different modes of antibacterial action.

### Quantitative structure–activity relationship analysis

To provide support for our hypothesis of different modes of antibacterial action, we conducted a simple, one-descriptor (logP) quantitative structure–activity relationship (QSAR) analysis. The data set was divided into two groups: results from aminoglycoside-susceptible and results from aminoglycoside-resistant strains (Figures 1 and 2). From the results against susceptible strains, a 'V-shape' relation was obtained instead of a linear, or at least a monotonous relationship. The latter was found in the cases of resistant strains.

### Hemolysis studies

Gram-positive bacteria differ from G- bacteria by the absence of an outer membrane found in the latter with abundant and structurally diverse lipopolysaccharides. Judging from the length of acyl groups and the activity profile of compounds **4d**, **4e** and **4f** with lower MICs against G+ bacteria, we postulate new modes of antibacterial action involving an interaction with bacterial inner membranes. Therefore, one of the concerns of using these newly synthesized compounds as an antibacterial is their potential toxicity to mammalian cells. Thus, we decided to conduct the hemolysis study by using compounds **4e** and **4f** with longer linear acyl chains. From the hemolysis data, it was found that compounds **4e** and **4f** cause an estimated 50% hemolysis at 0.2 and 0.3 mg mL<sup>-1</sup>, respectively (Figure 3).



**Figure 1** Quantitative structure–activity relationship (QSAR) analysis of minimum inhibitory concentrations (MICs) from aminoglycoside-susceptible strains.

### Combinational studies

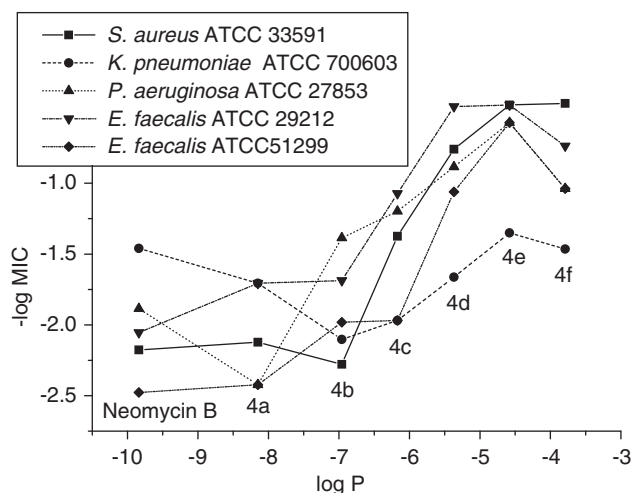
Using a combination of antibiotics is a common practice in the treatment of bacterial infection.<sup>16</sup> Such a practice has the advantages of potentially enhancing the efficacy of treatment, as in the case of synergism, lowering the possibility of inducing drug resistance from microbes and reducing the dose of antibiotics. An example of such synergism is the use of vancomycin (bacterial cell membrane action) in combination with gentamicin, an aminoglycoside.<sup>17</sup> Therefore, we explored the possible use of the new neomycin derivatives in combination with clinically used antibiotics. As **4e** is the most active neomycin derivative, we selected **4e** along with amikacin, neomycin B and vancomycin for combinational antibacterial studies.

We used a checkerboard assay for the antibiotic combinational study.<sup>18</sup> The selected antibiotics were used in combination with the lead, **4e**. The results are summarized in Table 2. The combinational effect can be evaluated on the basis of the fractional inhibitory concentration (FIC) index, which can be calculated on the basis of the following equation:

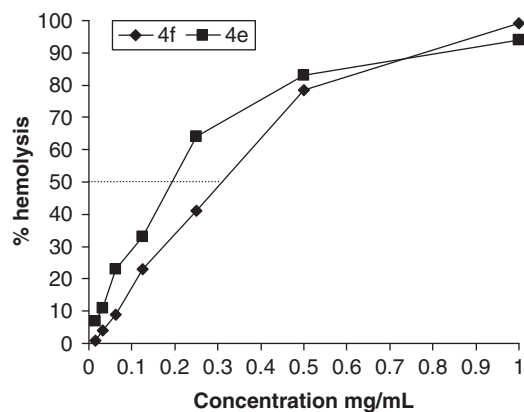
$$\text{FIC} = [\text{A}]/\text{MICA} + [\text{B}]/\text{MICB}, \text{ synergism : FIC} = 0.5;$$

$$\text{addition : FIC} = 0.5 - 1.0; \text{ indifference :}$$

$$\text{FIC} = 1 - 4; \text{ antagonism : FIC} \geq 4$$



**Figure 2** Quantitative structure–activity relationship (QSAR) analysis of minimum inhibitory concentrations (MICs) from aminoglycoside-resistant strains.



**Figure 3** Hemolysis of **4e** and **4f**.

**Table 2** FIC from combinational studies of **4e**

Strains of bacteria	Neomycin	Amikacin	Vancomycin
<i>E. coli</i> ATCC 25922	0.27–0.56 (synergism) <sup>a</sup>	0.38–0.53 (synergism)	ND
<i>S. aureus</i> ATCC 25923	ND	1.0 (addition or indifference)	0.53–1.5 (addition or indifference)
<i>K. pneumoniae</i> ATCC 13883	ND	0.08–0.16 (synergism)	ND
<i>K. pneumoniae</i> ATCC 700603	ND	0.38–0.53 (synergism)	ND
<i>S. aureus</i> ATCC 33591 (MRSA)	ND	0.28–0.53 (synergism)	0.53–0.56 (addition)
<i>P. aeruginosa</i> ATCC 27853	ND	0.38–0.50 (synergism)	ND
<i>E. faecalis</i> ATCC 29212	ND	0.53–0.56 (addition)	0.28–0.53 (synergism)

<sup>a</sup>FIC=(A)/MIC<sub>A</sub>+(B)/MIC<sub>B</sub>, synergism: FIC ≤ 0.5; addition: FIC=0.5–1.0; indifference: FIC=1–4; antagonism: FIC ≥ 4, ND, not determined.

## DISCUSSION

From the relationship between acyl chain length and MIC profile, it seems that when a shorter chain length was incorporated, the derivatives maintain the original mode of antibacterial action as does the parent neomycin (that is, binding to the A-site decoding region of 16S rRNA). The added acyl chain, however, reduces the activity of **4a**, likely because of the steric interference of the acyl group on the binding of the neomycin derivative to rRNA. As the chain length increases, steric hindrance increases, resulting in a further decrease in antibacterial activity. Nevertheless, when even longer acyl chains were incorporated, the derivatives showed significant antibacterial activity with SAR in the order of C16 ≈ C18 > C14 against both susceptible and resistant strains. As an elevated steric hindrance from these long acyl groups is expected, it is unlikely that compounds **4d**, **4e** and **4f** will regain their antibacterial activity by exerting the same mode of action. Our previous enzymatic and molecular modeling studies also revealed that AMEs can inactivate aminoglycosides with diverse structural motifs.<sup>7</sup> Thus, it is probable that these three neomycin derivatives have different modes of antibacterial action. The activity of **4d**, **4e** and **4f** against enterococci, which are known to be intrinsically resistant against traditional aminoglycosides, also supports this hypothesis.

The results from the QSAR analysis also support our hypothesis that different modes of actions are likely for compounds **4d**, **4e** and **4f** because of the increase in lipophilicity. The compounds presented in the plot of Figure 1 can be roughly divided into two groups: neomycin **4a** (C8) and **4b** (C10) as the first group and compounds **4c** (C12), **4d** (C14), **4e** (C16) and **4f** (C18) as the second group. The linear relationship of the first group suggests that when the sizes of acyl groups are relatively small, compounds **4a** and **4b** still exert the same antibacterial mode of action as that of neomycin. As the sizes of acyl groups increase, the second group shows a different linear relationship with a reverse dependence of the activity on lipophilicity unlike the first group. These two different linear relationships are consistent with our speculation that compounds **4d**, **4e** and **4f** regain their antibacterial activity by exerting different modes of action. The results from Figure 2 also manifest two roughly linear relationships from two groups of compounds as described above. As traditional aminoglycosides are inactive against these resistant bacteria, it is expected to obtain a rather leveling line for the first group. Once again, for the second group, the linear relationship implies a strong connection between the increased lipophilicity and the antibacterial activity.

On the basis of the results of hemolysis studies, the concentrations for 90% hemolysis for both compounds are expected to be higher than 1 mg ml<sup>-1</sup>. Compared with the MIC values of these two compounds (ranging from 2 to 16 μg ml<sup>-1</sup>), the concentrations that give significant hemolysis are 50- to 500-fold higher. Thus, these neomycin

derivatives are not hemolytic at their effective antibacterial concentrations.

According to the FIC index, compound **4e** shows a strong synergistic effect with amikacin and/or neomycin against G- bacteria, including *E. coli*, *K. pneumoniae* and *P. aeruginosa*. As compound **4e** alone is less active against G- bacteria, one possible reason could be that **4e** causes damage to the bacterial membrane and facilitates the entrance of amikacin into bacteria. The synergistic effect with **4e** and amikacin was consistently observed with all G- bacterial strains tested. With G+ bacteria, the synergistic effects with **4e** were inconsistent with the outcomes ranging from synergism to indifference with amikacin and vancomycin. The synergism of amikacin and **4e** against MRSA is of interest. As MRSA is equipped with various AMEs that should drastically reduce the activity of aminoglycosides, including amikacin, the observed synergism is unexpected. The synergism of vancomycin and **4e** against *E. faecalis* is difficult to explain. It is possible that the action of **4e** may not interfere with the action of vancomycin, and unlike the combinational effect against G- bacteria, it may depend on individual bacterial strain. The difference in the combinational effect of compound **4e** against G+ and G- could possibly reflect the action of compound **4e** toward inner and outer membranes, respectively.

In conclusion, we have completed the structural optimization of 5'-acylated neomycin derivatives. Interesting antibiotic combinational effects have been revealed. The lead, **4e**, alone has a prominent antibacterial activity against G+ bacteria such as MRSA and VRE. Facing the emergence of vancomycin-resistant *S. aureus*,<sup>19,20</sup> we believe that compound **4e** represents a possible countermeasure against this formidable pathogen. Judging from the data of the QSAR analysis, it is evident that lipophilicity is responsible for the observed antibacterial activity of the lead.

On the basis of the combinational study, compound **4e** will be more effective when used alone against G+ bacteria. Nevertheless, we have shown that it is possible to use **4e** in combination with aminoglycosides against G- bacteria. This finding is consistent with a novel mode of antibacterial action of **4e**. The synergism between **4e** and amikacin against *E. coli* and other G- bacteria may provide a potential application in counteracting food-borne bacterial outbreaks such as those caused by *E. coli* O157:H7, *Salmonella* spp. and other G- pathogens.

## METHODS

### General experimental procedures

**General procedure for coupling of compound 3 with carboxylic acids.** To a solution of compound **3** (0.20 g, 0.14 mmol) and carboxylic acids (0.28 mmol) in dimethyl formamide (10 ml) and Et<sub>3</sub>N (0.04 ml, 0.28 mmol), HOBT (0.030 g, 0.21 mmol) and EDC (0.040 g, 0.21 mmol) were added.<sup>1</sup> The reaction was

stirred at room temperature overnight. After completion of the reaction, the reaction was concentrated and diluted with EtOAc. The organic solution was washed with water, saturated NaHCO<sub>3(aq)</sub>, brine and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. After removal of the solvent, followed by a fast gradient column chromatography (eluted from hexane/EtOAc=1/1 to EtOAc/MeOH=9:1), the product was usually obtained as a solid, which was subjected to hydrogenation without further purification.

**General procedure for hydrogenation and purification.** The solids from acid/amine coupling reaction (0.1–0.2 mmol) were dissolved in degassed MeOH (9 ml), followed by the addition of 1 ml HOAc:H<sub>2</sub>O (1:4 ratio) degassed solution. A catalytic amount of Pd(OH)<sub>2</sub>/C powder was added and the system was well sealed and further degassed. The system was stirred under atmospheric H<sub>2</sub> at room temperature for 10 h. The reaction was then quenched by filtering through Celite and the residue was washed with H<sub>2</sub>O and the combined solutions were concentrated. The crude product was purified with Amberlite CG50 (NH<sub>4</sub><sup>+</sup>) eluted with a gradient of NH<sub>4</sub>OH solution (0–20%). After collecting the desired fractions and removing the solvent, the product was re-dissolved in water and loaded onto an ion-exchange column packed with Dowex IX8-200 (Sigma-Aldrich, St Louis, MO, USA) (Cl<sup>-</sup> form) and eluted with water. After removal of the solvent, the product was obtained as a white solid.

**5''-Deoxy-5''-decanamidoneomycin B (4b).** <sup>1</sup>H NMR (D<sub>2</sub>O, 300 MHz) δ 5.87 (d, *J*=5.0 Hz, 1H), 5.32 (d, *J*=4.1 Hz, 1H), 5.18 (d, *J*=1.7 Hz, 1H), 4.35 (t, *J*=5.2 Hz, 1H), 4.1–4.2 (m, 4H), 3.9–4.0 (m, 3H), 3.6–3.7 (m, 2H), 3.2–3.6 (m, 12H), 2.40 (dt, *J*=12.4 Hz, *J*=4.1 Hz, 1H), 2.18 (t, *J*=7.2 Hz, 2H), 1.8 (m, 1H), 1.5 (m, 2H), 1.1 (m, 12H), 0.72 (t, *J*=6.5 Hz, 3H); <sup>13</sup>C NMR (D<sub>2</sub>O, 75 MHz) δ 178.0, 109.1, 95.7, 94.9, 84.8, 80.7, 77.3, 75.0, 73.5, 72.3, 70.5, 70.2, 69.8, 68.1, 67.6, 67.4, 53.3, 50.9, 49.6, 48.6, 41.1, 40.6, 40.1, 36.0, 31.2, 28.7, 28.5 (2 carbons), 28.4, 28.0, 25.5, 22.1, 13.5; ESI/APCI Calcd for C<sub>33</sub>H<sub>66</sub>N<sub>7</sub>O<sub>13</sub><sup>+</sup> ([M+H]<sup>+</sup>) *m/e* 768.4713; measure *m/e* 768.4698.

**5''-Deoxy-5''-dodecanamidoneomycin B (4c).** <sup>1</sup>H NMR (D<sub>2</sub>O, 300 MHz) δ 5.86 (d, *J*=4.1 Hz, 1H), 5.31 (d, *J*=4.1 Hz, 1H), 5.18 (d, *J*=1.4 Hz, 1H), 4.34 (t, *J*=4.8 Hz, 1H), 4.1–4.2 (m, 4H), 3.9–4.0 (m, 3H), 3.6–3.7 (m, 2H), 3.2–3.6 (m, 12H), 2.40 (dt, *J*=8.2 Hz, *J*=4.1 Hz, 1H), 2.16 (t, *J*=7.2 Hz, 2H), 1.9 (m, 1H), 1.5 (m, 2H), 1.1 (m, 16H), 0.72 (t, *J*=6.5 Hz, 3H); <sup>13</sup>C NMR (D<sub>2</sub>O, 75 MHz) δ 177.9, 109.2, 95.7, 94.9, 84.8, 80.7, 77.3, 75.0, 73.5, 72.3, 70.5, 70.2, 69.8, 68.1, 67.6, 67.4, 53.3, 50.8, 49.6, 48.6, 41.1, 40.5, 40.1, 36.0, 31.2, 28.8 (2 carbons), 28.7, 28.6, 28.5, 28.4, 28.0, 25.5, 22.1, 13.5; ESI/APCI Calcd for C<sub>35</sub>H<sub>70</sub>N<sub>7</sub>O<sub>13</sub><sup>+</sup> ([M+H]<sup>+</sup>) *m/e* 796.5026; measure *m/e* 796.5016.

**5''-Deoxy-5''-tetradecanamidoneomycin B (4d).** <sup>1</sup>H NMR (D<sub>2</sub>O, 300 MHz) δ 5.86 (d, *J*=3.8 Hz, 1H), 5.31 (d, *J*=4.1 Hz, 1H), 5.18 (d, *J*=1.7 Hz, 1H), 4.34 (t, *J*=5.2 Hz, 1H), 4.1–4.2 (m, 4H), 3.9–4.0 (m, 3H), 3.6–3.7 (m, 2H), 3.2–3.6 (m, 12H), 2.40 (dt, *J*=12.4 Hz, *J*=4.1 Hz, 1H), 2.17 (t, *J*=7.2 Hz, 2H), 1.8 (m, 1H), 1.5 (m, 2H), 1.1 (m, 20H), 0.72 (t, *J*=6.5 Hz, 3H); <sup>13</sup>C NMR (D<sub>2</sub>O, 75 MHz) δ 178.0, 109.1, 95.7, 94.9, 84.8, 80.7, 77.3, 75.0, 73.5, 72.3, 70.5, 70.2, 69.8, 68.1, 67.6, 67.4, 53.3, 50.8, 49.6, 48.6, 41.1, 40.6, 40.1, 36.0, 31.2, 28.9 (2 carbons), 28.8 (2 carbons), 28.7, 28.6, 28.5, 28.4, 28.0, 25.5, 22.1, 13.5; ESI/APCI Calcd for C<sub>37</sub>H<sub>74</sub>N<sub>7</sub>O<sub>13</sub><sup>+</sup> ([M+H]<sup>+</sup>) *m/e* 824.5339; measure *m/e* 824.5333.

#### Minimum inhibitory concentration determinations

A solution of selected bacteria was inoculated into trypticase soy broth at 35 °C for 1–2 h.<sup>21</sup> The absorbance at 625 nm was measured and diluted with broth, if necessary, to an absorbance of 0.08–0.1. The adjusted inoculated medium (100 μl) was diluted with 10 ml broth and then applied to a 96-well microtiter plate (50 μl). A series of solutions (50 μl each in twofold dilution) of the tested compounds were added to the testing wells. The 96-well plate was incubated at 35 °C for 12–18 h. MIC is defined as the lowest concentration of compound needed to inhibit the growth of bacteria. Determinations were repeated at least three times.

#### Quantitative structure–activity relationship analysis

Minimum inhibitory concentrations presented in Table 1 have been recalculated to molar concentrations (μM). MICs represented as ranges in Table 1

(for example, '16–32') were calculated as arithmetic means of these values. Log P (logarithm of the partition coefficient octanol/water) values for neomycin B and for each of the new compounds were calculated using the HyperChem 7.0 package (HyperCube, Inc., Gainesville, FL, USA), with atomic charges being calculated using the AM1 semi-empirical method.

#### Combinational study

A block of 6×6 wells on a 96-well microtiter plate was created with a twofold serial dilution of **4e** and the selected antibiotic to locate the optimal range of inhibitory concentrations. Thereafter, a block of 8×8 wells on a 96-well microtiter plate with a twofold serial dilution of **4e** and the selected antibiotic was created to study the combinational studies. For each compound, there was a row or column with only one compound so that MIC could be determined. FIC index was calculated using the following equation: FIC=[A]/MIC<sub>A</sub>+ [B]/MIC<sub>B</sub>. The combinational effect was defined as synergism: FIC≤0.5; addition: FIC=0.5–1.0; indifference: FIC=1–4; antagonism: FIC≥4. The procedure for every combination of compound **4e** and selected antibiotic was repeated two to four times.

#### Hemolytic activity

Hemolytic activity was determined using methods described by Dartois *et al.*<sup>22</sup> and Sorensen *et al.*<sup>23</sup> with modification. Sheep erythrocytes were used to test hemolytic activities of **4e** and **5f**. Sheep red blood cells (RBCs) were obtained by centrifuging whole blood at 1000×g, washed four times with phosphate-buffered saline (PBS) and resuspended in PBS to a final concentration of 10<sup>8</sup> erythrocytes per ml. The RBC suspension (80 μl) was added to each well containing different concentrations of **4e** and **5f** (20 μl). The plate was incubated at 37 °C for 60 min. Wells with added deionized water and Triton X-100 (1%, w/v) served as negative (blank) and positive controls, respectively. The percentage of hemolysis was calculated using the following equation:

$$\% \text{ hemolysis} = \frac{[(\text{absorbance of sample}) - (\text{absorbance of blank})]}{\times 100 / (\text{absorbance of positive control})}$$

#### Supplementary data

Spectroscopic information for the synthesized compounds can be found in the supplementary information in the online version.

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