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Structure-Activity Relationships for 5”-Modifications of 4,5-Aminoglycoside Antibiotics

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Abstract

Modification at the 5”-position of 4,5-disubstituted aminoglycoside antibiotics (AGAs) to circumvent inactivation by aminoglycoside modifying enzymes (AMEs) is well known. Such modifications, however, unpredictably impact activity and affect target selectivity thereby hindering drug development. A survey of 5”-modifications of the 4,5-AGAs and the related 5-*O*-furanosyl apramycin derivatives is presented. In the neomycin and the apralog series, all modifications were well-tolerated, but other 4,5-AGAs require a hydrogen bonding group at the 5”-position for maintenance of antibacterial activity. The 5”-amino modification resulted in parent-like activity, but reduced selectivity against the human cytosolic decoding A site rendering this modification unfavorable in paromomycin, propylamycin, and ribostamycin. Installation of a 5”-formamido group and, to a lesser degree, a 5”-ureido group resulted in parent-like activity without loss of selectivity. These lessons will aid the design of next-generation AGAs capable of circumventing AME action while maintaining high antibacterial activity and target selectivity.

Graphical Abstract

Multiple modifications (**X**) are tolerated at the ribofuranosyl 5-position in neomycin B and the apralogs, but only amine-based derivatives are active in the paromomycins, propylamycins and ribostamycins. This is discussed in terms of total amino group count and the ring 1 functionality,

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Conflict of Interest

AV, ECB, SNH, and DC are cofounders of and equity holders in Juvabis AG, a biotech start-up developing aminoglycoside antibiotics.

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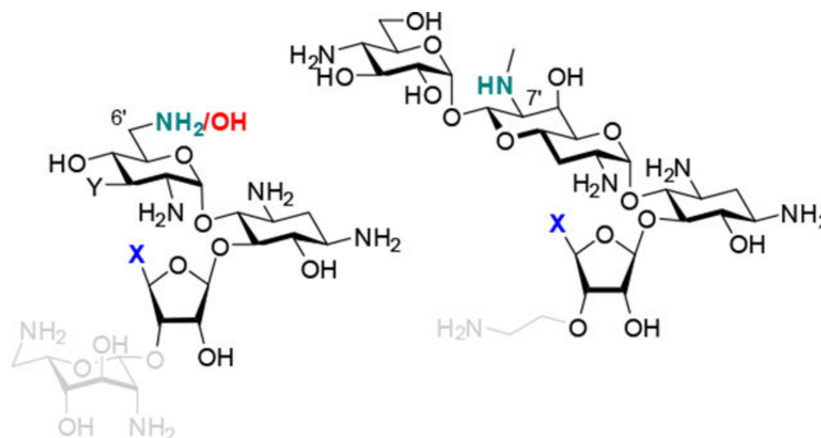
amino or **hydroxy**, with **amino** groups at the 6’- and 7’-positions in the neomycins and apralogs conferring greater flexibility



Keywords

aminoglycoside modifying enzymes; antibacterial; antiribosomal; ototoxicity

Introduction

The aminoglycoside phosphotransferases (APHs) are a class of aminoglycoside modifying enzymes (AMEs) that phosphorylate specific hydroxyl groups on aminoglycoside antibiotics (AGAs), thereby reducing their affinity for their biological target, the decoding A site on helix 44 in the 30S subunit of the bacterial ribosome, and resulting in AGA-resistant bacterial strains.[1] A case in point is the APH(3’) class, whose members phosphorylate the 3’-hydroxy group in ring I of both the 4,5- and 4,6-disubstituted classes of 2-deoxystreptamine-type AGAs (Figure 1).[1a] The action of the APH(3’)s at the 3’-hydroxy group can be thwarted by deoxygenation as illustrated by the clinical 4,6-class AGA tobramycin **2**, which unlike the parent kanamycin B **1** is active in the presence of APH(3’)s. Thus, 3’-deoxygenation is a strategy widely employed both by nature and by chemists.[2]

Some APH(3’) isozymes exhibit dual modes of reactivity at both the 3’- and 5”-hydroxy groups in the 4,5-AGAs, as was discovered by Courvalin and coworkers with their finding that certain APH(3’)s inactivate the 3’-deoxy-4,5-AGA lividomycin **5** in addition to the parent paromomycin **3**.[3] Subsequently, in order to combat the full range of APH(3’)s acting on the 4,5-AGAs it became necessary to develop derivatives modified at both the 3’- and 5”-positions to prevent phosphorylation. Chemistry at the 5”-position is easier than at the 3’-position as it is one of only two possible primary alcohols in paromomycin **3**, and the only one in neomycin **4** and in ribostamycin **6**, but early modifications resulted in a reduction in activity and were thus not promising. For example, it was found that both 5”-deoxy- and 5”-amino-5”- deoxy-lividomycin A showed broadly reduced antibacterial activity over the parent lividomycin A.[4] Similarly, the 5”-chloro-5”-deoxy and 5”-fluoro-5”-deoxy analogues of lividomycin B as well as 5”-deoxy lividomycin B itself were markedly less active than the parent.[5] Although antibacterial activities were not

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reported, 5”-deoxyneomycin B was shown to have approximately 10-fold less affinity for a 27-mer RNA fragment modelling the decoding A site than neomycin B itself.[6] Attempts to block the APH(3’)s by the construction of cyclic AGAs with bridges spanning the 3’- and 5”-, or 2’- and 5”-positions were similarly fruitless, giving compounds with much reduced activity.[6–7] A series of 5”-*O*-monosaccharyl derivatives of neomycin B showed either comparable or reduced antibacterial activity.[8] In contrast to these mostly negative observations, it was reported that 5”-amino-5”-butirosin essentially retains the full spectrum of antibacterial activity when compared to butirosin **7** itself.[9] Likewise, 5”-amino- and 5”-guanidino derivatives of 4’,5”-dideoxybutirosin were shown to retain strong, parent-like antibacterial activity against multiple strains.[10] In comparison to the parent ribostamycin, 3’,4’,5”-trideoxyribostamycin was notably less active in all cases except for strains carrying AMEs acting at those positions.[11] The preparation of 5”-chloro-5”-deoxyribostamycin and 5”-deoxyribostamycin has been described in the patent literature but to our knowledge no antibacterial data for these compounds has been published.[12]

Particularly in the neomycin series, where the 5”-position carries the only primary hydroxy group making it easy to derivatize selectively, many more substantial modifications have been made beyond the simple aminations, deoxygenations, and halogenations listed above.[13] Often, however, these neomycin 5”-modifications were carried out with simultaneous modification at other positions, making it difficult to tease out the contribution to activity of the 5”-modification itself. Nevertheless, the fact that these highly modified derivatives sometimes retain high activity indicates that 5”-functionalization is broadly tolerated in neomycin. For example, a study by Chang and coworkers revealed that a series of 5”-deoxy-5”-triazolyl derivatives **8** and **9** retained significant activity against wild-type *E. coli,* and that several 5”-deoxy-5”-amido derivatives had comparable activity to the parent, with the optimal members being 5”-deoxy-5”-glycinamido neomycin derivatives **10** and **11**, and 5”-deoxy-5”-palmitamido neomycin **12** (Figure 2).[14]

In our own laboratories we developed two series of 5-*O*-furanosyl apramycin derivatives, dubbed apralogs and advanced apralogs, carrying modifications at the primary position of the ribofuranosyl residue that is equivalent to the 5”-position in the 4,5-AGAs. We note that in apramycin the 5”-position is formally C5 in the terminal 4-aminoglucopyranosyl ring, such that the furanosyl ring in the apralogs and advanced apralogs should be numbered 1”’–5”’. However, to avoid confusion in this discussion, here we consistently designate positions in the furanose ring 1”–5” across all series of compounds We found that the 5-amino-5-deoxy modification in the ribose ring was optimal and afforded compounds (**16** and **18**) with excellent in vitro and in vivo antibacterial activity coupled with low ototoxicity in a cochlear explant model.[15] In contrast, the corresponding 5”-deoxy-5”-amino derivative **20** of the next-generation 4,5-AGA propylamycin **19**, while retaining strong activity, was not as active as the 5”-deoxy-5”-formamido modification **21** (Figure 3).[16]

In view of the somewhat disparate results reported by multiple groups for modifications at the 5”-position, and particularly of the differences between the propylamycin and apralog series in our own laboratories, we undertook a systematic study of a series of minimal modifications at the 5”-position in each of the neomycin, ribostamycin, paromomycin, propylamycin, apralog, and advanced apralog series of 4,5-AGAs with the

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aim of establishing structure activity relationships that would enable the informed choice of modification for use in the development of advanced AGAs circumventing the action of the APH(3')s.

Results and Discussion

Synthesis.

Literature compound **23**[17] was subjected to one-pot tritylation and acetylation followed by subsequent deprotection of the trityl group using $FeCl_3{\cdot}6H_2O$.[18] The resulting primary alcohol was oxidized using BAIB and TEMPO[19] to give a 33% yield of carboxylic acid **24**, which was immediately subjected to Barton decarboxylation[20] to generate intermediate **26** in 42% yield. Subsequent treatment with $Mg(OMe)_2$ to selectively cleave the trifluoroacetamide groups followed by NaOH gave the 4"-des(hydroxymethyl) neomycin derivative **27**. Final purification was achieved through chromatography over Sephadex C25 and lyophilization with acetic acid to generate the corresponding peracetate salt in 55% overall yield (Scheme 1).

All other neomycin derivatives were prepared from common intermediate **29**, which was generated through trisylation of literature compound **28**[21] in 61% yield. Displacement of the trisyl group with sodium iodide and potassium phthalimide afforded intermediates **30** and **32** in 74% and 50% yield respectively. Hydrogenolysis of **30** followed by chromatographic purification through Sephadex C-25 and lyophilization with acetic acid afforded 5"-deoxyneomycin **31** in 33% yield. Cleavage of the phthalimide group of **32** with hydrazine hydrate gave the free amine **33** in 73% yield, which was subsequently subjected to either hydrogenolysis or acylation followed by saponification of any undesired esters and reduction of the azido groups. Each of the fully deprotected neomycin derivatives was passed through Sephadex C-25 and lyophilized with acetic acid to generate the corresponding peracetate salts (Scheme 2).

Turning to modifications at the 5"-position of paromomycin, literature compound **37**[21] was subjected to one-pot tritylation and acetylation followed by trityl deprotection and oxidation of the resulting primary alcohol[18] to give carboxylic acid **38** in 36% overall yield. Decarboxylation[20] of **38** gave the intermediate **39** in 47% yield, which upon acidic hydrolysis of the benzylidene acetal and basic hydrolysis of the trifluoroacetamides and esters followed by purification by Sephadex C-25 chromatography and lyophilization with acetic acid generated the pentaacetate salt of des(hydroxymethyl) paromomycin **40** in 83% overall yield (Scheme 3).

Literature compounds **41**[22] and **42**[23] were treated with trisyl chloride to generate intermediates **43** and **44** in 53% and 58% yield respectively. Displacement of the trisyl group of **43** with sodium iodide gave alkyl iodide **45** in 68% yield, which was deprotected through acidic hydrolysis of the benzylidene acetal and hydrogenolysis, purified by Sephadex C-25 chromatography, and lyophilized with acetic acid to give 5"-deoxyparomomycin **46** in 54% overall yield. Displacement of the trisyl groups of **43** and **44** with sodium azide afforded 5"-azido derivatives **47** and **48** in 78% yield in each case. Intermediate **47** was subsequently subjected to hydrogenolysis, purification over Sephadex C-25, and lyophilization with acetic

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acid to give the hexaacetate salt of **49** in 57% overall yield. After subjection of **48** to Staudinger conditions to generate the 5”-amino derivative **50**, acylation of the free amine followed by global deprotection, Sephadex C-25 purification, and lyophilization with acetic acid gave the peracetate salts of 5”-*N*-acyl derivatives **51–53** (Scheme 4).

In the ribostamycin series, neamine derivative **54**[21] was glycosylated with erythrosyl donor **55**[15a] and boron trifluoride diethyl etherate as an activator to give **56** as a single isomer in quantitative yield. Subsequent basic hydrolysis of the benzoates, Staudinger reduction of the azides, and hydrogenolysis of the benzyl ethers generated the 4”-des(hydroxymethyl) ribostamycin derivative **57**, which was purified over Sephadex C-25 and lyophilized with acetic acid to form the peracetate salt in 35% overall yield (Scheme 5). The anomeric configuration at the newly generated glycosidic linkage of **57** was assigned based on the ^{13}C chemical shift of C1”’ (δ105.7) in accordance with well-established rules.[24]

As with the paromomycin series, azide-protected **58**, prepared in 57% yield from treatment of ribostamycin with triflyl azide and copper sulfate, and literature compound **59**[11] were each subjected to sulfonation at the 5”-hydroxyl group to form derivatives **60** and **61** in 41% and 26% yield respectively. Subsequent displacement of the sulfonate with sodium azide afforded compounds **62** and **63** in 74% and 47% yield respectively. Hydrogenolysis of **62** followed by Sephadex C-25 purification and lyophilization with acetic acid generated the peracetate salt of **64** in 24% yield. Deprotection of the 5”-azido group of **63** under Staudinger conditions gave intermediate **65** in 77% yield, which was subsequently formylated and saponified or acetylated, hydrogenated, purified over Sephadex C-25, and lyophilized with acetic acid to give the peracetate salts of **66** and **67** in 26% and 46% yield respectively (Scheme 6).

Finally, apramycin derivatives **75**, **76**, and **79** were synthesized to enable comparison between the apralogs, advanced apralogs and the 4,5-AGAs. Glycosylation of acceptor **68** and either donor **70** or donor **71** with boron trifluoride diethyl etherate as an activator gave exclusively the β-anomers of **72** and **73** in 76% and 45% yield respectively. Saponification of the esters (and cleavage of the phthalimide with sodium borohydride in the case of **72**) followed by formylation with **74**[25] and Staudinger reduction of azides generated final compounds **75** and **76**, each of which was purified by Sephadex C-25 chromatography and lyophilized in acetic acid to give the peracetate salts in 75% and 57% yield respectively. Glycosylation at room temperature of acceptor **69** with commercial donor **77** using boron trifluoride diethyl etherate afforded **78** in 27% isolated yield (α:β= 0.3:1), which was subsequently subjected to one-pot ester hydrolysis and Staudinger reduction, followed by subsequent purification via Sephadex C-25 and lyophilization with acetic acid to give the peracetate salt of **79** in 57% yield (Scheme 7). As above, the anomeric configurations at the newly generated glycosidic linkages of **72**, **73**, and **78** were assigned based on the ^{13}C chemical shifts of C1”’ (δ106.9, 106.7, and 105.5 respectively) in accordance with well-established rules.[24]

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Antiribosomal Activity and Selectivity.

To determine the influence of 5”-modification at the level of the drug target, all compounds (Figure 4) were screened in cell-free translation assays for their ability to inhibit luciferase production by wild type *Mycobacterium smegmatis* bacterial ribosomes. In parallel and as a measure of selectivity for binding to the bacterial ribosome, the more active compounds were also subjected to cell-free translation assays using engineered *M. smegmatis* ribosomes containing the human drug binding pocket, namely the mitochondrial decoding A site and its A1555G mutant, and the human cytosolic ribosome decoding A site (Figure 5, Table 1).[26] Lack of selectivity in binding of the AGA to the bacterial ribosome over the human mitochondrial ribosome is an established predictor of ototoxicity, as AGA-induced ototoxicity has been linked to translational inhibition of mitochondrial ribosomes in cochlear hair cells.[26b, 27] Moreover, hypersusceptibility to AGA-induced ototoxicity has been linked to translational inhibition of the A1555G mutant in genetically predisposed patients.[26d, 28] Lack of selective binding of the drug to bacterial ribosomes over human cytosolic ribosomes is an indicator of systemic toxicity.

It is apparent from Table 1 that modification of the 5”-position in neomycin has no significant impact on the ability of the drug to inhibit wild type bacterial ribosomes, consistent with the reported use of the neomycin 5”-position as attachment point for the preparation of multiple derivatives with retention of antibacterial activity.[13–14] In the paromomycin series, on the other hand, an approximately 3-fold loss of activity is seen on conversion of the 5”-hydroxy group to an amino group (**49**). Functionalization of the amino group in **49** in the form of a formamide (**51**) restores the lost activity while acetylation (**52**) is highly detrimental. The urea derivative **53**, which can be considered isosteric with the acetamide **52** but with greater hydrogen-bonding capabilities, in part regains activity. The complete removal of hydrogen-bonding capability at the 5”-position as in both **40** and **46** results in an approximately 30-fold loss of activity. In the propylamycin series no attempt was made to prepare the 5”-deoxy, 4”-des(hydroxymethyl), and 5”-acetamido-5”-deoxy derivatives in view of the detrimental nature of these modifications to paromomycin: essentially the same trend in activity against the bacterial ribosome as in the paromomycin series was seen with the 5”-amino **20**, 5”-formamido **21** and 5”-ureido **22** modifications to propylamycin with the formamide **21** retaining the greatest level of activity. In the ribostamycin series a different pattern was observed with the 5”-amino-5”-deoxy modification **64** having essentially the same activity as the parent, the formamide **66** with approximately 8-fold lower activity, and the des(hydroxymethyl) and acetamide derivatives **57** and **67** showing 20 and 40-fold losses of activity relative to the parent.

Moving to the apralog and advanced apralog series of compounds, replacement of the 5”-hydroxy group by an amino group (**16** and **18**) results in a minor increase in antiribosomal activity, which disappears on conversion to the corresponding formamido derivatives (**75** and **76**). In so far as the formamido derivatives are less active than the amino derivatives, this pattern in the apralogs resembles that seen in the neomycin series. The complete removal of functionality at the apralog 5”-position as in the deoxy derivative **79** or the des(hydroxymethyl) derivative **15** similarly has little impact on selectivity, as seen in the neomycin series.

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Overall, in the neomycin series with its six basic amines, mostly protonated at physiological pH[29] and providing a strong affinity for the negatively charged ribosomal decoding A site,[30] all modifications studied at the 5”-position are tolerated. In all other series except the apralogs the complete removal of functionality from the ribosyl side chain, as in the deoxy and des(hydroxymethyl) modifications results in a significant loss of activity indicating the need for a hydrogen bonding capable group (donating and/or accepting) at that position that is only offset by the presence of six basic amines. The amino modification is effective in all series resulting in compounds with only minor losses or minor gains in activity. The formamido modification is similarly effective in all series except the ribostamycins, where the parent is inherently less tolerant of modification. Whenever studied, except in the tightly bound neomycin series, the acetamido modification is detrimental, which we ascribe to the presence of the hydrophobic methyl group as activity is largely recovered with the isosteric but hydrophilic ureido group. While formamido groups are known to populate the Z-conformation to a much greater extent than acetamides,[31] and indeed are seen to do so in the NMR spectra of **35**, **51**, **66**, **21**, **75**, and **76** in free solution, we see no reason to invoke preferential binding of the formamides through this conformation in view of the activity of the ureido derivatives.

Turning to the hybrid ribosomes carrying the human mitochondrial ribosome (Mit13), with the exception of the tightly bound neomycin series where all modifications result in a minor reduction in activity, the amino modification stands out in causing a modest increase in activity, which in the paromomycin, propylamycin, and ribostamycin series results in a reduction in selectivity compared to the parent. In the A1555G mutant mitochondrial hybrid ribosomes a comparable pattern is seen.

In the Cyt14 series of hybrid ribosomes carrying the human cytosolic decoding A site, which is characterized by a C1409•A1491 mismatch at the base of the binding pocket as well as an A1409G substitution at the site of interaction with positions 4’ and 6’ of the drug, all substitutions at the 5”-position of the drug are similarly accommodated in the tightly bound neomycin B framework. In the other series of 4,5-compounds, the amino modification affords noticeably higher activity than any other changes made, which is attributable to the stronger H bond between the protonated 5”-amino group and N7 of A1491, such that the 5”-amino-5”-deoxy compounds have noticeably lower selectivity for the bacterial ribosome over the cytosolic variant. The observation that 5”-amino modified 4,5-AGAs show less selectivity for the bacterial over the A1555G mutant mitochondrial and cytoplasmic ribosomes than the parents suggests that compounds carrying this modification will suffer from increased toxicity over the parent compounds and will not be good candidates for development as antibacterial agents. The pattern of the relatively high activity of the 5”-amino modified compounds against the cytoplasmic ribosome accords with the status of the 5”-aminoribostamycin derivative **ELX-02** as a candidate drug for the treatment of genetic diseases arising from the replacement of an amino acid codon by a premature stop codon (Figure 6).[32] For the apralogs, the 5”-amino modification affects drug selectivity to a significantly lower extent. Notably, the 5”-amino-3”-*O*-(2-aminoethyl)apralog **18** shows increased selectivity over the mitochondrial and A1555G mutant mitochondrial ribosomes predictive of lower ototoxicity and as borne out previously by toxicity studies with mouse

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cochlear explants. Inspection of the data reveals that this apparent anomaly is not the result of breakdown of the interaction with the humanized hybrid ribosomes but is due to a larger increase in activity for the bacterial ribosome.

It is widely accepted that the affinity of AGAs for the decoding A site is dependent on the number of basic amines and their protonation state,[30, 33] begging the question of why installation of a further basic amine in the 5”-amino derivatives (to a total of seven in **18** and **34**, six in **16**, **20**, and **49**, and five in **64**) does not lead to a larger increase in activity over the parents. For this, we turn to the prototypical X-ray structure of the 4,5-AGA paromomycin in the drug binding pocket (Figure 7)[34] and the hydrogen bonding interactions involving the 5”-substituent, particularly the intramolecular hydrogen bond between the protonated N2’ in ring I and the 5”-hydroxy group in the ribofuranose ring.

This same interaction has been established to pre-organize 4,5-AGAs in free solution for binding to the target.[35] Clearly, in the 5”-amino derivatives it is not possible to fully protonate both N2’ and N5” at the same time as this would result in a strongly repulsive electrostatic interaction between two proximal ammonium ions, hence the breakdown in the rule of thumb relating activity to the number of basic amines. Rather we suggest that N2’ and N5” share a hydrogen bonded proton thereby maintaining the overall geometry apparent for the parent in Figure 7 and resulting in no net increase in positive charge. According to this hypothesis, N5” in the amino series nevertheless carries a partial positive charge making it a strong hydrogen bond donor in its interaction with N7 of G1491 (Figure 8 a).

The importance of the hydrogen bond between the protonated N2’ in ring I and the 5”-substituent is borne out by the limited range of modifications possible at the 5”-position, where only groups capable of hydrogen bonding are tolerated in all but the tightly bound neomycin series. The importance of this hydrogen bond in all but the neomycin series is further reflected in earlier studies on the modification of N2’ in which it was shown that the retention of a hydrogen bonding group was essential.[30a]

Finally in this section, we return to the differences in relative inhibitory activities of the various amino and formamido derivatives for the bacterial ribosome. Thus, in the neomycin and apralog series the amines are better inhibitors than the formamides. Conversely, in the paromomycin and propylamycin series the formamides are better inhibitors than the amines. We hypothesize that this difference in behavior is again related to the interaction between N2’ and the 5”-amines, and that this interaction is modulated by the functionality at the 6’-position and in the case of the apralogs the 7’-position. Thus, the paromomycin and propylamycin series of compounds carry a hydroxy group at the 6’-position (Figure 8a, X = O), whereas neomycin is a 6’-amine, whose protonated ammonium form interacts with A1408 in the decoding A site (Figure 8a, X = NH_2^+). Protonation of the 6’-amino group in neomycin necessarily inductively reduces the ability of the 2’-amino group to accept a hydrogen bond from a protonated amine at the 5”-position and so increases the ability of the latter to hydrogen bond with G1491. In contrast, hydrogen bond donation from the 6-hydroxy group of the paromomycin and propylamycin series of compounds to A1408 in the interaction with the target will tend to increase the basicity of N2’ and hence its interaction with a protonated amine at the 5”-position. The apralogs are 6’-hydroxy AGAs

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and so are nominally most closely related to the paromomycin and propylamycin series, but they also carry a secondary amino group at the 7'-position. This secondary amine does not contact the ribosome directly,[36] but is critical for activity[37] from which it follows that its protonated form donates a hydrogen bond to the adjacent 6'-hydroxy group, which in turn inductively modulates the hydrogen bond accepting ability of N2' (Figure 8b): in this manner the apralogs are functionally analogs to neomycin.

Antibacterial Activity Against Wild Type Bacterial Strains.

All compounds were screened for activity against a panel of Gram-negative pathogens (*Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Acinetobacter baumannii,* and *Pseudomonas aeruginosa*) as well as the Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA), all of which were obtained from the Diagnostic Department of the Institute of Medical Microbiology at the University of Zurich (Table 2).

On the whole, the observed antibacterial activities against wild type bacteria were consistent with the antiribosomal activities; thus, the 4"des(hydroxymethyl), 5"-deoxy, and 5"-acetamido derivatives of all parent compounds studied displayed decreased antibacterial activity, though to a significantly lesser degree in the case of neomycin. Also consistent with the antiribosomal activity data is the decreased antibacterial activity resulting from 5"s-NH_2 installation in neomycin, paromomycin, ribostamycin, and propylamycin, which mirrors previous observations from the Hanessian[38] and Baasov[32c] groups.

Antibacterial Activity against Resistant Bacterial Strains.

All compounds were additionally screened for activity against both engineered strains and clinical isolates of *Escherichia coli* expressing various APH(3') isozymes (Table 3). All compounds in the ribostamycin, paromomycin, and neomycin series remained susceptible to APH(3') isozymes, an unsurprising result in view of the free hydroxyl group at the 3'-position in each case. In the case of propylamycin, installation of a 5"-amino, 5"-formamido, and 5"-ureido groups resulted in significantly reduced susceptibility to the APH(3')-I isoforms which act on the 5"-position. The apralogs, lacking a 3'-hydroxy group and so retaining activity in the presence by APH(3')s acting only at that position, showed minimal susceptibility to the APH(3') isozymes acting at the 5"-position.

Conclusion

A systematic study of the impact of 5"-modifications on activity and selectivity at the target level in the neomycin, paromomycin, propylamycin ribostamycin, and the related 5-*O*-ribofuranosyl apramycin derivatives series of AGAs has been conducted. In the neomycin and apralog series, modifications at the 5"-position were well-tolerated, as any potential destabilizing interactions are outweighed by the significant Coulombic stabilization of the AGA-ribosome complex from the high number of protonated amines. In 4,5-AGAs with fewer basic amines, we find that a hydrogen bonding-capable group at the 5"-position is critical for maintenance of comparable antibacterial activity to the parent. Though antibacterial activity is maintained in the 5"-amino derivatives of paromomycin,

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ribostamycin, and propylamycin, reduced selectivity against the human cytosolic ribosome renders this modification generally unfavorable. No such reduction of selectivity is observed, however, for the comparably active 5”-formamido and 5”-ureido modifications. In contrast, for the apralog series all 5”-amino modifications affect selectivity minimally indicating ample room for further modification. In this respect the apralogs resemble neomycin but come with a much-improved selectivity profile. These lessons will inform the design of next-generation antibiotics exhibiting reduced toxicity, greater antibacterial activity, and reduced susceptibility to the aminoglycoside modifying enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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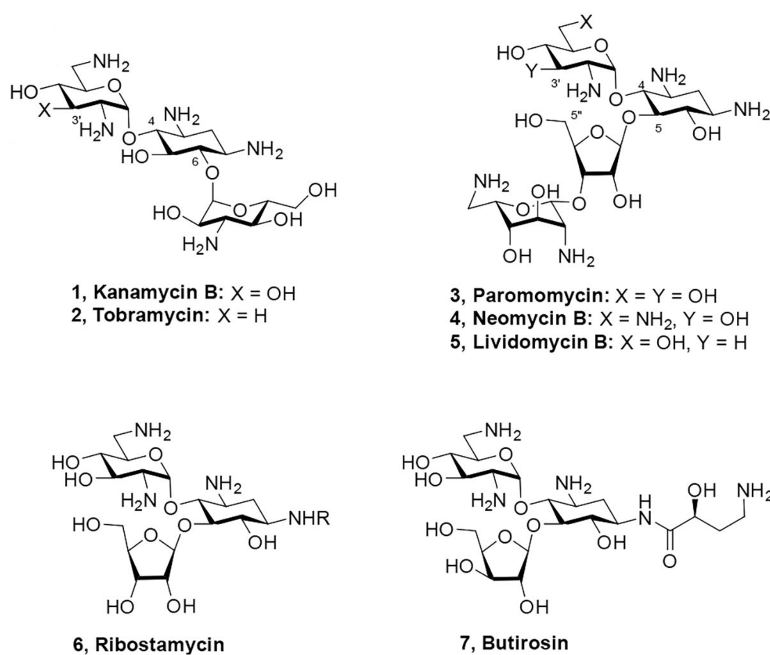

Figure 1.

Structures of select 4,5- and 4,6-AGAs.

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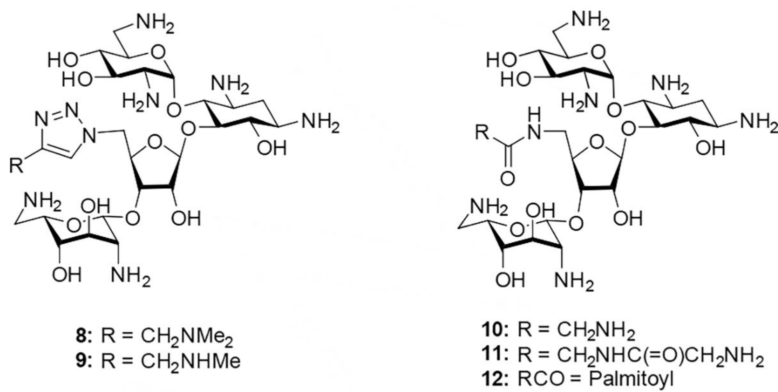

Figure 2.

Some established neomycin 5”-derivatives.

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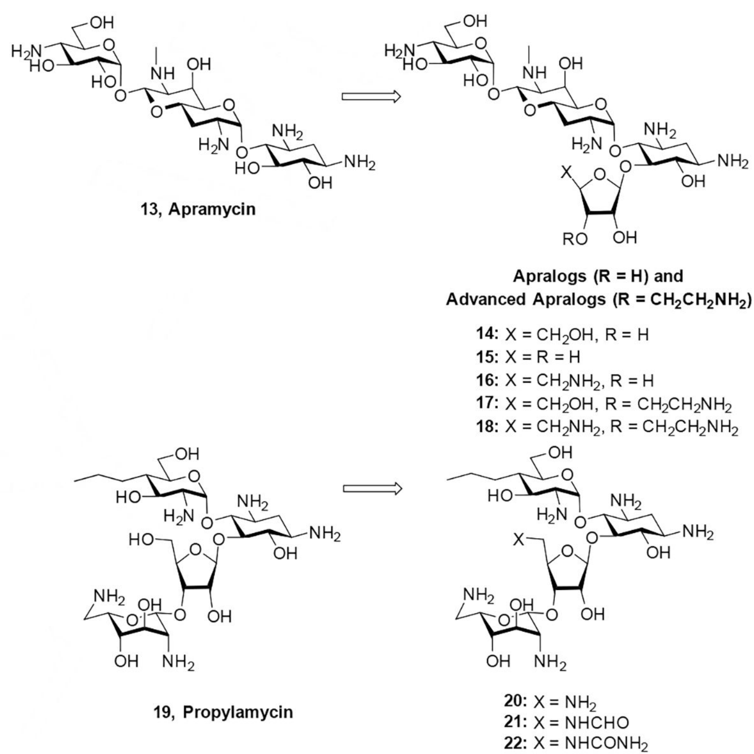

Figure 3.

Apralogs, advanced apralogs, and propylamycin derivatives.

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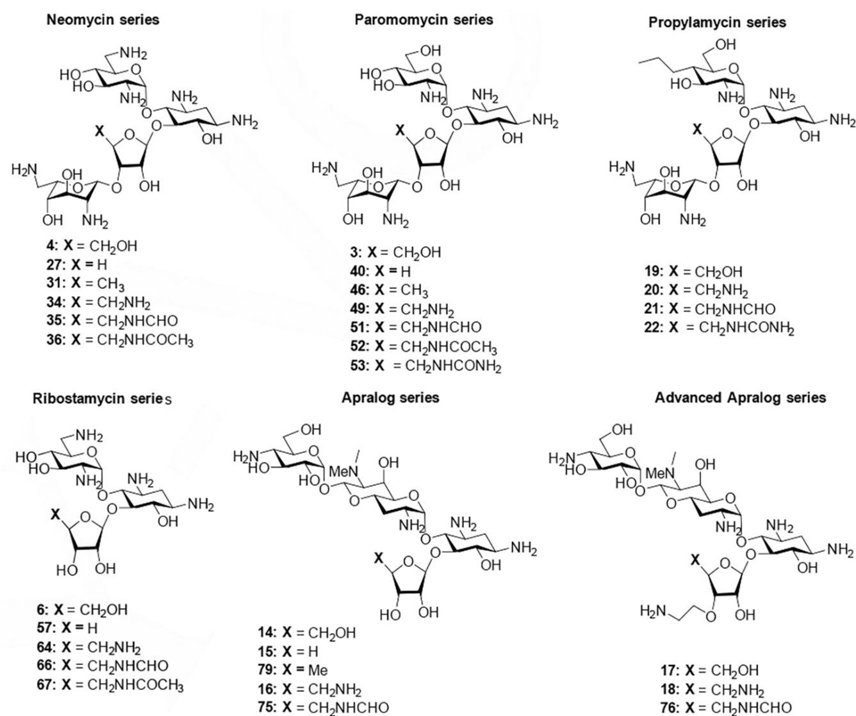

Figure 4.

Set of parent compounds and derivatives screened.

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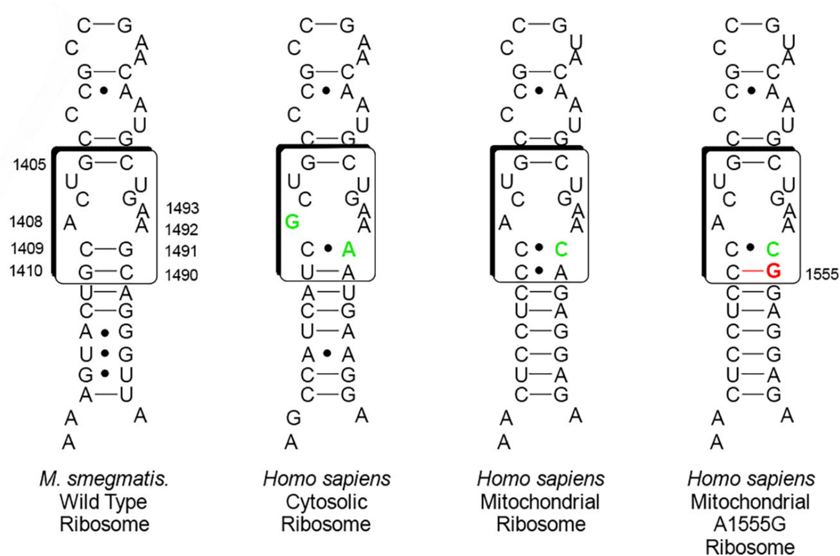

Figure 5.

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 colored green. The A1555G mutant conferring hypersusceptibility to AGA ototoxicity is colored red.

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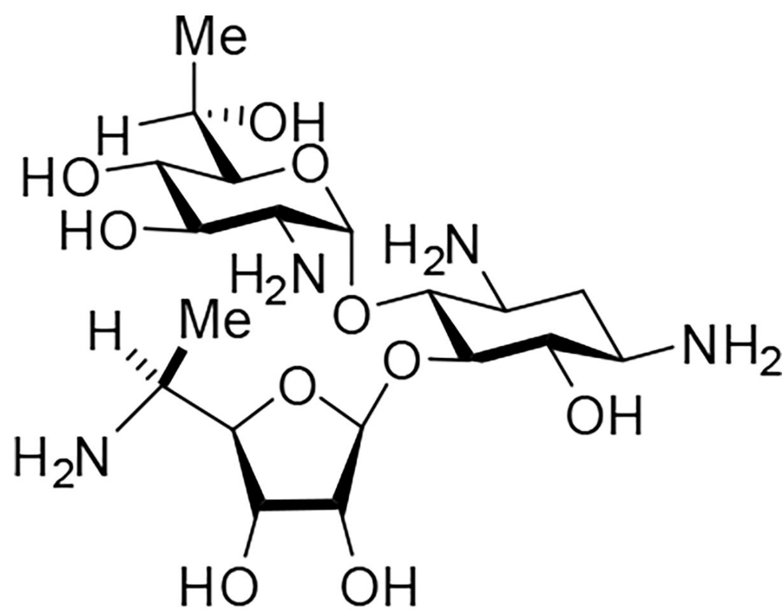

Figure 6.

Structure of the Experimental Drug **ELX-02**.

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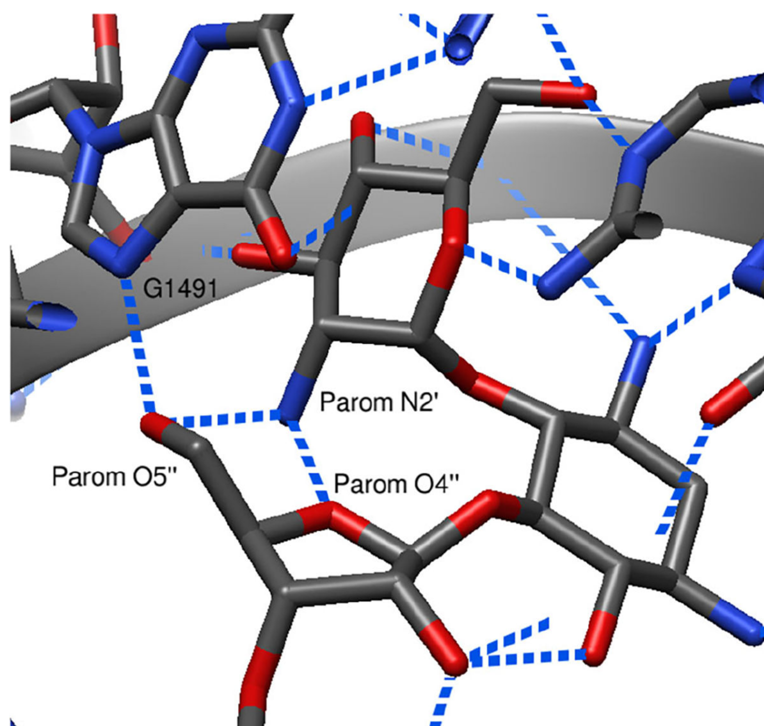

Figure 7.

Partial crystal structure of paromomycin bound to the decoding A site of *Thermus thermophilus* (PDB ID 1FJG), with dashed blue lines denoting hydrogen bonds.

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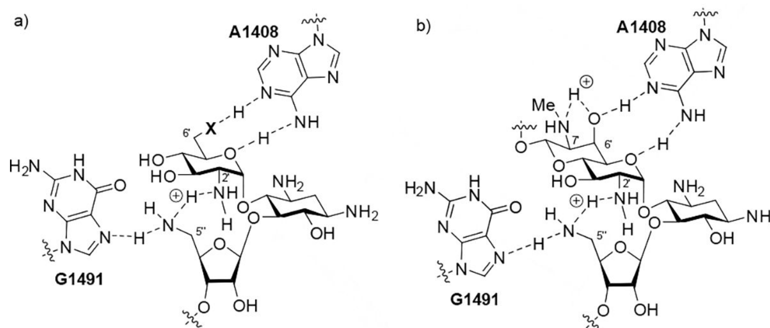
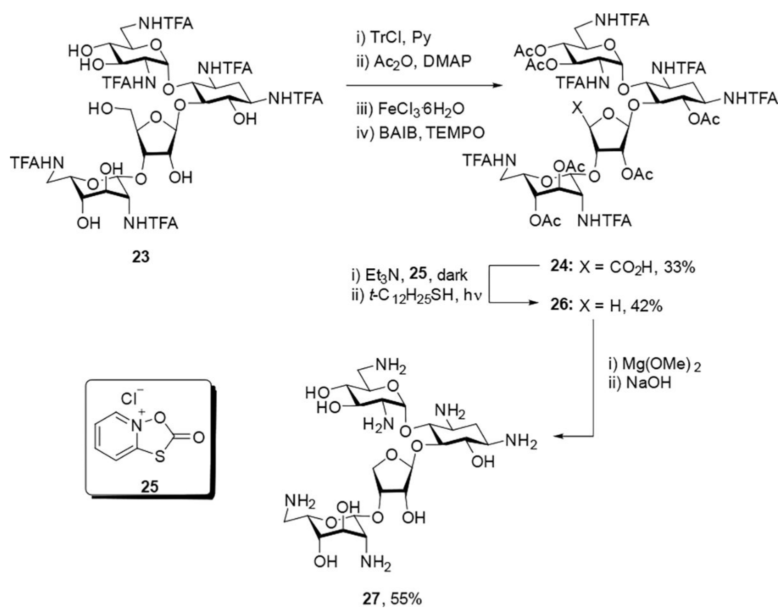


Figure 8.

a) Proposed hydrogen bonding scheme between N2’, N5”, and G1491, and the ring I A1408 pseudo-base pair in the 5”-deoxy-5”-amino of the 6’amino (X = NH_2^+) and 6’hydroxy AGAs (X = O), and b) Proposed hydrogen bonding scheme in the 5”-amino apralog interaction with the target.



Scheme 1.

Synthesis of 4”-des(hydroxymethyl)neomycin **27**.

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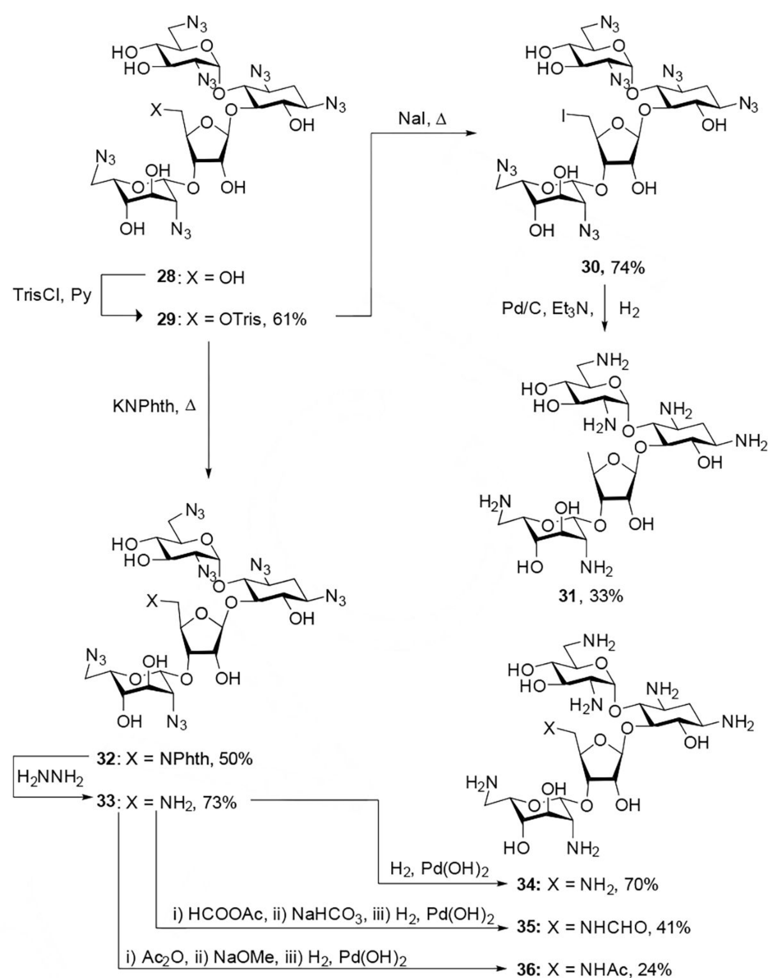

Scheme 2.

Synthesis of 5”-deoxy, 5”-deoxy-5”-amino, and 5”-deoxy-5”-amidoneomycin derivatives.

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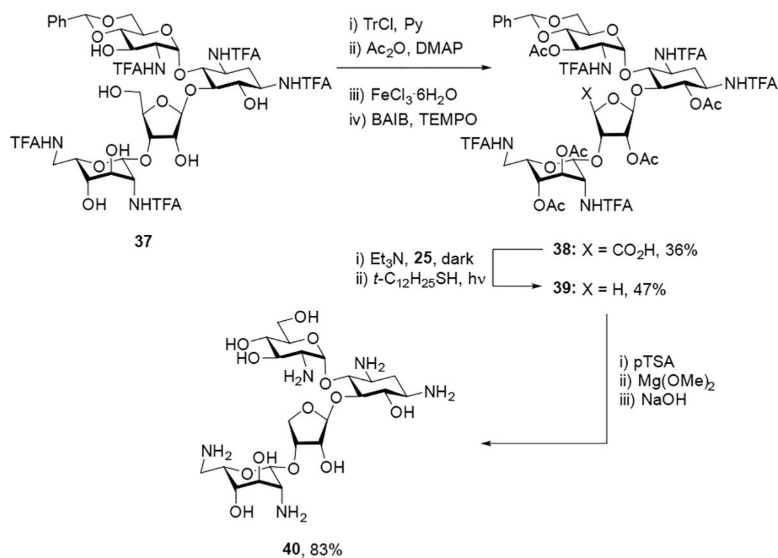

Scheme 3.

Synthesis of 4”-des(hydroxymethyl)paromomycin.

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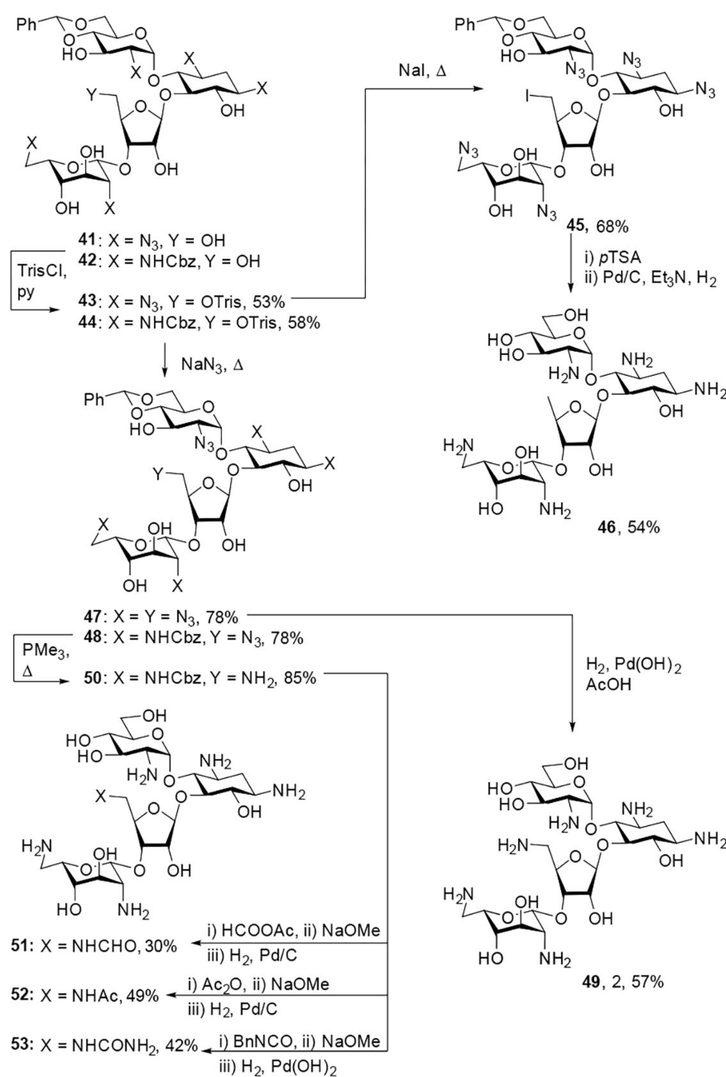
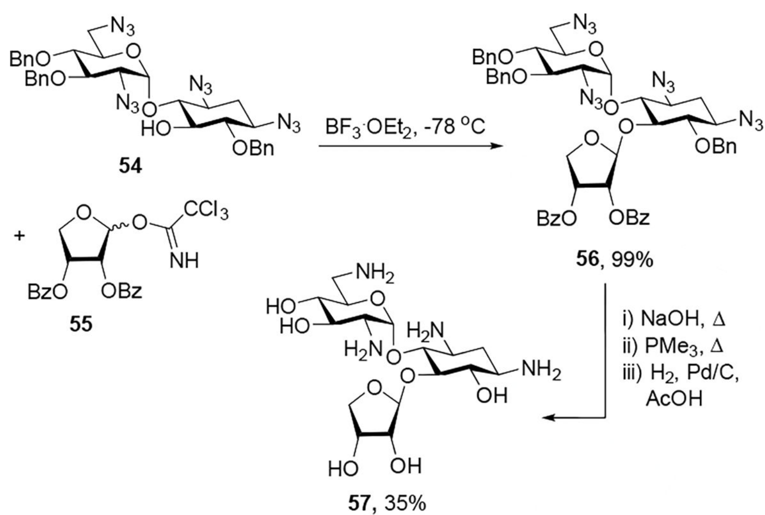


Scheme 4.

Synthesis of 5”-deoxy, 5”-deoxy-5”-amino, and 5”-deoxy-5”-amidoparomomycin derivatives.



Scheme 5.

Synthesis of 4”-des(hydroxymethyl)ribostamycin.

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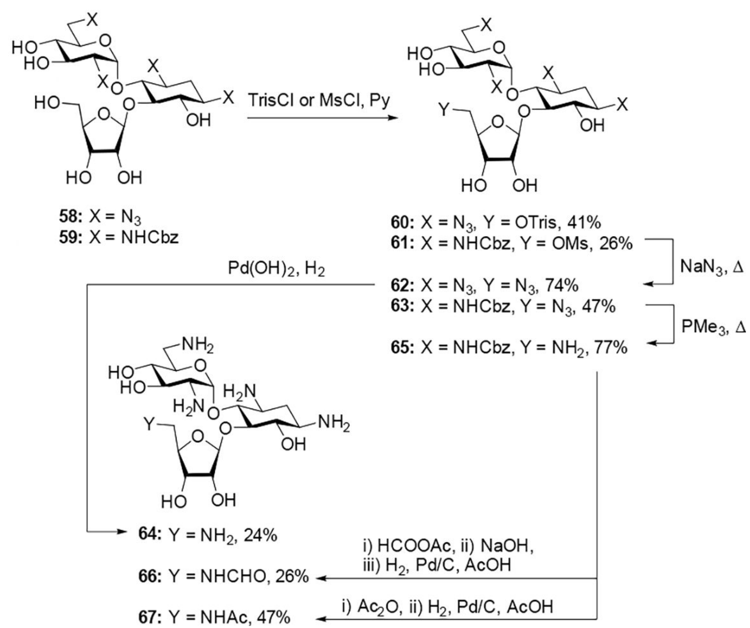

Scheme 6.

Synthesis of 5”-deoxy-5”-amino and 5”-deoxy-5”-amidoribostamycin derivatives.

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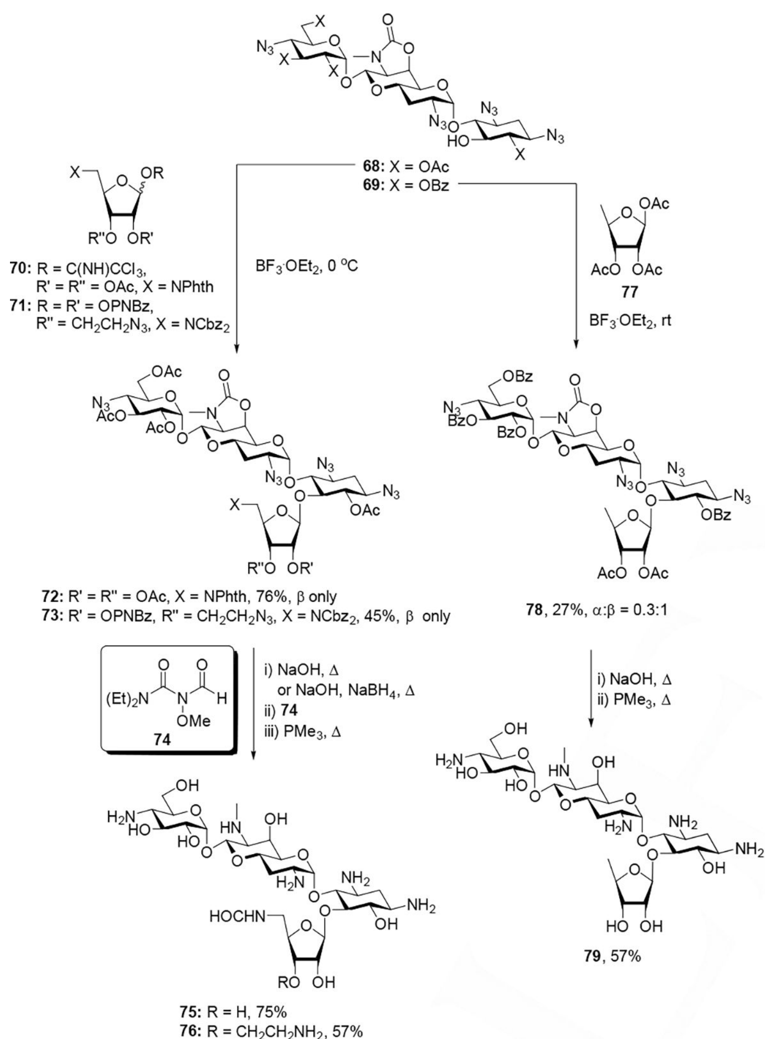

Scheme 7.

Synthesis of apralog and advanced apralog derivatives.

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Table 1.

Antiribosomal activities and selectivities of 4,5-AGA derivatives[a]

		IC_{50} (μM)				Selectivity		
Compound	**4”-Substituent**	**wt**	**Mit13**	**A1555G**	**Cyt14**	**Mit13**	**A1555G**	**Cyt14**
		Neomycin series (6 basic amines in parent)						
Neomycin, 4	CH_2OH	0.034	3.6	0.40	34	106	12	1000
27	H	0.038	5.0	0.077	32	132	2	865
31	CH_3	0.039	10.7	0.945	32	274	24	821
34	CH_2NH_2	0.025	14	0.470	32	560	19	1391
35	CH_2NHCHO	0.048	15	1.09	56	313	23	1167
36	CH_2NHAc	0.038	18	1.2	38	474	32	1000
		Paromomycin series (5 basic amines in parent)						
Paromomycin, 3	CH_2OH	0.033	131	12	37	3970	364	1121
40	H	1.059	-	-	-	-	-	-
46	CH_3	1.092	-	-	-	-	-	-
49	CH_2NH_2	0.090	36	6.3	6.6	400	70	73
51	CH_2NHCHO	0.040	166	27	114	4140	675	2850
52	CH_2NHAc	0.513	-	-	-	-	-	-
53	$CH_2NHCONH_2$	0.096	114	45	184	1188	469	1917
		Propylamycin series (5 basic amines in parent)						
Propylamycin, 19	CH_2OH	0.022	150	56	61	6818	2545	2773
20	CH_2NH_2	0.14	62	38	9.0	443	271	64
21	CH_2NHCHO	0.034	505	118	175	14853	3471	5147
22	$CH_2NHCONH_2$	0.078	318	189	194	4077	2423	2487
		Ribostamycin series (4 basic amines in parent)						
Ribostamycin, 6	CH_2OH	0.089	461	86	416	5180	966	4674
57	H	1.612	-	-	-	-	-	-
64	CH_2NH_2	0.088	76	34	50	861	386	570
66	CH_2NHCHO	0.686	-	-	-	-	-	-

Compound	4''-Substituent	IC$_{50}$ (μM)				Selectivity		
		wt	**Mit13**	**A1555G**	**Cyt14**	**Mit13**	**A1555G**	**Cyt14**
67	CH_2NHAc	3.542	-	-	-	-	-	-
Apralog series (5 basic amines in parent)								
14	CH_2OH	0.16	439	272	475	2815	1745	3045
15	H	0.128	332	211	444	2594	1648	3469
79	CH_3	0.28	562	323	462	2022	1162	1662
16	CH_2NH_2	0.12	113	81	111	941	675	925
75	CH_2NHCHO	0.31	572	232	567	1863	755	1847
Advanced Apralog series (6 basic amines in parent)								
17	CH_2OH	0.071	68	13	190	957	183	2676
18	CH_2NH_2	0.030	42	20	38	1400	667	1267
76	CH_2NHCHO	0.085	93	11	142	1096	130	1669

[a] All values were determined in at least duplicate using 2.5-fold dilution series

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Table 2.

Antibacterial Activity Against Relevant Pathogens (MIC, μg/mL)[a]

	Strain	AG212	AG215	AG290	AG225	AG220	AG038
Compound	**4”-Substituent**	***E. coli***	***K. pneumoniae***	***E. cloacae***	***A. baumanii***	***P. aeruginosa***	**MRSA**
	Neomycin series (6 basic amines in parent)						
Neomycin, 4	CH_2OH	1	0.5	1	1–2	32–64	1
27	H	1–2	0.5–1	1–2	2–4	>64	1
31	CH_3	2	1–2	1–2	2–4	>64	1–2
34	CH_2NH_2	2–4	2	2–4	8	16–32	1–2
35	CH_2NHCHO	1–2	0.5–1	1	2–4	64	1–2
36	CH_2NHAc	1–2	0.5–1	1–2	2–4	>64	1
	Paromomycin series (5 basic amines in parent)						
Paromomycin, 3	CH_2OH	2–4	1	2	2–4	>64	2
40	H	16	4–8	8–16	16	>64	8
46	CH_3	32	16	32	64	>64	16–32
49	CH_2NH_2	4	2–4	4	4	>64	2–4
51	CH_2NHCHO	2–4	1	2	2–4	>64	2–4
52	CH_2NHAc	16	8	8–16	8–16	>64	8
53	$CH_2NHCONH_2$	4–8	2	2–4	4–8	>64	2–4
	Propylamycin series (5 basic amines in parent)						
Propylamycin, 19	CH_2OH	1	0.25–0.5	0.5	1–2	8	1–2
20	CH_2NH_2	2–4	1	1	2	4	2
21	CH_2NHCHO	1–2	1	1	2	8	1–2
22	$CH_2NHCONH_2$	4	2–4	2–4	2–4	>64	2–4
	Ribostamycin series (4 basic amines in parent)						
Ribostamycin, 6	CH_2OH	4	2	2–4	4	>128	4
57	H	64	32	64	64	>128	32
64	CH_2NH_2	8	4	4	4	>32	8
66	CH_2NHCHO	16	8	8–16	16	>128	16–32

Compound	Strain / 4”-Substituent	AG212 *E. coli*	AG215 *K. pneumoniae*	AG290 *E. cloacae*	AG225 *A. baumanii*	AG220 *P. aeruginosa*	AG038 MRSA
67	CH_2NHAc	64–128	32	64	128	>128	64–128
	Apralog series (5 basic amines in parent)						
14	CH_2OH	4–8	1–2	2–4	8–16	32–64	4–8
15	H	8	2–4	2–4	8	16–32	2–4
79	CH_3	8–16	8	8–16	16	64	4
16	CH_2NH_2	4	1–2	2	8	4–8	2
75	CH_2NHCHO	8	2–4	4–8	16	>64	8–16
	Advanced Apralog series (6 basic amines in parent)						
17	CH_2OH	2	1–2	1–2	4–8	16	2–4
18	CH_2NH_2	1–2	0.5–1	1	2	2	1–2
76	CH_2NHCHO	2–4	1	1–2	4–8	16–32	4

[a] All values were determined in at least duplicate using 2-fold dilution series

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Table 3.

MIC assays on engineered (EC) and clinical (AG) strains of *E. coli* bearing specific resistance determinants (MIC, μg/mL) [a]

		Engineered (EC) Strains					Clinical (AG) Strains		
		EC026	**EC189**	**EC191**	**EC125**	**EC141**	**AG212**	**AG163**	**AG166**
Compound	**4''-Substituent**	**wt**	**APH(3')-Ia**	**APH(3')-IIa**	**APH(3')-IIb**	**APH(3')-VI**	**wt**	**APH(3')-I**	**APH(3')-II**
Neomycin series (6 basic amines in parent)									
Neomycin, 4	CH_2OH	1	32–64	16–32	16–32	8–16	1	64–128	64–128
27	H	1	128	128	128	64–128	1–2	>128	>128
31	CH_3	1	128	>128	128	64–128	2	>128	>128
34	CH_2NH_2	1–2	64–128	32	32	16–32	2–4	128	128
35	CH_2NHCHO	1	8–16	16	8–16	16	1–2	32–64	64
36	CH_2NHAc	0.5–1	128	128	128	128	1–2	>128	>128
Paromomycin series (5 basic amines in parent)									
Paromomycin, 3	CH_2OH	1	>128	32–64	64–128	64	2–4	>128	>128
40	H	4	>64	>64	>64	>64	16	>128	>128
46	CH_3	16	>128	>128	>128	>128	32	>128	>128
49	CH_2NH_2	4	16–32	16–32	32	16–32	4	>64	>64
51	CH_2NHCHO	1	8–16	32	64	32–64	2–4	64	>128
52	CH_2NHAc	4–8	>128	>128	>128	>128	16	>128	>128
53	$CH_2NHCONH_2$	2	32	32–64	>32	>32	4–8	>128	>128
Propylamycin series (5 basic amines in parent)									
Propylamycin, 19	CH_2OH	0.25–0.5	64–128	0.5–1	0.5–1	0.5	1	>128	1–2
20	CH_2NH_2	0.5–1	2–4	1–2	0.5–1	0.5–1	2–4	16	2–4
21	CH_2NHCHO	0.5–1	2	0.5–1	0.5	0.25	1–2	4–8	2–4
22	$CH_2NHCONH_2$	1	8	2–4	2	0.5–1	4	32	16–32
Ribostamycin series (4 basic amines in parent)									
Ribostamycin, 6	CH_2OH	1–2	>128	>128	>128	>128	4	>128	>128
57	H	16	>128	>128	>128	>128	64	>128	>128
64	CH_2NH_2	-	-	-	-	-	8	>128	>128

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Compound	4''-Substituent	Engineered (EC) Strains					Clinical (AG) Strains		
		EC026 wt	**EC189** APH(3')-Ia	**EC191** APH(3')-IIa	**EC125** APH(3')-IIb	**EC141** APH(3')-VI	**AG212** wt	**AG163** APH(3')-I	**AG166** APH(3')-II
66	CH_2NHCHO	4	>128	>128	>128	>128	16	>128	>128
67	CH_2NHAc	16–32	>128	>128	>128	>128	64–128	>128	>128
Apralog series (5 basic amines in parent)									
14	CH_2OH	1–2	1–2	1	1	1	4–8	8	4–8
15	H	2	1–2	1	1	1	8	8	8
79	CH_3	2–4	4–8	2–4	2–4	2–4	8–16	8–16	8–16
16	CH_2NH_2	1–2	1	1	1	1	4	2	8
75	CH_2NHCHO	2–4	-	-	-	-	8	16	8–16
Advanced Apralog series (6 basic amines in parent)									
17	CH_2OH	0.5–1	0.5–1	0.5	0.5	0.5	2	4	2
18	CH_2NH_2	0.25–0.5	0.5–1	0.5	0.25	0.5	1–2	1–2	2
76	CH_2NHCHO	0.5–1	0.5–1	0.25–0.5	0.25	0.25–0.5	2–4	2–4	2–4

[a] All values were determined in at least duplicate using 2-fold dilution series