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Apralogs: Apramycin 5-*O*-Glycosides and Ethers with Improved Antibacterial Activity and Ribosomal Selectivity and Reduced Susceptibility to the Aminoacyltranserferase (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 ototoxocity in animal models combine to make apramycin an excellent starting point for the development of next-generation aminoglycoside antibiotics for the treatment 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-eryrthofuranosides 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 bacterial 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.

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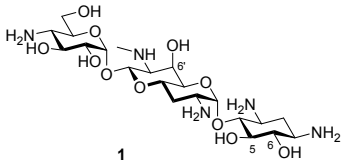
Introduction

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]



1

Figure 1. Structure of apramycin

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]

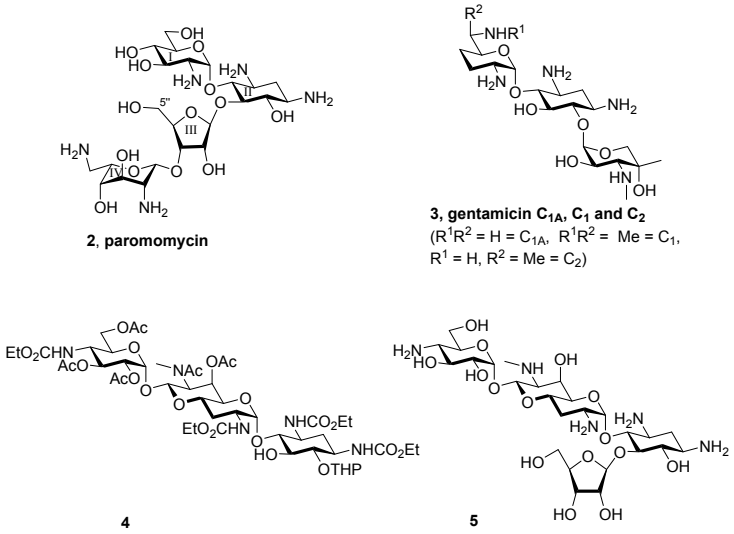
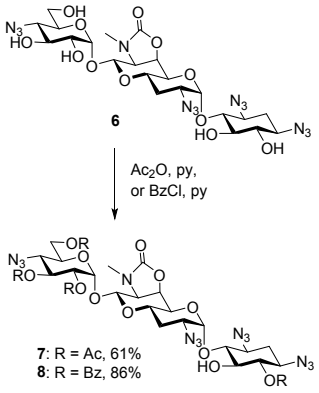


Figure 2. Structures of paromomycin, the gentamicins, and apramycin derivatives **4** and **5**

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 5,2'',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**

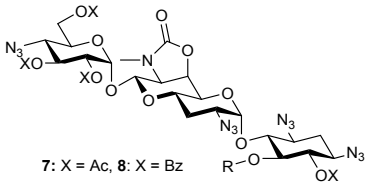
in 86 % yield. These efficient four-step preparations of selectively protected apramycin mono-ols for use in functionalization at the 5-position compare favorably to the methods employed in the synthesis of **4** by Abe and coworkers.[15]

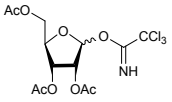
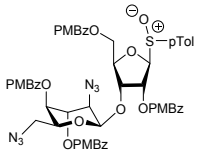
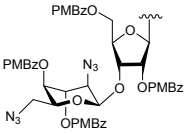
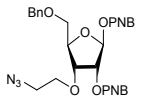
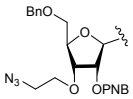
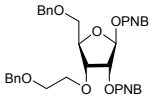
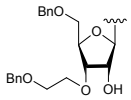
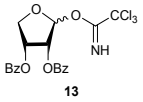
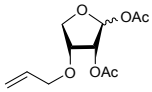
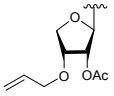
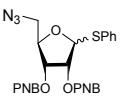
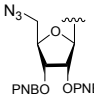
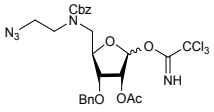
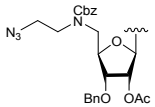
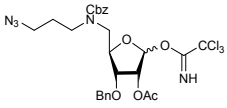
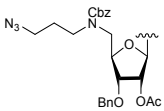


Scheme 1. Preparation of the Selectively Protected Apramycin Derivatives **7** and **8**.

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 ^{13}C NMR spectra consistent with established rules (Table 1).[72]

Table 1. Glycosylation Reactions[a,b]

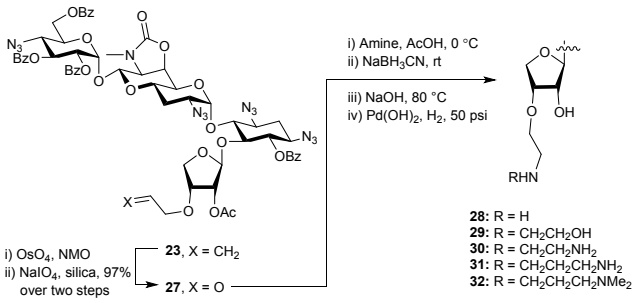


Donor	Conditions	R, Yield, Selectivity (β:α)	^{13}C NMR δ C1'''
 **9**	$BF_3.OEt_2$, 0 ºC, 4 h	 **18**, 95%, β:α = 9:1	106.1
 **10**	Tf_2O, rt, 6 h	 **19,** 48% β only	106.8
 **11**	$BF_3.OEt_2$, 0 ºC, 40 h	 **20,** 43% β:α = 1:1.15	α: 102.8 β: 106.9
 **12**	$BF_3.OEt_2$, 0 ºC, 40 h	 **21,** 25% β:α = 1:1	α: 102.9 β: 107.2
 **13**	$BF_3.OEt_2$, -78 ºC, 4 h	 **22,** 50% β only	106.4
 **14**[b]	$BF_3.OEt_2$, 0 ºC, 2 h	 **23,**[b] 34% β only	107.1
 **15**	NIS, AgOTf -20 - 0 ºC, 6 h	 **24,** 78% β:α = 1:0.6	α: 102.5 β: 106.7
 **16**	$BF_3.OEt_2$, -30 ºC, 6 h	 **25,** 93% β only	106.9
 **17**	$BF_3.OEt_2$, -30 ºC, 6 h	 **26,** 94% β only	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 donor.

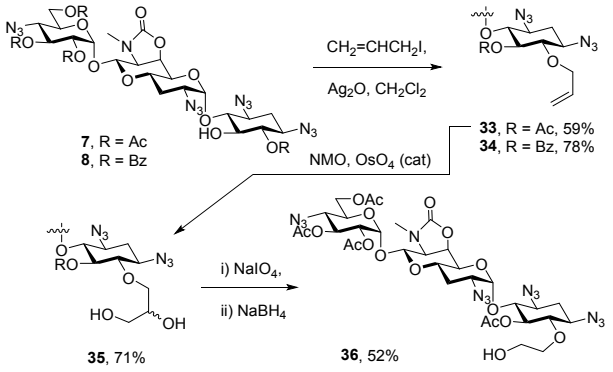
The allyl ether of glycoside **23** was further derivatized by treatment with catalytic osmium tetroxide in the presence of *N*-methyl morpholine *N*-oxide (NMO) according to the Van Rheenan protocol[73] to give the corresponding diol as a mixture of diastereomers. Oxidative cleavage with sodium metaperiodate on silica gel[74] then afforded the corresponding aldehyde **27** in 97% yield over two steps that was immediately subjected to reductive amination in the presence of sodium cyanoborohydride[75] 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).

Table 2. Post-Glycosylation Modifications and Deprotection of Erythrosyl Apramycin Derivatives



Amine	Product	Yield from 27
$BnNH_2$	**28**	18%
$H_2NCH_2CH_2OH$	**29**	3%
$H_2NCH_2CH_2NH_2$	**30**	18%
$H_2NCH_2CH_2CH_2NHCbz$	**31**	3%
$H_2NCH_2CH_2CH_2NMe_2$	**32**	8%

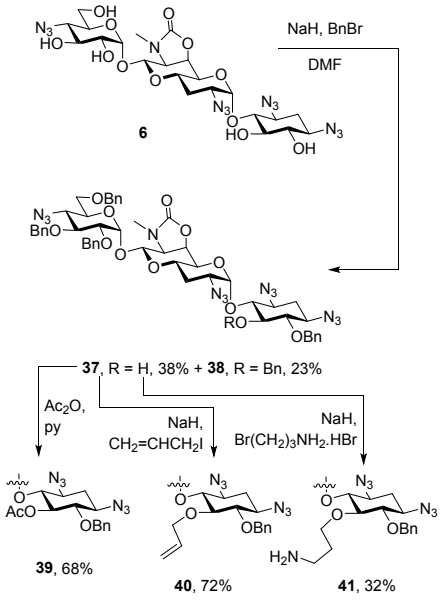
Initial attempts to prepare a series of acyclic variants of the above 5-*O*-glycosyl apramycin derivatives were thwarted by migration of the ester between the 5- and 6-positions under a variety of conditions for the installation of an allyl ether, resulting in complex reaction mixtures. The optimum conditions 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.



Scheme 2. Allylation and Subsequent Derivatization of Apramycin at the 6-Position.

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 4,2'',3'',6''-tetra-*O*-benzyl ether **37** could be isolated in 38% yield along with 23% of the

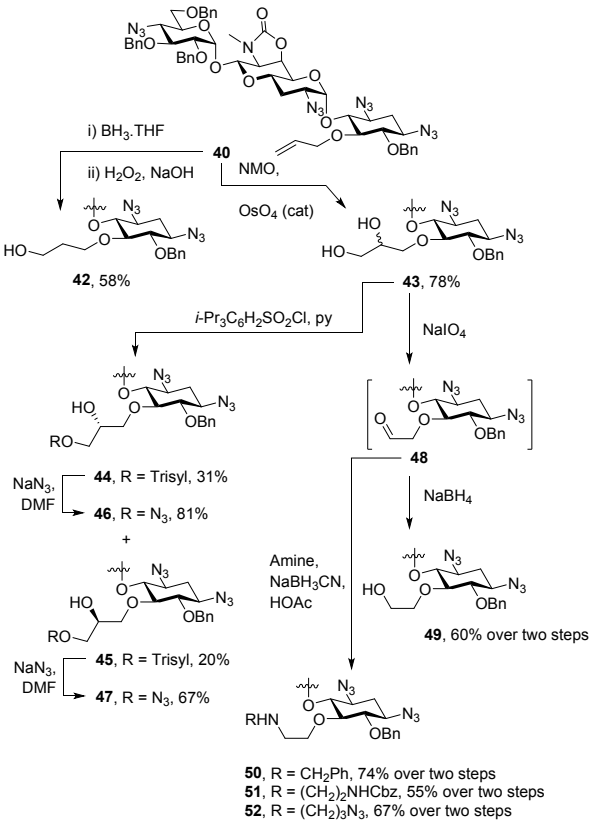
perbenzyl ether **38** (Scheme 3). The location of the free-alcohol in **37** was readily apparent following acetylation to give **39** and inspection of its 1 and 2D NMR spectra. Allylation of **37** with sodium hydride and allyl iodide then provided the 5-*O*-allyl ether **40**, while treatment with sodium hydride and 3-bromopropylamine hydrogen bromide gave the aminopropyl ether **41**, in 72 and 32% yield, respectively (Scheme 3).



Scheme 3. Partial Benzylation of Apramycin Giving Rise to 5-*O*-Alkyl Derivatives.

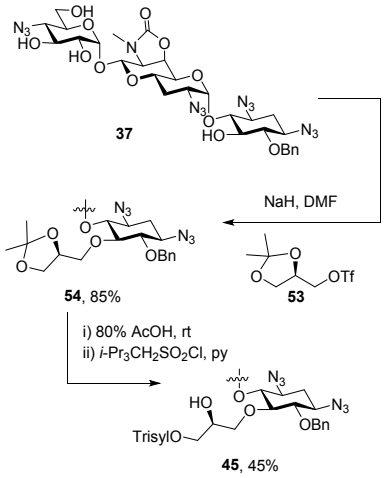
Hydroboration of **40** with oxidative workup gave the 3-hydroxypropyl ether **42** in 58% yield, while reaction with NMO and catalytic osmium tetroxide afforded an inseparable mixture of diastereomeric diols **43** in 78% yield. This mixture of diols was treated with 2,4,6-triisopropylbenzenesulfonyl chloride in pyridine to give the corresponding chromatographically separable sulfonates **44** and **45** in 66% 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).



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

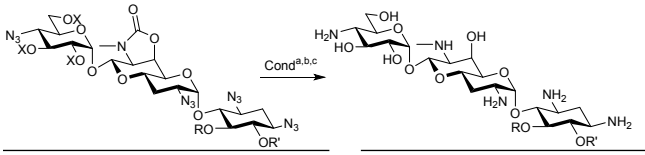
Finally, in order to assign the configuration of the diastereomeric azido alcohols alcohols **46** and **47**, and by extrapolation the precursor diols **44** and **45**, alcohol **37** was alkylated with the enantiomerically pure triflate **53**, obtained by triflation of commercial *S*-isopropylidene 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.



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

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).

Table 3. Deprotection of Apramycin 5- and 6-*O*-Derivatives



Subs	Product, Yield	Subs	Product, Yield
18	**55,** 69%	**49**	**65**, 12%
19	**56,** 35%	**43**	**66**, 14%
20α	**57α,** 85%	**50**	**67,** 36%
20β	**57β**, 95%	**41**	**68,** 19%
21	**58,** 48%	**46**	**69,** 11%
22	**59,** 71%	**47**	**70**, 18%
24	**60,** 24%	**51**	**71,** 12%
25	**61,** 66%	**52**	**72,** 7%
26	**62,** 72%	**33**	**73,** 75%
40	**63,** 20%	**36**	**74,** 63%
42	**64,** 10%	**35**	**75,** 39%

a) X = R' = Ac, R = glycoside: (i) NaOH or $Ba(OH)_2$; (ii) PMe_3, NaOH or H_2,Pd/C; (iii) AcOH; b) X = R' = Bn, R = alkyl: (i) H_2, $Pd(OH)_2$; (ii) $Ba(OH)_2$; (iii) AcOH; c) X = R = Ac, R' = alkyl: (i) NaOH; (ii) H_2, Pd/C; (iii) AcOH

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; erythrofuranosyl; 5-*O*-alkyl; and 6-*O*-alkyl (Figure 4).

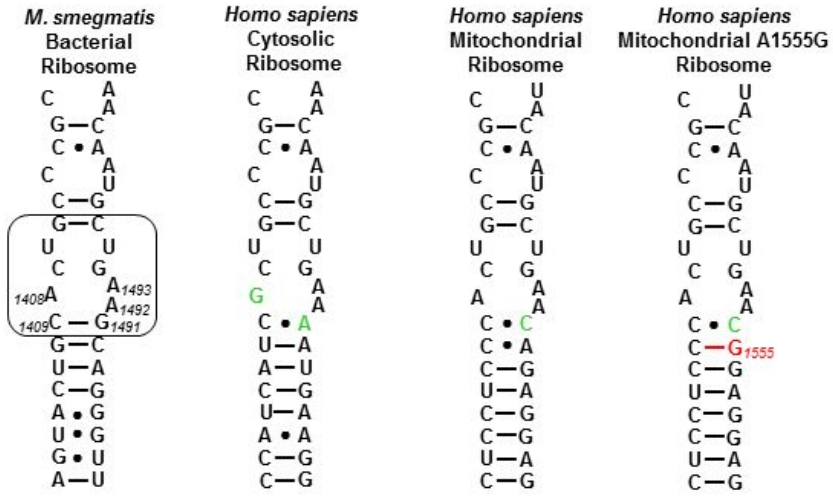


Figure 3. Decoding A sites of prokaryotic and eukaryotic ribosomes. The bacterial AGA binding pocket is boxed. The bacterial numbering scheme is illustrated for the AGA binding pocket. Changes from the bacterial ribosome binding pocket are coloured green. The A1555G mutant conferring hypersusceptibility to AGA ototoxicity is coloured red.

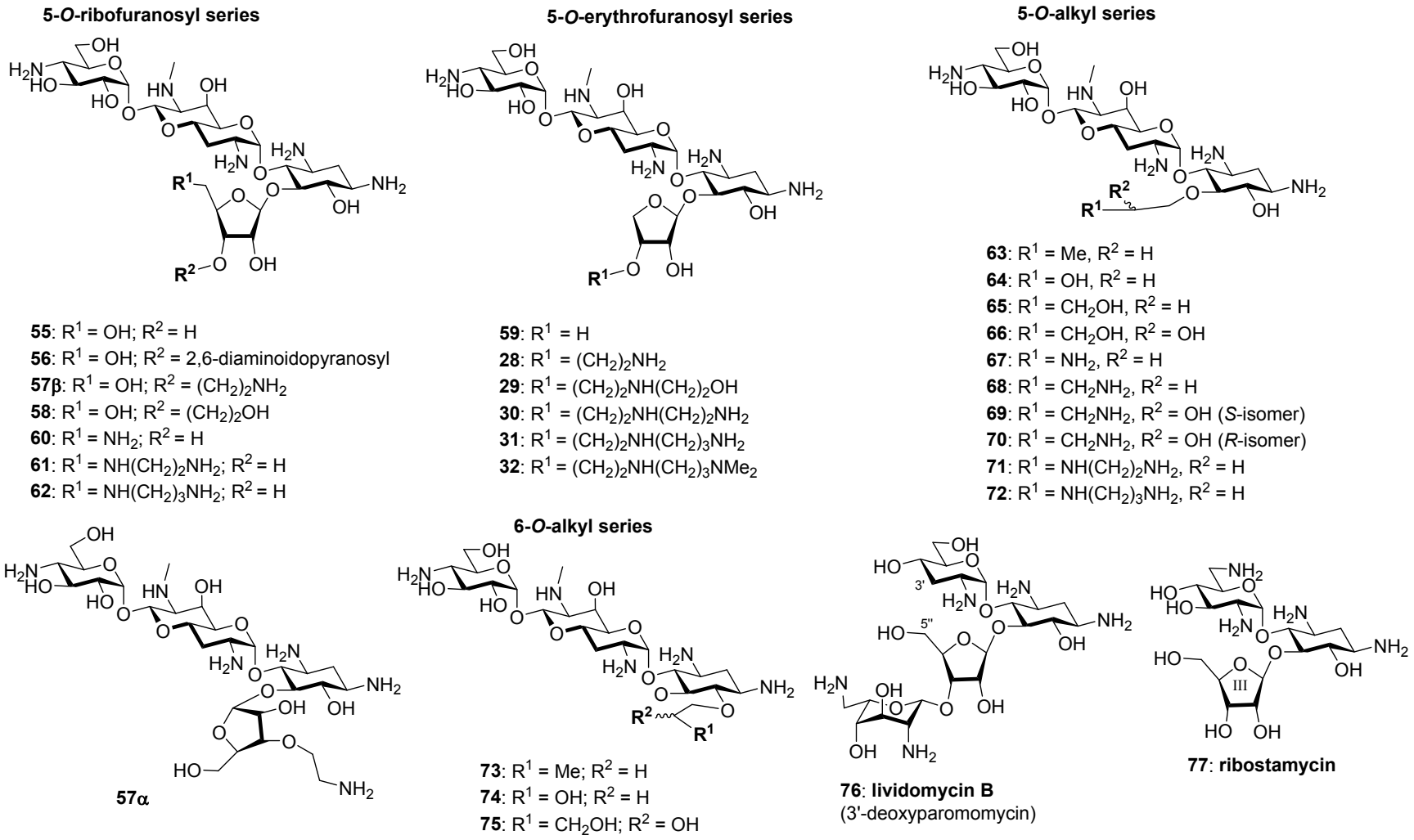


Figure 4. 5-*O*-Ribofuranosyl, 5-*O*-Erythrofuranosyl and 5-*O*-Alkyl Series of Apramycin Derivatives Studied, and the Comparators Lividomycin B and Ribostamycin.

Table 4. Antiribosomal Activities and Selectivities (IC_{50}, μM).[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
57α	0.35	192	285	257	549	814	735
57β	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.

Consideration of the 5-*O*-(D-ribofuranosyl) series of compounds (**55**, **56**, **57α**, **57β**, **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 (**57β**) 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 **57β** 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 eurkaryotic 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 erythrofuranose 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 erythrofuranosyl 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.

Table 5. Antibacterial Activities (MIC, μg/mL)[a]

Species	***E. coli***	***K. pneu.***	***Enterob.***	***A. baum.***	***P. aerug.***	**MRSA**	**MRSA**
Features	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
57α	16	4	16	16	64	16	16
57β	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

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

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*-ribofuranosyl derivatives of apramycin, and because phosphorylation at the 5''-site (the primary alcohol of the ribofuranosyl 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-ribofuranosyl ring to the 5-position of apramycin, as in **55**, **56**, **57β**, **58**, **61** and **62**, largely restores activity in the presence of AAC3-IV. This effect is maximal for **56** and **57β**, carrying the full

paromomycin rings III and IV disaccharide, or a 3-*O*-(2-aminoethyl)-β-D-ribofuranosyl 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 ribofuranosyl 5-position are not as effective as **56** and **57β** in overcoming AAC(3)-IV, this indicates that at least one correctly-placed basic amino group is required in addition to the β-D-ribofuranosyl 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-ribofuranosyl ring (**57β**) the corresponding 3-*O*-(2-aminoethyl)-β-D-erythrofuranosyl 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 **57β** 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.

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)-IId	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
57α	16	32	128	16	16
57β	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

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.

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
57α	>128	>128	>128	16	16	16	32	8
57β	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
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

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-amino-3-deoxyerythrosyl) 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 mice[43] were incubated with increasing concentrations of **30**, **61** and **69**, and of the controls apramycin and gentamicin, before staining with Alexa-594 594 phalloidin for actin and counting of outer hair cells. Plotting of dose response curves (Figure 5) then allowed the determination of the LD_{50} 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 IC_{50} values for the inhibition of mitochondrial and mutant mitochondrial hybrid ribosomes, apramcyin 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 IC_{50} values of the two compounds

for the mitochondrial ribosomes. Somewhat surprisingly in view of its relative IC_{50} values the potent erythrosyl apramycin derivative **30** had LD_{50} 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 of **61** exhibits an LD_{50} significantly less than the parent, clearly demonstrating that it is possible to make a potent derivative of apramycin that displays even further reduced ototoxocity.

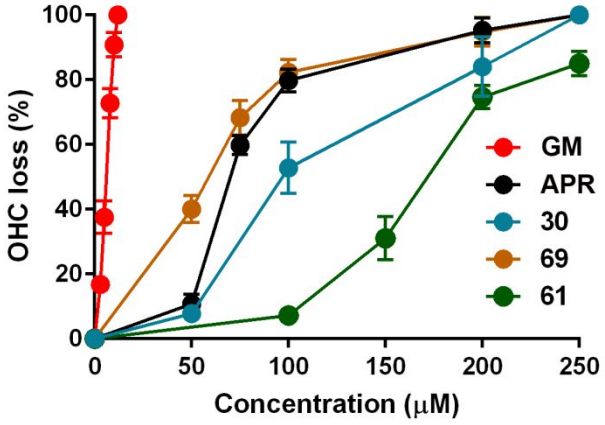


Figure 5. Dose response plots of the percentage of outer hair cell loss (OHC) versus concentration of aminoglycoside. Data are presented as means ± σ, *n* = 5–8 per point. GM = gentamicin, APR = apramycin.

Table 8. Ex-vivo Ototoxicity (LD_{50}, μM) and Antiribosomal Activity (μM) and Selectivity[a]

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

a) Data are presented as means ± σ

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 AAC3-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 $\log_{10}$ 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 **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.

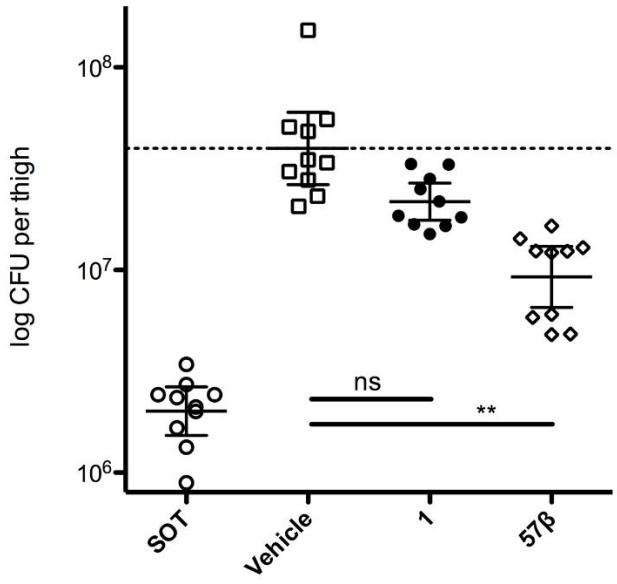


Figure 6. In-vivo efficacy of **57β** in comparison to apramycin **1** in a neutropenic mouse thigh infection model with a dose of 6 mg.kg^{-1}. Statistical significance was determined by an unpaired t test as P = 0.058 (ns, not significant) for **1** and P = 0.007 (**) for **57β**.

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 LD_{50} 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.

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Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI 10.1021/jacs…

Full experimental parts and copies of ^{1}H and ^{13}C 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

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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.

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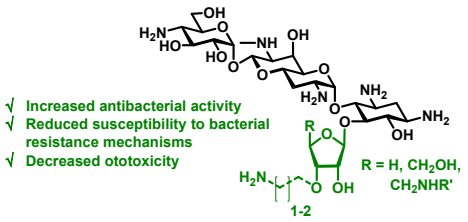
TOC Graphic


√ Increased antibacterial activity
√ Reduced susceptibility to bacterial resistance mechanisms
√ Decreased ototoxicity
R = H, CH2OH, CH2NHR'
1-2