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6-Hydroxy to 6‴-amino tethered ring-to-ring macrocyclic aminoglycosides as probes for APH(3′)-IIIa kinase

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Abstract—Based on molecular modeling and available X-ray structure data on aminoglycosides complexed with a bacterial ribosomal surrogate or with a kinase, two analogues of paromomycin were prepared by tethering the 6-OH and the 6‴-NH_2 group with a five-carbon bridge. Only one of two possible hydroxyl groups was phosphorylated by the kinase. The application of ring closure metathesis is presented for the first time to construct bridged macrocyclic analogues in the aminoglycoside series.

Antibiotics have been used as effective drugs to combat infection for nearly 75 years.[1] Among these, the aminoglycoside family holds a historically and clinically prominent position as potent therapeutic drugs against Gram positive and Gram negative infections.[2] Aminoglycosides are administered parenterally under a hospital regimen. However, their promiscuous use as broad spectrum antibiotics has caused the emergence of bacterial resistance. Resistance can develop by enzymatic deactivation of the drugs, by target modification, or by decreased intracellular concentration of the active entity by efflux.[3] Oto- and nephrotoxicity are also associated with dose-related practices of aminoglycoside therapy in the clinic.[4]

The cellular target for bactericidal aminoglycosides is the A-site of the 16S ribosomal RNA.[5] In recent years, elegant X-ray crystallographic[6] and NMR[7] studies of A-site—aminoglycoside complexes have shown highly conserved interactions, especially in the spatially common rings I and II of the pseudosaccharide portion of 4,5- and 4,6-disubstituted aminoglycosides (Fig. 1). In general, aminoglycosides such as paromomycin[6b] (**1**) and neomycin (**2**) bind to the A-site in their lowest energy conformation. Other conformations may also prevail for different inactivating enzymes.[8] Coincidentally,

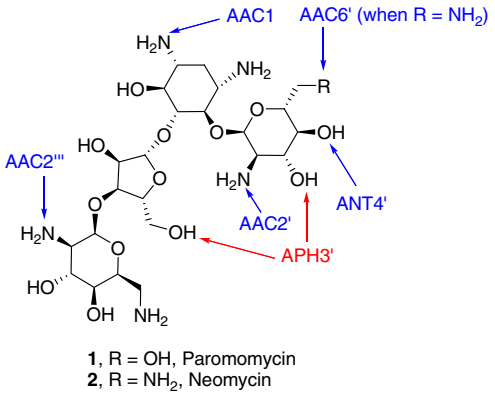

Figure 1. Susceptibility to enzymatic deactivation in the neomycin–paromomycin group of aminoglycosides.

aminoglycoside-modifying enzymes such as the kinase APH(3′)-IIIa, that catalyzes the phosphorylation of the 3′OH and 5″OH groups of ring I in paromomycin and related congeners, also recognize these lowest energy conformers of their substrates.[3,9] In fact, X-ray crystallographic studies have shown a virtual superposition of the conformations observed in the ternary complex APH(3′)-IIIa with neomycin B, and that observed in the crystal structure of the 30S ribosomal subunit with paromomycin (Fig. 2).[6b,9] Apart from specific van der Waals interactions, several functional groups utilized in binding to the bacterial ribosome are also identical to those required to act as a substrate of APH(3′)-IIIa.

Keywords: Aminoglycosides; Phosphotransferase; APH(3′)-IIIa kinase; Paromomycin.
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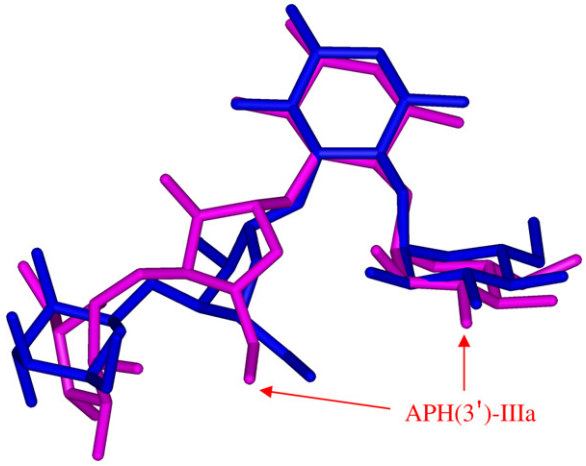


Figure 2. Superposition of X-ray structures of neomycin in the APH(3′)-IIIa ternary complex (magenta) and paromomycin in the A-site (blue). APH(3′)-IIIa can phosphorylate the 3′-OH of ring I or the 5″-OH of ring III.

In spite of the promiscuity of this enzyme in detoxifying several aminoglycosides containing a 3′-OH group, there is a fundamental topological feature that distinguishes it from its ribosomal RNA-based counterpart vis-à-vis the substrate. Thus, the face of the aminoglycoside that is exposed to APH(3′)-IIIa is opposite to that which interacts with the prokaryotic ribosomal site.[9,10] This is also observed in all available X-ray structures for aminoglycoside-deactivating enzyme complexes such as AAC(2′)-Ic,[11] AAC(6′)-Iy,[12] ANT(4′),[13] and APH(3′)-IIa.[14]

This fundamental difference based on van der Waals interactive forces constitutes an important observation that can be exploited in the design and synthesis of chemically modified aminoglycosides.[15] Considering the L-shaped topology of paromomycin and its bioactive conformation within the 16S ribosomal A-site,[6b,7] we considered the spatial proximity of 6-OH and 6‴-NH_2 to connect them within energetically favorable macrocyclic motifs (Fig. 3). Access to functionally distinct alkene appendages would provide the opportunity to explore ring closing metathesis reactions[16] in the aminoglycoside series for the first time. We were particularly interested to see if the macrocyclic variants of paromomycin would protect the ring I and/or ring III landscapes normally exposed to the kinase for phosphorylation. Recently, Tor,[17] Hermann,[18] Asencio,[19] and their respective coworkers have independently prepared conformationally constrained derivatives of neomycin B, and consisting of bridging the C2′ amino group with extended 5″-methano and ethano tethers, respectively. The specificity of these analogues toward different RNA targets was studied by NMR spectroscopy and molecular dynamics simulations.

The readily available 4′,6′-*O*-benzylidene penta-*N*-Cbz paromomycin **3**[20] was selectively *O*-benzoylated, then *O*-allylated at 6-OH which remained free to give **4** (Scheme 1). Treatment with NaOH in aqueous dioxane cleaved both the ester groups and the 6‴*N*-Cbz group, presumably via cyclic carbamate **5**, to give the corresponding 6‴ amine **6**. Amide formation with 3-butenoic acid followed by *O*-acetylation gave the diene **7**. Ring closing metathesis in the presence of Grubbs' second generation catalyst[21] followed by deacetylation gave macrocyclic amide **8** as a mixture of *cis*/*trans* isomers. Cleavage of the benzylidene group under acidic conditions and hydrogenation gave macrocyclic amide **9** in excellent yield (Scheme 1).

Treatment of penta-*N*-Cbz paromomycin **10**[20] with aqueous base effected a selective cleavage of the 6‴-*N*-Cbz group to give 11 (Scheme 2). *N*-3-Butenylation

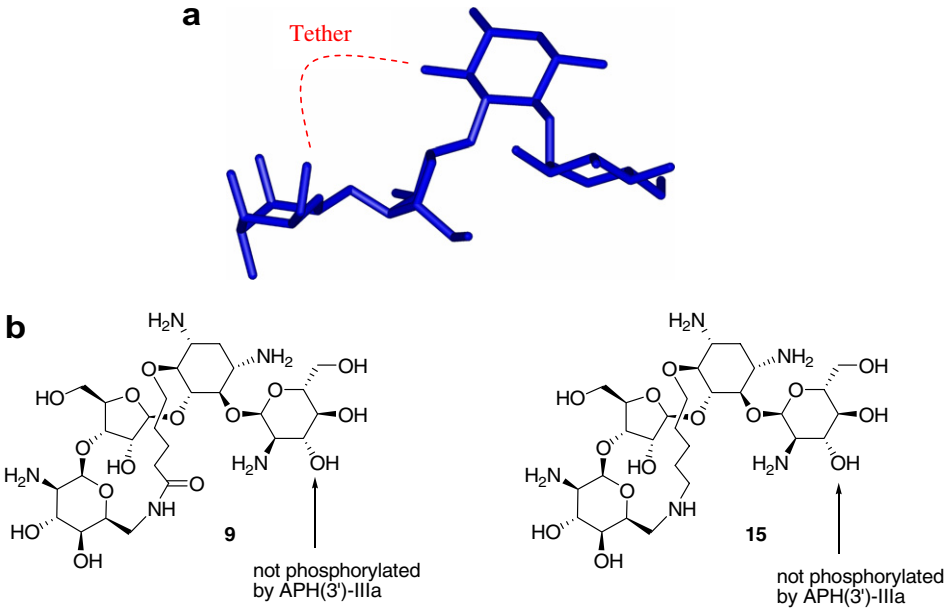
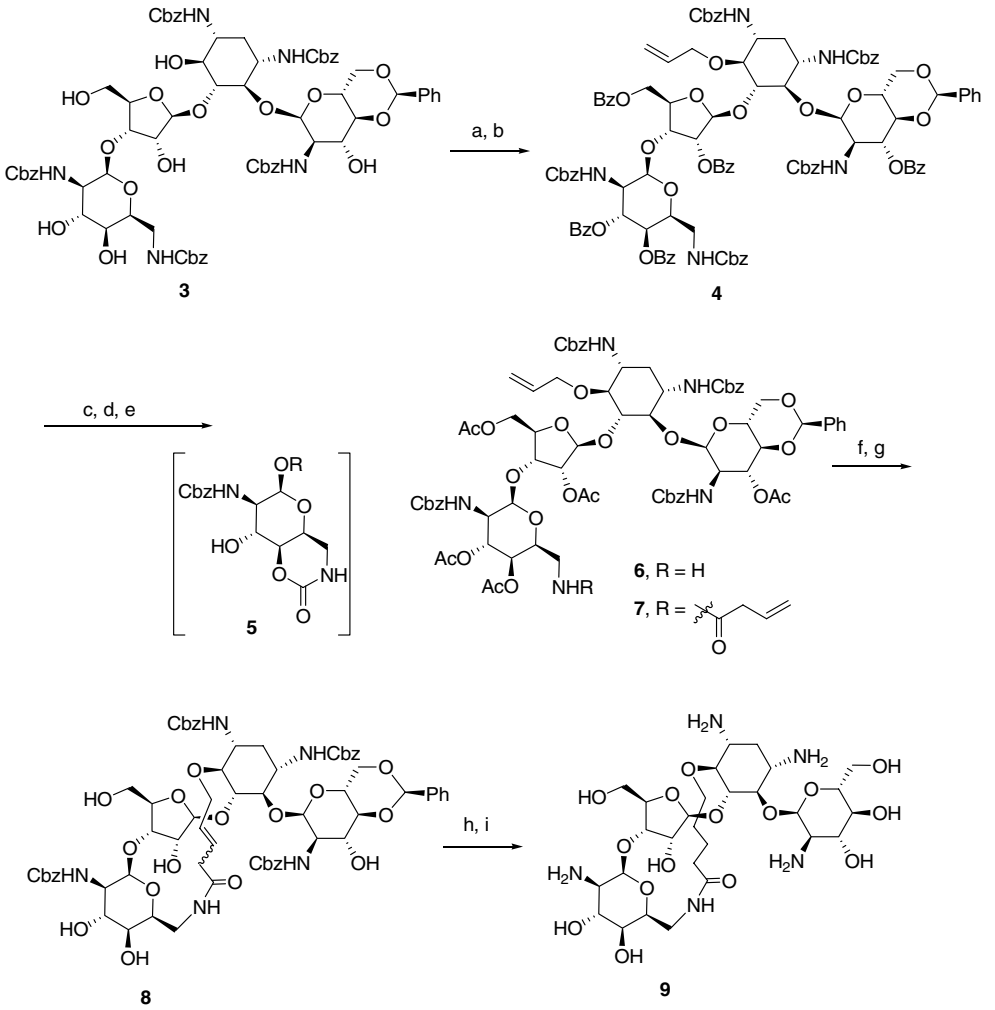


Figure 3. (a) Proposed ligation of selected functional groups within paromomycin to study the influence of the topology on the APH(3′)-IIIa-mediated phosphorylation; (b) structures of macrocycles **9** and **15**.

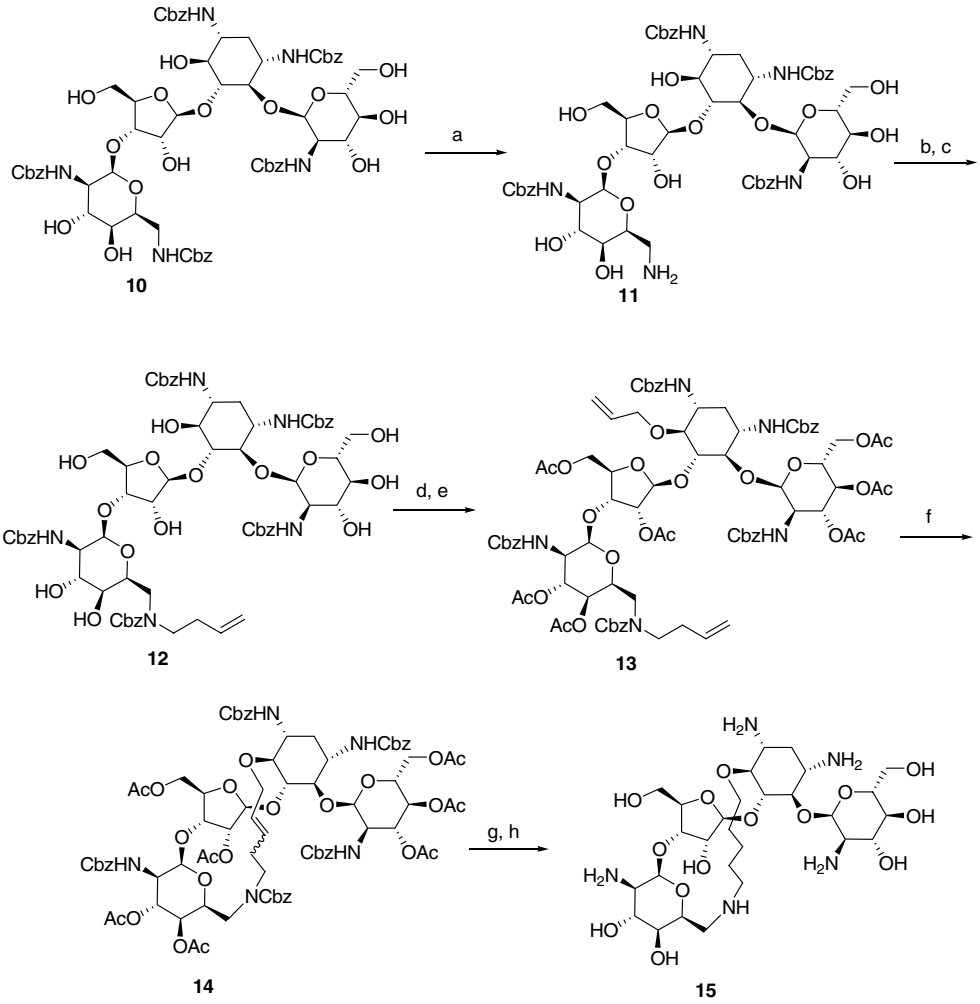


Scheme 1. Reagents and conditions: (a) BzCl, pyridine, 75%; (b) CH_2=$CHCH_2I$, KHMDS, THF, 43%; (c) 0.5 M NaOH, dioxane/H_2O (2:1); (d) EDCl, 3-butenoic acid, Et_3N, CH_2Cl_2/DMF (9:1); (e) Ac_2O, pyridine, 23% for three steps; (f) Grubbs' 2nd generation catalyst, CH_2Cl_2, reflux; (g) NaOMe/MeOH, 48% for two steps; (h) HOAc/H_2O (4:1), 60 °C, 4 h; (i) $Pd(OH)_2$/C, H_2, HOAc/H_2O (4:1), 85% for two steps.

followed by restoration of the *N*-Cbz group gave **12** which was selectively acetylated, then *O*-allylated at the remaining 6-OH group to give diene **13**. Ring closing metathesis as described above gave the macrocyclic olefin **14** as a mixture of *cis*/*trans* isomers. Deacetylation followed by hydrogenolysis gave the macrocyclic amine **15**.[22]

The main objective of this study was to explore the ability of the kinase APH(3′)-IIIa to phosphorylate 3′-OH and 5″-OH in the macrocyclic analogues **9** and **15** compared to the parent paromomycin.[23] Both compounds were found to be substrates for phosphorylation, albeit poorer than paromomycin, as shown in Figure 4a for compound **9** ($k_{cat} = 0.073\ s^{-1}$, $K_m = 21.8\ \mu M$, $k_{cat}/K_m = 3.35 \times 10^3\ M^{-1}\ s^{-1}$).[22] By way of comparison, we also measured the kinetic parameters for phosphorylation of paromomycin at 4 °C. As can be seen from Figure 4b, the initial rates of paromomycin phosphorylation showed biphasic hyperbolic dependence on substrate concentration. The consumption of *one* equivalent of ATP over the duration of this phosphorylation reaction demonstrates that these results correspond to two different modes of monophosphorylation of paromomycin. This behavior has been observed previously[24] and has been attributed to the phosphorylation of either the 3′-OH or the 5″-OH groups from two different Michaelis complexes formed from the same substrate that is bound in two different ways, with different affinities. Separate fitting of the low- and high-concentration data provided the kinetic parameters for the high-affinity phosphorylation of paromomycin ($k_{cat(1)} = 0.190\ s^{-1}$, $K_{m(1)} = 16.9\ \mu M$, $k_{cat(1)}/K_{m(1)} = 1.12 \times 10^4\ M^{-1}\ s^{-1}$) and the low-affinity phosphorylation, respectively ($k_{cat(2)} = 0.304\ s^{-1}$, $K_{m(2)} = 51.1\ \mu M$, $k_{cat(2)}/K_{m(2)} = 5.95 \times 10^3\ M^{-1}\ s^{-1}$). Our high-affinity kinetic parameters compare favorably to those measured at 37 °C and published previously (i.e., $k_{cat}^{37} = 3.62\ s^{-1}$, $K_m^{37} = 19.5\ \mu M$, $k_{cat}^{37}/K_m^{37} = 1.86 \times 10^5\ M^{-1}\ s^{-1}$).[23]

Unlike paromomycin, analogues **9** and **15** were phosphorylated only at 5″-OH which was determined by LC–MS with in-source fragmentation.[22] Mono- and bis-phosphorylated paromomycins were used as test samples to validate our experimental approach to these product studies.[24,25] The single phosphorylation at



Scheme 2. Reagents and condition: (a) 0.5 M NaOH, dioxane/H_2O (2:1); (b) CH_2=$CHCH_2CH_2Br$, $NaHCO_3$, DMF; (c) Cbz-Cl, $NaHCO_3$, MeOH/H_2O (20:1), 40% for three steps; (d) Ac_2O, pyridine, 65%; (e) CH_2=$CHCH_2I$, KHMDS, THF, 35%; (f) Grubbs' 2nd generation catalyst, CH_2Cl_2, reflux, 45%; (g) NaOMe/MeOH, 85%; (h) $Pd(OH)_2$/C, H_2, HOAc/H_2O (4:1), 80%.

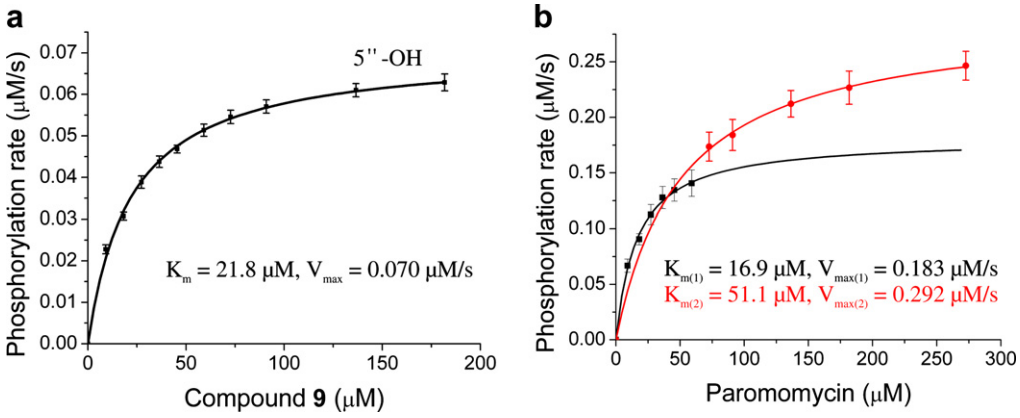


Figure 4. APH(3′)-IIIa-mediated phosphorylation of paromomycin and **9**. (a) Phosphorylation of **9** at 5″-OH; (b) phosphorylation of paromomycin at 3′-OH or 5″-OH.

5″-OH may be due to a shielding of the 3′-OH of ring I in **9** and **15** by the bridged tether. Ideally, one would wish to have an analogue that is modified in such a way that it is no longer recognized as a substrate by the kinase, while maintaining its ability to bind to the ribosomal A-site. Such an analogue would have the attributes for a potentially important antibacterial. Alternatively, one could design an analogue that is strongly bound by the kinase but not phosphorylated. Such an analogue would act as an inhibitor of the kinase

that could be co-administered with an aminoglycoside in a clinical setting, a strategy currently employed with beta-lactam antibiotics.[26]

Unfortunately, neither **9** nor **15** was found to inhibit growth of *Staphylococcus aureus* or *Escherichia coli* at concentrations lower than 40 μg/mL (MIC > 40 μg/mL in all cases). The incorporation of the five-carbon tether between the 6-OH and 6‴-NH_2 groups may alter the fidelity of binding of the crucial rings I and II in the A-site, possibly due to the exclusion of bound water molecules by the hydrophobic aliphatic motif. The reasons for the monophosphorylation at the 5″-OH, but not at the 3′-OH, are less evident, and may also involve subtle conformational changes. Nevertheless, this selectivity augurs well for a continued exploration of specific modifications toward effective aminoglycoside congeners. The macrocyclic analogues **9** and **15** represent the first attempts to chemically and topologically discriminate between A-site accommodation and kinase recognition[9]. Further studies in this area are in progress in our laboratories and will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.03.014.

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