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Singly Modified Amikacin and Tobramycin Derivatives Show Increased rRNA A-Site Binding and Higher Potency against Resistant Bacteria

Richard J. Fair,[a] Lisa S. McCoy,[a] Mary E. Hensler,[b] Bernice Aguilar,[b] Victor Nizet,*[b] and Yitzhak Tor*[a]

Semisynthetic derivatives of the clinically useful aminoglycosides tobramycin and amikacin were prepared by selectively modifying their 6′′ positions with a variety of hydrogen bond donors and acceptors. Their binding to the rRNA A-site was probed using an in vitro FRET-based assay, and their antibacterial activities against several resistant strains (e.g., *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, MRSA) were quantified by determining minimum inhibitory concentrations (MICs). The most potent derivatives were evaluated for their eukaryotic cytotoxicity. Most analogues displayed higher affinity for the bacterial A-site than the parent compounds. Although most tobramycin analogues exhibited no improvement in antibacterial activity, several amikacin analogues showed potent and broad-spectrum antibacterial activity against resistant bacteria. Derivatives tested for eukaryotic cytotoxicity exhibited minimal toxicity, similar to the parent compounds.

Introduction

The discovery of penicillin (a β-lactam) and streptomycin (an aminoglycoside) in the 1940s launched the golden age of antibiotics. Many of the antibiotics discovered in the ensuing decades are still used in the clinic today.[1] However, the extensive and frequently unnecessary use of antibiotics has contributed to the increase in resistant pathogens. Horizontal gene transfer between bacteria has played a significant role in conferring resistance.[2] Drug-resistant bacteria, especially the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *and Enterobacter* species), *Clostridium difficile*, and *Escherichia coli* now infect not only immunocompromised hospital patients but otherwise healthy individuals as well.[3] This trend has led to rising healthcare costs, often due to extended hospital stays and increased mortality.[4] Problematically, the number of new antibiotics approved by the U.S. Food and Drug Administration has been steadily decreasing, a reflection of the fact that many pharmaceutical companies have been abandoning or downsizing their antibacterial research and development.[5]

On a positive note, there have been a few new classes of antibiotics in recent years, all of which target Gram-positive bacteria.[6] Nevertheless, the emergence of multidrug-resistant bacteria, especially Gram-negative bacilli with no new treatment options, has led to reexamination of drugs from the early years of antibiotic discovery.[7] Aminoglycosides are effective against a broad range of bacteria, although the advent of safer, less toxic antibiotics resulted in their declined use. However, with the increase in resistant pathogens, especially severe Gram-negative infections, aminoglycosides remain clinically useful for certain infections.[8] Tobramycin (**1a**) is often used for *P. aeruginosa* infection in cystic fibrosis patients, amikacin (**2a**) is prescribed for highly resistant Gram-negative infections, and gentamicin is used for preventative measures, as well as for sepsis (Figure 1).[8a]

Most aminoglycosides bind to the ribosomal RNA (rRNA) A-site, the site of mRNA decoding, and cause translation infideli-

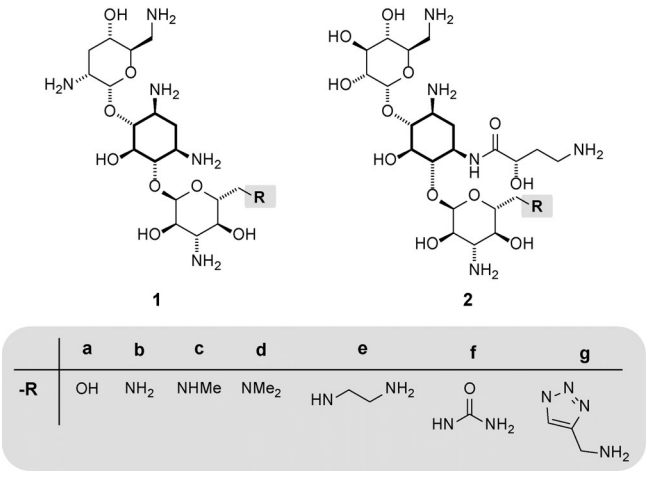

Figure 1. Tobramycin (**1a**), amikacin (**2a**), and derivatives that were prepared and studied. The 2-deoxystreptamine (2-DOS) ring is shown in bold. The 6′′ modification position is highlighted in grey.

[a] *Dr. R. J. Fair,[+] Dr. L. S. McCoy,[+] Prof. Dr. Y. Tor*
Department of Chemistry & Biochemistry
University of California, San Diego
9500 Gilman Drive, La Jolla, CA 92093 (USA)
E-mail: ytor@ucsd.edu

[b] *Dr. M. E. Hensler, B. Aguilar, Prof. Dr. V. Nizet*
Department of Pediatrics, University of California, San Diego
9500 Gilman Drive, La Jolla, CA 92093 (USA)
E-mail: vnizet@ucsd.edu

[+] *These authors contributed equally to this work.*

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ty.[9] The modes of action and resistance mechanisms have been well-studied, and the aminoglycoside scaffold has been established to bind RNA.[10] With this as a starting point, derivatization could lead to compounds that bind the A-site more avidly and show activity against otherwise drug-resistant bacteria.[11] Additionally, structural modifications could possibly diminish adverse side effects on host cells or physiology. With this in mind, we pursued the preparation and evaluation of minimally modified aminoglycosides in order to test their A-site affinity and, importantly, evaluate their effectiveness as potential antibiotics against important contemporary multidrug-resistant bacterial strains.

Here, we selectively modified two of the most common clinically used aminoglycoside antibiotics, amikacin and tobramycin. The primary alcohol in the 6″ position on these molecules was accessible to modification and was substituted for a variety of hydrogen bond donors and acceptors of different sizes (Figure 1). Most of the compounds showed increased in vitro affinity to the A-site as determined by a Förster resonance energy transfer (FRET)-based binding assay. Additionally, some of the derivatives showed equal to or better potency against certain resistant bacterial strains, while their eukaryotic cytotoxicity remains identical to that of the parent antibiotic.

Results

Design strategy

The 6″-hydroxy group is one of the few functional groups that appears to form no hydrogen bonds to the A-site RNA, either direct or water-mediated, in the crystal structures of tobramycin (**1a**) and amikacin (**2a**), though both are in close proximity to U1406 and C1407 (Figure 2).[12] Analogues with guanidinium groups replacing the 6″-hydroxy group have been shown to display increased A-site affinity and, in some cases, superior antibacterial activity.[13] This suggests that certain modifications to the 6″ position may indeed increase the affinity for the A-site and confer desirable antibacterial efficacy. We set out to test this hypothesis by making derivatives of both **1a** and **2a** with a variety of substituents differing in size, basicity, and number of hydrogen bond donors and acceptors. More basic functional groups could potentially increase the overall positive charge of the analogues, creating favorable electrostatic interactions with the polyanionic A-site rRNA. Hydrogen bond donors and acceptors could create new contacts to the A-site not observed in the parent compounds. Beyond imparting greater affinity for the A-site, some modifications could potentially disrupt recognition by aminoglycoside-modifying enzymes, the most common mechanism of aminoglycoside deactivation. Such derivatives could exhibit greater antibacterial potency against resistant bacteria.

Synthesis

The parent aminoglycosides were converted into three key intermediates using known procedures.[13,14] The synthetic approach for the conversion of the parent aminoglycosides into these intermediates is illustrated with tobramycin (**1a**) in Scheme 1. First, all amines were globally *tert*-butyloxycarbonyl (Boc)-protected using di-*tert*-butyldicarbonate. The single primary alcohol of $(Boc)_5$tobramycin (**3**) was then selectively converted into a sterically demanding sulfonate by treatment with

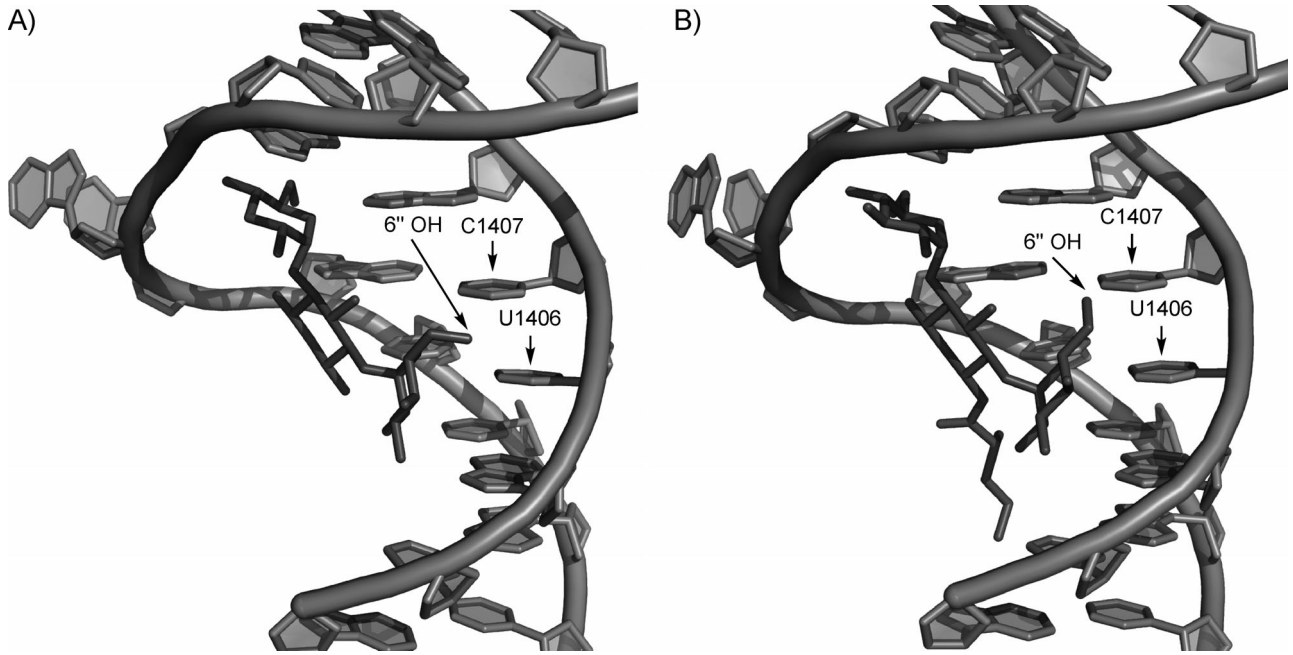


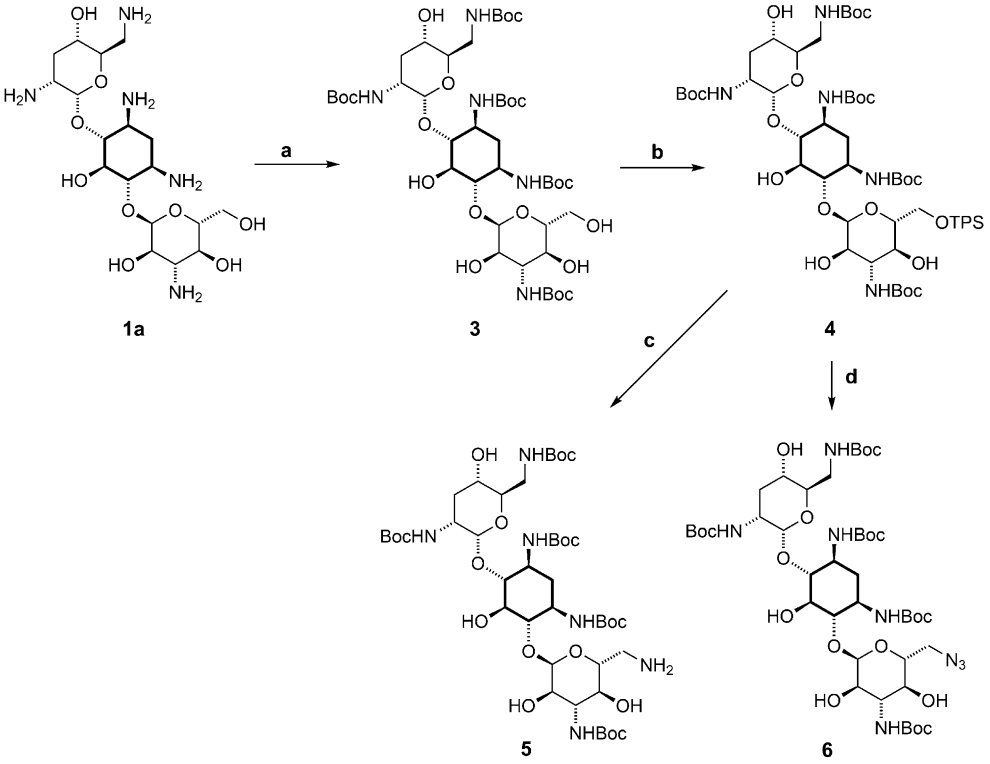
Figure 2. A) Crystal structure of tobramycin (**1a**) with A-site rRNA. B) Crystal structure of amikacin (**2a**) with A-site rRNA. Aminoglycoside 6″ alcohols and A-site bases U1406 and C1407 are labeled. Figures were adapted from PDB files for tobramycin (1LC4) and amikacin (2GSQ)[12] and were made using the PyMOL Molecular Graphics Systems, Version 1.4.1, Schrödinger LLC.

2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) in pyridine. Reflux in methanolic ammonia afforded 6″-deoxy-6″-amino-(Boc)$_5$tobramycin (**5**). Alternatively, the TPS derivative **4** could be converted into 6″-deoxy-6″-azido(Boc)$_5$tobramycin (**6**) by treating it with sodium azide.

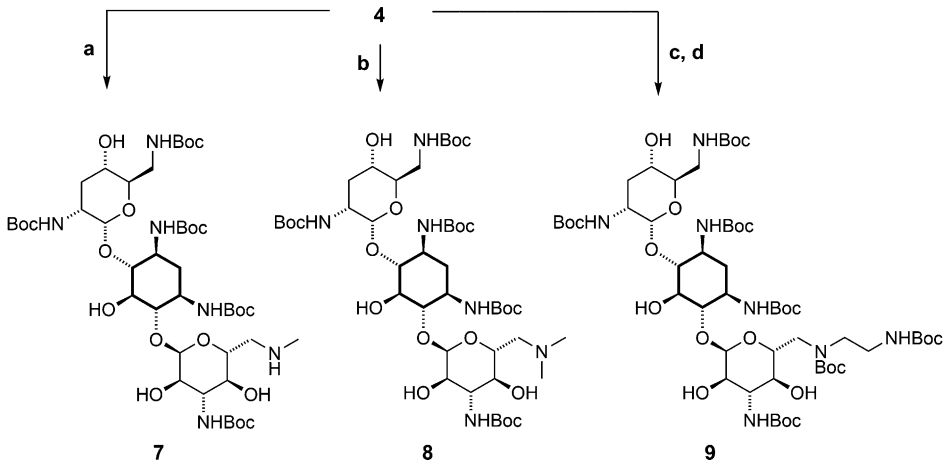
6″-Deoxy-6″-triisopropylbenzylsulfonyl(Boc)$_5$tobramycin (**4**) can also undergo substitution reactions with a variety of other nucleophiles (Scheme 2). Reflux in ethanolic methylamine yielded 6″-deoxy-6″-methylamino-(Boc)$_5$tobramycin (**7**). Reflux with dimethylamine in a tetrahydrofuran (THF) and dimethylformamide (DMF) mixture gave 6″-deoxy-6″-dimethylamino(Boc)$_5$tobramycin (**8**). 6″-Deoxy-6″-(2-(aminoethyl)-amino)-(Boc)$_7$tobramycin (**9**) was obtained by heating with ethylene diamine in methanol, followed by Boc protection using di-*tert*-butyldicarbonate to facilitate purification of this intermediate.

The free amine of 6″-deoxy-6″-amino(Boc)$_5$tobramycin (**5**) was used nucleophilically to react with 2,4-dimethoxybenzylisocyanate in the presence of pyridine to give a 2,4-dimethoxybenzyl (DMB)-protected urea. The DMB and Boc protecting groups were concurrently removed using a 1:1 mixture of trifluoroacetic acid (TFA) and dichloromethane with triisopropylsilane (TIPS) cation scavenger. HPLC purification afforded the analytically pure 6″-deoxy-6″-ureidotobramycin (**1 f**) (Scheme 3).

6″-Deoxy-6″-azido(Boc)$_5$tobramycin (**6**) was used in a cycloaddition reaction with propargyl(Boc)amine in the presence of copper sulfate and sodium ascorbate to give 6″-deoxy-6″-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)-(Boc)$_6$tobramycin (**10**) (Scheme 4). The intermediates **5**, **7**, **8**, **9**, and **10** were all deprotected using the aforementioned acidic conditions and HPLC purified to yield the tobramycin analogues **1 b**, **1 c**, **1 d**, **1 e**, and **1 g**. All of the amikacin derivatives were synthesized using the same reagents as for the tobramycin analogues.



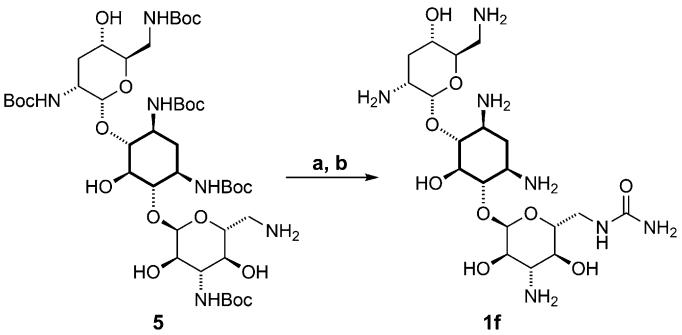
Scheme 1. Synthesis of key intermediates **4**, **5**, and **6**. *Reagents and conditions*: a) Boc_2O, Et_3N, H_2O, DMF, 55 °C, 2 days, 96%; b) TPSCl, pyridine, RT, overnight, 72%; c) NH_3, MeOH, 80 °C, 2 days, 94%; d) NaN_3, DMF, 55 °C, 2 days, 71%.



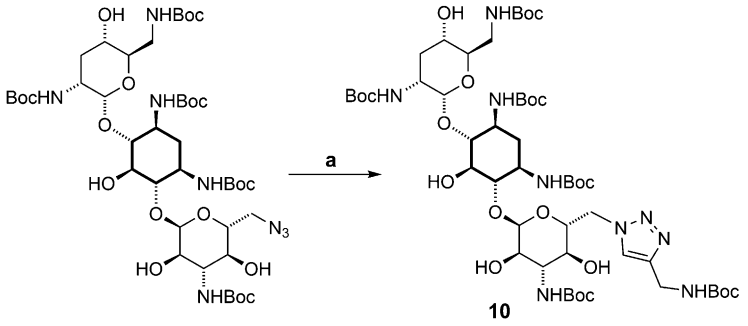
Scheme 2. Substitution reactions of 6″-deoxy-6″-triisopropylbenzylsulfonyl(Boc)$_5$tobramycin (**4**). *Reagents and conditions*: a) methylamine, EtOH, 80 °C, overnight, 88%; b) dimethylamine, THF, DMF, 80 °C, overnight, 91%; c) ethylene diamine, MeOH, 80 °C, 2 days; d) Boc_2O, Et_3N, H_2O, DMF, 55 °C, overnight, 58% (two steps).

Affinity for the bacterial 16S A-site RNA construct

To determine the affinity of all derivatives for the bacterial 16S A-site, we used a modified version of a FRET-based assay that was previously developed in our lab (Figure 3).[15] This modified version has been used previously to measure A-site affinities of modified aminoglycosides.[13] It consists of an aminoglycoside–coumarin conjugate (FRET donor), which binds to a Dy 547-labeled 16S A-site RNA hairpin construct (FRET acceptor) (Figure 3). The relative affinity of unlabeled ligands for the A-



Scheme 3. Synthesis 6′′-deoxy-6′′-ureidotobramycin (**1 f**). *Reagents and conditions*: a) 2,4-dimethoxybenzylisocyanate, pyridine, RT, overnight; b) TFA, TIPS, CH_2Cl_2, RT, 3.5 h, 58% (two steps).



Scheme 4. Synthesis 6′′-deoxy-6′′-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)-(Boc)$_6$tobramycin (**10**). *Reagents and conditions*: a) propargyl(Boc)amine, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, THF, *t*BuOH, H_2O, RT, overnight, 81%.

noglycoside conjugates can be used to cover distinct affinity ranges of A-site ligands. Plotting the fractional fluorescent saturation versus compound concentration generated titration curves.

Amikacin has a much lower affinity to the A-site as compared to tobramycin, so initial titrations of amikacin analogues were performed with a coumarin–kanamycin derivative, the lowest affinity aminoglycoside conjugate (Table 1). Tobramycin derivatives and higher affinity amikacin analogues were titrated against a coumarin–neomycin derivative (Table 2). In all cases, binding curves were generated by plotting the fractional fluorescence saturation of the FRET acceptor against the concentration of the molecule of interest. Representative curves of kanamycin–coumarin and neomycin–coumarin are shown in Figure 4.

All amikacin derivatives showed improved A-site binding, with the exception of 6′′-deoxy-6′′-ureidoamikacin (**2 f**), which had a much lower affinity than any other aminoglycoside tested. All amikacin analogues with modifications containing a single amine moiety, **2 b**–**e** and **2 g**, showed similar binding to each other and were also similar to tobramycin (**1 a**). 6′′-Deoxy-6′′-(2-(aminoethyl)amino)amikacin (**2 e**) showed superior binding relative to any of the other amikacin derivatives.

All tobramycin analogues showed improved binding over tobramycin (**1 a**). Like the amikacin derivatives, the urea modification resulted in the weakest binder. This urea tobramycin analogue (**1 f**) was the

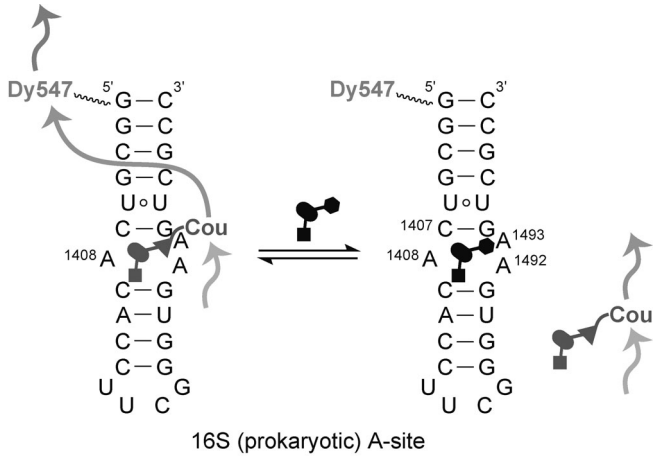


Figure 3. Secondary structure of the 16S (prokaryotic) A-sites RNA labeled with the FRET acceptor Dy 547 (grey). The place-holding coumarin-labeled aminoglycoside (neomycin–coumarin or kanamycin–coumarin) is shown in dark grey. As the aminoglycoside–coumarin conjugate is displaced by an unlabeled aminoglycoside, the affinity and selectivity of unlabeled aminoglycosides for the 16S A-site can be accurately monitored using FRET by following a decrease in emission of the acceptor (Dy 547).

site can be measured in a competition experiment in which the compound of interest is titrated into and displaces the coumarin–aminoglycoside conjugate, resulting in decreased emission of the FRET acceptor, Dy 547. Different coumarin–aminoglycoside conjugates can be used to cover distinct affinity ranges of A-site ligands. Plotting the fractional fluorescent saturation versus compound concentration generated titration curves.

Table 1. IC_{50} values for competition with kanamycin–coumarin.[a]

Compound	IC_{50} [μM]
tobramycin (**1 a**)	1.5 ± 0.2
amikacin (**2 a**)	6.7 ± 0.7
6′′-deoxy-6′′-aminoamikacin (**2 b**)	2.1 ± 0.2
6′′-deoxy-6′′-methylaminoamikacin (**2 c**)	1.5 ± 0.2
6′′-deoxy-6′′-dimethylaminoamikacin (**2 d**)	2.2 ± 0.2
6′′-deoxy-6′′-(2-(aminoethyl)amino)amikacin (**2 e**)	1.7 ± 0.03
6′′-deoxy-6′′-ureidoamikacin (**2 f**)	50.7 ± 5.5
6′′-deoxy-6′′-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)amikacin (**2 g**)	2.2 ± 0.1

[a] Conditions: A-site RNA (1 μM), kanamycin–coumarin (0.53 μM), cacodylate buffer pH 7.0 (20 mM), NaCl (100 mM), EDTA (0.5 mM).

Amikacin has a much lower affinity to the A-site as compared to tobramycin, so initial titrations of amikacin analogues were performed with a coumarin–kanamycin derivative, the lowest affinity aminoglycoside conjugate (Table 1). Tobramycin derivatives and higher affinity amikacin analogues were titrated against a coumarin–neomycin derivative (Table 2). In all cases, binding curves were generated by plotting the fractional fluorescence saturation of the FRET acceptor against the concentration of the molecule of interest. Representative curves of kanamycin–coumarin and neomycin–coumarin are shown in Figure 4.

All amikacin derivatives showed improved A-site binding, with the exception of 6′′-deoxy-6′′-ureidoamikacin (**2 f**), which had a much lower affinity than any other aminoglycoside tested. All amikacin analogues with modifications containing a single amine moiety, **2 b**–**e** and **2 g**, showed similar binding to each other and were also similar to tobramycin (**1 a**). 6′′-Deoxy-6′′-(2-(aminoethyl)amino)amikacin (**2 e**) showed superior binding relative to any of the other amikacin derivatives.

All tobramycin analogues showed improved binding over tobramycin (**1 a**). Like the amikacin derivatives, the urea modification resulted in the weakest binder. This urea tobramycin analogue (**1 f**) was the only one that was not superior to all of the amikacin derivatives. 6′′-Deoxy-6′′-aminotobramycin (**1 b**) and 6′′-deoxy-6′′-(2-(aminoethyl)amino)tobramycin (**1 e**) showed the highest affinities of all derivatives tested, followed by the methylamino- (**1 c**) and dimethylamino- (**1 d**) modified derivatives. 6′′-Deoxy-6′′-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)tobramycin (**1 g**) was worse than these, but still significantly better than the urea-modified analogue (**1 f**).

Table 2. IC_{50} values for competition with neomycin–coumarin.[a]

Compound	IC_{50} [μM]
tobramycin (**1a**)	53.0 ± 6.0
6″-deoxy-6″-aminotobramycin (**1b**)	4.7 ± 0.4
6″-deoxy-6″-methylaminotobramycin (**1c**)	7.4 ± 0.6
6″-deoxy-6″-dimethylaminotobramycin (**1d**)	6.8 ± 0.8
6″-deoxy-6″-(2-(aminoethyl)amino)tobramycin (**1e**)	5.3 ± 0.5
6″-deoxy-6″-ureidotobramycin (**1f**)	30.0 ± 4.0
6″-deoxy-6″-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)tobramycin (**1g**)	9.8 ± 1.0
amikacin (**2a**)	> 100
6″-deoxy-6″-aminoamikacin (**2b**)	46.7 ± 1.5
6″-deoxy-6″-methylaminoamikacin (**2c**)	45.7 ± 5.8
6″-deoxy-6″-dimethylaminoamikacin (**2d**)	46.4 ± 5.4
6″-deoxy-6″-(2-(aminoethyl)amino)amikacin (**2e**)	20.2 ± 2.6
6″-deoxy-6″-ureidoamikacin (**2f**)	> 100
6″-deoxy-6″-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)amikacin (**2g**)	47.6 ± 2.6

[a] Conditions: A-site RNA (1 μM), neomycin–coumarin (0.53 μM), cacodylate buffer pH 7.0 (20 mM), NaCl (100 mM), EDTA (0.5 mM).

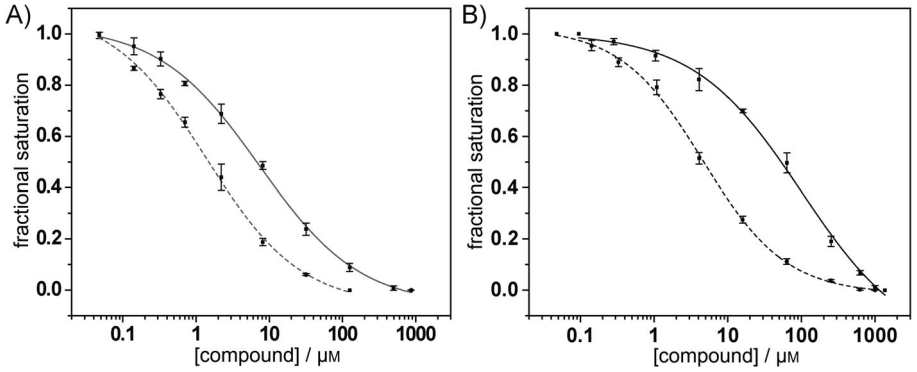


Figure 4. Representative displacement curves of A) kanamycin–coumarin by **2a** (solid grey line) and **2c** (dashed grey line), with respective IC_{50} values of 6.7 ± 0.7 and 1.5 ± 0.2 μM. B) Neomycin–coumarin by **1a** (solid black line) and **1b** (dashed black line), with respective IC_{50} values of 53.0 ± 6.0 and 4.7 ± 0.4 μM.

Antibacterial activities

To assess the relative antibacterial activities of the synthetic derivatives, minimum inhibitory concentration (MIC) values of both the modified and parent antibiotics were determined against an array of bacterial strains (Tables 3 and 4). Multiple Gram-positive and Gram-negative strains were chosen to establish a broad spectrum representation of antibacterial activity. The compounds were first tested against the antibacterial-susceptible control *E. coli* strain ATCC25922. No derivatives showed improvement against this strain, and only one compound, 6″-Deoxy-6″-aminoamikacin (**2b**), showed activity equivalent to that of its parent aminoglycoside, with an MIC value of 6.25–12.5 μg mL^{-1}.

The aminoglycosides were tested against three *P. aeruginosa* strains: P4, PA01, and ATCC27853. Tobramycin (**1a**) showed much better activity than amikacin (**2a**) against these *P. aeruginosa* strains. Unfortunately, only one tobramycin derivative, 6″-deoxy-6″-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)tobramycin (**1g**), showed activity equal to that of tobramycin (**1a**) against any of these strains. Both had MIC values of 0.39 μg mL^{-1} against PA01. However, the amikacin derivatives 6″-deoxy-6″-aminoamikacin (**2b**), 6″-deoxy-6″-methylaminoamikacin (**2c**), and 6″-deoxy-6″-(2-(aminoethyl)amino)amikacin (**2e**) showed improved activity over amikacin. 6″-Deoxy-6″-(2-(aminoethyl)amino)amikacin (**2e**) showed superior activity against all three strains, including a fourfold improvement (to 6.25 μg mL^{-1}) against P4. 6″-Deoxy-6″-aminoamikacin (**2b**) showed activity equal to that of amikacin, with MIC values of 1.56–3.13 μg mL^{-1} against PA01, but showed slight improvements in activity against ATCC27853, with an MIC value of 1.56–3.13 μg mL^{-1} compared with a parent MIC value of 3.13 μg mL^{-1}. Compound **2b** also showed a fourfold improvement against P4.

The aminoglycosides were also tested against two *K. pneumoniae* strains: ATCC700603 and the highly drug-resistant *K. pneumoniae* carbapenemase producer GNR1100. Amikacin demonstrated better activity than tobramycin against these strains. Again, the tobramycin derivatives were disappointing, with only **1e** showing activity similar to that of the parent anti-

Table 3. Inhibitory activities of tobramycin (**1a**) and derivatives against a panel of bacterial strains.[a]

Strain	MIC [μg mL^{-1}]						
	1a	**1b**	**1c**	**1d**	**1e**	**1f**	**1g**
E. coli (ATCC25922)	3.13	25–50	≥ 50	≥ 50	6.25–12.5	6.25	6.25
P. aeruginosa (P4)	0.78	25	> 50	> 50	3.13–6.25	3.13–6.25	1.56
P. aeruginosa (PA01)	0.39	12.5	50	50	1.56	0.78	*0.39*
P. aeruginosa (ATCC27853)	0.39	12.5–50	50	> 50	3.13	0.78–1.56	0.78
K. pneumoniae (ATCC700603)	6.25	12.5	25	25–50	*6.25*	12.5	12.5
K. pneumoniae (GNR1100)	> 50	> 50	> 50	> 50	> 50	> 50	> 50
MRSA (TCH1516)	0.78–1.56	6.25–12.5	12.5–25	25	6.25	3.13–6.25	6.25
MRSA (ATCC33591)	3.13	6.25	12.5–25	25	3.13–6.25	3.13–6.25	**0.78–1.56**

[a] Minimum inhibitory concentration (MIC) values equal to tobramycin are shown in italics; those less than tobramycin are shown in bold.

Table 4. Inhibitory activities of amikacin (**2a**) and derivatives against a panel of bacterial strains.[a]

Strain	MIC [μg mL^{-1}]						
	2a	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**
E. coli (ATCC25922)	6.25–12.5	*6.25–12.5*	12.5–25	25–50	12.5	50	12.5
P. aeruginosa (P4)	25	**6.25**	**12.5**	>50	**6.25**	>50	25–50
P. aeruginosa (PA01)	1.56–3.13	*1.56–3.13*	3.13	12.5–25	**1.56**	25–50	*1.56–3.13*
P. aeruginosa (ATCC27853)	3.13	**1.56–3.13**	3.13–6.25	25	**1.56**	25–50	*3.13*
K. pneumoniae (ATCC700603)	0.78	0.78–1.56	1.56	3.13	*0.78*	6.25–12.5	*0.78*
K. pneumoniae (GNR1100)	50	*50*	>50	>50	**12.5–25**	>50	**12.5–25**
MRSA (TCH1516)	6.25–12.5	12.5–25	50	>50	12.5–25	>50	12.5–25
MRSA (ATCC33591)	25	**12.5–25**	*25*	>50	**12.5**	>50	**12.5**

[a] Minimum inhibitory concentration (MIC) values equal to amikacin are shown in italics; those less than amikacin are shown in bold.

biotic. Both had MIC values of 6.25 μg mL^{-1} against ATCC700603. Compounds **2e** and **2g** showed activity equal to that of amikacin against ATCC700603 with MIC values of 0.78 μg mL^{-1}. Interestingly, they also both had improved activity (12.5–25 μg mL^{-1} compared with 50 μg mL^{-1} for amikacin) against GNR1100.

To test efficacy against Gram-positive bacteria, the aminoglycosides were tested against MRSA strains TCH1516 and ATCC33591. No amikacin or tobramycin derivatives showed any improvements or equal activity to their parents against TCH1516. There were, however, several compounds that showed improved activity against ATCC33591. Compound **1g** had an MIC value of 0.78–1.56 μg mL^{-1}, relative to a parent value of 3.13 μg mL^{-1}. Several amikacin derivatives showed increased potency over the parent MIC value of 25 μg mL^{-1}. These included **2b**, with a slight improvement to 12.5–25 μg mL^{-1}, and **2e** and **2g**, which both showed more significant improvements to 12.5 μg mL^{-1}.

Cytotoxicity

The amikacin and tobramycin derivatives with the most potent, broad spectrum antibacterial activities (**1g**, **2b**, **2e**, **2g**) were tested for eukaryotic cytotoxicity against HeLa cells compared with the parent compounds (**1a**, **2a**). The derivatives showed minimal toxicity, similar to the parent compounds, with little detrimental effects at concentrations up to 100 μM (~55–66 μg mL^{-1}) (Supporting Information Table S1).

Discussion

Tobramycin and amikacin analogues, modified at the 6″ position, were synthesized and evaluated for their A-site affinities and antibacterial activity. All tobramycin analogues showed superior affinity for the A-site relative to tobramycin, the parent antibiotic. There were significant variations in A-site affinity among the tobramycin analogues. The tightest binders were 6″-deoxy-6″-aminotobramycin (**1b**) and 6″-deoxy-6″-(2-(aminoethyl)amino)tobramycin (**1e**), and the worst tobramycin analogue was 6″-deoxy-6″-ureidotobramycin (**1f**). The general trend among the tobramycin analogues suggests that binders with smaller steric bulk or with greater overall potential charge show higher affinity.

All amikacin analogues showed improved A-site binding with the exception of 6″-deoxy-6″-ureidoamikacin (**2f**), which had by far the lowest A-site affinity of any compound tested. It was the only modification made without a basic functionality, which likely contributed to its lower RNA affinity. The amikacin analogues with one additional basic functional group showed similar IC_{50} values to one another, including the bulky 6″-deoxy-6″-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)amikacin (**2g**). 6″-Deoxy-6″-(2-(aminoethyl)amino)-amikacin (**2e**) has two additional basic amines relative to amikacin (**2a**) and, indeed, it displayed the highest A-site affinity among the amikacin analogues. In contrast to the tobramycin analogues, the RNA affinity of the amikacin analogues appears to result mostly from sensitivity to electrostatic effects, with no apparent steric preference among the analogues tested.

The tobramycin analogues generally showed disappointing antibacterial activity. The most successful analogue was 6″-deoxy-6″-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)tobramycin (**1g**), which showed no eukaryotic toxicity up to 100 μM, similar to the parent antibiotic tobramycin. It showed better activity than tobramycin against a MRSA strain and equal activity against one *P. aeruginosa* strain. In most other cases, the MIC values for **1g** were twofold worse than that of its parent. This particular modification was also one of the more successful among the amikacin analogues. 6″-Deoxy-6″-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)amikacin (**2g**) showed activity equal to or better than amikacin against five of nine strains tested, and in all other cases, its MIC value was within one serial dilution. It is intriguing that this modification was so efficacious, particularly as it was the most structurally significant alteration made.

6″-Deoxy-6″-aminoamikacin (**2b**) was also promising, with equivalent or improved activity compared with the parent against six of nine strains tested, including all three *P. aeruginosa* strains. It is interesting to note that the antibacterial activity was decreased across the entire panel for 6″-deoxy-6″-methylaminoamikacin (**2c**) and even more so for 6″-deoxy-6″-dimethylaminoamikacin (**2d**). This trend was also present for the tobramycin derivatives. This suggests that hydrogen bonding may play a role in the increased activity of **2b**.

The most successful derivative synthesized, however, was 6″-deoxy-6″-(2-(aminoethyl)amino)-amikacin (**2e**). This compound showed increased activity against five strains and equal activity against one compared with the parent compound. It

was universally better against the *P. aeruginosa* strains, and it showed equivalent or better activity against both *K. pneumoniae* strains, including an improvement against GNR1100. This makes the broad spectrum improvement of some of the amikacin derivatives particularly fascinating, given that amikacin itself is a semisynthetic aminoglycoside with an amino 2-hydroxybutyryl (AHB) side chain, which lowers its susceptibility to aminoglycoside-modifying enzymes.[16] It is possible that the AHB and 6″ modifications operate synergistically to further decrease its affinity for these enzymes. This is a hypothesis that we have previously posited when we observed increased antibacterial activity in an analogue with a guanidinium group in this position.[13]

When analyzing MIC values, it is important to appreciate that the affinity of an antibiotic to the A-site does not necessarily correlate with ribosome susceptibility, as determined by in vitro translation assays, or with antibacterial potency.[17] Interestingly, all but one derivative, **2b**, showed inferior antibacterial activity against the control *E. coli* strain ATCC25922. This suggests that improvement observed in activity against resistant strains is at least partially due to overcoming bacterial resistance mechanisms.

Conclusions

A series of 6″-modified tobramycin and amikacin analogues were synthesized. In all cases, the derivatives showed improved A-site affinity compared with their parent antibiotics when tested in an in vitro FRET-based assay, with the exception of 6″-deoxy-6″-ureidoamikacin (**2f**), which showed greatly decreased binding affinity. The tobramycin analogues generally showed disappointing antibacterial activity. In contrast, several amikacin analogues exhibited promising antibacterial potency against resistant strains. The most potent antibacterial derivatives tested did not show toxicity toward eukaryotic cells. Most notably, 6″-deoxy-6″-(2-(aminoethyl)amino)amikacin (**2e**) showed greater potency than amikacin (**2a**) against the majority of strains that were tested in MIC assays. Our results illustrate the potential utility of modifying the native antibiotics, as well as their established semisynthetic analogues, as a pathway to new agents of an altered, yet effective, therapeutic spectrum.

Experimental Section

Materials: Unless otherwise specified, materials purchased from commercial suppliers were used without further purification. Tobramycin (**1a**) and amikacin (**2a**) were obtained from Sigma–Aldrich (St. Louis, MO, USA) as their free bases. Propargyl(Boc)amine was synthesized according to an established procedure.[18] Anhydrous NH_3 was purchased from Airgas. All other anhydrous solvents and reagents, as well as ion exchange resins, were purchased from Sigma–Aldrich. NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

The Dy547-labeled A-site construct was purchased from Thermo Scientific and purified by gel electrophoresis.[7d,13,15b] Kanamycin–coumarin and neomycin–coumarin conjugates were synthesized and purified according to established procedures.[15] Chemicals for preparing buffer solutions (enzyme grade) were purchased from Fisher Biotec (Wembley, Western Australia). Autoclaved water was used in all fluorescence titrations.

Mueller-Hinton broth used for sensitivity testing was obtained from Hardy Diagnostics (Santa Maria, CA, USA). Polystyrene 96-well microplates for MIC testing were purchased from Corning Inc. (Corning, NY, USA). Bacterial strains for sensitivity testing included five strains from the American Type Culture Collection (ATCC; Manassas, VA, USA): hospital-associated MRSA strain 33591, rendered resistant to rifampicin by serial passage; USA300 MRSA strain TCH1516 (BAA-1717); *K. pneumoniae* strain 700603, *P. aeruginosa* strain 27853, and *E. coli* strain 25922. *P. aeruginosa* strain PA01 was used as a general antibiotic-sensitive *P. aeruginosa* strain.[19] Other Gram-negative strains used were clinical isolates obtained from a tertiary academic hospital in the New York metropolitan area: *K. pneumoniae* strain GNR1100 (respiratory isolate) and *P. aeruginosa* strain P4 (sputum isolate).

Instrumentation: NMR spectra were recorded on Varian Mercury 300 and 400 MHz, Varian VX 500 MHz, and Jeol ECA 500 MHz spectrometers. Mass spectra (MS) were recorded at the University of California, San Diego, Chemistry and Biochemistry Mass Spectrometry Facility, using an Agilent 6230 HR-ESI-TOF mass spectrometer. Reverse-phase HPLC (Vydac C_{18} column) purification and analyses were carried out using an Agilent 1200 series instrument. Products were lyophilized using a Labconco FreeZone 2.5 freeze drier. Steady-state fluorescence experiments were carried out in a microfluorescence cell with a path length of 1.0 cm (Hellma GmbH & Co. KG, Müllheim, Germany) on a Jobin Yvon Horiba FluoroMax-3 luminescence spectrometer. A background spectrum (buffer) was subtracted from each sample. A VersaMax plate reader (Molecular Devices, Mountain View, CA, USA), set at 600 nm wavelength, was used for MIC assays.

Aminoglycoside desalting: Aminoglycoside·TFA salts obtained upon global deprotection reactions (up to 40 mg) were dissolved in autoclaved H_2O (0.6 mL) in a sterile Eppendorf tube. Dowex Monosphere 550 A (100 mg) was added, and the suspension was shaken lightly overnight. The resin was removed by centrifugal filtration and washed twice with autoclaved H_2O. The desalted solutions were lyophilized, and the complete removal of TFA counterions was confirmed by ^{13}C NMR spectroscopy.

A-site binding assay: Aminoglycoside titration procedures, binding curves, and the curve-fitting equation can be found in the Supporting Information.

MIC determinations: Minimum inhibitory concentration (MIC) values for aminoglycosides were determined using broth microdilution in accordance with Clinical Laboratory Standards Institute guidelines.[20]

Cytotoxicity: The aminoglycosides were tested for mammalian cell cytotoxicity by measuring lactate dehydrogenase (LDH) release into the media. Briefly, HeLa cells (ATCC) were seeded at 2×10^4 cells per well in sterile tissue culture-treated microtiter plates (Sigma–Aldrich). The cells were allowed to attach for 24 h and were then incubated with the aminoglycosides in fresh media. LDH was assayed in the supernatant at 72 h using the CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA).

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R. J. Fair, L. S. McCoy, M. E. Hensler, B. Aguilar, V. Nizet, Y. Tor**

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Singly Modified Amikacin and Tobramycin Derivatives Show Increased rRNA A-Site Binding and Higher Potency against Resistant Bacteria