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Structure-Based Design of Highly Crowded Ribostamycin/Kanamycin Hybrids as a New Family of Antibiotics

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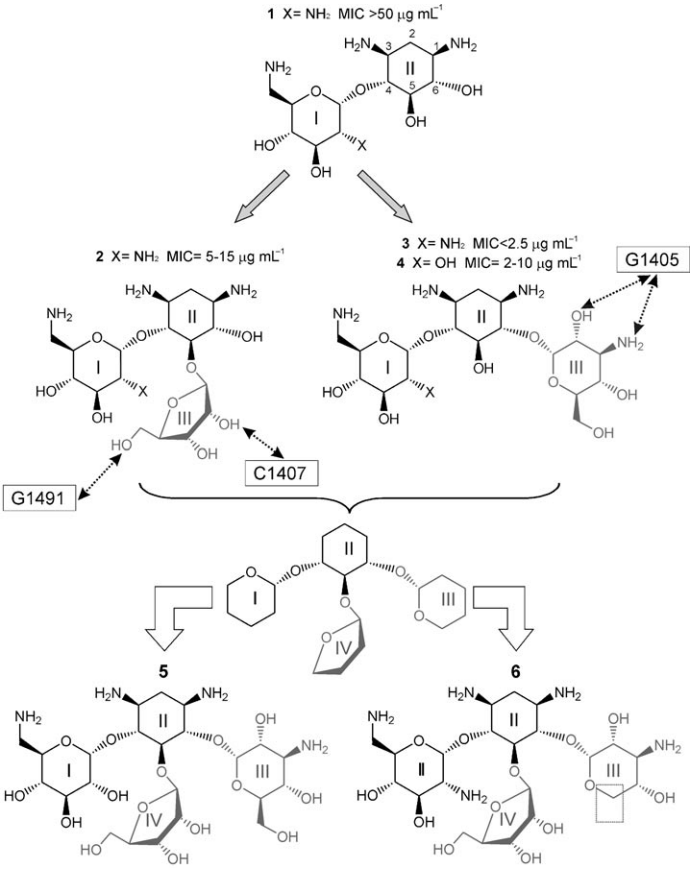
Aminoglycoside antibiotics are highly potent, wide-spectrum bactericidals. These drugs bind to the aminoacyl-tRNA site (A-site) in the ribosome decoding region inducing codon misreading and inhibiting translocation, which eventually results in cell death.[1] Unfortunately, their use in clinical practice has been seriously limited as a result of their toxicity and susceptibility to enzymatic inactivation[2] prompting the search for new active derivatives.[3]

Most aminoglycosides incorporate an aminocyclitol unit called 2-deoxystreptamine (2-DOS) that can exhibit two alternative glycosidation patterns, giving rise to the so-called 4,5-DOS and 4,6-DOS subfamilies (Scheme 1). The relevance of the different sugar units for biological activity has been firmly established from structural and functional studies.[4–6] Thus, the pseudodisaccharide fragment I/II (see Scheme 1), with slightly different patterns of OH/NH_2 distribution in unit I, is present in most aminoglycosides and has been shown to be essential for specific complex formation with the prokaryotic 16S RNA.[4] Despite their secondary importance, sugar units at the 5- or 6-positions in the 2-DOS ring provide additional contacts with the lower and upper stems, respectively, of the RNA receptor as shown by detailed NMR and crystallographic studies carried out in recent years (represented in Scheme 1 and Figure 1).[5]



Scheme 1. The general strategy employed in the design of a new family of antibiotics. The pseudodisaccharide fragment common to most aminoglycosides is represented at the top. Addition of a sugar ring (grey) at the 5- or 6-position of the aminocyclitol unit gives rise to the 4,5-DOS (left) and 4,6-DOS (right) subfamilies, respectively. The functional relevance of the additional drug/RNA contacts established through ring III (indicated in each case) is reflected in the antibiotic activities (typical ranges for the MIC values are also presented for comparison). We propose the synthesis and evaluation of the highly crowded new derivatives **5** and **6**, which incorporate the trisubstituted 2-DOS scaffold. The ribose unit, numbered as III in ribostamycin (**2**), has been renumbered as IV in the non-natural pseudotetrasaccharides.

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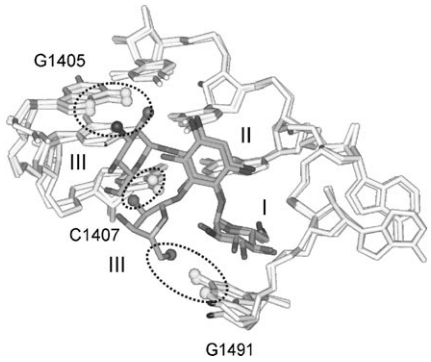


Figure 1. As shown by X-ray studies, the disaccharides I/II, present in both kanamycin-A and ribostamycin, occupy nearly identical positions within the RNA binding pocket. A superposition of kanamycin-A and ribostamycin I/II fragments in the complex is shown. Aminoglycoside/RNA contacts established through sugar unit III in both cases are highlighted.

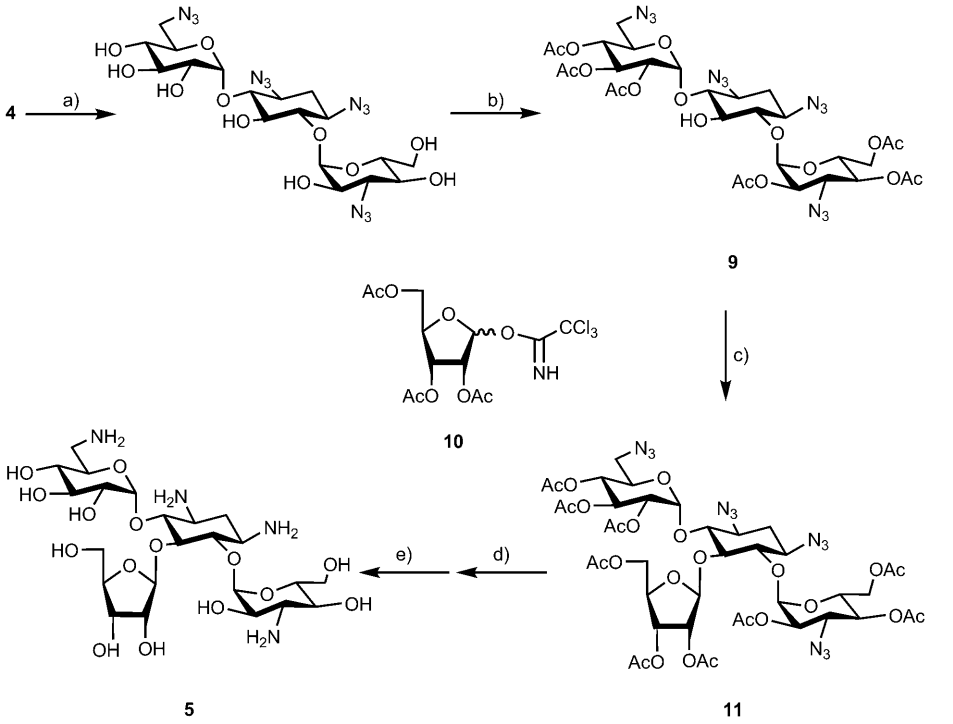
Bearing in mind these experimental findings, we analysed the viability of a new family of antibiotics incorporating the pseudodisaccharide fragment common to most aminoglycosides (rings I and II in Scheme 1) with a neutral ribose unit and a pyranose unit at the 5- and 6-positions, respectively, of the 2-DOS moiety (see Scheme 1). These novel derivatives could be considered as hybrids between those antibiotics pertaining to the 4,5- and 4,6-DOS subfamilies and simultaneously exploit the ligand/RNA contacts typical of them both. In principle, their lower flexibility and more optimized occupancy of the RNA binding pocket should translate into an increased specificity and reduced toxicity.

It could be hypothesised that steric crowding, inherent to the 4,5-/4,6-DOS hybrid scaffold, could have a negative influence on its RNA binding properties and antibiotic activity. However, extensive molecular dynamics simulations on model pseudotetrasaccharides in complex with RNA (see Supporting Information) show that, despite their increased dimensions, these ligands should, with a minor conformational adjustment, be tolerated within the RNA binding pocket. Moreover, according to these data, the ligand/receptor contacts established through the three sugar units (I, II and III), previously observed in natural complexes, are compatible with each other. Finally, it should be considered that the unfavourable contacts between the different sugar units might be alleviated by adequate modifications of the drug.

Preliminary conformational studies carried out in our group support the idea that the incorporation of a xylosamine unit, instead of glucosamine, as ring III should drastically reduce the contacts between the different sugar rings, providing an improved pseudotetrasaccharide structure for the RNA binding pocket. According to the X-ray data the glucose hydroxymethyl group is not involved in any interaction with the RNA receptor and therefore this substitution should not destabilize the complex. To prove the viability of the proposed new family of aminoglycosides, we have addressed the synthesis and evaluation of pseudotetrasaccharides **5** and **6** (Scheme 1). From a synthetic perspective, the preparation of such derivatives is challenging as it involves the glycosidation of an extremely hindered hydroxyl function (at the 5-position of the aminocyclitol ring). Despite this potential limitation, we have demonstrated that such reactions can be successfully accomplished in moderate yields.

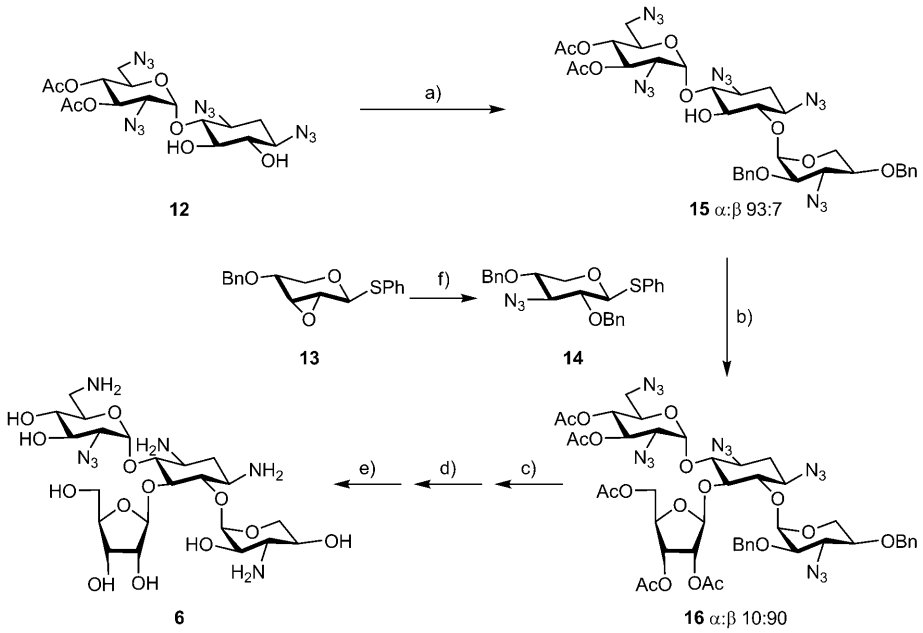
Compound **5** was prepared from kanamycin-A (**4**) following the five-step procedure in Scheme 2. The free amine groups were transformed into azides by means of a diazo-transfer reaction with freshly prepared triflyl azide (TfN_3).[7] The subsequent acetylation with acetic anhydride led to derivative **9** in excellent yield.

Glycosyl donor **10** was prepared by using a well-established procedure.[8] Glycosidation of **9** with **10** was carried



Scheme 2. Methodology employed for the synthesis of **5**. a) 1. Amberlite 400 (OH^-), H_2O, RT, 3 h; 2. TfN_3, $CuSO_4$, toluene/MeOH/py, RT, 16 h; b) Ac_2O, DMAP, py, RT, over night, yield: 80 % (global yield for steps a and b); c) $BF_3{\cdot}Et_2O$, CH_2Cl_2, −25 to −20 °C, glycosyl donor **10**, yield: 30 %; d) MeONa, MeOH/THF, 2 h; e) Me_3P, THF, NaOH, 24 h, 55 °C, yield: 37 % (global yield for steps d and e).

out under conventional conditions (−20/−25 °C, $BF_3{\cdot}OEt_2$ catalyst and CH_2Cl_2 solvent). Close inspection of minimized models of the acceptor **9** suggests that the reactive OH function at the 5-position of the 2-DOS ring is, to a large extent, hidden by the protected vicinal units I and III. We were pleased to discover that, despite its presumably low accessibility, the stereospecific glycosidation proceeded successfully to give the pseudotetrasaccharide **11** in moderate yields. Finally, hydrolysis of the acetyl groups followed by reduction of the azides, employing the Staudinger reaction, yielded derivative **5**.



Scheme 3. Methodology employed for the synthesis of **6**. a) Glycosyl donor **14**, TfOH, NIS, CH_2Cl_2/Et_2O, −40 °C, yield: 89 %; b) $BF_3{\cdot}Et_2O$, CH_2Cl_2, −25 to −20 °C, glycosyl donor **10**, yield 41 %; c) MeONa, MeOH/THF, 2 h; d) Me_3P, THF, NaOH, 24 h, 55 °C; e) H_2, Pd/C, H_2O/AcOH; yield 35 % (global yield for steps c–e); f) 1. NaN_3/DMF; 2. benzylation; yield 42 %.

Pseudotetrasaccharide **6** was prepared from the neamine acceptor **12** employing two consecutive glycosidation reactions (Scheme 3). First, the readily accessible 6-position of the aminocyclitol was regioselectively glycosidated with donor **14**, employing the methodology described by Chang and coworkers, to give the corresponding pseudotrisaccharide **15**.[9] The reaction proceeded stereospecifically and in good yields. Donor **14** was obtained from epoxide **13**[10] in just two steps: opening of the oxirane ring with sodium azide in DMF followed by the protection of the resulting OH functions with benzyl groups. As a final step, a second glycosidation reaction was performed at the 5-position of the 2-DOS unit employing the glycosyl donor **10.** After hydrolysis of the acetyl groups the final product was obtained from the Staudinger reaction followed by hydrogenolysis of the benzyl protecting groups. The final pseudotetrasaccharide was purified by ion-exchange chromatography.

Subsequently, the binding properties of the non-natural derivatives, **5** and **6,** and the parent natural compounds, **2**–**4**, to ribosomal RNA were analysed. For this we employed the fluorescence-based approach developed independently by Pilch and Hermann.[11] This methodology makes use of a modified 27-mer RNA fragment (A-site(2AP)) consisting of the sequence of the ribosomal A-site with the fluorescent base analogue, 2-aminopurine (2AP), replacing key residue A1492 (Figure 2a). The destacking of the 2AP base that accompanies complex formation causes a significant increase in the fluorescence emission at 370 nm that can be monitored by titration experiments to derive the K_d values (Figure 2b). Keeping in mind that, for this family of antibiotics, RNA binding and biological activity are frequently poorly correlated[12] we also measured minimal inhibitory concentration (MIC) values against the bacterial strain *E. coli (DH5α)*. Typical binding curves obtained for aminoglycosides **3**–**6** are shown in Figure 2. The RNA binding affinities and biological activities measured for the hybrid aminoglycosides **5** and **6**, together with those exhibited by related natural antibiotics, are shown in Table 1 and Figure 3.

It can be observed that, for the natural antibiotics, the introduction of a ribose moiety at the 5-position of the amino-

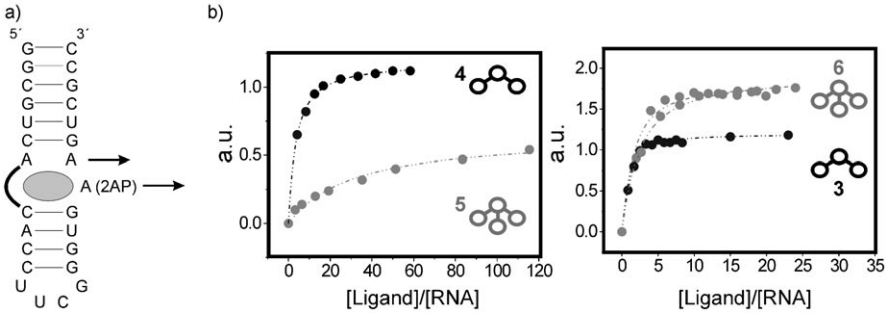


Figure 2. a) Schematic representation of the modified RNA fragment employed in our binding studies and the structural change induced by the ligand binding. b) Some representative binding curves obtained for natural aminoglycosides **3** and **4** (black) and the related pseudotetrasacchárides **5** and **6** (grey).

Table 1. RNA-binding affinities (K_d) and biological activities (MIC) of aminoglycosides **1**–**6**.

	K_d [μM][a]	MIC [μg mL^{-1}][b]
1	–/(7.39)[c]	–/(64)[c]
2	15.16/(16.35)[c]	5–10/(8)[c]
3	0.88	2.0–2.5
4	6.39	2.0–2.5
5	67.62	25–35
6	2.68	1.5–2.0

[a] Dissociation constants, K_d were measured at 25 °C in 100 mM NaCl, 10 mM phosphate and pH 7.0. [b] Experimental MIC values were measured against *E. coli (DH5α)*. [c] K_d and MIC values in brackets were taken from the literature.[13]

cyclitol ring of neamine (ribostamycin, **2**, vs. neamine, **1**, see Table 1 and Figure 3) causes a twofold increase in the dissociation constant K_d, which represents a 0.41 kcal mol^{-1} unfavourable contribution to ΔG. Despite this reduction in the aminoglycoside/RNA complex stability, ribostamycin exhibits an enhanced biological activity (almost an eightfold decrease in the MIC value). Piltch and co-workers have shown that aminoglycoside biological activity strongly correlates with the degree of conformational restriction that the drug imposes on the key RNA residues, A1492 and A1493, on complexation and not with the complex stability itself. According to this view, the additional ligand/RNA contacts established by the ribose unit in ribostamycin (**2**) may further decrease the internal mobility of the RNA. Although merely speculative, the entropic penalty associated with the extra freezing of the receptor may compensate for the enthalpic benefit derived from the additional RNA/ligand contacts (enthalpy/entropy compensation).[12,13]

Different behaviour was observed for the non-natural aminoglycosides. Thus, the introduction of a ribose ring to kanamycin-A, to yield derivative **5** (kanamycin-A, **4**, vs derivative **5**), has a negative impact on both complex formation and antibiotic activity. More specifically, the K_d value increases by one order of magnitude. This represents a +1.40 kcal mol^{-1} destabilisation of the aminoglycoside/RNA complex at 25 °C, almost an extra 1.0 kcal mol^{-1} energy penalty in comparison with that caused by the ribose ring in natural ribostamycin (**2**). As a consequence the antibiotic activity is now drastically reduced (from ca. 2.5 μg mL^{-1} in kanamycin-A, **4**, to ca. 30 μg mL^{-1} in derivative **5**). A speculative but plausible explanation for this behaviour is that the close proximity of the III and IV sugar units in **5** prevents an optimal adaptation of the ligand to the receptor binding pocket.

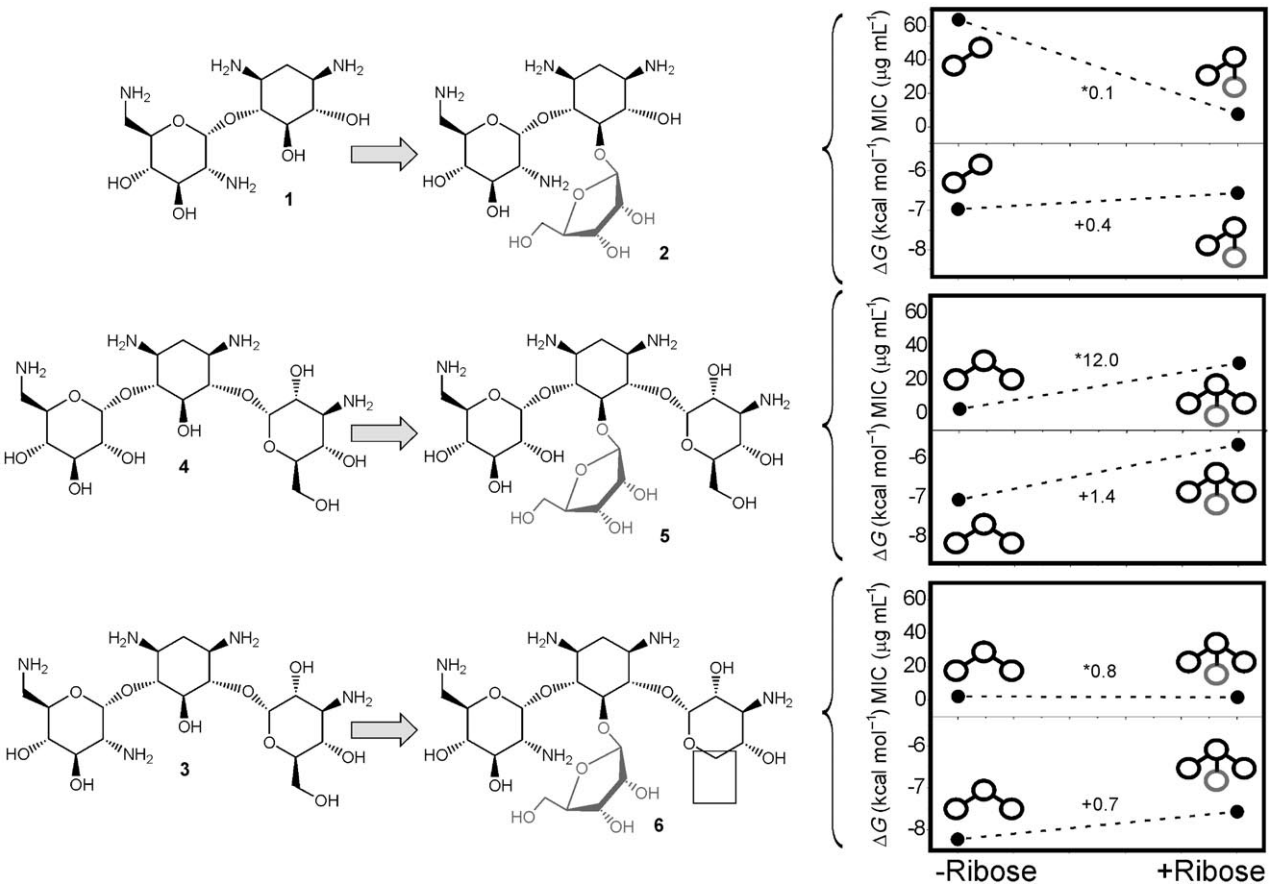


Figure 3. The aminoglycoside/RNA complex stabilities (ΔG) and biological activities (MIC values) measured for several natural and non-natural aminoglycosides, with (right) and without (left) a neutral ribose unit (grey) at the 5-position of the 2-DOS ring, are represented.

This hypothesis is fully supported by the experimental data measured for the xylose derivative (**6**). In this particular case, the RNA binding affinity is reduced by only a factor of three with respect to the natural compound kanamycin-B (**3**), implying a destabilisation of the aminoglycoside/RNA complex of only 0.68 kcal mol^{-1}. This energy penalty is similar to that caused by the ribose ring in natural ribostamycin (**2**, +0.40 kcal mol^{-1}). Moreover, the biological activity is not reduced by the presence of the additional ring IV. In contrast, derivative **6** exhibits a slightly improved MIC value (ca. 1.7 μg mL^{-1}). It seems that the removal of the hydroxymethyl function from ring III alleviates the steric conflict between rings III and IV allowing a more optimal adaptation of the antibiotic to the RNA binding pocket.

In summary, our analysis shows that the simultaneous presence of ribose and glucose sugar units at 5- and 6-positions, respectively, of the aminocyclitol ring leads to a significant increase in both the K_d (dissociation constant for the ligand/RNA complex) and MIC values. However, the steric conflict between sugar rings III and IV can be drastically reduced by the removal of a single hydroxymethyl function in unit III (replacement of glucose by xylose) that is not involved in direct drug/RNA contacts. According to our data, this simple chemical modification produces a dramatic improvement in both the ligand/RNA binding strength and biological activity.

Overall, our study demonstrates the viability of the proposed new family of 4,5-/4,6-DOS hybrid aminoglycosides. Considering its improved complementarity with the RNA A-site, the 4,5-/4,6-DOS hybrid scaffold constitutes an interesting basis for further modifications aiming to increase RNA binding affinity by establishing simultaneous contacts with the RNA upper and lower stems. Thus, the incorporation of a 2,6-diaminoidose at the 3-position of the ribose would lead to neomycin/kanamycin hybrids with high RNA binding affinity. Moreover, the replacement of the OH function at 2-position of the ribose ring by a smaller hydrogen-bonding acceptor, such as a fluorine atom, might further reduce the interaction between rings III and IV leading to additional improvements in biological activity. The larger steric volume of these derivatives, together with their limited flexibility and better occupation of the RNA binding pocket might translate into an improved selectivity for the prokaryotic ribosome and a reduction in toxicity. Current efforts to explore the potential of the 4,5-/4,6-DOS scaffold in the design of new aminoglycosides are currently underway in our laboratory.

Experimental Section

A detailed description of the synthetic protocols together with the characterisation of products and intermediates is included in the Supporting Information. Binding experiments and biological activities were performed as previously described.[3c]

Acknowledgements

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