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Synthesis and Antiribosomal Activities of 4′-*O*-, 6′-*O*-, 4″-*O*-, 4′,6′-*O*- and 4″,6″-*O*-Derivatives in the Kanamycin Series Indicate Differing Target Selectivity Patterns between the 4,5- and 4,6-Series of Disubstituted 2-Deoxystreptamine Aminoglycoside Antibiotics

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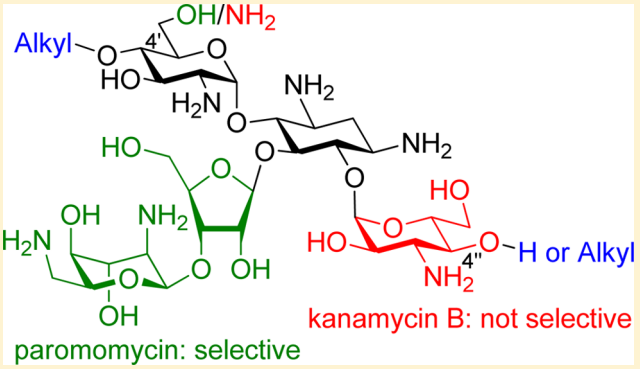
Supporting Information

ABSTRACT: Chemistry for the efficient modification of the kanamycin class of 4,6-aminoglycosides at the 4′-position is presented. In all kanamycins but kanamycin B, 4′-*O*-alkylation is strongly detrimental to antiribosomal and antibacterial activity. Ethylation of kanamycin B at the 4″-position entails little loss of antiribosomal and antibacterial activity, but no increase of ribosomal selectivity. These results are contrasted with those for the 4,5-aminoglycosides, where 4′-*O*-alkylation of paromomycin causes only a minimal loss of activity but results in a significant increase in selectivity with a concomitant loss of ototoxicity.



KEYWORDS: *ototoxicity, decoding A site, mitochondrial rRNA, antibacterial activity, ribosomal selectivity*

Drug-induced hearing loss, or ototoxicity, is a common side effect of the aminoglycoside antibiotics (AGAs), whose origin has been traced to AGAs binding to the decoding A site in eukaryotic ribosomal RNA (rRNA),[1−5] following drug uptake into inner ear hair cells via mechanotransducer channels.[6−10] Given our previous success in ameliorating ototoxicity in the 4,5-disubstituted deoxystreptamine class of AGAs by modifying drug selectivity at the ribosomal target level,[11] we describe here the extension of our studies to the kanamycins in the 4,6-disubstituted series of AGAs.

Mutations A1555G and C1494U in the base of the mitoribosomal decoding A site are associated with inherited hypersusceptibility to aminoglycoside ototoxicity.[12−14] The ability to incorporate the complete decoding A site domains of human mitochondrial (wild-type and mutant) and cytosolic rRNA into bacterial rRNA by means of domain shuffling experiments[14,15] has enabled the development of cell-free translation assays with which to probe AGA inhibition of mitochondrial, cytosolic, and bacterial protein synthesis.[1] These assays predict both AGA antibacterial activity and drug selectivity at the target level, facilitating the development of potent AGAs with reduced ototoxicity and systemic toxicity. Screening of commercially available AGAs with these cell-free translation assays first enabled the identification of the unusual mono-substituted 2-deoxystreptamine apramycin **1** (Figure 1) as an AGA with reduced ototoxic potential, as demonstrated in murine

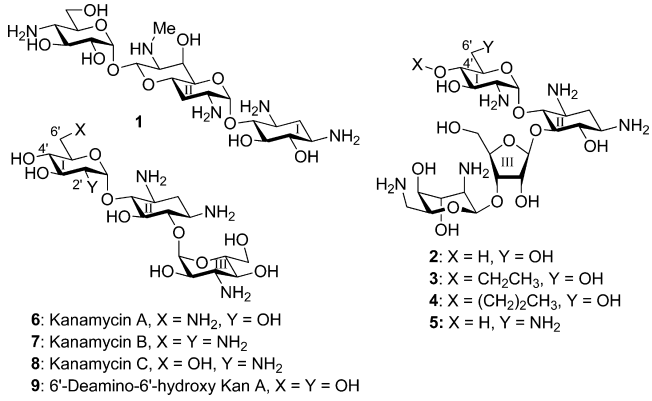


Figure 1. Apramycin, kanamycin, neomycin, and paromomycin analogues.

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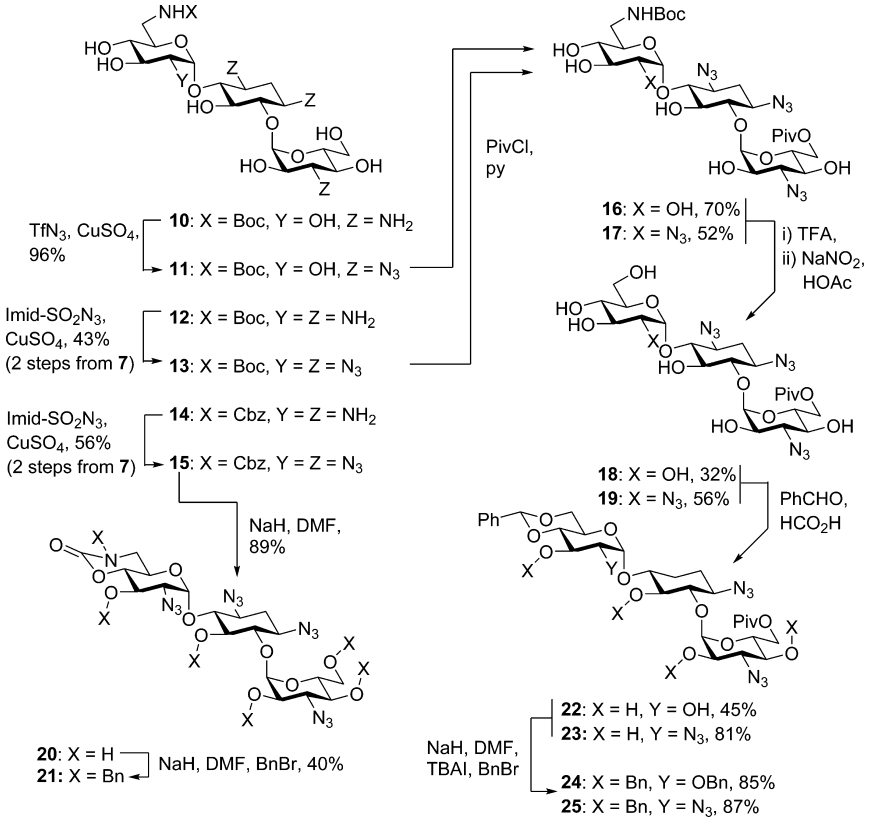
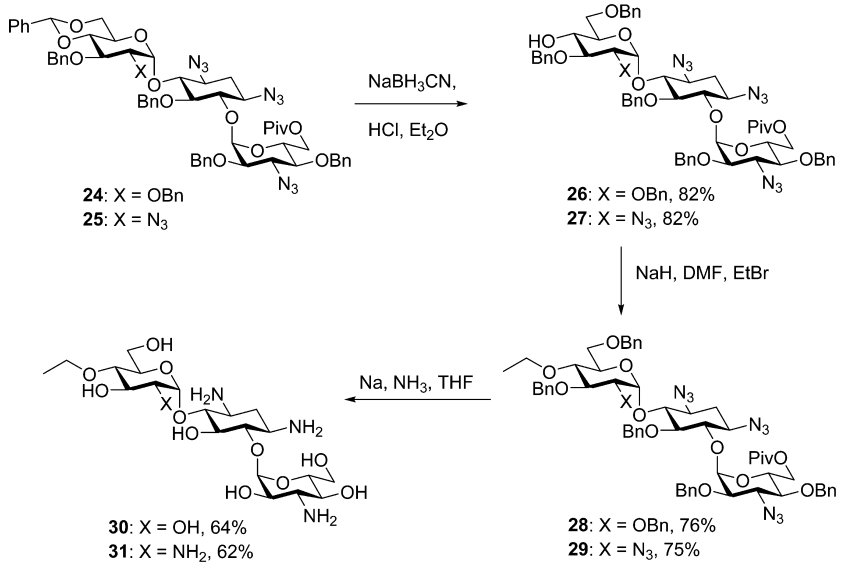
Scheme 1. Preparation of Key Cyclic Intermediates



Scheme 2. Preparation of 6′-Deamino-4′-*O*-ethyl-6′-hydroxykanamycin A and of 4′-*O*-Ethylkanamycin C



and guinea pig models.[16] The same cell-free translation assays were instrumental in the subsequent identification and optimization of 4′-*O*-substituted derivatives of paromomycin **2** in the 4,5-disubstituted class of AGAs as next-generation AGAs being essentially devoid of ototoxicity, again as borne out for **3** and **4** in animal models.[17−19] Similarly, key features of apramycin required for binding to the pro- and eukaryotic decoding A sites were revealed through use of these assays.[20]

In this paper we describe our efforts to extend the favorable characteristics conferred on paromomycin **2** by 4′-*O*-alkylation to the kanamycin (Kan) series of the 4,6-disubstituted class of AGAs **6**−**9**. The influence of such modifications in the kanamycin series is of particular interest in view of the recent description of 4′-*O*-kanamycin B derivatives with activity against various AGA resistant bacterial strains.[21] Literature reports[22−24] on the synthesis of various derivatives of **7** functionalized at the 4″-position prompted us to also synthesize and evaluate a simple 4″-*O*-alkyl and a 4″,6″-*O*-benzylidene derivative of kanamycin B.

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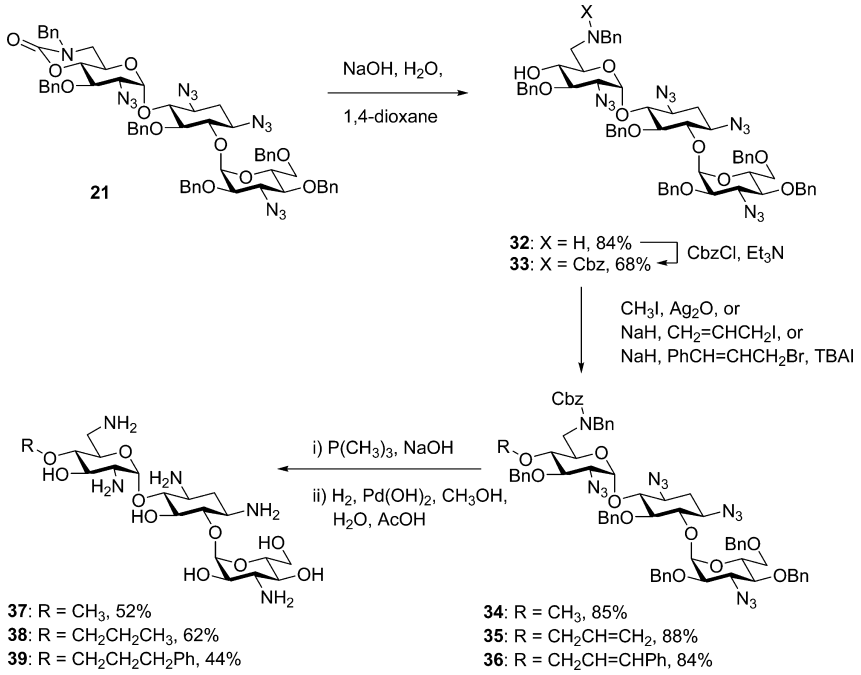
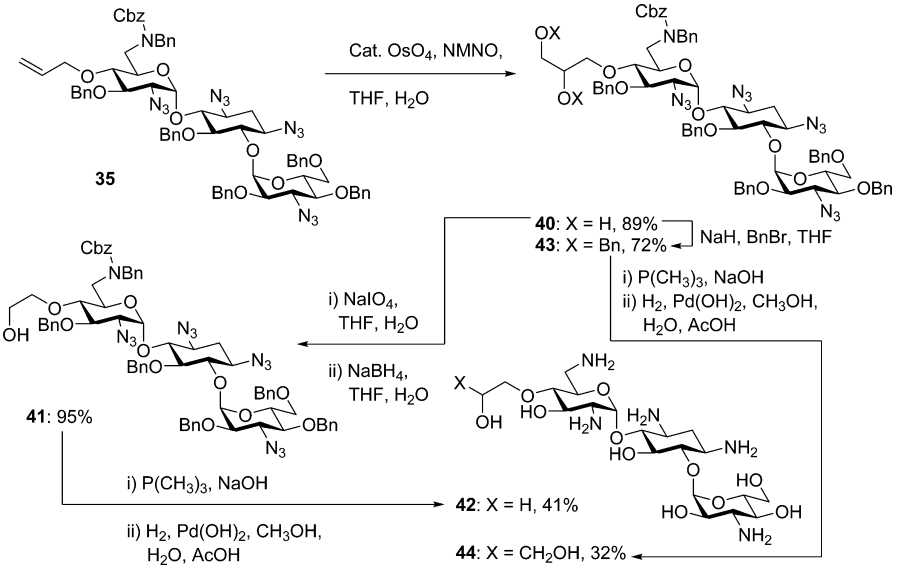
Scheme 3. Synthesis of 4′-*O*-Alkylkanamycin B Derivatives



Scheme 4. Preparation of 4′-*O*-Hydroxyethyl and Dihydroxypropyl Derivatives of Kanamycin B



RESULTS

Synthesis. As in the paromomycin series,[17,19] the synthesis of derivatives at the 4′-position of the kanamycins requires selective protection of the 4′-hydroxy group, which is best achieved by formation of cyclic derivatives spanning the 4′- and 6′-positions. To this end, the known 6′-*N*-Boc Kan A **10**,[25] 6′-*N*-Boc Kan B **12**,[25] and 6′-*N*-Cbz Kan B **14**[26] were obtained by selective acylation of kanamycins A **6** and B **7**, respectively, and then transformed into the corresponding perazides **11**, **13**, and **15** with either triflyl azide[27−29] or imidazole-1-sulfonyl azide (Stick's reagent)[30−32] (Scheme 1). Reaction of **11** and **13** with pivaloyl chloride then gave the 6″-*O*-pivalates **16** and **17** from which the Boc groups were removed with trifluoroacetic acid and the resulting amines exposed to sodium nitrite in aqueous acetic acid to give the 6′-deamino-6-hydroxy-Kan derivatives **18** and **19** (Scheme 1). Treatment of **15** with an excess of sodium hydride in DMF gave the Kan B oxazinone **20**, which was then perbenzylated to afford **21** (Scheme 1). The benzylidene acetals **22** and **23** were obtained from **18** and **19**, respectively, on treatment with benzaldehyde and formic acid and were subsequently converted into the perbenzyl ethers **24** and **25** by standard methods (Scheme 1).

In the 6′-deamino-6′-hydroxy Kan A series a 4′-*O*-ethyl derivative was obtained from **24** by regioselective reduction of the benzylidene acetal with sodium cyanoborohydride and HCl in ether[33−36] (Scheme 2), selectively giving the 6′-*O*-benzyl-4′-ol **26** (Scheme 2). Application of the analogous protocol to the Kan C benzylidene acetal **25** gave **27**. 4′-*O*-Alkylation of **26** and **27** with sodium hydride and ethyl bromide then afforded **28** and **29**, both of which were converted to the target 4′-*O*-ethyl derivatives

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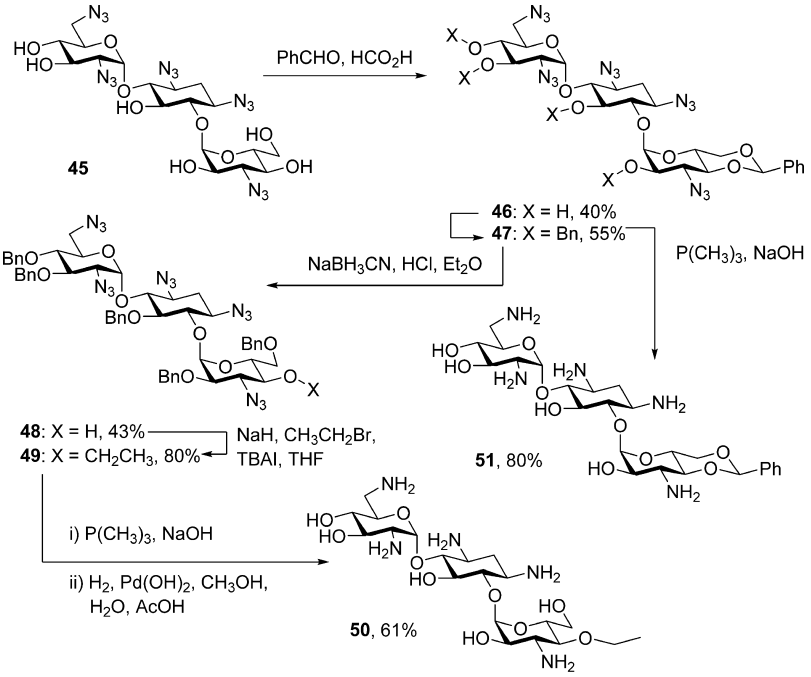
Scheme 5. Preparation of 4″-*O*-Ethylkanamycin B and of 4″,6″-*O*-Benzylidenekanamycin B



30 and **31** by treatment with sodium in liquid ammonia (Scheme 2).

In the Kan B series, hydrolytic cleavage of the oxazinone **21** gave the amino alcohol **32**, which was converted to the carbamate **33**. Subsequent alkylation with methyl iodide in the presence of silver oxide then gave **34**, whereas treatment with sodium hydride and either allyl iodide or cinnamyl bromide and tetrabutylammonium iodide gave the allyl and cinnamyl derivatives **35** and **36**, respectively. Deprotection of **34**−**36** was achieved by a two-step protocol involving Staudinger reduction of the azides with aqueous trimethylphosphine,[37] followed by hydrogenolysis, and affording **37**−**39**, respectively (Scheme 3).

Two 4′-*O*-hydroxyalkyl derivatives of Kan B were accessed from the 4′-*O*-allyl derivative **35**, beginning with dihydroxylation according to the Van Rheenan protocol[38] to give the diol **40** as an inseparable mixture of diastereomers (Scheme 4). Periodate cleavage of **40** followed by reduction with sodium borohydride afforded **41**, which on Staudinger reduction of the azides followed by hydrogenolysis gave the 4′-*O*-(2-hydroxyethyl) Kan B derivative **42** (Scheme 4). Attempted Staudinger reaction of **40** gave a complex reaction mixture from which the desired product could only be obtained in low yield. The diol was consequently first subjected to benzylation giving **43**, which was then converted by Staudinger reaction and subsequent hydrogenolysis to the dihydroxypropyl Kan B derivative **44**, isolated in the form of an inseparable 3:2 mixture of diastereomers (Scheme 4).

Finally, the chemistry employed to manipulate the 4′-position of 6′-deamino-6′-hydroxy Kan A derivatives was adapted to the 4″- and 6″-positions of Kan B. Accordingly, Kan B **7** was converted to the pentaazide **45**[21] with Stick's reagent in 40% yield. The 4″,6″-*O*-benzylidene acetal was then installed with benzaldehyde and formic acid giving **46**, which was converted to the perbenzyl derivative **47** in the usual manner (Scheme 5). Reduction with sodium cyanoborohydride and HCl in ether then afforded the 4″-monohydroxy derivative **48**, which was converted to the 4″-*O*-ethyl derivative **49** by alkylation with ethyl bromide, tetrabutylammonium iodide, and sodium hydride. The two-step Staudinger reduction and hydrogenolysis protocol then gave the 4″-*O*-ethyl-Kan derivative **50**, whereas simple Staudinger reduction of **46** gave 4″,6″-*O*-benzylidene-Kan B **51** (Scheme 5).

Determination of Antiribosomal and Antibacterial Activities. The activity at the target level of the various kanamycin derivatives prepared was assayed by means of cell-free luciferase translation assays, employing either wild-type bacterial ribosomes or hybrid ribosomes carrying the complete decoding A site cassettes from human mitochondrial ribosomes (Mit13), from the A1555G allele of human mitochondrial ribosomes (A1555G), and from human cytosolic ribosomes (Cyt14) (Figure 3).[14,15] The antiribosomal activities of the kanamycin derivatives (Figures 1 and 2) determined in this manner are presented in Table 1. With the exception of compounds **30** and **31**, which were omitted because of their poor activities at the ribosomal target level, all compounds were screened for antibacterial activity against clinical isolates of the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative

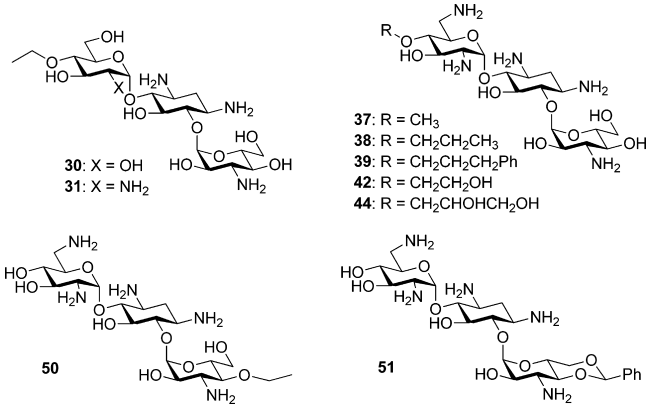


Figure 2. New kanamycin derivatives studied.

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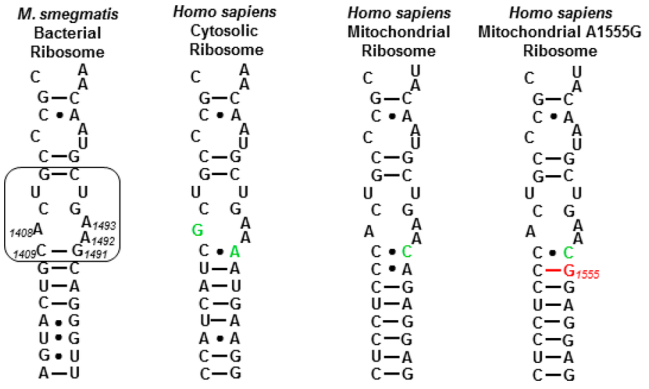

Figure 3. Decoding A sites of the bacterial and hybrid ribosomes employed in cell-free translation assays. The AGA binding pocket is boxed. The bacterial numbering scheme is illustrated for the AGA binding pocket. Changes from the bacterial ribosome binding pocket are colored green. The A1555G mutant conferring hypersusceptibility to AGA ototoxicity is colored red.

bacterium *Escherichia coli* obtained from the Diagnostic Department of the Institute of Medical Microbiology at the University of Zurich (Table 2). The *S. aureus* strains are all methicillin-resistant, and two of them (AG042 and AG044) are additionally resistant to gentamicin.

DISCUSSION

Influence of the Number and Location of Amino Groups in the 4,5 and 4,6 Series of Aminoglycoside Antibiotics. Previously, on the basis of cell-free translation assays carried out with wild-type bacterial ribosomes and recombinant ribosomes carrying single-point mutations representative of the rRNA polymorphisms present in the decoding A site of eukaryotic ribosomes, we studied how the number and position of amino groups in ring I of the 4,5 and 4,6 classes of AGAs affect drug affinity for the ribosome.[39] Guided by X-ray crystallographic studies of AGAs in complex with bacterial ribosomes[40,41] and by studies with mutant ribosomes,[42−44] ring I amination patterns were analyzed for the 4,5- and 4,6-AGAs in terms of the contributions of the individual amino groups to drug interaction with the ribosome.[39] The present studies using wild-type bacterial and a series of mutant hybrid bacterial ribosomes (Figure 3) carrying the complete A site cassettes of the human mitochondrial ribosome and its A1555G deafness allele and of the human cytoplasmic ribosome extend the earlier observations of substitution pattern influence on drug affinity and selectivity (Table 1). We find consistent differences between the 4,5 and 4,6 series of AGAs. Thus, paromomycin **2**, a 4,5-type AGA, and kanamycin C **8**, a 4,6-type AGA, share a common ring I with a single amino group at the 2′-position, yet their activity against the bacterial ribosome differs by >1 $\log_{10}$ unit (Table 1). In contrast, neomycin **5** and kanamycin B **7**, 4,6- and 4,5-AGAs with two amino groups in their identical ring I at the 2′- and 6′-positions, have the same affinity for the bacterial ribosome (Table 1). Apparently, the exchange of amino by hydroxy groups at the 6′-position has a far greater influence on activity in the kanamycins (**6**−**9**) than in the paromomycin/neomycin series (**2** and **5**).[39]

These observations suggest that in the kanamycin series of 4,6-aminoglycosides the highly basic[45] 6′-amino group is more important for binding to the target rRNA decoding A site than the 2′-amino group. This may reflect the overall lower degree of amination in the 4,6-AGAs (five amino groups) than in the 4,5-AGAs (six amino groups) and the location of specific amino groups.[39] With respect to the location of amino groups, ring IV of paromomycin and neomycin, with its two basic amino groups,[46] exerts its influence electrostatically and not through any important directional interaction with the ribosome.[47] Indeed, it is possible to replace ring IV of neomycin by simple aminoalkyl and diaminoalkyl groups and retain most of the antibacterial activity of the parent.[48] In contrast, the monobasic 3″-amino-D-glucopyranosyl ring III of the kanamycins extends into a different binding pocket in which the 3″-amino group is involved in a specific hydrogen bond with N7 of the ribosomal G1405. Consistent with this argument, replacement of ring III in the kanamycin series by simple aminoalkyl groups reveals the importance of aminoalkyl chain length on the affinity for a model decoding A site and on antibacterial activity.[49−51]

Influence of Alkylation at the 4′-Position on Antiribosomal Activity and Selectivity in the Kanamycins. Alkylation at the 4′-position of paromomycin (**3** and **4**) benefits drug selectivity.[17,19] In the kanamycin series, the introduction of a 4′-*O*-ethyl group **30** was found to completely abolish the activity of the weakly active 6′-hydroxykanamycin A **9** (Table 1). Similarly, 4′-*O*-ethylkanamycin C **31** showed a 70-fold loss of

Table 1. Antiribosomal Activities (IC_{50}, μg/mL) and Selectivities[a]

compound	bacterial activity	Mit13 activity (selectivity)	A1555G activity (selectivity)	Cyt14 activity (selectivity)
paromomycin (**2**)	0.02	50.19 (2506)	5.39 (267)	9.41 (471)
neomycin (**5**)	0.02	1.62 (81)	0.22 (11)	17.12 (856)
kanamycin A (**6**)	0.03	40.19 (1340)	0.83 (28)	173.36 (5779)
kanamycin B (**7**)	0.03	13.38 (446)	0.47 (16)	40.74 (1358)
kanamycin C (**8**)	0.27	>240 (−)	21.32 (79)	62.30 (231)
6′-hydroxykanamycin A (**9**)	10.83	>240 (−)	>240 (−)	>240 (−)
4′-*O*-ethyl-6′-hydroxykanamycin A (**30**)	207.5	>240 (−)	>240 (−)	>240 (−)
4′-*O*-methylkanamycin B (**37**)	0.09	34.85 (387)	6.79 (75)	57.26 (636)
4′-*O*-propylkanamycin B (**38**)	0.14	111.55 (797)	7.15 (51)	229.27 (1638)
4′-*O*-ethylkanamycin C (**31**)	19.53	>240 (−)	>240 (−)	>240 (−)
4′-*O*-(3-phenylpropyl)kanamycin B (**39**)	0.16	43.48 (272)	11.25 (70)	98.97 (619)
4′-*O*-(2-hydroxyethyl)kanamycin B (**42**)	0.07	21.05 (301)	2.09 (30)	98.89 (1413)
4′-*O*-(2,3-dihydroxypropyl)kanamycin B (**44**)	0.04	6.59 (165)	0.74 (19)	50.94 (1274)
4″,6″-*O*-benzylidenekanamycin B (**51**)	0.90	104.86 (117)	34.34 (38)	95.26 (106)
4″-*O*-ethylkanamycin B (**50**)	0.07	45.27 (647)	1.86 (27)	153.69 (2196)

[a]Selectivities are obtained by dividing the eukaryotic activity by bacterial activity.

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Table 2. Antibacterial Activities (MIC, μg/mL)

	MRSA				*E. coli*			
compound	AG038	AG039	AG042	AG044	AG006	AG001	AG055	AG003
paromomycin (**2**)	4	>256	>256	4–8	2–4	16–32	8	8–16
neomycin (**5**)	0.5–1	128	128	0.5–1	1	8–16	4	4
kanamycin A (**6**)	4	≥128	256	64	1–2	8	4	8
kanamycin B (**7**)	2	≥256	256	8–16	0.5	4	4	4
kanamycin C (**8**)	16–32	≥256	>256	256	8	64	32–64	64–128
6′-hydroxykanamycin A (**9**)	>128	>128	>256	>256	>256	>128	>128	>256
4′-*O*-methylkanamycin B (**37**)	4–8	8–16	128	32–64	2–4	16–32	16	64
4′-*O*-propylkanamycin B (**38**)	16	128	>128	64–128	4	16	16	64
4′-*O*-(3-phenylpropyl)kanamycin B (**39**)	8	>64	>64	32–64	2–4	8	8	32–64
4′-*O*-(2-hydroxyethyl)kanamycin B (**42**)	16	32–64	>64	32–64	4	32	16	32–64
4′-*O*-(2,3-dihydroxypropyl)kanamycin B (**44**)	8	16–32	>64	32	2	16	8–16	32
4″,6″-*O*-benzylidenekanamycin B (**51**)	16	>128	>128	128	1	4	4	16–32
4″-*O*-ethylkanamycin B (**50**)	8	>128	>128	64–128	2	8	8	16

activity compared to kanamycin C **8** (Table 1). This loss of activity upon alkylation of kanamycin C is noteworthy in view of the much smaller 4-fold reduction of activity caused by the same modification of paromomycin. In view of the significant loss of activity observed upon 4′-*O*-ethylation of 6′-hydroxykanamycin A and kanamycin C, all subsequent efforts were directed at kanamycin B with the expectation that the presence of two amino groups in ring I would better palliate any loss of affinity due to the alkylation. Indeed, the loss of activity against the bacterial ribosome on either 4′-*O*-methylation **37**, propylation **38**, or 3-phenylpropylation **39** of kanamycin B (Table 1) is largely consistent with those seen for the analogous changes in the paromomycin series. However, 4′-*O*-alkylation of kanamycin B only little benefits drug selectivity. Whereas 4′-*O*-hydroxyethylation **42** and especially dihydroxypropylation **44** afford an increase in activity over the simple alkyl derivatives, they also bring a significant increase in activity against the mitochondrial, mutant A1555G mitochondrial, and cytoplasmic ribosomes, resulting in an overall reduction in selectivity (Table 1). Again, this observation is in contrast with the paromomycin series in which the 4′-*O*-hydroxyethyl and dihydroxypropyl modifications result in an increase in selectivity against the A1555G mutant mitochondrial and cytosolic ribosomes.[19] Overall, in contrast to the paromomycin series, 4′-*O*-alkylation of kanamycin B does not afford an exploitable increase in ribosomal selectivity.

Influence of Modification at the 4″- and 4″,6″-Positions of Kanamycin B on Antiribosomal Activity and Selectivity. The influence of kanamycin ring III modifications was briefly examined for kanamycin B. The introduction of a 4″,6″-*O*-benzylidene acetal **51** into kanamycin B causes a significant loss of affinity for the bacterial ribosome (Table 1). In contrast, ethylation at the 4″-position **50** resulted in a compound with little loss of bacterial antiribosomal activity (Table 1), thereby revealing the 4″-position to be less susceptible to the introduction of a small alkyl group than the 4′-position. However, the 4″-*O*-ethylkanamycin B derivative **50** showed only a minor loss of activity for the deafness allele (A1555G) compared to kanamycin B itself. As no increase in selectivity for the bacterial over the human mutant A1555G mitochondrial ribosomes was observed, 4″-*O*-alkylation of kanamycin B was not pursued further.

Influence of Substitution on Antibacterial Activity. The 4′-*O*-alkylkanamycin derivatives **37**–**39** and the hydroxyalkyl analogues **42** and **44** showed moderate antibacterial activity compared to kanamycin B (Table 2). In addition, compounds **37**–**39**, **42**, and **44** exhibited modest activity against a clinical isolate of MRSA resistant to kanamycin B (Table 2, strain AG039), suggesting that derivatization at the 4′-position affords some protection against the aminoglycoside modifying enzyme (AME) responsible for resistance to kanamycin B in this strain. Similar effects have been noted by the Ye laboratory for a series of 4′-amido-4′-deoxykanamycin B derivatives.[21] The comparable phenomenon is observed in the 4,5-series of AGAs with the 4′-*O*-alkylparomomycin derivatives retaining activity against MRSA strain AG039 for which paromomycin itself is devoid of activity (Table 2).[19] The 4′-*O*-glycosylparomomycins[18] also retain activity against this strain, suggesting that the phenomenon is general. All of the 4′-*O*-substituted kanamycin B derivatives retained moderate activity against *E. coli*, albeit at a lower level than kanamycin B itself.

Ethylation at the 4″-position, or benzylidenation at the 4″,6″-positions, of kanamycin B affords compounds **50** and **51** that retain good activity against *E. coli* and partly against MRSA (Table 2). However, these modifications afford no protection against the AMEs active in the kanamycin B-resistant strains of MRSA. Nevertheless, and consistent with reports form the Chang laboratory,[22,23] compounds **50** and **51** identify the 4″-position of kanamycin B as a viable locus for the modification and possible improvement of activity in this class of 4,6-AGAs.

Conclusions. Efficient chemistry has been developed for the modification of the kanamycins at the 4′- and 4″-positions enabling the evaluation of such derivatives at the target level, through the use of cell-free translation assays, and as antibacterials. Modifications at the 4′-position are fatal to the antiribosomal acitivity of the already only weakly active kanamycin C and 6′-hydroxylated kanamycin A but lead to only a modest reduction in activity in the kanamycin B series. Modification of kanamycin B at the 4′-position does not afford greater selectivity, suggesting that this class of modifications will not be of use in engineering less ototoxic 4,6-AGAs, but may afford protection against certain strains of MRSA that are resistant to kanamycin.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Full experimental details and copies of ^{1}H and ^{13}C NMR spectra for the new AGAs (**37**−**39**, **42**, **44**, **50**, and **51**) (PDF)

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Notes

The authors declare no competing financial interest.

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