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Apralogs: Apramycin 5-O-Glycosides and Ethers with Improved Antibacterial Activity and Ribosomal Selectivity and Reduced Susceptibility to the Aminoacyltransferase (3)-IV Resistance Determinant.

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Abstract

Apramycin is a structurally unique member of the 2-deoxystreptamine class of aminoglycoside antibiotics characterized by a mono-substituted 2-deoxystreptamine ring that carries an unusual bicyclic eight-carbon dialdose moiety. Because of its unusual structure apramycin is not susceptible to the most prevalent mechanisms of aminoglycoside resistance including the aminoglycoside-modifying enzymes and the ribosomal methyltransferases whose widespread presence severely compromises all aminoglycosides in current clinical practice. These attributes coupled with minimal ototoxicity in animal models combine to make apramycin an excellent starting point for the development of next-generation aminoglycoside antibiotics for the treatment

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Full experimental parts and copies of ¹H and ¹³C NMR spectra for the synthesis of all new compounds. Experimental protocols for the ribosome inhibition assays. Experimental protocol for the antimicrobial susceptibility testing. Table of bacterial strains employed and their source. Experimental protocol for the animal efficacy studies. Experimental protocols for the ex-vivo ototoxicity studies

Conflict of Interest. SNH, AV, ECB and DC are cofounders of and equity holders in Juvabis AG, a biotech start-up working in the area of aminoglycoside development.

of multidrug-resistant bacterial infections, particularly the ESKAPE pathogens. With this in mind, we describe the design, synthesis, and evaluation of three series of apramycin derivatives, all functionalized at the 5-position, with the goals of increasing the antibacterial potency without sacrificing selectivity between bacterial and eukaryotic ribosomes, and of overcoming the rare aminoglycoside acetyltransferase (3)-IV class of aminoglycoside-modifying enzymes that constitutes the only documented mechanism of antimicrobial resistance to apramycin. We show that several apramycin-5-*O*- β -D-ribofuranosides, 5-*O*- β -D-erythrofuransides and even simple 5-*O*-aminoalkyl ethers are effective in this respect through the use of cell-free translation assays with wild-type bacterial and humanized bacterial ribosomes, and extensive antibacterial assays with wild-type and resistant Gram negative bacteria carrying either single or multiple resistance determinants. Ex-vivo studies with mouse cochlear explants confirm the low levels of ototoxicity predicted on the basis of selectivity at the target level, while the mouse thigh infection model was used to demonstrate the superiority of an apramycin-5-*O*-glycoside in reducing the bacterial burden in-vivo.

Graphical Abstract

Introduction

Apramycin **1** (Figure 1), originally known as nebramycin factor 2 and produced by *Streptomyces tenebrarius*, is an atypical 2-deoxystreptamine (DOS) aminoglycoside antibiotic (AGA) first reported by workers at Eli Lilly,^{1–3} and patented for use in veterinary medicine,⁴ where it continues to find application.⁵ Structurally, apramycin is characterized by an unusual eight-carbon dialdose in the form of a bicyclic hemiacetal that is linked by glycosidic bonds to the 4-position of 2-deoxystreptamine and, via an unusual α,β -1,1'-linked disaccharide motif, to 4-amino-4-deoxy- α -D-glucopyranose.^{6, 7} The antibacterial activity of apramycin and its unusual bicyclic dialdose motif spurred several synthetic studies^{8–11} culminating in a total synthesis by the Tatsuta group in 1984.¹² Most work in the area, however, has focused on derivatization and modification of the natural product with the goal of improving antibacterial activity. Thus, the effect of limited modifications at the 5-, 6-, 3'-, N1-, N2'-, N7'-, N4'', O8'- and O6''-positions,^{12–27} and most recently of double and triple modifications at the 5-, 6-, and 4''-positions,²⁸ have been widely described.

The unusual structure of apramycin is such that it is not modified by the majority of the aminoglycoside-modifying enzymes (AMEs),^{21, 29–32} with the exception of the aminoglycoside N-acetyl transferase AAC(3)-IV.³⁰ The AAC(3) N-acetyltransferases are present in various isoforms and constitute a primary mechanism of resistance to AGAs.^{33–38}

Because of its structure, the antibiotic activity of apramycin is not thwarted by the presence of the ribosomal methyltransferases (RMTases) acting on N7 of G1405 in the drug binding pocket of the bacterial ribosome,^{21, 30, 39} whose presence blocks the action of all AGAs in current clinical practice,⁴⁰ including the recently introduced plazomicin.^{41–43} Consequently, apramycin displays broad spectrum activity against a wide range of Gram negative and Gram positive pathogens in vitro and in-vivo, including drug-resistant pathogens carrying the most prevalent AGA resistance determinants.^{30–32, 39, 44–50}

Using a series of cell-free translation assays with wild-type and humanized bacterial ribosomes,^{51–53} we have discovered that apramycin enjoys excellent selectivity for prokaryotic over eukaryotic and especially over the mitochondrial and A1555G mutant mitochondrial ribosomes,^{21, 54} whose inhibition is considered to be the root cause of AGA-induced ototoxicity.^{38, 52, 55–57} This was reinforced by ex-vivo cochlear explant and in-vivo ototoxicity studies in the guinea pig model, which testified to a low ototoxic potential of apramycin.²¹

The ready availability by fermentation, lack of susceptibility to most common resistance determinants, and low levels of ototoxicity displayed in the guinea pig model combine to make apramycin an attractive candidate for use in the clinic to combat multidrug resistant infections including carbapenem-resistant enterobacteriaceae (CREs) and other ESKAPE pathogens.⁵⁸ Apramycin is also an excellent substrate on which to base the development of improved next-generation AGAs. Such a compound would ideally i) not suffer from susceptibility to the AAC(3)-IV resistance determinant, ii) display improved activity levels compared to the parent, and iii) retain excellent selectivity between prokaryotic and eukaryotic ribosomes predictive of reduced ototoxicity. With this in mind, we began such a program several years ago reporting first on the importance of the 6'-hydroxyl group on activity,²⁷ since when several groups,^{45, 54} but most notably that of Kirby,^{31, 32, 39, 46–50, 59} have drawn attention to the potential of apramycin as a substrate for the development of a next-generation AGA. We now report on an extensive program of work conducted with these aims in mind, and culminating in a series of derivatives at the 5-position of apramycin that display all of the requisite characteristics.

Results and Discussion

Design

Apramycin derives its antibacterial activity from binding to the decoding A site in helix 44 of the 30S subunit of the bacterial ribosome in the same manner as both the more common 4,5-disubstituted DOS series of AGAs and the isomeric 4,6-series that find current application in the clinic, exemplified by paromomycin **2** and gentamicin **3**, respectively.^{21, 60–63} Alternative modes of binding of apramycin to the bacterial ribosome, demonstrated by crystallographic and NMR studies with short sequences of nucleotides,^{64, 65} we consider to be of only minor relevance in view of the importance of the 6'-hydroxy group and its axial location on the bicyclic system.²⁷ Consideration of the structures of apramycin, paromomycin, and gentamicin suggested that appendage of an aminosaccharide or disaccharide to the 5-position of apramycin would provide a derivative that benefits from the largely electrostatic attraction for the decoding A site that rings III and IV are known to

provide for the 4,5-series AGAs,⁶⁶ without incurring susceptibility to the RMTs acting on G1405. Further, consideration of the existing data on AGA susceptibility to resistance determinants suggested that substitution at the 5-position might afford protection against the AAC(3)-IV from which apramycin suffers, as typical 4,5-AGAs are little targeted by AAC(3) N-acetyltransferases.³⁵ We elected not to target the apramycin 6-position for derivatization, and so not to take advantage of the extra affinity provided by ring III of the 4,6-AGAs, as we anticipated that such compounds would fall victim to the A1405 RMTs, whose effectiveness arises from blocking the direct hydrogen bond between ring III of the 4,6-series and A1405 N7. More pertinently, we were encouraged by the 1981 report of Abe and coworkers that 5-*O*- β -D-ribofuranosylation of the apramycin derivative **4**, providing **5** after deprotection, did not incur any reduction of antibacterial activity (Figure 2).¹⁵

Synthesis

The apramycin derivative **6** was prepared in three straightforward steps from apramycin as described previously.²⁷ Consistent with previous reports on the regioselective derivatization of 4-*O*-monosubstituted derivatives of 2-deoxystreptamine at the 6-position,^{15, 67, 68} treatment of **6** with a controlled amount of acetic anhydride in pyridine gave the 6,2'',3''-6''-tetra-*O*-acetate **7** in 61% yield (Scheme 1). Likewise, controlled exposure of **6** to benzoyl chloride in pyridine afforded the corresponding tetrabenzoate **8** in 86 % yield. These efficient four-step preparations of selectively protected apramycin mono-ols for use in functionalization at the 5-position compare favorably to the methods employed in the synthesis of **4** by Abe and coworkers.¹⁵

Alcohols **7** and/or **8** were then subjected to glycosylation by a series of glycosyl donors, prepared as described in the Supporting Information, to give the glycosides described in Table 1. Of note, the paromobiosyl donor **10** was readily prepared by Lewis acid-mediated cleavage of perazido-peracetyl paromomycin in the presence of 4-thiocresol, adapting methods described earlier by Hanessian, Swayze, and Wong and coworkers,^{69–71} followed by the adjustment of protecting groups and oxidation levels. All donors carried ester protecting groups at the 2-position so as to benefit from stereodirecting neighboring group participation, and correspondingly high levels of selectivity were observed in most cases. The anomeric configuration of the newly introduced glycosidic bonds was assigned based on the chemical shift of the anomeric carbon in the ¹³C NMR spectra consistent with established rules (Table 1).⁷²

The allyl ether of glycoside **23** was further derivatized by treatment with catalytic osmium tetroxide in the presence of *N*-methyl morpholine *N*-oxide (NMO) according to the Van Rhee protocol⁷³ to give the corresponding diol as a mixture of diastereomers. Oxidative cleavage with sodium metaperiodate on silica gel⁷⁴ then afforded the corresponding aldehyde **27** in 97% yield over two steps that was immediately subjected to reductive amination in the presence of sodium cyanoborohydride⁷⁵ to give, after saponification of the esters and carbamate, the desired aminoalkyl ethers. Finally, hydrogenolysis followed by chromatography over Sephadex C25 and lyophilization of aqueous acetic acid provided the fully deprotected AGAs **28–32** in the form of their peracetate salts suitable for assay (Table 2).

Initial attempts to prepare a series of acyclic variants of the above 5-*O*-glycosyl apramycin derivatives were thwarted by migration of the ester between the 5- and 6-positions under a variety of conditions for the installation of an allyl ether, resulting in complex reaction mixtures. The optimum conditions employed silver oxide and allyl iodide when a clean ether could be isolated in 59 and 78% yield in the acetyl and benzoyl series, respectively. Unfortunately, extensive 2D NMR experiments (Supporting Information) revealed ester migration to have taken place and the products to be the 6-*O*-allyl ethers **33** and **34** (Scheme 2). Although this ester migration with derivatization complicated the synthesis of the desired derivatives at the 5-position, it may prove useful in future work at the 6-position; in this spirit the peracetate **33** was converted to the dihydroxyl propyl and hydroxyethyl derivatives **35** and **36** suitable for eventual deprotection as set out in Scheme 2.

Fortunately, the lower reactivity of the 5-OH group apparent in the preparation of the esters **7** and **8** (Scheme 1) was also operative under more basic conditions, such that benzylation of **6** with sodium hydride and a controlled amount of benzyl bromide in DMF such that the requisite 6,2'',3'',6''-tetra-*O*-benzyl ether **37** could be isolated in 38% yield along with 23% of the perbenzyl ether **38** (Scheme 3). The location of the free-alcohol in **37** was readily apparent following acetylation to give **39** and inspection of its 1 and 2D NMR spectra. Allylation of **37** with sodium hydride and allyl iodide then provided the 5-*O*-allyl ether **40**, while treatment with sodium hydride and 3-bromopropylamine hydrogen bromide gave the aminopropyl ether **41**, in 72 and 32% yield, respectively (Scheme 3).

Hydroboration of **40** with oxidative workup gave the 3-hydroxypropyl ether **42** in 58% yield, while reaction with NMO and catalytic osmium tetroxide afforded an inseparable mixture of diastereomeric diols **43** in 78% yield. This mixture of diols was treated with 2,4,6-triisopropylbenzenesulfonyl chloride in pyridine to give the corresponding chromatographically separable sulfonates **44** and **45** in 51% combined yield. The sulfonate group was displaced from the individual diastereomers with sodium azide to afford derivatives **46** and **47** in 81 and 67% yield, respectively. Further, diol **43** was cleaved with sodium metaperiodate to give the aldehyde **48**, which upon reduction with sodium borohydride delivered alcohol **49** in 60% yield over two steps. Alternatively, aldehyde **48** was subjected to reductive amination with acetic acid and sodium cyanoborohydride to give compounds **50**, **51**, and **52** in 74, 55, and 67% yield over two steps, respectively (Scheme 4).

Finally, in order to assign the configuration of the diastereomeric azido alcohols **46** and **47**, and by extrapolation the precursor diols **44** and **45**, alcohol **37** was alkylated with the enantiomerically pure triflate **53**, obtained by triflation of commercial *S*-isopropylidene glycerol, giving **54** in 85% yield. Cleavage of the acetonide, followed by selective sulfonylation then gave **45** (Scheme 5) with the *S*-configuration in the pendant chain, whose NMR spectra correlated with the more polar of the two isomers prepared in Scheme 4.

All glycosides and 6-*O*-ethers were deprotected by a sequence of saponification, followed by either hydrogenolysis or Staudinger reduction of the azides as detailed in the Supporting Information. Final purification was achieved by ion exchange chromatography over Sephadex C25 followed by lyophilization from acetic acid to give the final compounds in the form of their peracetate salts (Table 3). In the case of the 5-*O*-ethers the sequence of

saponification and hydrogenolysis was reversed, such that the benzyl ethers and azides were removed before saponification (Table 3).

Activity and Selectivity at the Drug Target

To assess the influence of modification on activity at the level of the target, all compounds were tested for their ability to inhibit the production of luciferase in cell-free translation assays with isolated bacterial ribosomes. To determine the selectivity for bacterial over eukaryotic ribosomes parallel screening was conducted against a series of engineered bacterial ribosomes carrying the complete thirty base pair decoding A site of the human mitochondrial ribosome, its A1555G mutant, and the human cytoplasmic ribosome (Figure 3, Table 4).^{51, 52, 76–78} Selectivity for inhibition of the bacterial over mitochondrial ribosomes is particularly important as it has been demonstrated that inhibition of the mitochondrial ribosome in the cochlear hair cells, where AGAs have been shown to persist up to thirty days after administration in spite of their otherwise rapid clearance from the body in the urine,⁷⁹ is a primary cause of AGA-induced ototoxicity.^{52, 55} Further, inhibition of the mutant A1555G mitochondrial ribosome is known to be a cause of hypersusceptibility to AGA-induced ototoxicity in genetically predisposed subjects.^{53, 77, 80} Selectivity for inhibition of the bacterial over the mitochondrial ribosomes is consequently a feature that is predictive of reduced ototoxicity.^{21, 54, 81, 82} Selectivity over the cytoplasmic ribosome on the other hand is viewed as indicative of low levels of systemic toxicity. For ease of comparison, in Table 4 and all subsequent tables, compounds are grouped into four distinct sets according to the type of modification: ribofuranosyl; erythrofuransyl; 5-*O*-alkyl; and 6-*O*-alkyl (Figure 4).

Consideration of the 5-*O*-(D-ribofuranosyl) series of compounds (**55**, **56**, **57a**, **57b**, **58**, **60**, **61**, and **62**) indicates that the β -D-ribofuranosyl modification (**55**) provokes a relatively insignificant reduction in inhibitory activity of the bacterial ribosome, but a 3–4-fold reduction in inhibition of all three eukaryotic ribosomes, leading to significantly increased selectivity. This observation is consistent with work from the Fridman lab, in which the installation of a β -D-ribofuranosyl moiety at the 5-position of 4,6-AGAs led to an increase in selectivity for bacterial over cytosolic ribosomes without any increase in antibacterial activity.⁸³ The antibacterioribosomal activity in this series can be improved by the incorporation of a further basic amino group in the form of an aminoethyl appendage at the ribose 3-position (**57b**) but, surprisingly, not by addition of a paromobiosyl moiety with two basic amino groups (**56**) to the same locus. The antibacterioribosomal activity in this series also can be increased by the placement of a single basic amino group at the ribose 5-position (**60**). Extrapolating from this modification, the appendage of an ethylene diamine and to a lesser extent a propylene diamine moiety to the ribose 5-position affords compounds **61** and **62**, which both display increased antibacterioribosomal activity compared to apramycin. With respect to selectivity, the single basic amino group attached to the ribofuranosyl ring of **57b** moderates the effect of the ribofuranosyl ring itself on selectivity over the mitochondrial ribosomes (wild-type and mutant) but does not detract from selectivity over the cytosolic ribosome. The two basic amino groups of the paromobiosyl moiety of **56** result in significantly increased activity against all three eukaryotic ribosomes leading to an across the board notable reduction in selectivity. In addition to their increased

antibacterioribosomal activity compounds **60** and **62** display moderately increased activity against the mutant mitochondrial ribosomes, such that they display overall comparable selectivity to apramycin itself. In contrast, for **61** increased antibacterioribosomal activity comes without a comparable increase in activity for the eukaryotic ribosome, resulting in an excellent selectivity, particularly against the mitochondrial ribosomes. The relatively low selectivity of the 5-amino-5'-deoxy-ribosyl derivatives **60** and **62** for the bacterial over the cytoplasmic ribosome is due to increased inhibition of the latter, consistent with the influence of the 5"-amino-5"-deoxy modification on a series of ribostamycin compounds under consideration for the treatment of read-through diseases.⁸⁴ The contrast in selectivity between the homologs **61** and **62** must arise from differences in basicity of the monoprotonated diamine moiety, with the additional methylene group in **62** better insulating the second amine from the electron-withdrawing effect of the ammonium group.⁸⁵

It is noteworthy that the 5-*O*- β -D-erythrofuranosyl apramycin derivative **59**, which lacks the hydroxymethyl side chain of the cognate ribofuranosyl series, retains the excellent selectivity of the ribofuranosyl analog **55** and displays antibacterioribosomal activity that is comparable with the parent. This observation indicates that the potential inter-residue hydrogen bond from the 5"-hydroxy group to the 2'-amino group in **55**, inferred by extrapolation from the X-ray crystal structures of numerous 4,5-AGAs in complex with h44 of the bacterial decoding A site^{60, 86, 87} is of little consequence. As in the ribofuranosyl series, the appendage of a monobasic aminoethyl moiety to the erythrofurano-3-position results in a compound **28** with increased activity against the bacterial and eukaryotic ribosomes such that excellent selectivity is retained overall. Replacement of the aminoethyl moiety in **28** by a hydroethylaminoethyl group (**29**) or by aminoalkylaminoethyl groups (**30-32**) did not result in any further increase in activity or favorable change in selectivity.

In contrast to the appendage of a D-erythro- or D-ribofuranosyl ring with the correct β -anomeric configuration, the introduction of a simple alkyl (**63**), hydroxyethyl (**64**), or hydroxypropyl chain (**65**) to the 5-position of apramycin has a detrimental effect on the ability to inhibit the bacterial ribosome. The placement of a dihydroxypropyl chain (**66**) or an aminoethyl (**67**) or aminopropyl chain (**68**) however causes little or no loss of activity. The incorporation of a 3-amino-2-hydroxy propyl ether at the 5-position results in an increase in antibacterioribosomal activity in a configuration dependent manner, with the *S*-isomer **69** displaying 2–3-fold better activity than the *R*-isomer **70** – presumably the result of a favorable hydrogen bonding interaction in the more active isomer. As the same modification causes little change in the inhibition of the eukaryotic ribosomes, the *S*-isomer exhibits notably improved selectivity over the parent apramycin. The incorporation of a dibasic ethylenediamine (**71**) and especially a propylenediamine (**72**) residue results in a modest increase in antibacterioribosomal activity, without detriment to selectivity. The incorporation of simple propyl (**73**), hydroxyethyl (**74**) and dihydroxypropyl (**75**) chains at the 6-position leads to a significant reduction in activity.

Antibacterial Activity Against Wild-type Bacterial Strains

All compounds were assayed for activity against a panel of wild-type Gram negative pathogens (*Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Acinetobacter*

baumannii, *Pseudomonas aeruginosa*) obtained from the diagnostic department of the Institute of Medical Microbiology, at the University of Zurich. All compounds were also assayed for activity against a Gram positive Methicillin-resistant *Staphylococcus aureus* (MRSA) strain and an aminoglycoside-resistant MRSA strain from the same source (Table 5). For the most part all compounds displayed antibacterial activity in these screens consistent with their inhibition of the bacterial ribosome discussed above. Thus, multiple compounds had MIC values similar to and in some instances even increased MIC activity compared to the parent apramycin across the range of pathogens screened. A notable exception to this general trend was *Pseudomonas aeruginosa* for which markedly lower activity was seen in the ribo- and erythrofuransyl series, with the exception of those compounds with mono- or dibasic chains at the ribose 5-position (**60–62**) or at the erythrose 3-position (**30** and **31**). These compounds also mostly enjoyed increased MIC activities comparable to apramycin across the whole panel of pathogens tested. In the acyclic ether series of compounds the 3-amino-2-hydroxypropyl ethers of apramycin (**69** and **70**) displayed excellent activity across the panel of strains, including *P. aeruginosa*, as did the diaminoethers **71** and **72**. In particular, it is noteworthy that **69** with the 3-amino-2-(*S*)-hydroxy propyl ether at the 5-position of apramycin consistently displayed increased activity across the entire panel of pathogens as compared to the parent apramycin.

Antibacterial Activity Against Resistant Bacterial Strains

In view of the already outstanding profile of apramycin in the face of common AMEs, we concentrated on assessments of activity against a panel of clinical *E. coli* isolates carrying genes for two isoforms of AAC(3), of which only AAC(3)-IV is commonly considered to act on apramycin (Table 6).^{30, 32, 88, 89} Because several of the analogs are 5-*O*-ribofuransyl derivatives of apramycin, and because phosphorylation at the 5''-site (the primary alcohol of the ribofuransyl side chain) by the promiscuous APH(3')-I isoform^{90–95} of the APH(3')s is an established mechanism of resistance in the 4,5-series AGAs,⁹⁶ we also screened for activity against isolates carrying the different APH(3') isoforms (Table 6). The 4,5-AGAs ribostamycin, paromomycin and lividomycin B were included as positive controls.

Consistent with the insensitivity of the 4,5-AGAs ribostamycin and paromomycin to AAC(3) resistance determinants, the appendage of a β -D-ribofuransyl ring to the 5-position of apramycin, as in **55**, **56**, **57 β** , **58**, **61** and **62**, largely restores activity in the presence of AAC(3)-IV. This effect is maximal for **56** and **57 β** , carrying the full paromomycin rings III and IV disaccharide, or a 3-*O*-(2-aminoethyl)- β -D-ribofuransyl ring at the 5-position, respectively, neither of which suffer any significant loss of activity. Together with the observation that **61** and **62** carrying dibasic amino groups at the ribofuransyl 5-position are not as effective as **56** and **57 β** in overcoming AAC(3)-IV, this indicates that at least one correctly-placed basic amino group is required in addition to the β -D-ribofuransyl ring to fully compensate for the presence of AAC(3)-IV. In contrast to the beneficial influence of the 3-*O*-(2-aminoethyl)- β -D-ribofuransyl ring (**57 β**) the corresponding 3-*O*-(2-aminoethyl)- β -D-erythrofuransyl derivative **28** suffers a 16-fold loss of activity in the presence of AAC(3)-IV indicating that, in addition to the correctly placed aminoethyl chain, the hydroxymethyl ribose side chain is important in blocking the action of this AME. The positive influence of the hydroxymethyl group in **57 β** is presumably related to its

conformation-limiting hydrogen bond to N2' anticipated from structural studies of the 4,5-AGAs.^{61, 83, 97, 98} Whatever the reason for the loss of activity in the presence of AAC(3)-IV on going from the ribofuranoside **57β** to the corresponding erythrofuranoside **28**, it may be largely overcome by the replacement of the monobasic side chain in **28** by a dibasic one as in the erythrofuranosides **30-32**. All of the acyclic 5-*O*-alkyl ethers of apramycin fall victim to the AAC(3)-IV resistance determinant indicating that the conformational restriction and/or steric bulk of the furanose ring in the ribo and erythrofuranosyl series is an important factor in overcoming this class of AME. As expected on the basis of the resilience shown by apramycin itself, none of the derivatives studied suffered a significant loss of activity in the presence of the AAC(3)-IId isoform. As the antiribosomal activity of the 6-*O*-alkyl series of compounds (Table 3) and their MIC values against the standard panel of strains was disappointingly low, they were not profiled against the resistant strains.

Turning to the APH(3') AMEs, the 5-*O*-ribosylated derivatives of apramycin **55** and **57β** both showed a slight 2–4-fold reduction in activity in the presence of strains carrying APH(3')-I, reflecting the ability of this AME to phosphorylate the 5''-primary hydroxyl group. However, these compounds clearly are poor substrates as the reduction in activity does not match that seen with the 5-*O*-parombiosyl derivative **56** (8–16-fold) or with the classical substrate lividomycin B (>32-fold). Replacement of the 5''-hydroxy group in this series of compounds by an amino group restores full activity in the presence of APH(3')-I as indicated by **57β** and **62**. As expected, none of the 5-*O*-erythrosyl apramycin derivatives, **59**, and **28-32**, nor any of the apramycin 5-*O*-alkyl derivatives **63**, **64**, and **67-72**, experienced a notable loss of activity in the presence of this AME. None of the compounds tested showed any significant loss of activity in the presence of the APH(3')-IIa isoform when compared to the parent apramycin.

Overall, in the 5-*O*-β-D-ribofuranosyl series of compounds, derivatives **57β**, **61** and **62** are optimal showing little or no loss of activity in the presence of the AAC(3)-IV and APH(3')-Ia AMEs, while in the erythrofuranosyl series **30-32** show the most promise.

Finally, we assessed activity against a series of clinical *E. coli* isolates carrying two or more resistance determinants including the AAC(3)s, the APH(3')s, and the RMTs (Table 7). While the combination of AAC(3)-IV and APH(3')-I is detrimental to most compounds, it is noteworthy that **57β**, and **61** in the 5-*O*-ribosyl series are only affected to a minor extent by this combination. Similarly, compounds **30-32** in the erythrosyl series show only modest reductions in activity in the presence of this combination of AMEs. The majority of compounds is not affected by any of the various isoforms of AAC(3)-II, APH(3'), AAC(6') or by the ribosomal methyltransferase RmtB, even when present in combination.

Ex-vivo Ototoxicity Studies with Mouse Cochlear Explants

On the basis of target selectivity and antibacterial activity in the presence and absence of resistance determinants, one compound from each of the three distinct classes of apramycin derivatives, the 5-*O*-(5-amino-5-deoxyribosyl), 5-*O*-(3-*O*-aminoalkylerythrosyl) series, and the 5-*O*-(amino-hydroxypropyl) series, was selected for ex-vivo ototoxicity studies using the mouse cochlear explant model. Thus, explants from postnatal 3 day FVB mice⁴³ were

incubated with increasing concentrations of **30**, **61** and **69**, and of the controls apramycin and gentamicin, before staining with Alexa-594 phalloidin for actin and counting of outer hair cells. Plotting of dose response curves (Figure 5) then allowed the determination of the LD₅₀ values presented in Table 8 together with the antiribosomal activities of each compound for ease of comparison. Consistent with the earlier work and with their relative IC₅₀ values for the inhibition of mitochondrial and mutant mitochondrial hybrid ribosomes, apramycin was found to be significantly less cochleotoxic than the clinical benchmark gentamicin. The apramycin derivative **69** carrying a 3-amino-3-hydroxypropyl ether at the 5-position displayed cochleotoxicity similar to that of apramycin consistent with the similar IC₅₀ values of the two compounds for the mitochondrial ribosomes. Somewhat surprisingly in view of its relative IC₅₀ values the potent erythrosyl apramycin derivative **30** had LD₅₀ comparable to that of apramycin. Thus, while our results corroborate the general importance of selectivity for the mitochondrial ribosome with respect to ototoxicity, our data also indicate that this relationship may be non-linear. Most important, however, is the demonstration that the aminoribosyl derivative **61** exhibits an LD₅₀ significantly less than the parent, clearly demonstrating that it is possible to make a potent derivative of apramycin that displays even further reduced ototoxicity.

In-vivo Efficacy

Finally, we selected one compound, **57β**, to challenge the in-vitro MIC data for in-vivo efficacy. We chose compound **57β**, as this compound in-vitro is inherently more potent than apramycin and in addition shows resilience towards the action of AAC(3)-IV. We determined the in-vivo efficacy of **57β** in a mouse thigh infection model for *E. coli*. Compound **57β** displayed a significantly better clearance of the infection as compared to the parent apramycin. At a dose level of 6 mg/kg **57β** reduced the bacterial load by approximately 0.5 log₁₀ units more than the parent apramycin at the same dose, consistent with the relative MIC values of the two compounds for wild-type-*E. coli* (Figure 6). We note in passing that although the toxicity of **57β** was not investigated in the ex-vivo cochlear explant study, it is expected to exhibit comparable ototoxicity to **30** in view of the comparable structures and ribosomal selectivities (Table 4) of the two compounds.

Conclusion

We have prepared a number of modifications of apramycin by the installation of glycosides or ethers at the 5-position in such a manner as to make chimeras of apramycin and the 4,5-AGAs ribostamycin and paromomycin. The synthesis of these compounds was made possible by the development of short practical routes for the selective functionalization of the apramycin 5-hydroxy group. We show that modification in this manner can lead to apramycin derivatives with excellent target selectivity and exhibiting increased antibacterial activity for ESKAPE pathogens, particularly when the appendage contains one or more suitably placed basic amino groups. Two series of compounds based on the 5-*O*-(5-*O*-aminoalkyl-5-deoxy-β-D-ribofuranosyl)-apramycin and 5-*O*-(3-*O*-aminoalkyl-β-D-erythrofuranosyl)-apramycin motifs showed excellent across the board activity and in part resilience towards the AAC(3)-IV class of AMEs that is currently the only known resistant determinant operating on the parent compound. Exemplary compounds from both of these

series were also screened for ototoxic effects in the ex-vivo mouse cochlear explant model, where they displayed LD₅₀ values significantly less than the parent. Finally, one compound was selected for in-vivo efficacy studies revealing improved in-vivo efficacy over the parent in a mouse thigh infection model consistent with the relative MIC values. This study therefore demonstrates for the first time that it is possible to synthesize derivatives of apramycin that i) increase antibacterial activity in vitro and in-vivo against a wide range of Gram negative pathogens, ii) confer reduced susceptibility to the AAC(3)-IV resistance determinant, and iii) do so without giving rise to increased ototoxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Thompson RQ; Presti EA, Nebramycin, a new broad-spectrum antibiotic complex. III. Isolation and chemical-physical properties. *Antimicrob. Agent. Chemother* 1968, 332–340.
2. Stark WM; Hoehn MM, Nebramycin, a new broad-spectrum antibiotic complex. I. Detection and biosynthesis. *Antimicrob. Agent. Chemother* 1968, 314–323.
3. Wick WE; Welles JS, Nebramycin, a new broad-spectrum antibiotic complex. IV. In vitro and in vivo laboratory evaluation. *Antimicrob. Agent. Chemother* 1968, 341–348.
4. Ose EE (1975) Treatment of swine dysentery with apramycin. US Patent 3,876,767.
5. Dowling PM, Aminoglycosides and aminocyclitols In *Antimicrobial Therapy in Veterinary Medicine*, 5 ed.; Giguère S; Prescott JF; Dowling PM, Eds. Wiley-Blackwell: Chichester, 2013; pp 233–255.
6. O'Connor S; Lam LKT; Jones ND; Chaney MO, Apramycin, a unique aminocyclitol antibiotic. *J. Org. Chem* 1976, 41, 2087–2092. [PubMed: 932851]
7. Dorman DE; Paschal JW; Merkel KE, ¹⁵N nuclear magnetic resonance spectroscopy. The nebramycin aminoglycosides. *J. Am. Chem. Soc* 1976, 98, 6885–6888. [PubMed: 965656]
8. Jarrell HC; Szarek WA, Synthesis related to the octadiose in apramycin. Part iii. *Can. J. Chem* 1979, 57, 924–932.
9. Martin OR; Szarek WA, Approach to the pseudodisaccharides present in (oxy)apramycin. Synthesis of a 4-O-amino-octodiosyl-2-deoxystreptamine from paromamine J. *Chem. Soc., Chem. Commun* 1983, 926–928.
10. Canas R,A; Coronel-Borges LA, Synthesis of apramycin analogues. *Carbohydr. Res* 1987, 165, 129–133.
11. Canas R,A; Ruiz-Poveda SG; Coronel-Borges LA, Synthesis of apramycin analogues. *Carbohydr. Res* 1987, 159, 217–227.
12. Tatsuta K; Akimoto K; Takahashi H; Hamatsu T; Annaka M; Kinoshita M, Total synthesis of aminoglycoside antibiotics, apramycin and saccharocin (KA-5685). *Bull. Chem. Soc. Jpn* 1984, 57, 529–538.
13. Igarashi K; Honma T; Sugawara T (1982) 5-Deoxyapramycin. US Patent 4,358,585.
14. Igarashi K; Itami T; Honma T; Sugawara T (1983) Apramycin derivatives. US Patent 4,370,475.
15. Abe Y; Nakagawa S; Naito T; Kawaguchi H, Synthesis and activity of 6-O-(3-amino-3-deoxy- α -D-glucopyranosyl)- and 5-O-(β -D-ribofuranosyl)apramycins. *J. Antibiotics* 1981, 34, 1434–1446. [PubMed: 7319906]

16. Kirst HA (1984) 7-N-Substituted apramycin antibiotic derivatives and intermediates therefor. US Patent 4,458,065.
17. Kirst HA; Truedell BA (1984) 1-N-Acylated and 1-N-alkylated derivatives of 4-O-substituted-2-deoxystreptamine aminoglycosides. US Patent 4,468,512.
18. Kirst HA; Truedell BA (1984) 2'-N-Acylated and 2'-N-alkylated derivatives of 4-O-substituted 2-deoxystreptamine aminoglycosides. US Patent 4,468,513.
19. Kirst HA (1982) 4''-N-(Substituted)-apramycin antibiotic derivatives and intermediates therefor. US Patent 4,360,665.
20. Kirst HA (1983) 6''-(Substituted)-apramycin antibiotic derivatives and intermediates and starting materials therefor. US Patent 4,379,917.
21. Matt T; Ng CL; Lang K; Sha S-H; Akbergenov R; Shcherbakov D; Meyer M; Duscha S; Xie J; Dubbaka SR; Perez-Fernandez D; Vasella A; Ramakrishnan V; Schacht J; Böttger EC, Dissociation of antibacterial activity and aminoglycoside ototoxicity in the 4-monosubstituted 2-deoxystreptamine apramycin. *Proc. Natl. Acad. Sci., USA* 2012, 109, 10984–10989. [PubMed: 22699498]
22. Allen NE; Alborn WE; Kirst HA; Toth JE, Comparison of aminoglycoside antibiotics with respect to uptake and lethal activity in *Escherichia coli*. *J. Med. Chem* 1987, 30, 333–340. [PubMed: 3543366]
23. Igarashi K; Honma T (1982) Aprosamine derivatives. US Patent 4,362,866.
24. Awata M; Sato S; Muto N; Hayashi M; Sagai H; Sakakibara H, Saccharocin, a new aminoglycoside antibiotic. Fermentation, isolation, characterization and structural study. *J. Antibiotics* 1983, 36, 651–655.
25. Kamiya K; Deushi T; Iwasaki A; Watanabe I; Itoh H; Mori T, New aminoglycoside antibiotic KA-5685. *J. Antibiotics* 1983, 36, 738–741. [PubMed: 6874595]
26. Sarpe VA; Pirrone MG; Haldimann K; Hobbie SN; Vasella A; Crich D, Synthesis of saccharocin from apramycin and evaluation of its ribosomal selectivity. *Med. Chem. Commun* 2019, 10, 554–558.
27. Mandhapat AR; Shcherbakov D; Duscha S; Vasella A; Böttger EC; Crich D, Importance of the 6'-hydroxy group and its configuration for apramycin activity. *ChemMedChem* 2014, 9, 2074–2083. [PubMed: 25045149]
28. Takahashi Y; Umemura E; Ida T; Igarashi M; Nagai H (2017) Novel aminoglycoside antibiotic effective against multidrug-resistant bacteria and a pharmaceutical composition containing the same. WO Patent Appl 2017/018258 A1.
29. Ryden R; Moore BJ, The in vitro activity of apramycin, a new aminocyclitol antibiotic. *J. Antimicrob. Chemother* 1977, 3, 609–613. [PubMed: 340441]
30. Juhas M; Widlake E; Teo J; Huseby DL; Tyrrell JM; Polikanov Y; Ercan O; Petersson A; Cao S; Aboklaish AF; Rominski A; Crich D; Böttger EC; Walsh TR; Hughes DE; Hobbie SN, In-vitro activity of apramycin against multidrug-, carbapenem-, and aminoglycoside-resistant enterobacteriaceae and *Acinetobacter baumannii*. *J. Antimicrob. Chemother* 2019, 74, 944–952. [PubMed: 30629184]
31. Galani I; Nafplioti K; Chatzikonstantinou M; Giamarellou H; Souli M, Evaluation of apramycin activity against carbapenem-resistant enterobacteriaceae and *Acinetobacter baumannii*. *ECCMID* 2018, P 0096.
32. Smith KP; Kirby JE, Evaluation of apramycin activity against carbapenem-resistant and -susceptible strains of enterobacteriaceae. *Diagn. Microbiol. Infect. Dis* 2016, 86, 439–441. [PubMed: 27745736]
33. Magnet S; Blanchard JS, Molecular insights into aminoglycoside action and resistance. *Chem. Rev* 2005, 105, 477–497. [PubMed: 15700953]
34. Yang L; Ye XS, Development of aminoglycoside antibiotics effective against resistant bacterial strains. *Curr. Top. Med. Chem* 2010, 10, 1898–1926. [PubMed: 20615188]
35. Bacot-Davis VR; Bassenden AV; Berghuis AM, Drug-target networks in aminoglycoside resistance: Hierarchy of priority in structural drug design. *Med. Chem. Commun* 2016, 7, 103–113.

36. Garneau-Tsodikova S; Labby KJ, Mechanisms of resistance to aminoglycoside antibiotics: Overview and perspectives. *Med. Chem. Commun* 2016, 7, 11–27.
37. Zárate SG; De la Cruz Claire ML; Benito-Arenas R; Revuelta R; Santana AG; Bastida A, Overcoming aminoglycoside enzymatic resistance: Design of novel antibiotics and inhibitors. *Molecules* 2018, 23, 284, doi: 10.3390/molecules23020284.
38. Armstrong ES; Kostrub CF; Cass RT; Moser HE; Serio AW; Miller GH, Aminoglycosides In *Antibiotic Discovery and Development*, Dougherty TJ; Pucci MJ, Eds. Springer Science+Business Media: New York, 2012; pp 229–269.
39. Brennan-Krohn T; Kirby JE, Synergistic combinations and repurposed antibiotics active against the pandrug-resistant *Klebsiella pneumoniae* Nevada strain. *Antimicrob. Agent. Chemother* 2019, 63, e01374–19.
40. Doi Y; Wachino JI; Arakawa Y, Aminoglycoside resistance: The emergence of acquired 16S ribosomal RNA methyltransferases. *Infect. Dis. Clin. North Am* 2016, 30, 523–537. [PubMed: 27208771]
41. Livermore DM; Mushtaq S; Warner M; Zhang J-C; Maharjan S; Doumith M; Woodford N, Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant enterobacteriaceae isolates. *J. Antimicrob. Chemother* 2011, 66, 48–53. [PubMed: 21078604]
42. Cox G; Ejim L; Stogios PJ; Koteva K; Bordeleau E; Evdokimova E; Sieron AO; Savchenko A; Serio AW; Krause KM; Wright GD, Plazomicin retains antibiotic activity against most aminoglycoside modifying enzymes. *ACS Infect. Dis* 2018, 4, 980–987. [PubMed: 29634241]
43. Sonousi A; Sarpe VA; Brilkova M; Schacht J; Vasella A; Böttger EC; Crich D, Effects of the 1-N-(4-amino-2S-hydroxybutyryl) and 6'-N-(2-hydroxyethyl) substituents on ribosomal selectivity, cochleotoxicity and antibacterial activity in the sisomicin class of aminoglycoside antibiotics. *ACS Infect. Dis* 2018, 4, 1114–1120. [PubMed: 29708331]
44. Meyer M; Freihofer P; Scherman M; Teague J; Lenaerts A; Böttger EC, In-vivo efficacy of apramycin in murine infection models. *Antimicrob. Agents Chemother* 2014, 58, 6938–6941. [PubMed: 25136009]
45. Hu Y; Liu L; Zhang X; Feng Y; Zong Z, In vitro activity of neomycin, streptomycin, paromomycin and apramycin against carbapenem-resistant enterobacteriaceae clinical strains. *Front. Microbiol* 2017, 8, 2275, doi: 10.3389/fmicb.2017.02275.
46. Kang AD; Smith KP; Eliopoulos GM; Berg AH; McCoy C; Kirby JE, In vitro apramycin activity against multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Diagn. Microbiol. Infect. Dis* 2017, 88, 188–191. [PubMed: 28341099]
47. Holbrook SYL; Garneau-Tsodikova S, Evaluation of aminoglycoside and carbapenem resistance in a collection of drug-resistant *Pseudomonas aeruginosa* clinical isolates. *Microb. Drug Resist* 2018, 24, 1020–1030. [PubMed: 29261405]
48. Kang AD; Smith KP; Berg AH; Truelson KA; Eliopoulos GM; McCoy C; Kirby JE, Efficacy of apramycin against multidrug-resistant *Acinetobacter baumannii* in the murine neutropenic thigh model. *Antimicrob. Agent. Chemother* 2018, 62, e02585–17.
49. Truelson KA; Brennan-Krohn T; Smith KP; Kirby JE, Evaluation of apramycin activity against methicillin-resistant, methicillin-sensitive, and vancomycin-intermediate *Staphylococcus aureus* clinical isolates. *Diagn. Microbiol. Infect. Dis* 2018, 92, 168–171. [PubMed: 29934071]
50. Riedel S; Vijayakumar D; Berg G; Kang AD; Smith KP; Kirby JE, Evaluation of apramycin against spectinomycin-resistant and -susceptible strains of *Neisseria gonorrhoeae*. *J. Antimicrob. Chemother* 2019, 74, 1311–1316. [PubMed: 30689929]
51. Hobbie SN; Kalapala SK; Akshay S; Bruell C; Schmidt S; Dabow S; Vasella A; Sander P; Böttger EC, Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria. *Nucl. Acids Res* 2007, 35, 6086–6093. [PubMed: 17766247]
52. Hobbie SN; Akshay S; Kalapala SK; Bruell C; Shcherbakov D; Böttger EC, Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity. *Proc. Natl. Acad. Sci., USA* 2008, 105, 20888–20893. [PubMed: 19104050]
53. Qian Y; Guan M-X, Interaction of aminoglycosides with human mitochondrial 12S rRNA carrying the deafness-associated mutation. *Antimicrob. Agent. Chemother* 2009, 53, 4612–4618.

54. Ishikawa M; García-Mateo N; Usak A; López-Hernández I; Fernández-Martínez M; Müller M; Rüttiger L; Singer W; Löwenheim H; Kosec G; Fujs S; Martínez-Martínez I.; Schimmang T; Petkovi H; Knipper M; B. D-A, Lower ototoxicity and absence of hidden hearing loss point to gentamicin c1a and apramycin as promising antibiotics for clinical use *Sci. Rep* 2019, 9, 2410. [PubMed: 30787404]
55. Böttger EC; Schacht J, The mitochondrion: A perpetrator of acquired hearing loss. *Hearing Res* 2013, 303, 12–19.
56. Huth ME; Ricci AJ; Cheng AG, Mechanisms of aminoglycoside ototoxicity and targets of hair cell protection. *Int. J. Otolaryngol* 2011, 937861. [PubMed: 22121370]
57. Jiang M; Karasawa T; Steyger PS, Aminoglycoside-induced cochleotoxicity: A review. *Front. Cell. Neurosci* 2017, 11, 308; Doi: 10.3389/fncel.2017.00308. [PubMed: 29062271]
58. Tackling antimicrobial resistance: Enable selects first clinical candidate. <https://www.imi.europa.eu/projects-results/project-factsheets/enable>; accessed Feb 12, 2019.
59. Nafplioti K; Galani I; Adamou P; Moraitou E; Giannopoulou P; Chra P; Damala M; Vogiatzakis E; Triikka-Graphakos E; Baka V; Prifti E; Souli M, Epidemic dissemination of a carbapenem-resistant *Acinetobacter baumannii* clone carrying armA. *ECCMID P1105* 2018.
60. Carter AP; Clemons WM; Brodersen DE; Morgan-Warren RJ; Wimberly BT; Ramakrishnan V, Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* 2000, 407, 340–348. [PubMed: 11014183]
61. Vicens Q; Westhof E, Molecular recognition of aminoglycoside antibiotics by ribosomal RNA and resistance enzymes: An analysis of x-ray crystal structures. *Biopolymers* 2003, 70, 42–57. [PubMed: 12925992]
62. Han Q; Zhao Q; Fish S; Simonsen KB; Vourloumis D; Froelich JM; Wall D; Hermann T, Molecular recognition by glycoside pseudo base pairs and triples in an apramycin–RNA complex. *Angew. Chem. Int. Ed* 2005, 44, 2694–2700.
63. Balenci D; D’Amelio N; Gaggelli E; Gaggelli N; Cellai L; Molteni E; Valensin G, Structural features of apramycin bound at the bacterial ribosome A site as detected by NMR and CD spectroscopy. *ChemBioChem* 2010, 11, 166–169. [PubMed: 20024973]
64. Kondo J; François B; Urzhumtsev A; Westhof E, Crystal structure of the homo sapiens cytoplasmic ribosomal decoding site complexed with apramycin. *Angew. Chem. Int. Ed* 2006, 45, 3310–3314.
65. Tsai A; Uemura S; Johansson M; Puglisi EV; Marshall RA; Echeverría Aitken C; Korlach J; Ehrenberg M; Puglisi JD, The impact of aminoglycosides on the dynamics of translation elongation. *Cell Reports* 2013, 3, 497–508. [PubMed: 23416053]
66. Hermann T; Westhof E, Docking of cationic antibiotics to negatively charged pockets in RNA folds. *J. Med. Chem* 1999, 42, 1250–1261. [PubMed: 10197968]
67. Alper PB; Hung S-C; Wong C-H, Metal catalysed diazo transfer for the synthesis of azides from amines. *Tetrahedron Lett.* 1996, 37, 6029–6032.
68. Sati GC; Sarpe VA; Furukawa T; Mondal S; Mantovani M; Hobbie SN; Vasella A; Böttger EC; Crich D, Modification at the 2'-position of the 4,5-series of 2-deoxystreptamine aminoglycoside antibiotics to resist aminoglycoside modifying enzymes and increase ribosomal target selectivity. *ACS Infect. Dis* 2019, 5, 1718–1730. [PubMed: 31436080]
69. Hanessian S; Massé R; Ekborg G, Aminoglycoside antibiotics: The formation and characterization of dihydrooxazine derivatives in the paromomycin series. *Can. J. Chem* 1978, 56, 1492–1499.
70. Wu B; Yang J; He Y; Swayze EE, Reexamination of neomycin B degradation: Efficient preparation of its CD and D rings as protected glycosyl donors. *Org. Lett* 2002, 4, 3455–3458. [PubMed: 12323042]
71. Fridman M; Belakhov V; Lee LV; Liang F-S; Wong C-H; Baasov T, Dual effect of synthetic aminoglycosides: Antibacterial activity against *Bacillus anthracis* and inhibition of anthrax lethal factor. *Angew. Chem. Int. Ed* 2005, 44, 447–452.
72. Taha HA; Richards MR; Lowary TL, Conformational analysis of furanoside-containing mono- and oligosaccharides. *Chem. Rev* 2013, 113, 1851–1876. [PubMed: 23072490]
73. Van Rheen V; Cha DY; Hartley WM, Catalytic osmium tetroxide oxidation of olefins: cis-1,2-Cyclohexanediol. *Org. Syn. Coll. Vol* 1988, 6, 342–348.

74. Zhong Y-L; Shing TKM, Efficient and facile glycol cleavage oxidation using improved silica gel-supported sodium metaperiodate. *J. Org. Chem* 1997, 62, 2622–2624. [PubMed: 11671605]
75. Borch RF, Reductive amination with sodium cyanoborohydride. *Org. Synth. Coll. Vol* 1988, 6, 499–501.
76. Perez-Fernandez D; Shcherbakov D; Matt T; Leong NC; Kudyba I; Duscha S; Boukari H; Patak R; Dubbaka SR; Lang K; Meyer M; Akbergenov R; Freihofer P; Vaddi S; Thommes P; Ramakrishnan V; Vasella A; Böttger EC, 4'-O-substitutions determine aminoglycoside selectivity at the drug target level. *Nature Commun.* 2014, 5, 3112/doi: 10.1038/ncomms4112. [PubMed: 24473108]
77. Hobbie SN; Bruell CM; Akshay S; Kalapala SK; Shcherbakov D; Böttger EC, Mitochondrial deafness alleles confer misreading of the genetic code. *Proc. Natl. Acad. Sci., USA* 2008, 105, 3244–3249. [PubMed: 18308926]
78. Hobbie SN; Kaiser M; Schmidt S; Shcherbakov D; Janusic T; Brun R; Böttger EC, Genetic reconstruction of protozoan rRNA decoding sites provides a rationale for paromomycin activity against leishmania and trypanosoma. *PLoS Negl. Trop. Dis* 2011, 5, e1161. [PubMed: 21629725]
79. Henley CM; Schacht J, Pharmacokinetics of aminoglycoside antibiotics in blood, inner-ear fluids and tissues and their relationship to ototoxicity. *Audiology* 1988, 27, 137–146. [PubMed: 3044302]
80. Prezant TR; Agapian JV; Bohlman MC; Bu X; Öztas S; Qiu W-Q; Arnos KS; Cortopassi GA; Jaber L; Rotter JJ; Shohat M; Fischel-Ghodsian N, Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. *Nat. Genetics* 1993, 4, 289–294. [PubMed: 7689389]
81. Duscha S; Boukari H; Shcherbakov D; Salian S; Silva S; Kendall A; Kato T; Akbergenov R; Perez-Fernandez D; Bernet B; Vaddi S; Thommes P; Schacht J; Crich D; Vasella A; Böttger EC, Identification and evaluation of improved 4'-O-(alkyl) 4,5-disubstituted 2-deoxystreptamines as next generation aminoglycoside antibiotics. *mBio* 2014, 5, 10.1128/mBio.01827-14.
82. Matsushita T; Sati GC; Kondasinghe N; Pirrone MG; Kato T; Waduge P; Kumar HS; Cortes Sanchon A; Dobosz-Bartoszek M; Shcherbakov D; Juhas M; Hobbie SN; Schrepfer T; Chow CS; Polikanov YS; Schacht J; Vasella A; Böttger EC; Crich D, Design, multigram synthesis, and in vitro and in vivo evaluation of propylamycin: A semisynthetic 4,5-deoxystreptamine class aminoglycoside for the treatment of drug-resistant enterobacteriaceae and other Gram-negative pathogens. *J. Am. Chem. Soc* 2019, 141, 5051–5061. [PubMed: 30793894]
83. Herzog IM; Louzoun Zada S; Fridman M, Effects of 5-O-ribosylation of aminoglycosides on antimicrobial activity and selective perturbation of bacterial translation. *J. Med. Chem* 2016, 59, 8008–8018. [PubMed: 27509271]
84. Sabbavarapu NM; Pienko T; Zalman B-H; Trylska J; Baasov T, Exploring eukaryotic versus prokaryotic ribosomal RNA recognition with aminoglycoside derivatives. *Med. Chem. Commun* 2018, 9, 503–508.
85. Morgenthaler M; Schweizer E; Hoffmann-Roder A; Benini F; Martin RE; Jaeschke G; Wagner B; Fischer H; Bendels S; Zimmerli D; Schneider J; Diederich F; Kansy M; Muller K, Predicting and tuning physicochemical properties in lead optimization: Amine basicities. *ChemMedChem* 2007, 2, 1100–1115. [PubMed: 17530727]
86. Vicens Q; Westhof E, Crystal structure of paromomycin docked into the eubacterial ribosomal decoding A site. *Structure* 2001, 9, 647–658. [PubMed: 11587639]
87. François B; Russell RJM; Murray JB; Aboul-ela F; Masquid B; Vicens Q; Westhof E, Crystal structures of complexes between aminoglycosides and decoding A site oligonucleotides: Role of the number of rings and positive charges in the specific binding leading to miscoding. *Nucleic Acids Res.* 2005, 33, 5677–5690. [PubMed: 16214802]
88. Davies J; O'Connor S, Enzymatic modification of aminoglycoside antibiotics: 3-N-acetyltransferase with broad specificity that determines resistance to the novel aminoglycoside apramycin. *Antimicrob. Agent. Chemother* 1978, 14, 69–72.
89. Shaw KJ; Rather PN; Hare RS; Miller GH, Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol. Rev* 1993, 57, 138–163. [PubMed: 8385262]

90. Trieu-Cuot P; Courvalin P, Nucleotide sequence of the *Streptococcus faecalis* plasmid gene encoding the 3'5"-aminoglycoside phosphotransferase type iii. *Gene* 1983, 23, 331–341. [PubMed: 6313476]
91. Courvalin P; Davies J, Plasmid-mediated aminoglycoside phosphotransferase of broad substrate range that phosphorylates amikacin. *Antimicrob. Agents Chemother* 1977, 11, 619–624. [PubMed: 856013]
92. Thompson PR; Hughes DW; Wright GD, Regiospecificity of aminoglycoside phosphotransferase from enterococci and staphylococci (APH(3')-IIIa). *Biochemistry* 1996, 35, 8686–8695. [PubMed: 8679631]
93. McKay GA; Thompson PR; Wright GD, Broad spectrum aminoglycoside phosphotransferase type III from enterococcus: Overexpression, purification, and substrate specificity. *Biochemistry* 1994, 33, 6936–6944. [PubMed: 8204627]
94. Thompson PR; Schwartzenhauer J; Hughes DW; Berghuis AM; Wright GD, The COOH terminus of aminoglycoside phosphotransferase (3')-IIIa is critical for antibiotic recognition and resistance. *J. Biol. Chem* 1999, 274, 30697–30706. [PubMed: 10521458]
95. Hon W-C; McKay GA; Thompson PR; Sweet RM; Yang DSC; Wright GD; Berghuis AM, Structure of an enzyme required for aminoglycoside antibiotic resistance reveals homology to eukaryotic protein kinases. *Cell* 1997, 89, 887–895. [PubMed: 9200607]
96. Wright GD; Thompson PR, Aminoglycoside phosphotransferases: Proteins, structure, and mechanism. *Front. Biosci* 1999, 4, d9–21. [PubMed: 9872733]
97. Revuelta J; Vacas T; Bastida A; Asensio JL, Structure-based design of highly crowded ribostamycin/kanamycin hybrids as a new family of antibiotics. *Chem. Eur. J* 2010, 16, 2986–2991. [PubMed: 20162651]
98. Corzana F; Cuesta I; Freire F; Revuelta J; Torrado M; Bastida A; Jiménez-Barbero J; Asensio JL, The pattern of distribution of amino groups modulates the structure and dynamics of natural aminoglycosides: Implications for RNA recognition. *J. Am. Chem. Soc* 2007, 129, 2849–2865. [PubMed: 17298063]

Figure 1.
Structure of apramycin

Figure 2.
 Structures of paromomycin, the gentamicins, and apramycin derivatives **4** and **5**

Figure 3.

Decoding A sites of prokaryotic and eukaryotic ribosomes. The bacterial AGA binding pocket is boxed. The bacterial numbering scheme is illustrated for the AGA binding pocket. Changes from the bacterial ribosome binding pocket are coloured green. The A1555G mutant conferring hypersusceptibility to AGA ototoxicity is coloured red.

Figure 4.
 5-*O*-Ribofuranosyl, 5-*O*-Erythrfuranosyl and 5-*O*-Alkyl Series of Apramycin Derivatives
 Studied, and the Comparators Lividomycin B and Ribostamycin.

Figure 5.

Dose response plots of the percentage of outer hair cell loss (OHC) versus concentration of aminoglycoside. Data are presented as means $\pm$ σ , $n = 5-8$ per point. GM = gentamicin, APR = apramycin.

Figure 6.

In-vivo efficacy of **57β** in comparison to apramycin **1** in a neutropenic mouse thigh infection model with a dose of 6 mg.kg⁻¹. Statistical significance was determined by an unpaired t test as P = 0.058 (ns, not significant) for **1** and P = 0.007 (**) for **57β**.

Scheme 1. Preparation of the Selectively Protected Apramycin Derivatives **7** and **8**.

Scheme 2.

Alkylation and Subsequent Derivatization of Apramycin at the 6-Position.

Scheme 3.

Partial Benzylation of Apramycin Giving Rise to 5-O-Alkyl Derivatives.

Scheme 4.
Preparation of 5-*O*-alkyl apramycin derivatives.

Scheme 5.
Alternative Preparation of *S*-45.

Table 1.

Glycosylation Reactions^{a,b}

<p>7: X = Ac, 8: X = Bz</p> <p>R = H $\xrightarrow{\text{Glycosylation}}$ R = glycosyl</p>			
donor	conditions	R, yield, selectivity ($\beta:\alpha$)	¹³ C NMR δ C1 ^c
<p>9</p>	BF ₃ ·OEt ₂ , 0 °C, 4 h	<p>18, 95%, $\beta:\alpha = 9:1$</p>	106.1
<p>10</p>	Tf ₂ O, rt, 6 h	<p>19, 48% β only</p>	106.8
<p>11</p>	BF ₃ ·OEt ₂ , 0 °C, 40 h	<p>20, 43% $\beta:\alpha = 1:1.15$</p>	α : 102.8 β : 106.9

donor	conditions	R, yield, selectivity ($\beta:\alpha$)	^{13}C NMR δ C1''
<p>12</p>	$\text{BF}_3 \cdot \text{OEt}_2$, 0 °C, 40 h	<p>21, 25% $\beta:\alpha = 1:1$</p>	α : 102.9 β : 107.2
<p>13</p>	$\text{BF}_3 \cdot \text{OEt}_2$, -78 °C, 4 h	<p>22, 50% β only</p>	106.4
<p>14^b</p>	$\text{BF}_3 \cdot \text{OEt}_2$, 0 °C, 2 h	<p>23,^b 34% β only</p>	107.1
<p>15</p>	NIS, AgOTf -20 – 0 °C, 6 h	<p>24, 78% $\beta:\alpha = 1:0.6$</p>	α : 102.5 β : 106.7

donor	conditions	R, yield, selectivity ($\beta:\alpha$)	^{13}C NMR δ C1 ^a
<p>16</p>	BF ₃ ·OEt ₂ , -30 °C, 6 h	<p>25, 93% β only</p>	106.9
<p>17</p>	BF ₃ ·OEt ₂ , -30 °C, 6 h	<p>26, 94% β only</p>	107.0

^a) Unless otherwise noted the tetraacetate **7** was employed as glycosyl acceptor;

^b) In this example the tetrabenzoate **8** was employed as glycosyl acceptor.

Table 2.

Post-Glycosylation Modifications and Deprotection of Erythrosyl Apramycin Derivatives

amine	product	yield from 27
BnNH ₂	28	18%
H ₂ NCH ₂ CH ₂ OH	29	3%
H ₂ NCH ₂ CH ₂ NH ₂	30	18%
H ₂ NCH ₂ CH ₂ CH ₂ NHCbz	31	3%
H ₂ NCH ₂ CH ₂ CH ₂ NMe ₂	32	8%

Table 3.

Deprotection of Apramycin 5- and 6-*O*-Derivatives

subs	product, yield	subs	product, yield
18	 55 (=5), 69%	49	 65, 12%
19	 56, 35%	43	 66, 14%
20 α	 57a, 85%	50	 67, 36%
20 β	 57b, 95%	41	 68, 19%
21		46	 69, 11%
22	 59, 71%	47	 70, 18%

^{a)} X = R' = Ac, R = glycoside: (i) NaOH or Ba(OH)₂; (ii) PMe₃, NaOH or H₂, Pd/C; (iii) AcOH;

^{b)} X = R' = Bn, R = alkyl: (i) H₂, Pd(OH)₂; (ii) Ba(OH)₂; (iii) AcOH; c) X = R = Ac, R' = alkyl: (i) NaOH; (ii) H₂, Pd/C; (iii) AcOH

Table 4.Antiribosomal Activities and Selectivities (IC₅₀, μ M).^{a,b}

	Antiribosomal Activity				Selectivity		
	wt	Mit13	A1555G	Cyt14	Mit13	A1555G	Cyt14
Apramycin	0.11±0.02	127±29	134±42	130±3.5	1123	1186	1155
Ribosyl series							
55	0.16	439	272	475	2815	1745	3045
56	0.12	2.7	0.49	22	22	4	184
57a	0.35	192	285	257	549	814	735
57b	0.08	79	30	253	994	288	3173
58	0.31	904	456	1227	2940	1483	3992
60	0.12	113	109	81	949	916	679
61	0.046±0.008	191±67	150±60	133±38	4152	3261	2898
62	0.09	80	72	61	917	820	696
Erythrosyl series							
59	0.11	329	208	474	2931	1857	4230
28	0.02	90	12	194	4471	573	9646
29	0.14	180	65	495	1285	464	3536
30	0.030±0.006	30±0.5	11.9±3.6	72±14	1010	397	2400
31	0.07	46	7.4	204	657	106	2914
32	0.05	21	4.8	95	403	92	1827
5-O-alkyl series							
63	1.8	nd	nd	nd	nd	nd	nd
64	0.35	299	nd	nd	755	nd	nd
65	0.60	2393	nd	nd	3998	nd	nd
66	0.21	434	375	nd	2077	1798	nd
67	0.11	99	119	100	906	1084	914
68	0.21	327	236	210	1553	1120	998
69	0.040±0.013	124±32	86±23	152±46	3100	2155	3813
70	0.10	158	145	112	1565	1428	1110
71	0.10	103	132	91	1053	1357	938
72	0.07	86	85	63	1235	1227	902
6-O-alkyl series							
73	2.50	449	829	646	179	331	258
74	0.32	650	396	655	2055	1254	2071
75	0.57	403	566	463	707	992	812

^{a)} All values are single point determinations. For apramycin, **30**, **61**, and **69** the mean and standard deviation of 3–5 determinations were assessed as these compounds were selected for further ototoxicity studies.

^{b)} Selectivities are obtained by dividing the eukaryotic by the bacterial values.

Table 5.

Antibacterial Activities (MIC, $\mu\text{g/mL}$)^a

Species	<i>E. coli</i>	<i>K. pneu.</i>	<i>Enterob.</i>	<i>A. baum.</i>	<i>P. aerug.</i>	MRSA	MRSA
Resistance det	WT	WT	WT	WT	WT	WT	APH(2') ANT(4')-I AAC(6')-I
Strain	AG001	AG215	AG290	AG225	AG220	AG038	AG042
Apramycin	4	1–2	2–4	4	4	4	8
Ribosyl series							
55	4	2	4	16	64	64	64
56	2	1	2	4	16	2–4	2
57 α	16	4	16	16	64	16	16
57 β	2–4	1–2	1–2	4–8	16–32	2–4	8
58	32	4	8	16	>64	32	32
60	4	1–2	1–2	4–8	4	2	nd
61	2	1	1–2	4–8	4–8	4	8
62	2–4	2	4	4	2–4	2	2
Erythrosyl series							
59	8–16	4	4	8	32	16–32	16
28	1–2	0.5	2–4	4	16	2	4–8
29	2–4	2	4	8	32	8	8–16
30	1–2	1	2–4	4–8	4–8	1–2	4
31	2	1	1–2	4	8	2	4
32	1–2	0.5	1	4	16	2	2–4
5-O-alkyl series							
63	32	16	64–128	64	64	32–64	64
64	8	2–4	16	16	16–32	8–16	8–16
65	16	8	16	32	64	32	16–32
66	8	4	4	16	16	8–16	nd
67	4	1–2	4–8	4	4	2	2
68	8	2	4–8	8	8	4	2–4
69	2	1	1	4	2–4	1–2	2–4
70	4–8	2	4	4	4	4	2
71	4–8	2–4	8–16	8	1	4	2–4
72	4	2	2–4	4	2	4	4
6-O-alkyl series							
73	64–128	nd	nd	nd	nd	nd	64–128
74	16	nd	nd	nd	nd	nd	64
75	32	nd	nd	nd	nd	nd	64

^aAll values were determined in duplicate using twofold dilution series

Table 6.Antibacterial Activities (MIC, mg/L) Against *E. coli* Strains with Acquired AAC(3) and APH(3') Resistance^a

Resistance Determinant	WT	AAC(3)-IIId	AAC(3)-IV	APH(3')-I	APH(3')-IIa
Strain	AG001	AG170	AG173	AG164	AG166
Apramycin	4	4–8	128–256	4–8	4–8
Ribostamycin	4–8	4–8	4–8	>256	>256
Paromomycin	2	4	2–4	>256	>256
Lividomycin B	4–8	4–8	16	>256	4–8
Ribosyl series					
55	4	8	16–32	8–16	8
56	2	4–8	2	8–16	2–4
57a	16	32	128	16	16
57b	2–4	2	4	4	2
58	16	16	32–64	8–16	8
60	4	nd	nd	nd	nd
61	2	2	16	2	4
62	2–4	4	16	2–4	2–4
Erythrosyl series					
59	8–16	16	128–256	8	8
28	1–2	1	16–32	1	2
29	2–4	4	64	4–8	4
30	1–2	4	8	1–2	2
31	2	2–4	4	1–2	4
32	1–2	1	4–8	1–2	2–4
5-O-alkyl series					
63	32	64	>128	32	32
64	8	8	128	8	4–8
65	16	16	>128	16	16
66	8	nd	>64	nd	nd
67	4	2	128	2	2–4
68	8	4	128	4	2–4
69	2	4	16–32	2	4
70	4–8	4	64	4	4
71	4–8	4	64	4	4
72	4	4	32	nd	8

^a) All values were determined in duplicate using twofold dilution series

Table 7.

Antibacterial Activities (MIC, mg/L) Against *E. coli* Strains with Two or More Acquired Resistance Determinants.^a

Resistance Det	AAC(3)-IV, APH(3')-I	AAC(3)-IV, APH(3')-I	AAC(3)-IV, APH(3')-I	AAC(6')-Ib, AAC(3)-IId	APH(3')-Ia, AAC(3)-IId	AAC(3)-IIa, AAC(6')-Ib, RmtB	AAC(3)-IIa, APH(3')-IIa, RmtB	AAC(3)-II, APH(3')-II, RmtB
Strain	AG182	AG183	AG184	AG157	AG180	AG341	AG386	AG153
Apramycin	>256	>256	>256	8	4	2-4	8	8-16
Ribosyl series								
55	64	64	64	8	16	8	16	8
56	64	16-32	32-64	2	8	1-2	2-4	4
57a	>128	>128	>128	16	16	16	32	8
57b	8-16	4-8	4	4	4	1-2	nd	2-4
58	>128	>128	>128	16	32	16	32	8-16
60	nd	nd	nd	nd	nd	nd	nd	8
61	32	16-32	32	4	4	1-2	8	8
62	32-64	32	64	2	4-8	4	8	8
Erythrosyl series								
59	>128	>128	>128	8	32	8	16	4-8
28	32-64	32	nd	2	2-4	2	2	4-8
29	128-256	128	>64	nd	8	2-4	8	32
30	16	16	32	2	2	2-4	4-8	2-4
31	16	8	16	4	8-16	2	2	4
32	8	16	8	2	2-4	1	2-4	4
5-O-alkyl series								
63	>64	>64	>64	32-64	64	32	64	>64
64	>64	>64	>64	8	8	8	32	64
65	>128	>64	>64	32	16	16	16	8
66	nd	nd	nd	nd	nd	nd	nd	8
67	128-256	>128	128-256	4	8	4	8	nd
68	>128	>64	>64	8	4	16	32	32
69	64	32-64	32	4	4	4	8	nd

Resistance Det	AAC(3)-IV, APH(3')-I	AAC(3)-IV, APH(3')-I	AAC(3)-IV, APH(3')-I	AAC(6')-Ib, AAC(3)-IIId	APH(3')-Ia, AAC(3)-IIId	AAC(3)-IIa, AAC(6')-Ib, RmtB	AAC(3)-IIa, APH(3')-IIa, RmtB	AAC(3)-II, APH(3')-II, RmtB
Strain	AG182	AG183	AG184	AG157	AG180	AG341	AG386	AG153
70	>128	>64	>64	8	8	16	32	32
71	128–256	>64	>64	8	4	8	8–16	8
72	64	64	>64	nd	nd	nd	nd	4–8

^{a)} All values were determined in duplicate using twofold dilution series

Table 8.Ex-vivo Ototoxicity (LD₅₀, μ M) and Antiribosomal Activity (μ M) and Selectivity^a

	LD ₅₀	Antiribosomal Activity			
		WT	Mit13	1555G	Cyt14
Apramycin, 1	59 $\pm$ 3	0.113 $\pm$ 0.019	127 $\pm$ 29	134 $\pm$ 42	130 $\pm$ 3.5
Gentamicin, 3	6 $\pm$ 4	0.028 $\pm$ 0.003	17 $\pm$ 2	0.95 $\pm$ 1.5	58
30	115 $\pm$ 7	0.030 $\pm$ 0.006	30 $\pm$ 0.5	11.9 $\pm$ 3.6	72 $\pm$ 14
61	182 $\pm$ 4	0.046 $\pm$ 0.008	191 $\pm$ 67	150 $\pm$ 60	133 $\pm$ 38
69	56 $\pm$ 5	0.040 $\pm$ 0.013	124 $\pm$ 32	86 $\pm$ 23	152 $\pm$ 46

^a) Data are presented as means $\pm$ σ