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A Simple Structural-Based Approach to Prevent Aminoglycoside Inactivation by Bacterial Defense Proteins. Conformational Restriction Provides Effective Protection against Neomycin-B Nucleotidylation by ANT4

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The emergence of bacterial resistance to the major classes of antibiotics has become a serious problem over recent years.[1] For aminoglycosides, the major biochemical mechanism for bacterial resistance is the enzymatic modification of the antibiotic.[1] The search for new derivatives not susceptible to modification by bacterial defense proteins constitutes an active field of research.[2−5] Herein, we describe how the conformational differences exhibited by these flexible ligands within the binding pockets of the ribosome and of those enzymes involved in bacterial resistance can be exploited for designing new antibiotic derivatives with improved activity in resistant strains.

Staphylococcus aureus ANT4, an enzyme involved in the bacterial defense against aminoglycosides, catalyzes the transfer of one adenyl group from ATP to position O4 (see Figure 1) of the glucose (Glc) unit present in most aminoglycosides. This transfer leads to a sharp decrease in the drug affinity for its target RNA. The 3D structure of ANT4 complexed to kanamycin has been determined by using X-ray.[6] In addition, the structures of related analogues, such as gentamycin, tobramycin, and paromomycin, bound to the target ribosomal RNA have also been determined recently.[7] Interestingly, the oligosaccharide conformation recognized by the RNA and the enzyme are remarkably different (Figure 1).

In particular, the Glcα(1-4)-2-deoxy streptamine fragment adopts a syn-Ψ conformation (Φ/Ψ = −40/−30) for tobramycin and paromomycin in the RNA-bound state. This pseudodisaccharide moiety, with different patterns of amination/hydroxylation at the Glc unit, is present in all aminoglycosides. The syn-Ψ geometry also constitutes the most populated minimum for the free antibiotics, according to NMR data.[8] In contrast, the analogous glycosidic linkage of kanamycin bound to ANT4 is defined by an anti-ψ geometry (ϕ/ψ = −21/152). This conformation represents a high-energy minimum that must be stabilized by specific aminoglycoside−ANT4 interactions. The different 3D shape adopted by these aminoglycosides in the RNA- and enzyme-bound states suggests a possible structure-based chemical strategy to overcome bacterial resistance. Assuming that some degree of conformational distortion at the Glcα(1-4)-2-deoxy streptamine fragment is required for enzymatic activity, it should be possible to design a conformationally locked oligosaccharide that still retains antibiotic activity, but that is not susceptible to inactivation by ANT4.[9] Following this strategy (Scheme 1), we have designed and synthesized the conformationally constrained neomycin-B derivative **5**. In **5**, a direct covalent bond has been formed between positions $O5_{Rib}$ and $N2_{Glc}$ that, for the natural antibiotic, are hydrogen bonded in the ribosome-bound state.[7a,b]

Compound **5** was prepared following the simple four-step procedure shown in Scheme 1. Thus, hexa-*N*-(carbobenzyloxy) derivative **2**, readily prepared from neomycin-B **1**, was treated with 2,4,6-triisopropylbenzenesulfonyl chloride in pyridine to yield the primary sulfonate **3** regioselectively.[10] Deprotection of the amino groups by hydrogenation in MeOH/TFA gave crude sulfonate **4**

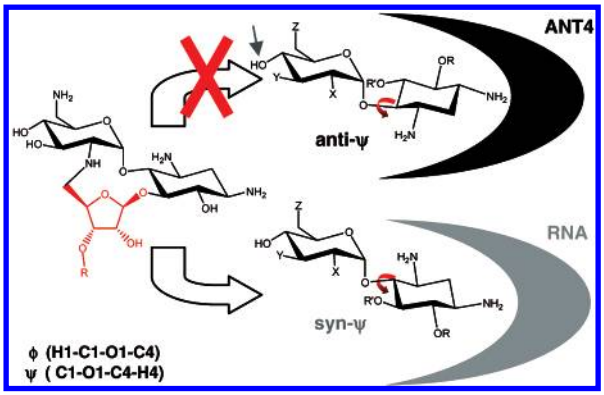
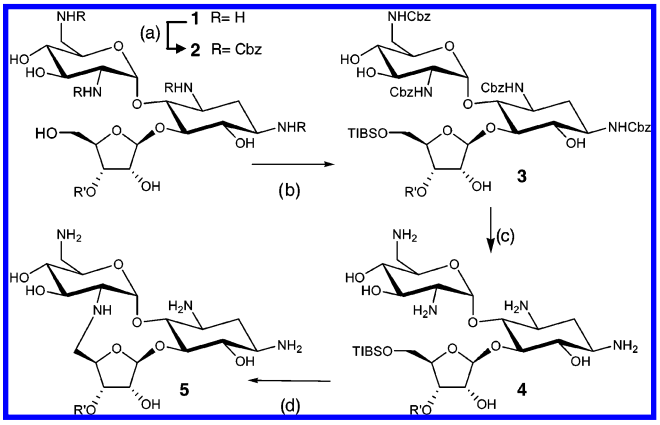


Figure 1. (Bottom right) Schematic representation of the Glcα(1-4)-2-deoxy streptamine fragment of tobramycin (X = NH_2, Y = H, Z = NH_2, R = 3-amino-3-deoxy-α-Glc, R′ = H) and paromomycin (X = NH_2, Y = OH, Z = OH, R = H, R′ = 2,6-diamino-2,6-dideoxy-L-Idoβ(1-3)Rib) in their ribosome-bound states. (Top right) Schematic representation of the same disaccharide fragment in kanamycin (X = OH, Y = OH, Z = NH_2, R = 3-amino-3-deoxy-α-Glc, R′ = H) in the ANT4-bound state. The OH group modified by the enzyme is marked with a gray arrow. The two receptors recognize different conformations of the ligand. (Left) Our design is based on locking this disaccharide fragment in its RNA-bound conformation by using a ribose (Rib) bridge (in red) to form the neomycin-B analogue **5**. This modification might prevent inactivation of the antibiotic by ANT4, while preserving antibiotic activity. The definition employed for the glycosidic torsion angles (ϕ/ψ) is shown at the bottom left corner of the figure.

Scheme 1 [a]



[a] (a) CbzCl, Na_2CO_3, MeOH/H_2O 3:1, 0 °C, 3 h, 92%; (b) 2,4,6-triisopropylbenzenesulfonyl chloride (TIBSCl), Py, rt, 72 h, 44%; (c) H_2 (1 atm), Pd/C, MeOH, trifluoroacetic acid, rt, 8 h, 90%; (d) H_2O, pH 7.0, 60 °C, 7 days, 56% (see Supporting Information). In **1**, **4**, and **5**, R′ = 2,6-diamino-2,6-dideoxy-β-L-idose. In **2** and **3**, R′ = 2,6-diamino-2,6-di-*N*-(carbobenzyloxy)-2,6-dideoxy-β-L-idose.

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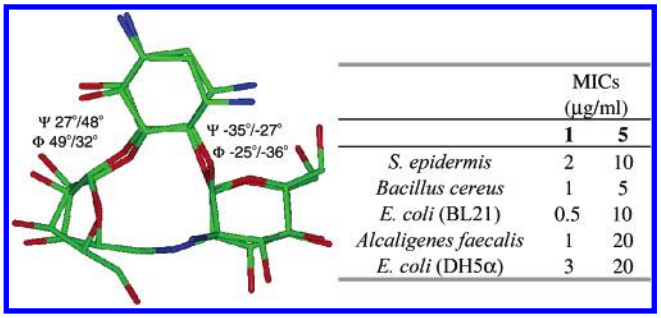

	MICs (μg/ml)	
	1	**5**
S. epidermis	2	10
Bacillus cereus	1	5
E. coli (BL21)	0.5	10
Alcaligenes faecalis	1	20
E. coli (DH5α)	3	20

Figure 2. (Left) Average NMR conformation (see Supporting Information) of neomycin-B analogue **5** superimposed onto the X-ray structure of paromomycin complexed to ribosomal RNA.[7a,b] The 3D structure of the constrained aminoglycoside closely resembles the paromomycin bioactive conformation (ϕ and ψ values for both structures are also shown). (Right) Experimental MIC values (μg/mL) measured for neomycin-B **1** and its conformationally constrained mimic **5**.

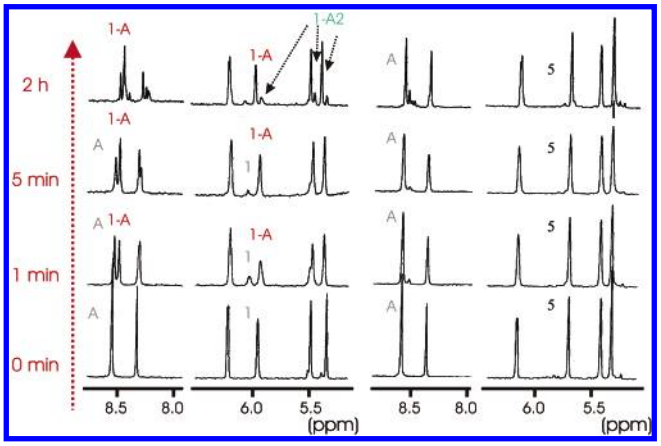

Figure 3. Evolution of neomycin-B **1**/ATP (left) and mimic **5**/ATP (right) mixtures in 20 mM phosphate buffer, pH 7.0, 10 mM $MgCl_2$, 310 K, after addition of ANT4 (1 μM). A is for ATP; 1-A and 1-A2 are for monoadenylated and diadenylated derivatives of **1**, respectively.

that smoothly cyclized in a highly regioselective way by mild heating in water to give **5** in 56% yield.

According to NMR and MD calculations, the conformational constraint imposed by the covalent bond of $C5_{Rib}$ to $N2_{Glc}$ in **5** is especially severe (see Supporting Information). In fact, only very minor fluctuations around the glycosidic linkages and one unique puckering for the Rib ring are allowed. Moreover, the geometry of **5** closely resembles the bioactive conformation of the natural antibiotic. The average NMR structure of **5** superimposed on the X-ray structure of paromomycin in complex with A-site RNA[7b] is shown in Figure 2. Deviations in ϕ/ψ values with respect to the bound structure are less than 11 and 21° for the Glc/Strp and Rib/Strp linkages, respectively. In addition, the furanose ring is locked in a very similar conformation to that present in the RNA-bound geometry, with an identical orientation of the key polar groups, O3 and O4.

The biological activity of **5** was tested against different bacteria. The obtained MIC values are shown in Figure 2. It is important to bear in mind that $OH5_{Rib}$ is involved in RNA recognition, participating in the hydrogen bond to G1491.[7a,b] Cyclization of neomycin-B (**1**) to give **5** requires removal of this hydroxyl group. Interestingly, despite this modification, **5** displays significant activity against both Gram-positive and Gram-negative bacteria.

In a second step, the activity of ANT4 toward neomycin-B (**1**) and the locked derivative **5** as substrates was tested. Enzymatic reactions were monitored in NMR tubes. ATP (1.5−3.0 mM) and the aminoglycoside (1.5 mM) were dissolved in phosphate buffer. After addition of the enzyme (1 μM), the evolution of the reaction mixture at 310 K was monitored by 1D NMR, and the final products were characterized by 2D NMR and MALDI-TOF MS. As shown in Figure 3, under these experimental conditions, adenylation of **1** is completed within 2 min. NMR analysis permitted the confirmation that ANT4 selectively modifies $OH4_{Glc}$. In fact, if only 1 equiv of ATP is employed, this is the only product detected. Interestingly, if 2 equiv of ATP is used, nonselective secondary adenylations are detected, but at much longer reaction times. Indeed, under the employed experimental conditions, the enzyme requires more than 12 h to consume the second ATP equivalent once the primary adenylation is completed. The presence of diadenylated neomycin-B derivatives was confirmed by MALDI-TOF.

A completely different behavior was exhibited by the conformationally constrained analogue **5**. In this case, under identical experimental conditions, no reaction was detected even 30 min after enzyme addition. At longer reaction times, slow nonselective adenylation processes (similar to those described above for **1**) were observed. Nevertheless, NMR analysis of the reaction mixture confirmed that 2 h after addition of the enzyme, more than 95% of **5** remained unmodified.

Finally, the in vivo activities of neomycin-B (**1**) and mimic **5** were tested employing the bacteria *Escherichia coli* DH5α (pBBRIMCS-2), which expresses the resistance enzyme ANT4. As expected, the MIC value for the natural antibiotic is significantly increased (from 3 to 60 μg/mL). In contrast, the cyclic derivative **5** maintains the same activity observed for the nonresistant bacteria (20 μg/mL). In conclusion, this simple modification leading to the conformationally restricted **5** provides an effective protection against aminoglycoside inactivation by *S. aureus* ANT4, both in vivo and in vitro, while maintaining a significant antibiotic activity.

Thus, in our opinion, this example represents a test case of the validity of a structure-based approach for designing and preparing ligands that specifically interact with a given receptor and might potentially be used as antibiotics.

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Supporting Information Available: Extended experimental details about the design, synthesis, NMR and computational analysis, and enzymatic and biological testing of **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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