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Synthesis and Combinational Antibacterial Study of 5″-Modified Neomycin

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Abstract

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.

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 (AME's).1-3 Structural modification of aminoglycosides remains to be 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 AME's but are still being able to bind to the targeted site on rRNA.4,5 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. While such modification strategies may lead to development of novel broad spectrum antibiotics, X-ray structural and enzymatic studies suggest that higher concentrations of AME's as compared to that of rRNA and the substrate promiscuity of AME's could limit the success of this approach.6

Recently, we discovered that structural modifications of aminoglycosides may alter the traditional mode of action of aminoglycosides and lead to revived activities against resistant

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Supplementary data: Spectroscopic information for the synthesized compounds can be found in the online version.

bacteria.7 Encouraged by these findings and prompted by an article that reported a similar discovery,8 we conducted further syntheses of derivatives based on 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.

Materials and Methods

General Experimental Procedures

General Procedure for Coupling of Compound 31 with Carboxylic Acids—To a solution of compound **3** (0.20 g, 0.14 mmol) and carboxylic acids (0.28 mmol) in DMF (10 mL) and Et_3N (0.04 mL, 0.28 mmol), HOBt (0.030 g, 0.21 mmol) and EDC (0.040 g, 0.21 mmol) were added. 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 $NaHCO_{3(aq)}$, brine and dried over anhydrous Na_2SO_4. 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_2O (1/4 ratio) degassed solution. Catalytic amount of $Pd(OH)_2/C$ powder was added and the system was well sealed and further degassed. The system was stirred under atmospheric H_2 at room temperature for 10 hours. The reaction was then quenched by filtering through Celite and the residue was washed with H_2O and the combined solutions were concentrated. The crude product was purified with Amberlite CG50 (NH_4^+) eluted with a gradient of NH_4OH solution (0% – 20%). After collection of the desired fractions and removal of solvent, the product was re-dissolved in water and loaded to an ion-exchange column packed with Dowex 1X8-200 (Cl^- form), and eluted with water. After removal of solvent, the product was obtained as white solid.

5″-Deoxy-5″-decanamidoneomycin B (4b)—^{1}H NMR (D_2O, 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); ^{13}C NMR (D_2O, 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 $C_{33}H_{66}N_7O_{13}^+$ ($[M+H]^+$) m/e 768.4713; measure m/e 768.4698.

5″-Deoxy-5″-dodecanamidoneomycin B (4c)—^{1}H NMR (D_2O, 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); ^{13}C NMR (D_2O, 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 $C_{35}H_{70}N_7O_{13}^+$ ([M+H]$^+$) m/e 796.5026; measure m/e 796.5016.

5″-Deoxy-5″-tetradecanamidoneomycin B (4d)—^{1}H NMR (D_2O, 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); ^{13}C NMR (D_2O, 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 $C_{37}H_{74}N_7O_{13}^+$ ([M+H]$^+$) m/e 824.5339; measure m/e 824.5333.

Minimum Inhibitory Concentration (MIC) Determinations9—A solution of selected bacteria was inoculated into trypticase soy broth at 35°C for 1 - 2hrs. The absorbance at 625 nm was measured, and diluted with broth, if necessary, to an absorbance of 0.08 to 0.1. The adjusted inoculated medium (100 μL) was diluted with 10 mL broth, and then applied to a 96-well microtilter plate (50 μL). A series of solutions (50 μL each in 2-fold dilution) of the tested compounds was added to the testing wells. The 96-well plate was incubated at 35°C for 12 - 18 hrs. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of compound needed to inhibit the growth of bacteria. The determinations were repeated at least three times.

Quantitative Structure–Activity Relationship (QSAR) Analysis—MIC's presented in Table 1 have been recalculated to molar concentrations (μM). MIC's represented as ranges in Table 1 (e.g. “16-32”) were calculated as arithmetic means of these values. Log P (logarithm of the partition coefficient octanol/water) values for neomycin B and each of the new compounds were calculated using the HyperChem™ 7.0 package, with atomic charges calculated using the AM1 semi-empirical method.

Combinational Study—A block of 6×6 wells on a 96-well microtiter plate was created with 2-fold serial dilution of **4e** and the selected antibiotic to locate the optimal range of inhibitory concentrations. Then a block of 8×8 wells on a 96-well microtitter plate with 2-fold 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 the MIC could be determined. The fractional inhibitory concentration (FIC) index was calculated following the equation: $FIC = [A]/MIC_A + [B]/MIC_B$. 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 2-4 times.

Hemolytic Activity—Hemolytic activity was determined using methods described by Dartois et al.10 and Sorensen et al.11 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 1,000 × *g*, washed four times with phosphate-buffered saline (PBS), and resuspended in PBS to a final concentration of 10^8 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 percent of hemolysis was calculated using the following equation: % hemolysis = [(absorbance of sample) – (absorbance of blank)]×100/ (absorbance of positive control).

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). While the derivative with a heptanoyl group (**4a**)7 maintains its traditional antibacterial profile, derivatives with a hexadecanoyl group from palmitic acid (C16, **4e**)7 and an octadecanoyl group from stearic acid (C18, **4f**)7 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.13 VRE contains *vanB, ant(6)-I*, and *aac(6′)-aph(2″)* resistance genes with high levels of resistance to aminoglycosides and vancomycin.12,15 The *vanB* gene and other genes encode for proteins that produce abnormal D-Ala- D-Lac terminal ends of peptidoglycan precursors.16 Vancomycin binds to D-Ala- D-Lac with much lower affinity as compared to the normal D-Ala- D-Ala allowing the bacteria to become resistant to vancomycin. Since both MRSA and VRE are known to possess high levels of resistance against traditional aminoglycosides,12-14 we decided to further explore the SAR regarding the linear acyl groups by synthesizing **4b, 4c** and **4d** with C10 (decanoyl), C12 (dodecanoyl) and C14 (tetradecanoyl) chain length, respectively. The synthesis of these 5″-acylated neomycin derivatives proceeded via the reported method using neomycin B as the starting material (Scheme 1).7

Following the protection of amino groups of neomycin B with a carbobenzyloxy (Cbz or Z) group, the 5″-OH was selectively substituted via compound **1**17 with azide forming compound **2**. After the Staudinger reduction of azide, compound **3**7 with 5″-NH_2 was employed 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 modest resistance toward aminoglycosides,18 MRSA (ATCC 33591), *Enterococcus faecalis* (G+, ATCC51299,

VRE), and a *E. faecalis* strain (ATCC 29212) which is susceptible to vancomycin but moderately resistance to aminoglycosides. The minimum inhibitory concentrations (MIC's) are summarized in Table 1.

From the MIC values, the 5″-acylated neomycin derivatives are generally more active against G+ bacteria than G- bacteria. When comparing the antibacterial activity of each compound, compound **4a** displayed a similar MIC profile as neomycin although it was 4-8 fold less active than neomycin against aminoglycoside susceptible strains while inactive against aminoglycoside resistant strains. Increasing the acyl chain length from heptanoyl (**4a**) to decanoyl (**4b**) led to a decrease in antibacterial activity: 8-16 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.

QSAR Analysis—To provide support for our hypothesis of different modes of antibacterial action, we conducted a simple, one-descriptor (logP) 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 monotonous relationship. The latter was found in the cases of the resistant strains.

Hemolysis Studies—G+ 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 interaction with bacterial inner membranes. Therefore, one of the concerns of employing 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, compounds **4e** and **4f** cause an estimated 50% hemolysis at 0.2 and 0.3 mg/mL, respectively (Figure 3).

Combinational Studies—Employing a combination of antibiotics is a common practice in the treatment of bacterial infection.19 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.20 Therefore, we explored the possible use of the new neomycin derivatives in combination with clinically used antibiotics. Since **4e** is the most active neomycin derivative, we selected **4e** along with amikacin, neomycin B and vancomycin for combinational antibacterial studies.

We employed a checkerboard assay for the antibiotic combinational study.21 The selected antibiotics were used in combination with the lead, **4e**. The results are summarized in Table

2. The combinational effect can be evaluated based on the fractional inhibitory concentration (FIC) index, which can be calculated based on the following equation:

FIC = [A]/MIC_A + [B]/MIC_B, Synergism: FIC≤0.5; Addition: FIC = 0.5–1.0; Indifference: FIC = 1-4; Antagonism: FIC ≥ 4.

Discussion

From the relationship between acyl chain length and MIC profile, it appears that when a shorter chain length was incorporated, the derivatives maintain the original mode of antibacterial action as the parent neomycin (i.e. binding to the A-site decoding region of 16S rRNA). The added acyl chain, however, reduces the activity of **4a**, likely due to the steric interference of the acyl group on the binding of the neomycin derivative to rRNA. As the chain length increases, the steric hindrance will increase resulting in further decrease in the antibacterial activity. Nevertheless, when even longer acyl chains were incorporated, the derivatives displayed significant antibacterial activity with the SAR in the order of C16 ≈ C18 > C14 against both susceptible and resistant strains. Since elevated steric hindrance from these long acyl groups are expected, it is unlikely that compounds **4d, 4e** and **4f** regain their antibacterial activity by exerting the same mode of action. Our previous enzymatic and molecular modeling studies also revealed that AME's can inactivate aminoglycosides with diverse structural motifs.7 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 QSAR analysis also support our hypothesis that different modes of actions are likely for compounds **4d, 4e** and **4f** due to 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 neomycin. As the sizes of acyl groups increase, the second group shows a different linear relationship with a reverse dependence of the activity on the 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. Since 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.

Based on the results of hemolysis studies, the concentrations for 90% hemolysis for both compounds are expected to be greater than 1 mg/mL. Compared to the MIC values of these two compounds (ranging from 2 to 16 μg/mL) the concentrations that give significant hemolysis are 50 – 500 fold higher. Thus, these neomycin derivatives are not hemolytic at their effective antibacterial concentrations.

According to the fractional inhibitory concentration (FIC) index, compound **4e** displays strong synergistic effect with amikacin and/or neomycin against G- bacteria including *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. Since 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. Since MRSA is equipped with various AME's which should drastically reduce the activity of aminoglycosides including amikacin, the observed synergism is unexpected. The synergism of vancomycin and **4e** against *E. faecali* 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 dependent on the individual bacterial strain. The difference in the combinational effect of compound **4e** against G+ and G- could possibly reflect the action of compound **4e** towards inner and outer membranes, respectively.

In conclusion, we have completed the structural optimization of the 5″-acylated neomycin derivatives. Interesting antibiotic combinational effects have been revealed. The lead, **4e,** alone has a prominent antibacterial activity against G+ bacteria like MRSA and VRE. Facing the emergence of vancomycin-resistant *S. aureus* (VRSA),22,23 we believe that compound **4e** represents a possible countermeasure against this formidable pathogen. Judging from the data of QSAR analysis, it is evident that the lipophilicity is responsible for the observed antibacterial activity of the lead.

Based on 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 potential application in counteracting food-borne bacterial outbreaks such as those caused by *E coli* O157:H7, *Salmonella* spp. and other G- pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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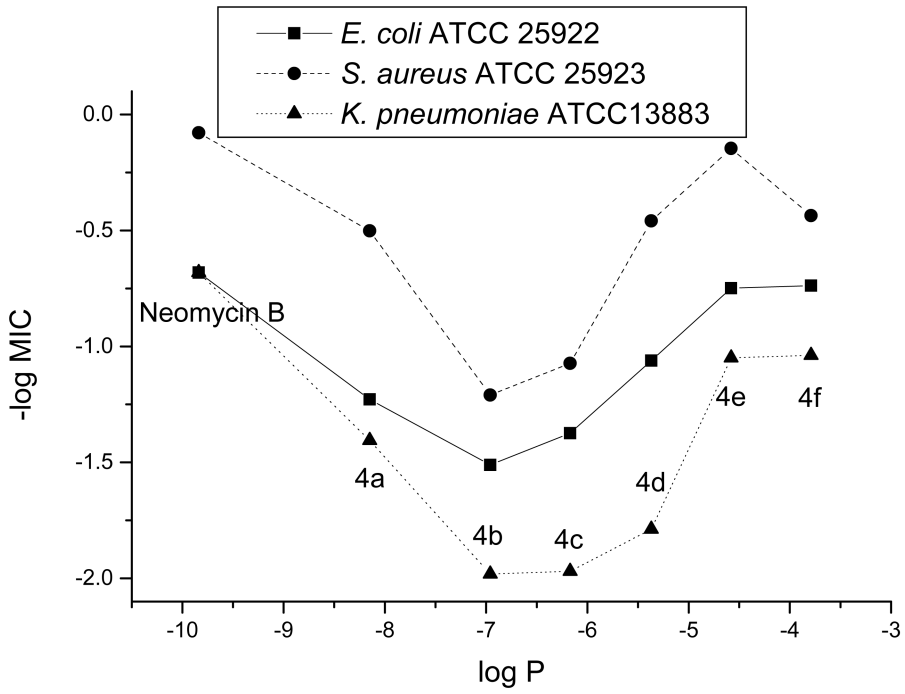


Figure 1.
QSAR Analysis for MIC's from Aminoglycoside Susceptible Strains

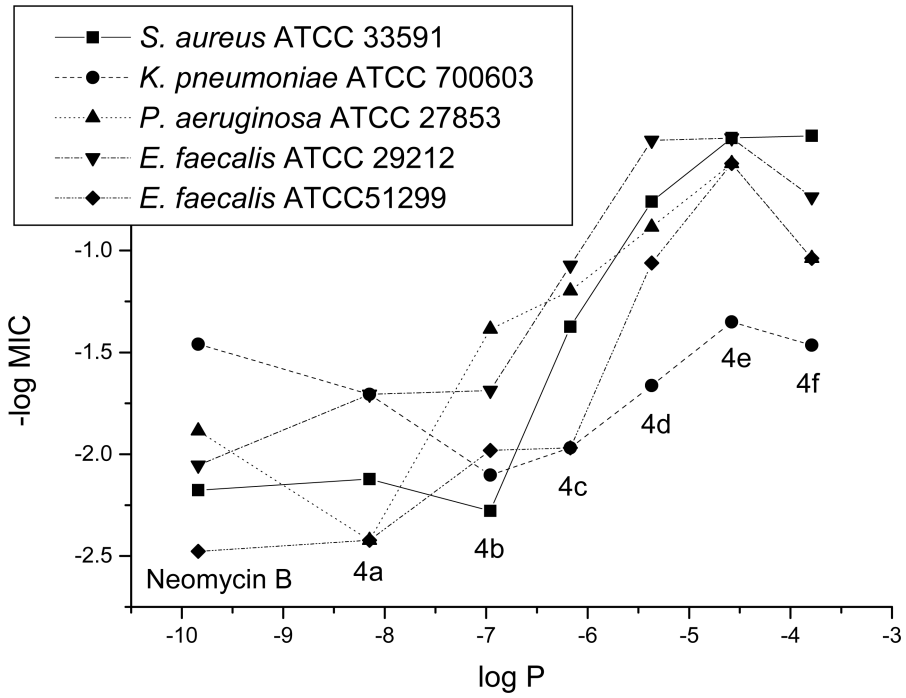


Figure 2.
QSAR Analysis for MIC's from Aminoglycoside Resistant Strains

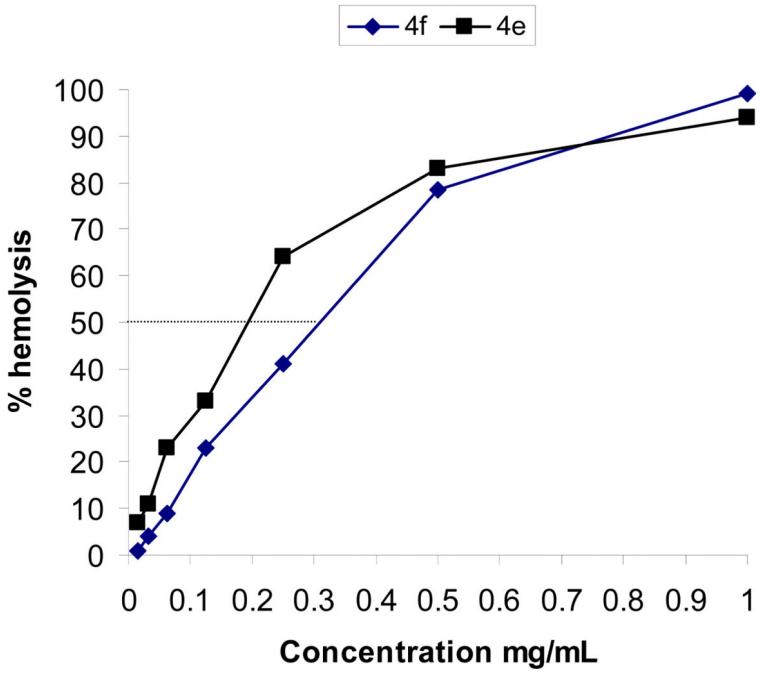
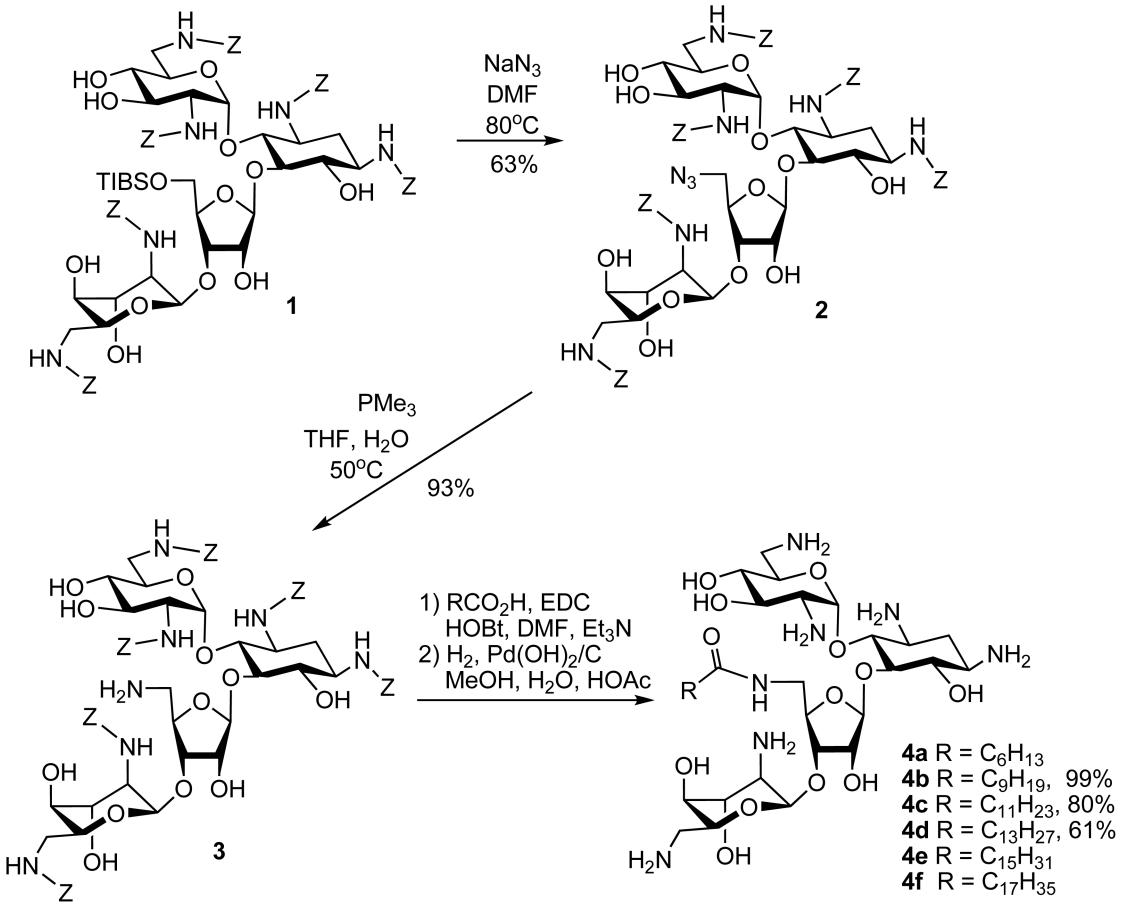


Figure 3.
Hemolysis of **4e** and **4f**



Scheme 1.
Synthesis of Neomycin Derivatives

Table 1

MIC of the 5″-Modified Neomycin Derivatives[a]

Compounds	Antibiotic susceptible strains			Antibiotic resistant strains				
	***E. coli* ATCC 25922**	***S. aureus* ATCC 25923**	***K. pneumoniae* ATCC13883**	***S. aureus* ATCC 33591 (MRSA)**	***K. pneumoniae* ATCC 700603**	***P. aeruginosa* ATCC 27853**	***E. faecalis* ATCC 29212**	***E. faecalis* 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

[a]Unit: μg/mL, ND: Not Determined.

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Table 2

FIC from Combinational Studies of **4e**

Strains of Bacteria	Neomycin	Amikacin	Vancomycin
E. coli ATCC 25922	0.27 – 0.56 (Synergism) [a]	0.38 - 0.53 (Synergism)	ND
S. aureus ATCC 25923	ND	1.0 (Addition or Indifference)	0.53 – 1.5 (Addition or Indifference)
K. pneumoniae ATCC 13883	ND	0.08 - 0.16 (Synergism)	ND
K. pneumoniae ATCC 700603	ND	0.38 - 0.53 (Synergism)	ND
S. aureus ATCC 33591 (MRSA)	ND	0.28 – 0.53 (Synergism)	0.53 – 0.56 (Addition)
P. aeruginosa ATCC 27853	ND	0.38 – 0.50 (Synergism)	ND
E. faecalis ATCC 29212	ND	0.53 – 0.56 (Addition)	0.28 – 0.53 (Synergism)

[a] $FIC = [A]/MIC_A + [B]/MIC_B$, Synergism: FIC≤0.5; Addition: FIC = 0.5–1.0; Indifference: FIC = 1-4; Antagonism: FIC ≥ 4, ND: Not Determined.