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Inhibition of Aminoglycoside-Deactivating Enzymes APH(3′)-IIIa and AAC(6′)-Ii by Amphiphilic Paromomycin O2″-Ether Analogues

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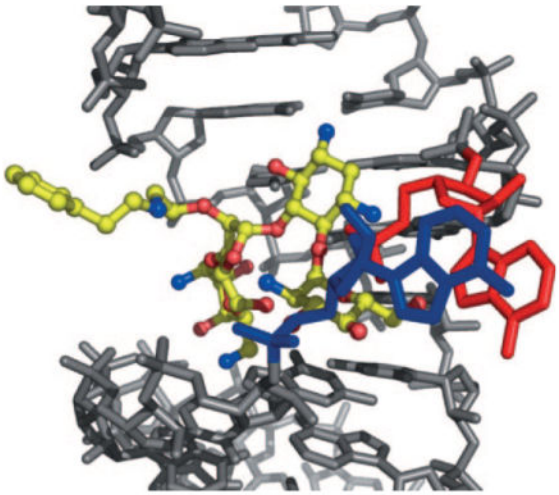
Paromomycin analogue activity

Novel amphiphilic aminoglycosides are shown to inhibit clinically relevant deactivating enzymes, without undergoing significant deactivation themselves.



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Keywords

aminoglycosides; antibacterial agents; antibiotics; APH(3′)-IIIa; drug discovery; paromomycin

Aminoglycoside antibiotics have been in clinical use for the last sixty years. Following the discovery of streptomycin in 1944, many other broad-spectrum aminoglycosides have been discovered (Figure 1).[1,2] Typically, aminoglycosides are classified according to the substitution pattern of the deoxystreptamine unit that forms the core of these antimicrobial compounds. 4,5-Disubstituted deoxystreptamine compounds are comprised in class A aminoglycosides (Figure 1a), whereas 4,6-disubstituted derivatives are in class B (Figure 1b). The isolation of new aminoglycosides declined rapidly in the early seventies, when efforts were diverted to the preparation of semi-synthetic analogues intended to counteract increasing bacterial resistance to these useful drugs.[3] This led to dibekacin (**11**),[3a] a deoxygenated analogue of kanamycin B (**7**), and to ar-bekacin (**12**),[3c] by modifying the N1 group of dibekacin with the 2*S*-4-amino-2-hydroxybutanoyl moiety originally found in butirosin (**2**). Although it has been known for decades that aminoglycosides interfere with protein biosynthesis by binding to the prokaryotic ribosome,[4] the lack of precise structural information hampered the identification of beneficial drug modification until the late nineties. A better understanding of the mode of action of aminoglycosides, exemplified by paromomycin (**3**), was obtained from biochemical[5] and spectroscopic[6] approaches, as well as by mass spectrometry[7] and nuclear magnetic resonance.[8] Definitive confirmation was provided by X-ray structures of the 30S ribosomal subunit bound to amino-glycosides, as well as kinetic studies of protein biosynthesis.[9,10] This long-awaited information led to an increase in structure-based modifications of aminoglycosides, leading to many of semisynthetic analogues from our laboratory and elsewhere.[11,12]

Aminoglycoside therapy is usually limited to a clinical environment since parenteral injection of these highly hydrophilic drugs is required to obtain the desired plasma concentration in a patient.[2] Their use is also limited by their oto- and nephrotoxicity. Since well-studied dosage strategies are used to maximize their antibiotic potential while minimizing their toxicity,[13] the future of these antibiotics will eventually be compromised by the emergence of bacterial resistance. In order to overcome this threat to human health, the structures of some other classes of antibiotics have also been substantially modified. For example, the β-lactam family has “evolved” remarkably since the first report of penicillin resistance.[1,14] However, clinically effective aminoglycosides have been only minimally modified since their first use.[1,2] Bacteria have developed two general strategies to resist aminoglycosides: 1) diminution of intracellular concentration of the antibiotic, mainly by efflux; and 2) chemical modification of the drug itself or its biological target.[15] Fortunately, bacterial responses influencing aminoglycoside intracellular concentration, as well as the chemical modification of the ribosomal A-site, are still not widespread. However, modification of aminoglycosides by deactivating enzymes is a major threat to the continued clinical efficacy of these antibiotics.[15]

Aminoglycoside deactivating enzymes can be divided into three categories: nucleotidyltransferases (ANTs), acetyltransferases (AACs), and phosphotransferases

(APHs).[15] Once adenylated, acetylated or phosphorylated, the affinity of an aminoglycoside for its biological target is drastically attenuated. There are multiple ANTs, AACs and APHs, that can each target different amino or hydroxy groups on the various aminoglycosides. APH(3′)-IIIa mediates the phosphorylation of aminoglycosides at their 3′-OH position by a sequential mechanism where ATP binds first and ADP is the last species to leave the active site.[16] X-ray structures of APH(3′)-IIIa bound to kanamycin A or neomycin B are available.[17] The *Enterococcus faecium* enzyme AAC(6)-Ii′ catalyzes the acetylation of most aminoglycosides at the 6′-N position. This isoform proceeds via an ordered bi bi mechanism, with acetyl coenzyme A (AcCoA) binding first.[18] Crystal structures have been reported for AAC(6′)-Ii in complex with AcCoA,[19] CoA,[20] and some inhibitors.[21e] A number of inhibitors of AAC(6′)-Ii have been reported.[21]

Based on mechanistic and structural information regarding the mode of action of aminoglycosides as well as the enzymes that deactivate them,[15] aminoglycoside analogues have recently been prepared in an attempt to overcome bacterial resistance.[22] In this regard, we reported the preparation of paromomycin analogues with hydrophobic substituents at the O2″ position.[11a,23] The persistent antimicrobial activities of some of these amphiphilic O2″ analogues compared to the parent paromomycin prompted us to further investigate their properties. Indeed, of paramount interest was the realization that these O2″-ether analogues have a unique and altered mode of binding to the bacterial ribosomal A-site, whereby rings I and II maintain their original position, but rings III and IV assume a 90° twist around the glycosidic bond, as evidenced by X-ray cocrystal structure analysis.[11a,c,23] Herein, we describe our results relating to the in vitro inhibition of two aminoglycoside-deactivating enzymes, APH(3′)-IIIa and AAC(6)-Ii,′ by O2″-ether analogues of paromomycin.

In view of their altered binding mode to the ribosomal A-site, we were interested to see whether the O2″-ether analogues are substrates of APH(3′)-IIIa, a promiscuous aminoglycoside-deactivating enzyme. It is known from the available crystal structures of APH(3′)-IIIa bound to neomycin that the enzyme interacts with the aminoglycoside in a manner that is different to the ribosomal A-site.[17] We were pleased to observe that the initial phosphorylation rates (v_0) of all the O2″-ether analogues tested (compounds **14**–**38**) were significantly slower than that of paromomycin and even undetectable in some cases (compounds **18**, **20**, **24** and **35**; Table 1).

X-ray structural analysis of several O2″-paromomycin analogues revealed that the O2″ substituents are oriented outside of the deep major groove of the ribosomal A-site.[11a,c,23] This observation was also confirmed by the X-ray structure of analogue **20**, a key compound in the present study, bound in the A-site of rRNA (Figure 2). The phenylethylamino side chain of compound **20** is outside of the rRNA major groove. A1492 and A1493 are bulged out, as typically observed when an amino-glycoside is bound in the A-site. These observations inspired us to introduce large ether-linked groups at the O2″ position in paromomycin, hoping that these groups could potentially reduce the affinity of the compound for the aminoglycoside-deactivating enzymes. Consistent with our design hypothesis, slower initial phosphorylation rates were observed for analogues **26**, **27**, **28**, **33** and **34**, bearing large groups. However, the observation that some analogues with relatively

smaller groups, such as **14, 18** and **20**, were also phosphorylated significantly more slowly than paromomycin required further investigation.

To understand why APH(3′)-IIIa was not efficient at catalyzing the phosphorylation of analogues such as **18**, **20**, **33** and **35**, we first verified whether these compounds were indeed bound in the active site of the enzyme by evaluating their ability to inhibit the reaction of APH(3′)III a with its known substrate, amikacin.[24,25] The APH(3′)-IIIa-catalyzed phosphorylation of amikacin was significantly inhibited by low concentrations of the O2″-paromomycin analogues **18** and **20** (Figure 3). From the linear regression (see Figure S1 in Supporting Information), the K_i values determined for compounds **18** and **20** were 3.9 μM and 1.0 μM, respectively (Table 2). The mode of inhibition was shown to be predominantly competitive ($K_i \ll K_i'$) with respect to amikacin. Furthermore, K_m(app) was found to increase with increasing inhibitor concentration, whereas V_{max} (app) was relatively insensitive. The observed competitive inhibition suggests that compounds **18** and **20** are bound in the active site of APH(3′)-IIIa.

IC_{50} values were then determined for a variety of O2″ paromomycin analogues as inhibitors of APH(3′)-IIIa (Table 3). Considering the steric, electronic and hydrophobic variability of the analogues studied, the similarity of the IC_{50} values indicates that the O2″-ether groups have a relatively small effect on the inhibitory potency of these paromomycin analogues, suggesting they do not contribute greatly to active site affinity, relative to the aminoglycoside portion.

In view of our observation that the APH(3′)-IIIa-catalyz ed phosphorylation of compounds **18**, **20**, **33** and **35** was experimentally undetectable, we suspected that the O2″-side chain of the inhibitors may obstruct binding of the ATP co-factor to APH(3′)-IIIa. The mode of inhibition of compound **20** with respect to ATP was therefore determined (Figure 4). At low inhibitor concentrations of compound **20** (<5 μM), a K_i value of 2.7 μM and a K_i' of 0.8 μM were obtained from the linear regression (see Figures S2 and S3 in Supporting Information). Also at low inhibitor concentration, inhibition was observed to be mixed uncompetitive with respect to ATP, indicating that **20** cannot be bound in the ATP site before the binding of ATP. At higher concentrations, the inhibitor might also be bound to the E·ADP complex (Scheme 1), as observed with other APH(3′)-IIIa substrates.[16] According to this model, compounds **18** and **20** are in competition with the aminoglycoside substrate for each form of the enzyme known to bind an aminoglycoside.[16] Inhibition could be due to an altered orientation of the pseudotetrasaccharide harboring the O2″-ether chain in the active site, which causes a 90° twist of the glycosidic bond linking rings III–IV to I–II. Several attempts to obtain X-ray quality crystals of **20** with APH(3′)-IIIa were unsuccessful.

Compound **20** was also found to be an inhibitor of AAC(6′)-Ii. A K_i value of 8 ± 3 μM was determined using a known protocol.[21e] This result was expected since AAC(6′)-Ii is known not to modify aminoglycosides lacking a 6′-amino group.[18] Moreover, the K_i value of compound **20** (8 μM) is within error of the reported K_m for neomycin C (5 μM),[18] which is the 6′-NH_2 analogue of paromomycin. This suggests that the O2″-substituent of compound **20** on its own is not contributing significantly to the inhibition observed.

The promising results in reducing the rate of in vitro O3′ phosphorylation in amikacin in the presence of **20** prompted us to test the antimicrobial activity of compound **20** against a variety of resistant bacterial strains expressing efflux pumps and aminoglycoside-deactivating enzymes. Compound **20** was not effective against most of these strains (Table 4), much like paromomycin and neomycin B. These disappointing results, albeit against some of the most recalcitrant strains, are probably due to the existence of different isoforms of APH(3′)-IIIa and AAC(6′)-Ii and also efflux. It is also possible that these strains express aminoglycoside-deactivating enzymes other than APH(3′)-III and AAC(6′)-I that can deactivate compound **20**. Nevertheless, it is of interest that amphiphilic analogues of paromomycin provided insights into a new mode of binding to the ribosomal A-site while maintaining activity against sensitive strains of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*. No synergistic effect was observed for varying concentrations (up to 64 μg mL^{-1}) of compound **20** in the presence of amikacin and paromomycin.

Amphiphilic O2″-ether analogues of paromomycin such as compound **20**, demonstrating a new mode of binding to the ribosomal A-site and showing inhibitory activity similar to the parent aminoglycoside, were used as a model to understand the impact of the O2″-modification on two aminoglycoside-deactivating enzymes, APH(3)-IIIa ′ and AAC(6′)-Ii. Compound **20** was not a substrate for these clinically relevant enzymes, but was capable of inhibiting both with K_i values in the low micromolar range. Competitive inhibition was observed for APH(3′)- IIIa relative to the natural substrate amikacin, and uncompetitive inhibition was observed for APH(3′)-IIIa with respect to ATP at low inhibitor concentration. The IC_{50} values determined for a series of paromomycin O2″ analogues indicate that the ether moiety introduced at the O2″ position is not a determining factor for APH(3′)-IIIa affinity. Further work will involve incorporation of deoxygenation and N1-substitution with the 2*S*-4-amino-2-hydroxybutanoyl moiety, known to impart activity against resistant strains as in the case of amikacin.[3b]

Experimental Section

Experimental details are given in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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25. For amikacin, the K_m value of 245 μM reported by McKay et al. in Reference [24] (we observed K_m = 150 μM) is higher than for other commercially available aminoglycosides and facilitates the detection of the phosphorylation at the concentrations used in the assay. Also, amikacin is only singly phosphorylated whereas aminoglycosides, such as paromomycin, are phosphorylated twice, and their biphasic phosphorylation curves make analysis more complicated.

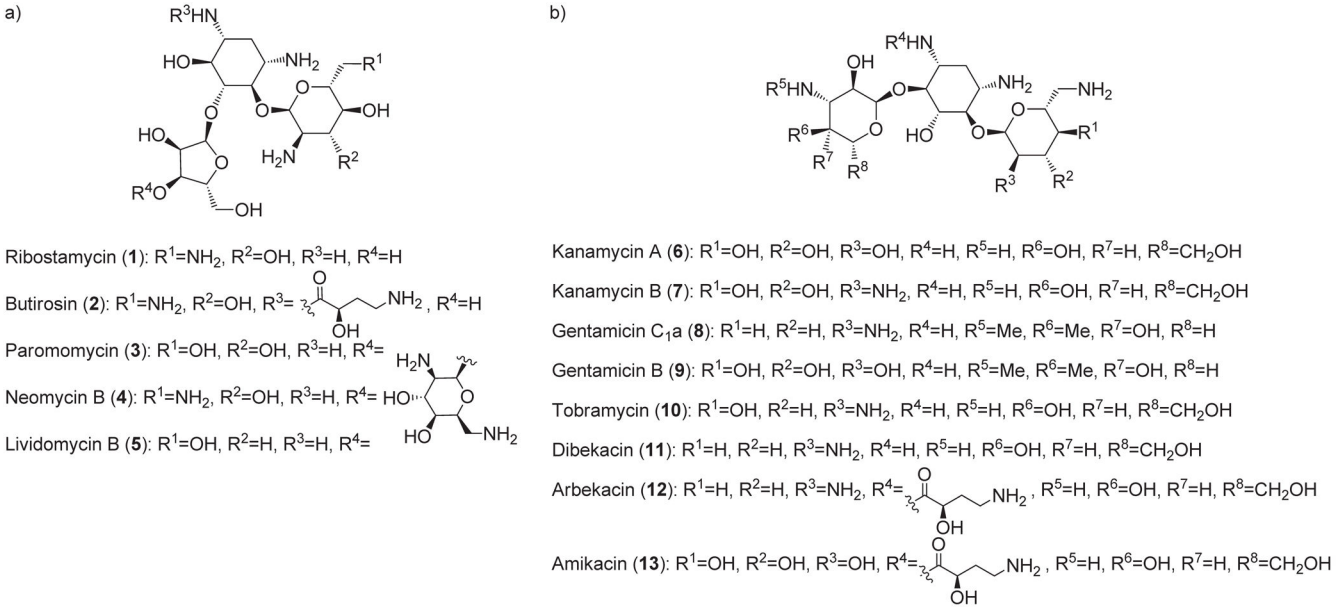


Figure 1.

a) Representative class A aminoglycosides; b) representative class B aminoglycosides.

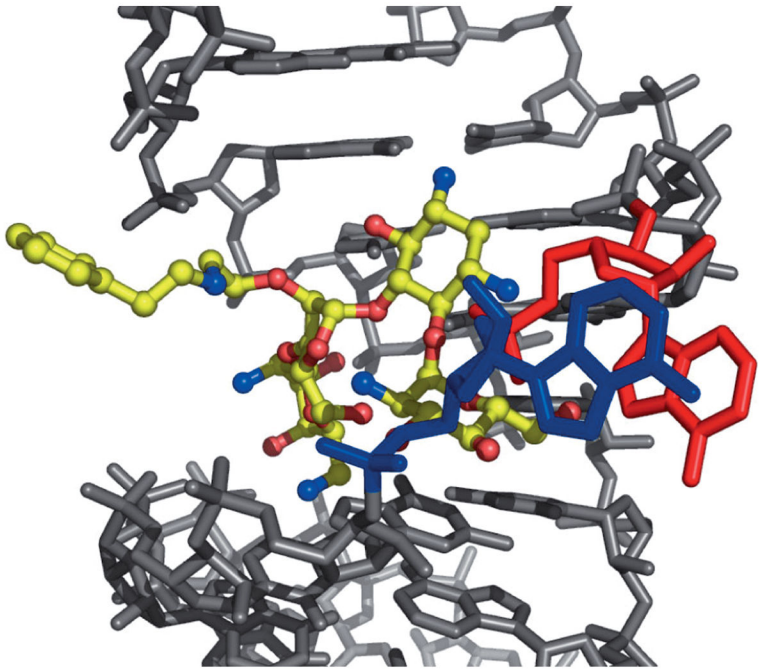


Figure 2.

X-ray structure of analogue **20** (yellow) bound in the A-site of rRNA (grey; A1492 (blue) and A1493 (red) are highlighted).

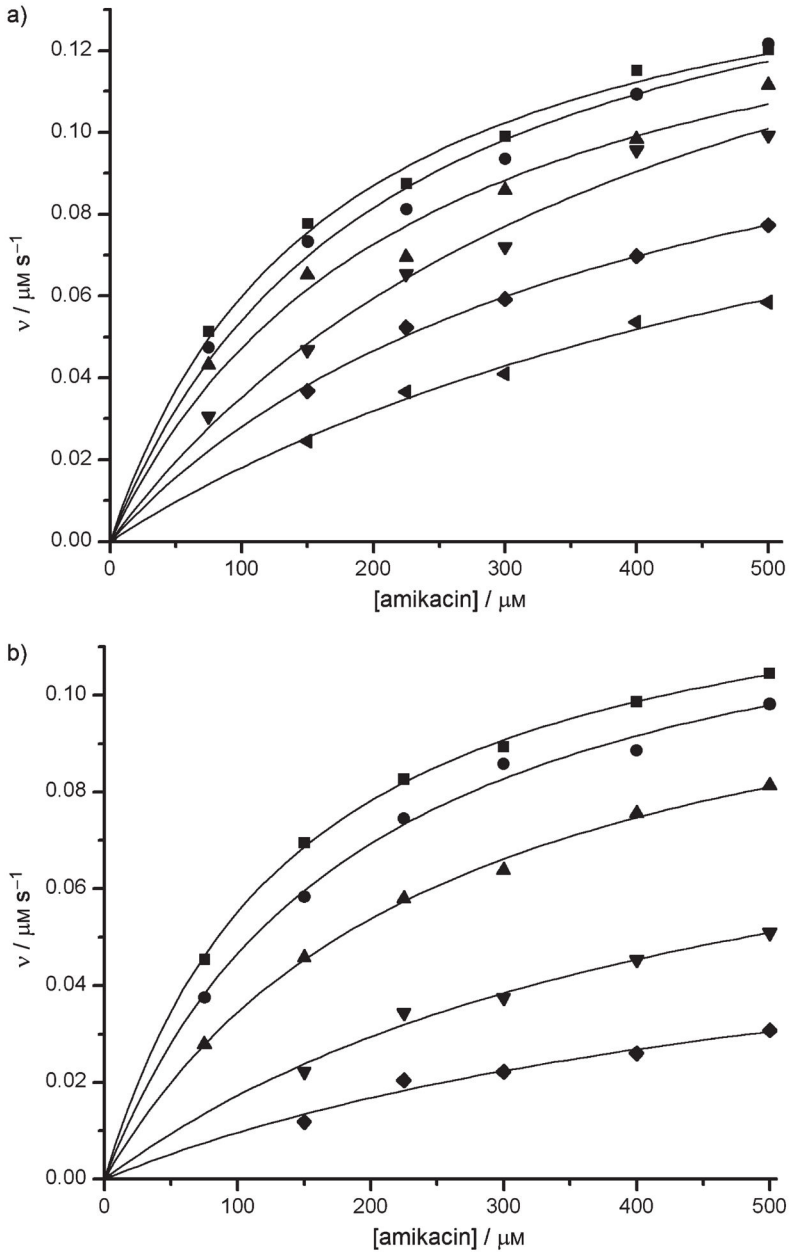


Figure 3.

Inhibition of amikacin phosphorylation rates (*v*) by a) analogue **18** (■=0 μM, ●=1 μM, ▲=2.5 μM, ▼=5 μM, ◆ =10 μM, ◀ =15 μM) and b) analogue **20** (■=0 μM, ●=1 μM, ▲=5 μM, ▼=10 μM, ◆ =15 μM).

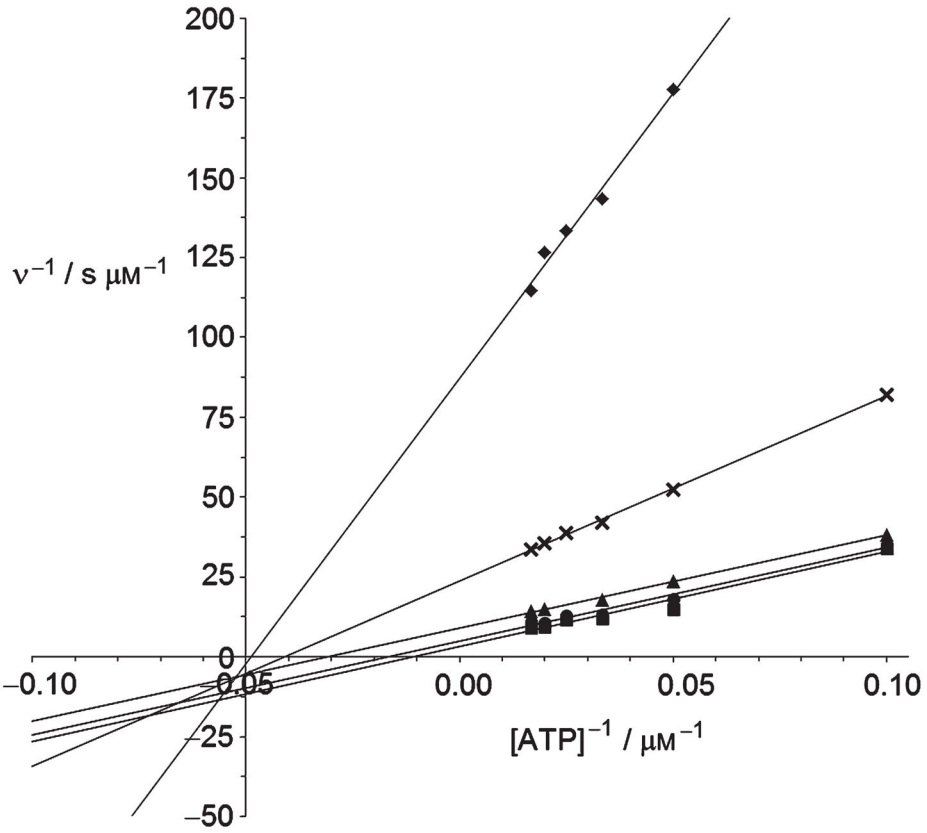
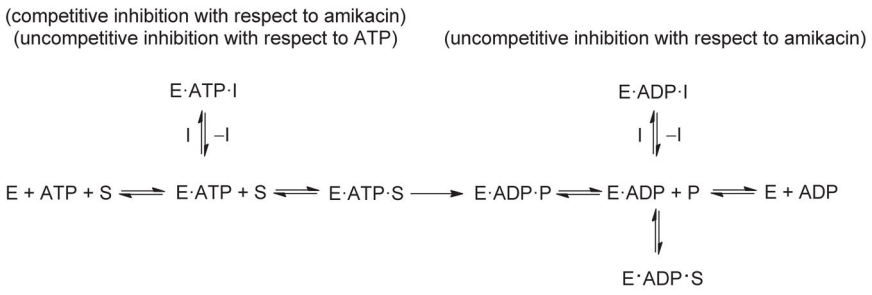


Figure 4.

Inhibition of APH(3′)-IIIa-catalyzed amikacin phosphorylation by analogue **20** as a function of ATP concentration. *v*=phosphorylation rates, ◆ =0 μM, × =1 μM, ▲=2.5 μM, ●=5 μM, ■=10 μM.

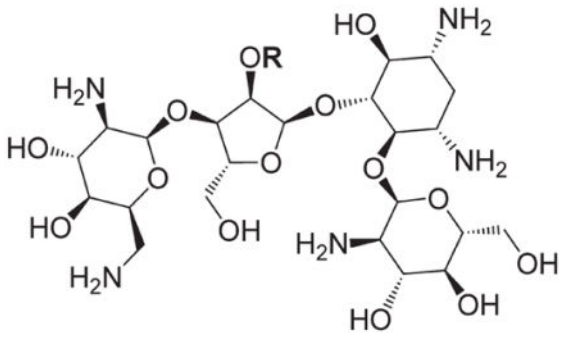


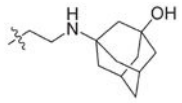
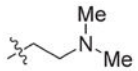
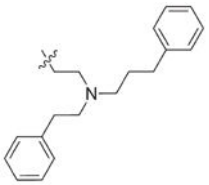
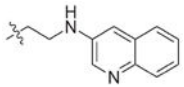
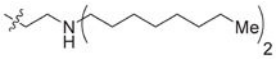
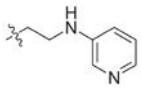
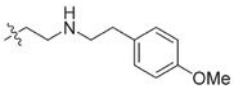
Scheme 1.

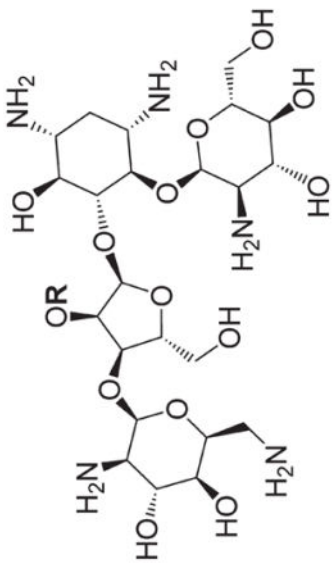
Proposed mechanism for the inhibition of APH(3′)-IIIa by compounds **18** and **20**. E =APH(3′)-IIIa, S =aminoglycoside substrate of APH(3′)-IIIa, I =inhibitor, P =aminoglycoside product after modification by APH(3′)-IIIa.

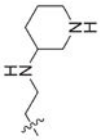
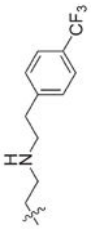
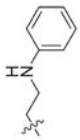
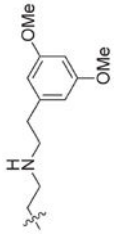
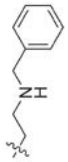
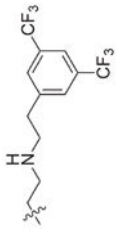
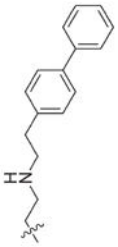
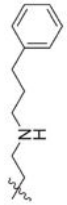
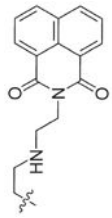
Table 1

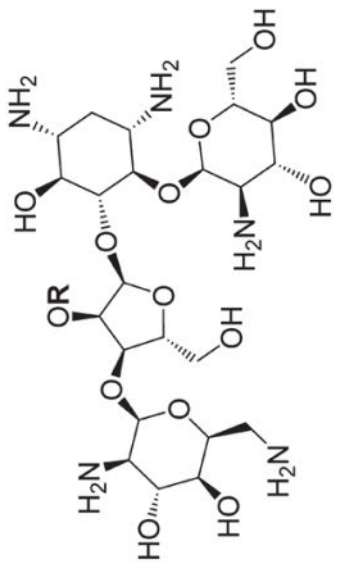
APH(3′)-IIIa-catalyzed phosphorylation rates determined at the same concentration for all paromomycin analogues.

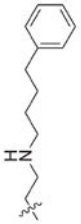
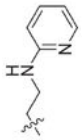
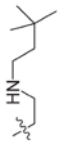
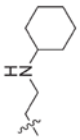
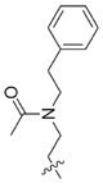
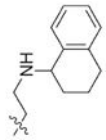
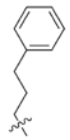


Compd	R	v_0[a] [nM s^{-1}]	Compd	R	v_0[a] [nM s^{-1}]
3	H	54.9	**26**		7.6
14		9.1	**27**		8.7
15		12.2	**28**		9.5
16		8.3	**29**		11.1



Compd	R	v_0[a] [nM s^{-1}]	Compd	R	v_0[a] [nM s^{-1}]
17		9.8	**30**		10.9
18		ND[b]	**31**		6.8
19		6.4	**32**		5.6
20		ND[b]	**33**		ND[b]
21		7.0	**34**		5.4



Compd	R	v_0[a] [nM s^{-1}]	Compd	R	v_0[a] [nM s^{-1}]
22		8.3	**35**		ND[b]
23		6.2	**36**		6.6
24		7.2	**37**		11.7
25		5.4	**38**		5.2

[a] v_0 : initial phosphorylation rate;

[b] ND: not detectable.

Table 2

K_i and K_i' values of compounds **18** and **20** for inhibition of APH(3′)-IIIa.

Compd	K_i [μM]	K_i' [μM]
18	3.9	69.4
20	1.0	14.2

Table 3

Inhibition (IC_{50}) of APH (3′)-IIIa by paromomycin analogues functionalized at the O2″ position.[a]

Compd	IC_{50} [μM]	Compd	IC_{50} [μM]
14	17.2 ± 2.3	**27**	30.3 ± 5.6
15	30.6 ± 5.7	**28**	37.1 ± 3.5
16	2.0 ± 0.2	**29**	6.0 ± 0.7
17	4.3 ± 0.5	**30**	2.3 ± 0.3
18	12.3 ± 1.7	**31**	12.4 ± 1.9
19	16.1 ± 2.5	**32**	21.1 ± 1.6
20	9.5 ± 0.7	**33**	2.8 ± 0.3
21	18.5 ± 1.8	**34**	39.2 ± 7.3
22	24.2 ± 4.5	**35**	5.5 ± 0.1
23	11.7 ± 0.1	**36**	4.3 ± 0.5
24	3.1 ± 0.4	**37**	12.7 ± 1.6
25	16.0 ± 2.0	**38**	2.2 ± 0.3
26	5.0 ± 0.7		

[a]Values represent the mean ± SD of n =3 experiments.

Table 4

Antibacterial activities of compound **20** and other aminoglycosides (**3**, **4**, **9**, **10** and **13**) against a variety of different bacterial strains.

Species	Phenotype	20	3[a]	4[a]	9	10	13
		MIC [μg mL^{-1}]					
Staphylococcus aureus	ATCC 29213	0.5	2	0.5	0.5	0.5	4
Escherichia coli	ATCC 25922	4	2	1	0.5	1	2
Klebsiella pneumoniae	ATCC 10031	2	2	0.25	0.25	0.5	0.25
Pseudomonas aeruginosa	ATCC 27853	>64	>64	64	0.5	0.5	2
P. aeruginosa	Wild-type pump	>64	>64	8	1	0.5	2
Acinetobacter baumannii	Susceptible	2	2	1	0.5	0.5	2
P. aeruginosa	MexXY up	>64	>64	16	4	0.5	4
A. baumannii	ATCC 19606	>64	16	4	8	4	16
P. aeruginosa	Efflux	>64	>64	> 64	4	2	16
P. aeruginosa	Efflux	>64	>64	> 64	8	2	32
Providencia stuartii	AAC(2′)-I	>64	>64	32	64	32	4
E. coli	APH(3′)-Ib	>64	>64	> 64	0.25	1	0.5
S. aureus	APH(3′)-III	>64	>64	> 64	0.5	1	8
A. baumannii	ANT(2″)-I, APH(3′)-VI, Perm/Efflux	>64	>64	> 64	>64	>64	> 64
Acinetobacter spp.	APH(3′)-VI	>64	>64	16	0.5	1	> 64
S. aureus	ANT(4′)-I	>64	>64	> 64	0.5	>64	> 64
P. aeruginosa	ANT(4′)-II	>64	>64	8	1	64	64
Serratia marcescens	AAC(6′)-I	8	4	4	2	64	64
P. aeruginosa	AAC(6′)-I	>64	>64	> 64	64	>64	> 64
P. aeruginosa	AAC(6′)-II	>64	>64	8	64	32	4
E. coli	ANT(2″)-I	8	8	2	>64	>64	4
Enterobacter cloacae	AAC(6′)-I, ANT(2″)-I, APH(3′)-I	>64	>64	> 64	>64	>64	64
S. aureus	AAC(6′)-I, APH(2″)	>64	>64	> 64	>64	>64	> 64
P. aeruginosa	AAC(3)-I	>64	>64	8	64	0.5	4
E. coli	AAC(3)-IV	16	16	8	32	>64	2

[a]Paromomycin (**3**); neomycin B (**4**).