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Molecular Recognition of RNA by Neomycin and a Restricted Neomycin Derivative**

*Fang Zhao, Qiang Zhao, Kenneth F. Blount, Qing Han, Yitzhak Tor, and Thomas Hermann**

Aminoglycoside antibiotics such as neomycin (**1a**) and paromomycin (**1b**) bind to ribosomal RNA (rRNA) at the decoding site and thereby interfere with the accuracy of protein synthesis, ultimately leading to bacterial cell death (Figure 1). In addition to the decoding site in 16S rRNA, several other RNA motifs form well-defined complexes with individual aminoglycosides, which makes these antibiotics excellent model ligands for the study of RNA recognition.[1,2] The target "promiscuity" of the aminoglycosides has been attributed to two major factors: 1) their highly charged nature, which is responsible for their eletrostatically driven RNA-binding mode and 2) their conformational adaptability. Although rotation around the glycosidic bonds that link the saccharide building blocks is restricted, the remaining limited flexibility explains some adaptability toward diverse RNA targets.[2] This restricted conformational flexibility attenuates

[*] F. Zhao, Dr. K. F. Blount, Prof. Dr. Y. Tor, Dr. T. Hermann
Department of Chemistry and Biochemistry
University of California, San Diego
La Jolla, CA 92093 (USA)
Fax: (+1) 858-534-0202
E-mail: tch@chem.ucsd.edu
Dr. Q. Zhao, Dr. Q. Han
Department of Structural Chemistry
Anadys Pharmaceuticals, Inc.
3115 Merryfield Row, San Diego, CA 92121 (USA)

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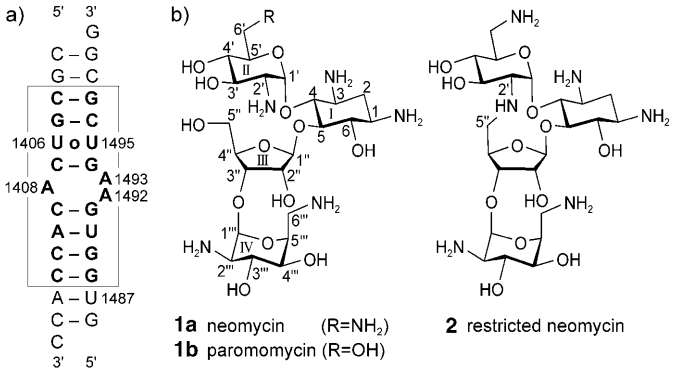


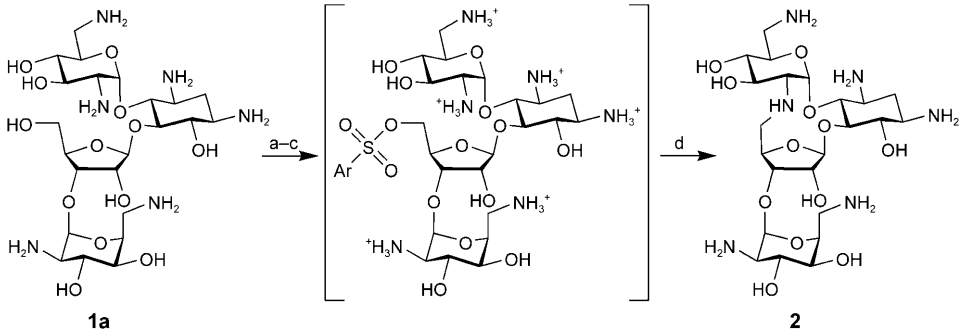
Figure 1. a) Secondary structure of the decoding-site oligonucleotide used for X-ray crystallography and structure determination. The box indicates the region that corresponds to the bacterial RNA sequence. Flanking nucleotides were added to enhance crystal packing. Residues are numbered according to the *Escherichia coli* 16S rRNA sequence. For multiwavelength anomalous diffraction experiments, U1487 was replaced by 5-Br-U. b) The natural aminoglycosides neomycin (**1a**) and paromomycin (**1b**) which differ at the 6′ position, and the semisynthetic restricted neomycin **2** in which rings II and III are linked through the 2′ and 5″ positions.

the contribution of charged interactions between RNA and the aminoglycosides, resulting in the formation of well-defined drug complexes that are distinct from nonspecific interactions of nucleic acids with flexible polyamines such as spermidine. The term structural electrostatic complementarity has been coined for this promiscuous yet target-specific binding of aminoglycosides to RNA.[2]

Aminoglycosides derived from 4,5-disubstituted 2-deoxystreptamine (2-DOS, ring I), including neomycin (**1a**) and paromomycin (**1b**), bind to a variety of RNA sequences. Examination of structurally characterized aminoglycoside complexes reveals that the relative orientation of rings I and II is very similar, whereas the conformation around the linkages to rings III and IV is significantly variable depending on the RNA target.[3] The apparent rigidity of the ring I/II system underlines the importance of this module for RNA recognition, attested by the fact that the 2-DOS and ring II moieties participate in key interactions that are responsible for target binding in decoding-site complexes with aminoglycosides.[1,4–6] Whereas higher thermal factors of rings III and IV in the crystal structure of paromomycin might suggest that these sugars generally contribute less to target-specific interactions,[4,5] the results presented herein suggest that the affinity of neomycin for the cognate decoding-site RNA and thus the antibacterial activity of this compound depend on an exquisitely balanced interplay of all four rings.

Previous investigations of aminoglycoside mimetics derived from neamine and paromamine, both of which lack rings III and IV, confirm the key role that the ring I/II core plays in decoding-site binding.[7,8] In the crystal structure of the related aminoglycoside paromomycin (**1b**) complexed with bacterial decoding-site RNA,[4,5] the distance between the 2′-amino group of ring II and the 5″-C atom of ring III is ≈3.7 Å, which suggests that cross-linking these positions may yield an aminoglycoside that retains the decoding-site-bound conformation of the parent drug. To support this hypothesis, we determined the X-ray crystal structures of neomycin (**1a**), among the most potent aminoglycoside antibiotics of therapeutic relevance, and its conformationally restricted analogue **2** bound to a decoding-site oligonucleotide (Figure 1a). Herein, we analyze the structural characteristics of both RNA–small-molecule complexes and discuss the structural basis of aminoglycoside ligand affinity along with general implications on the understanding of RNA recognition.

For the synthesis of restricted neomycin **2** we devised an intramolecular and regioselective cyclization strategy of an unprotected 5″-activated neomycin intermediate under high-dilution conditions that exploits the proximity of the 2′-amino and 5″-hydroxymethyl groups (Scheme 1).[3] The sulfonate-activated intermediate was obtained after Boc protection of the amino groups of neomycin, reaction of the primary alcohol at the 5″ position with 2,4,6-triisopropylbenzene sulfonyl chloride,[9] and cleavage of the Boc protecting groups with trifluoroacetic acid. High dilution of the activated intermediate to favor the intramolecular reaction and neutralization to deprotonate the ammonium groups for subsequent nucleophilic substitution eventually furnished the restricted aminoglycoside **2**.[3]



Scheme 1. Synthesis of restricted neomycin **2**: selective formation of **2** from neutralization of the unprotected sulfonate intermediate relied on the proximity of the 2′-amino and 5″-hydroxy groups. Neomycin (**1a**) was fully Boc-protected (a: Boc_2O, NEt_3, dioxane/H_2O; 75%). and activated as an aryl sulfonate at the 5″ position (b: 2,4,6-triispropylbenzenesulfonyl chloride, pyridine; 70%).[7] After acidic removal of the Boc protecting groups (c: TFA/$CHCl_3$ (1:1)), the sulfonate intermediate was highly diluted and neutralized to yield **2** (d: NEt_3, DMF, 10 days; 12% for steps c and d; a final Boc protection (not shown) was performed to facilitate purification).[3] Ar = 2,4,6-triisopropyl benzene; Boc = *tert*-butoxycarbonyl; TFA = trifluoroacetic acid; DMF = *N*,*N*-dimethylformamide.

Both neomycin (**1a**) and restricted neomycin **2** were co-crystallized with an oligonucleotide that contains the decoding-site sequence and flanking bases, which facilitated crystal packing (Figure 1a). Similar small RNAs have been shown to provide authentic model systems that retain the structural and dynamic characteristics of the ribosomal decoding site.[5,6,10,11] Three-dimensional structures of the RNA complexes of **1a** and **2** were determined by X-ray diffraction by using anomalous dispersion of the halogen atom in the 5-Br-U1487 residue. For comparison, the structure of the unbound decoding site was solved as well (Supporting Information).

The electron density of the decoding-site complexes clearly revealed the identity of the bound aminoglycoside ligands (Figure 2a,b). Specifically, the 5″-hydroxymethyl group in neomycin gave rise to a characteristic extension of the electron density envelope which was lacking for the cyclized derivative **2**. Both **1a** and **2** bind to the decoding site at the same position as paromomycin (**1b**).[4,5] Complex formation induces a more compact structure of the RNA around the ligand-binding site as indicated by the base of A1408 and the sugar–phosphate backbone of A1492 and A1493 pulled closer to the aminoglycoside (Supporting Information). The hydrogen-bonding contacts between neomycin and the RNA are identical to those of paromomycin, including the interaction of the distinctive 6′ substituent (Figure 3a). The N1 atom of A1408 in the decoding site forms a hydrogen bond with either the 6′-hydroxy group of paromomycin or the 6′-amino substituent of neomycin which helps stabilize the base-pair-like interaction between ring II and the A1408 residue.[5]

The position of rings I and II within restricted neomycin **2** relative to the decoding-site RNA coincides with the binding site of these rings in the neomycin–RNA complex (Figure 2c). Consequently, the hydrogen-bonding network between the RNA and rings I and II is identical for the restricted derivative and neomycin as well as paromomycin (Figure 3). Distinct patterns are, however, observed for the interactions of rings III and IV. Intramolecular cyclization imparts to **2** with a slightly more compact overall structure than that of the parent neomycin, reflected by differences in the positioning of rings III and IV (see also Supporting Information). Ring IV of **2** interacts with the phosphate group of G1491, whereas hydrogen bonds are formed to the phosphate group of G1405 in the opposite RNA strand for the complexes formed with neomycin and paromomycin[5] (Figure 3). The covalent link between rings II and III in **2** pulls ring III away from the bottom of the RNA deep groove which leads to disruption of a hydrogen-bond interaction to C1407 that involves the 2″-hydroxy group; this interaction is present for complexes with neomycin and paromomycin. Another contact of the 5″-hydroxy substituent in the natural aminoglycosides with G1491 is absent for the restricted neomycin. Interestingly, the contacts to rings III and IV, which were considered to play a secondary role for decoding-site binding, are sacrificed upon binding of **2** to the decoding site. This is not the case for the interactions with rings I and II, the importance of which in RNA target recognition is underscored once again.

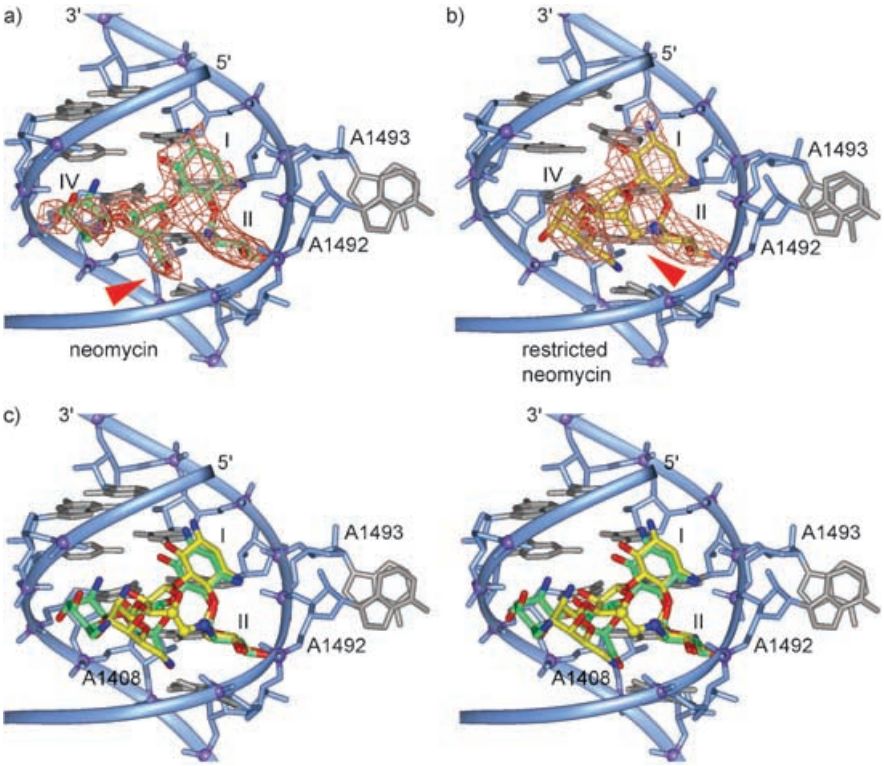


Figure 2. X-ray crystal structures of a) neomycin (**1a**) and b) restricted neomycin **2** complexed with decoding-site RNA. Aminoglycoside rings are numbered according to the structure shown in Figure 1b. Electron density ($2F_o-F_c$, contoured at 1.0σ around the ligands) reveals the identity of the aminoglycosides, specifically the extended 5″-hydroxymethylene group in neomycin, which is absent in the restricted ligand (red arrows). c) Stereo view of neomycin (**1a**, green) superimposed with its restricted derivative **2** (yellow) bound to the decoding site. (The superimposition is based on phosphate coordinates; only the RNA of the neomycin complex is shown.) The link between the 2′ and 5″ positions in **2** is highlighted in ball-and-stick representation.

Comparison of the binding affinities of neomycin (**1a**) with its cyclized derivative **2** for the decoding site revealed a 20-fold lower K_d value for the restricted derivative at pH 7.5,[3] in line with a similarly decreased antibacterial potency under physiological conditions.[12,13] The overall loss of direct hydrogen bonds between ring III and the RNA might explain the lower target affinity of **2**. However, at a lower pH value (5.8) similar to the crystallization conditions (pH 6.2), the K_d value of **2** decreased as a result of increasing protonation of the amino groups and thus domination of the ligand–RNA interaction by electrostatic forces.[3] It is well-documented

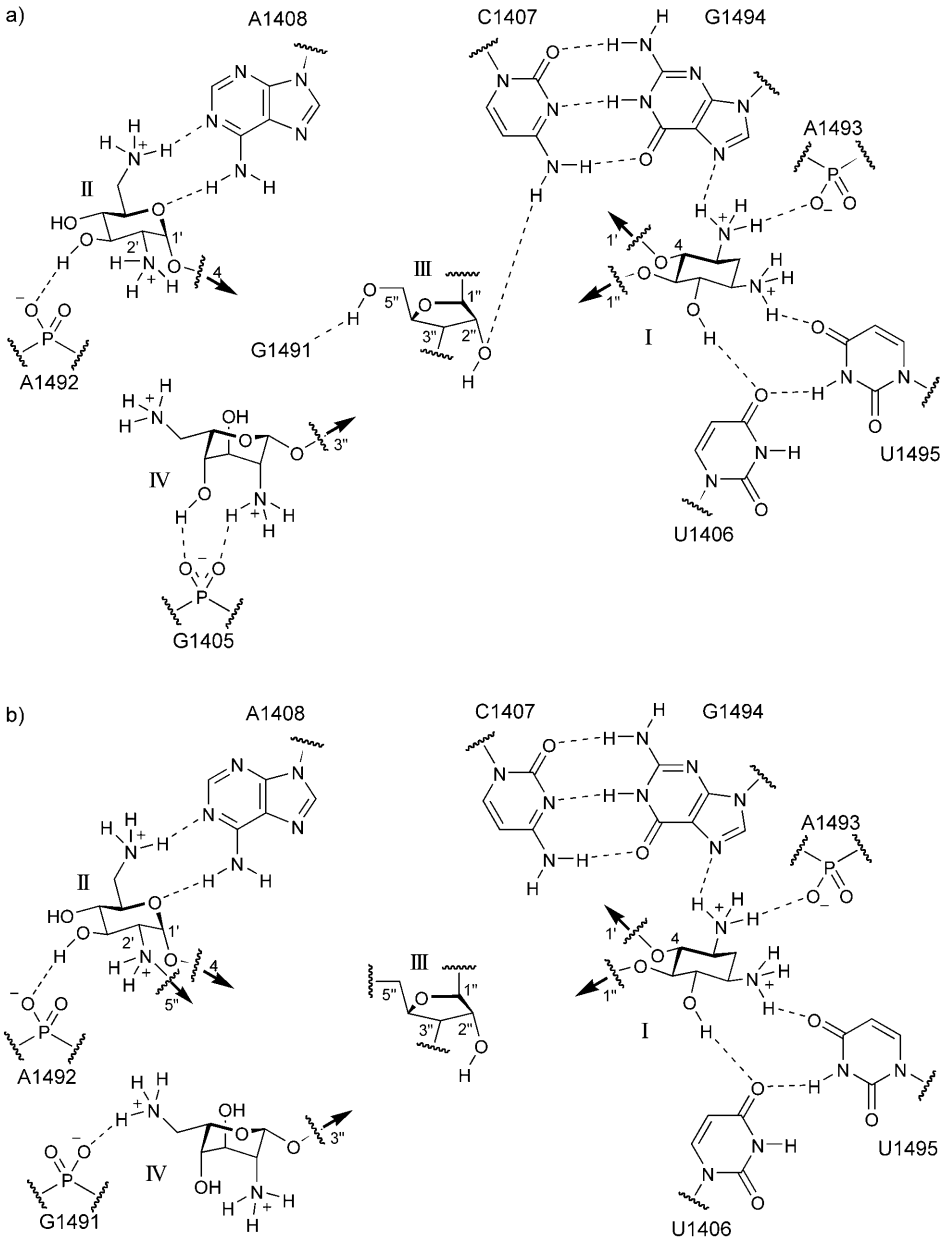


Figure 3. Schematic illustration of the dominant interactions of a) neomycin (**1a**) and b) restricted neomycin **2** with the decoding-site RNA. Unlike the case for the neomycin complex, the ribose moiety III of the restricted derivative does not participate in direct contacts with the target. The hydrogen-bonding network in the neomycin complex is identical to that previously observed in the decoding-site complex of paromomycin (**1b**).[4,5] Whereas there is a considerable range of pK_a values for the amino groups in **1a** and **2**,[3] protonated states dominate at the buffer conditions used (pH 6.2) which contribute an important electrostatic component to hydrogen-bonding interactions with the RNA.[3,21]

that the differences in RNA-binding affinities of various aminoglycosides carrying the same number of amino groups disappear at lower pH values, as electrostatic interactions of the protonated amino groups are the primary factors in complex formation.[3,19,20] Interestingly, the basicity of the secondary 2′-amino group, which covalently links rings II and III, is the lowest (pK_a 6.4 in **2**, versus pK_a 8.1 for the corresponding amine in **1a**).[3] Under physiological conditions, it is likely a combination of the decreased number of hydrogen-bond interactions between **2** and the decoding site, and diminished electrostatic contribution by the only partially protonated 2′-amino group that might cause the decreased binding affinity and antibacterial potency of the restricted neomycin derivative.[13]

A second aminoglycoside-binding site was observed in the crystal structure of both neomycin (**1a**) and restricted neomycin **2**. This site is located in the deep groove at the interface of two continuously stacking RNA constructs and displays features of ligand recognition that are radically different than those of the decoding site (Figure 4; and Supporting Information). Therefore, the complexes of **1a** and **2** described herein allow a direct comparison between distinct ligand-

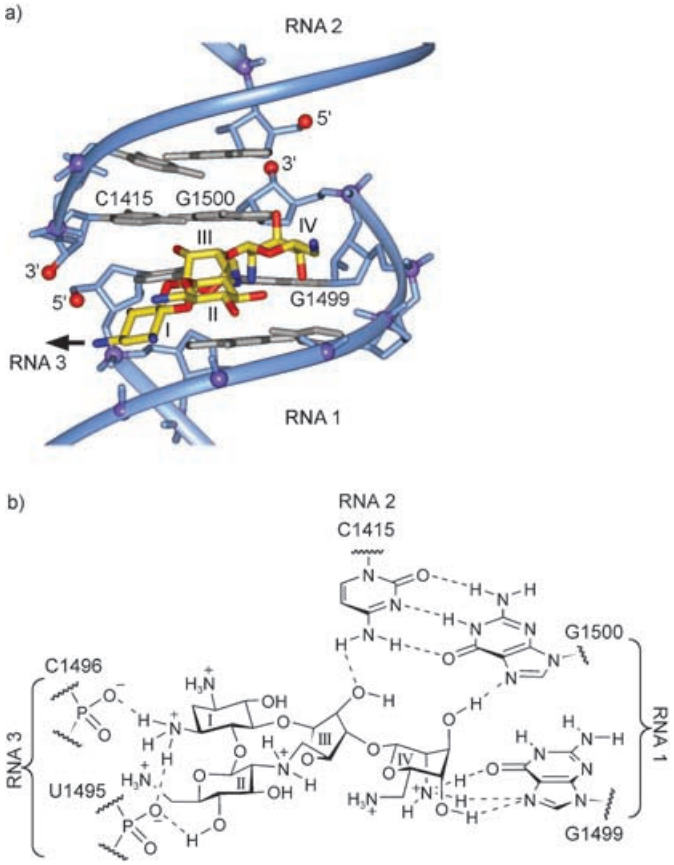


Figure 4. a) The second binding site of neomycin and restricted neomycin in the deep groove at the interface of coaxially stacked RNA helices (RNA 1 and 2). Only the structure of the complex with restricted neomycin is shown. The binding geometry at this site is identical to that of the complex with neomycin. Base-pair formation between the 3′-overhanging nucleotides of the decoding-site constructs promotes formation of pseudo-continuous helices in the crystal. A third, laterally docking RNA provides contacts with rings I and II of the aminoglycoside (RNA 3 not shown). b) Hydrogen-bonding interactions of **2** with RNA at the second binding site, which involves three RNA molecules.

recognition sites within the same RNA target. This provides the first "intramolecular" showcase for the promiscuity of aminoglycoside–RNA interactions.

At the interfacial site, the aminoglycoside forms hydrogen bonds with two mutually stacking RNA molecules and a third RNA helix that docks laterally. The interaction pattern differs remarkably from that observed at the decoding site in which rings I and II are involved in base-specific contacts, whereas rings III and IV play accessory roles. At the secondary binding site, ring IV docks in a coplanar manner between G1499 and G1500 and forms hydrogen bonds to the Hoogsteen edges of these bases. This is facilitated by a pyranose conformation that projects the 2‴, 3‴ and 4‴ substituents in an axial position (Figure 4). An additional hydrogen bond to the base of C1415 in a second RNA construct is provided by the 2″-hydroxy group of ring III. Rings I and II are projected outward from the RNA deep groove and are aligned along the sugar–phosphate backbone where they form hydrogen bonds exclusively with phosphate groups of a laterally docked third RNA helix (Figure 4). The geometry of the secondary aminoglycoside binding site is identical in the neomycin and restricted neomycin complexes. The small differences in the orientation of rings I and II that are induced by the intramolecular cyclization in **2** are accommodated by nonspecific interactions of these rings with phosphate groups. Interestingly, the docking site of ring IV at G1499 and G1500 in the aminoglycoside complexes corresponds to a magnesium ion binding site at the Hoogsteen face of consecutively stacked guanine residues. This metal ion binding site has been observed in RNA crystal structures, including the group I self-splicing intron[14] and 5S rRNA[15] (Supporting Information). The alternative occupancy of a magnesium binding site by neomycin, and the restricted derivative **2** is a further elegant example of structural electrostatic complementarity between cation binding sites in RNA and aminoglycosides.[2]

In summary, we have used X-ray crystallography to determine the three-dimensional structure of decoding-site RNA complexes of the aminoglycoside antibiotic neomycin (**1a**) and a synthetic restricted derivative **2** that was designed as a rigidified analogue of the natural product.[16] Conformational restriction by intramolecular cyclization is an established principle of drug design,[17] which we have applied to RNA-directed ligands.[3] The key requirement for conformational restriction is maintenance or induction of an active ligand conformation that allows target recognition. Our structural studies on RNA decoding-site complexes of neomycin (**1a**) and the restricted derivative **2** demonstrate that the intramolecular 2′–5″ cross-link introduced into the natural product is compatible with target binding. Comparison of the crystal structures of **1a** and **2** complexed to the decoding site reveals the exquisite sensitivity of aminoglycoside target recognition toward even slight modifications of the architecture of the natural product. Whereas key interactions of the aminoglycoside ligand are undisturbed by the modification, minimal differences in the overall conformation of **2** relative to **1a** disable hydrogen-bond contacts to the target.

Structural analysis revealed that neomycin, which is among the most potent aminoglycoside translation inhibitors, binds to the decoding site in the same conformation and at the same site as the structurally similar paromomycin, which is a considerably weaker inhibitor of bacterial translation.[7,18] The identical geometries for the decoding-site-bound neomycin and paromomycin suggest that the higher binding affinity of neomycin for the RNA target[7,18] may be due to the stronger N6′−H···$N1_{A1408}$ H bond relative to the O6′−H···$N1_{A1408}$ interaction in the paromomycin complex. A similar conclusion was drawn by Böttger and co-workers, who analyzed aminoglycoside binding to decoding-site mutants.[19] The identity of the 6′ substituent might also impact the basicity of other amino groups in the antibiotic, leading to a synergistic enhancement of neomycin binding over paromomycin. The interplay of amino group substitution pattern and basicity in aminoglycosides is well-established,[20] as is the importance of amino group protonation for ligand–RNA binding interactions.[21]

As an unexpected finding, a secondary aminoglycoside-binding site was discovered in the decoding-site complexes of both neomycin and the restricted derivative **2**. Interactions of the aminoglycosides at the secondary site involve the docking

of an amino-sugar moiety of the ligand at a metal ion binding site of the RNA, following the principle of structural electrostatic complementarity.[2]

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