Organic & Biomolecular Chemistry

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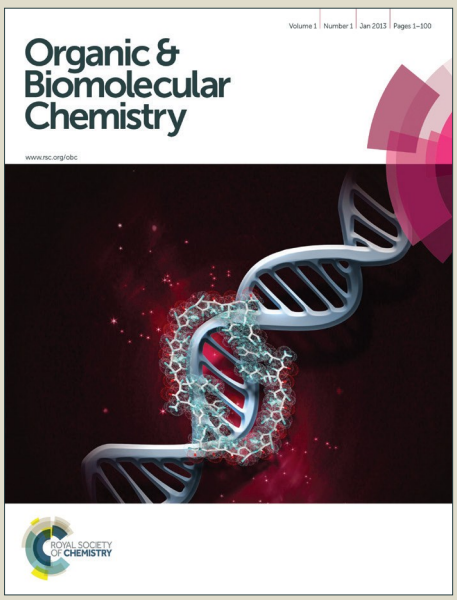

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Journal Name

ARTICLE

Selective modification of the 3''-amino group of Kanamycin prevents significant loss of activity in resistant bacterial strains

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Aminoglycosides are highly potent, wide-spectrum bactericidals. *N*-1 modification of aminoglycosides has thus far been the best approach to regain bactericidal efficiency of this class of antibiotics against resistant bacterial strains. In the present study we have evaluated the effect that both, the number of modifications and their distribution on the aminoglycoside amino groups (*N*-1, *N*-3, *N*-6' and *N*-3''), have on the antibiotic activity. The modification of *N*-3'' in the antibiotic kanamycin A is key towards the design of new aminoglycoside antibiotics. This derivative maintains the antibiotic activity against aminoglycoside acetyl-transferase- and nucleotidyl-transferase-expressing strains, which are two of the most prevalent modifying enzymes found in aminoglycoside resistant bacteria.

Introduction

Aminoglycosides are clinically relevant antibiotics that participate in a large variety of molecular recognition processes involving different RNA and protein receptors. Their potent bactericidal activity relies upon binding specifically to the rRNA in the bacterial small ribosomal subunit, thus interfering with protein synthesis.[1] However, many of the originally identified antibiotics are no longer clinically useful as they are compromised by bacterial resistance mechanisms, which include chemical modification of the drug, mutation of the molecular targets, or increased cellular drug efflux by small-molecule transporters.[2] In this context, from a clinical point of view, the most relevant source of resistance is the enzymatic inactivation of the drug by modification of its functional groups; these enzymes have been broadly classified as *N*-acetyltransferases (AACs), *O*-adenyltransferases (ANTs) and *O*-phosphotransferases (APHs).[3]

Aminoglycosides are multifunctional hydrophilic oligosaccharides that possess several amino and hydroxyl functionalities (Fig. 1). The amino groups are mostly protonated in biological medium; hence, these antibiotics can be considered as polycationic species for the purpose of understanding their biological interactions.

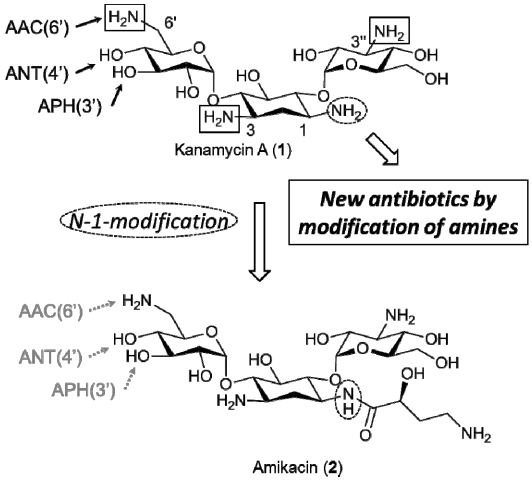

Fig. 1 Structures of kanamycin A (**1**) and amikacin (**2**). The positions vulnerable to aminoglycoside modifying enzymes (AACs, ANTs and APHs) are indicated with arrows. Addition of a substituent at the *N*-1 position (highlighted in blue) of **1** gives rise to the non-resistant antibiotic **2**. Herein we propose the synthesis and evaluation of other kanamycin derivatives by modification of the other amino groups (highlighted in green).

In fact, it has been demonstrated that these antibiotics exert their antibacterial activity mainly through these functional groups, which in turn play a key role also during enzymatic inactivation.[4]

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Electronic Supplementary Information (ESI) available: Figures S1-S6 and ^{1}H, ^{13}C spectrums of the compounds. See DOI: 10.1039/x0xx00000x

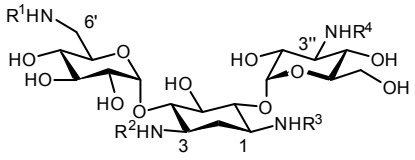
The emergence of resistance towards the first generation of aminoglycosides has led to increased efforts to identify similar antibiotics that were not susceptible to resistance.[5] As such, the *N*-1 modification of natural 4,6-aminoglycosides has undoubtedly proven to be the best approach to obtain semi-synthetic derivatives with improved biological activity against resistant strains.[6] In fact, **2** (Fig. 1), a *N*-1 kanamycin A derivative, reported in 1974, is still today included on the World Health Organization's List of Essential Medicines.[7] Compound **2** bears an (*S*)-α-hydroxy-γ-amino-*n*-butyryl (AHB) group at the *N*-1 position, which prevents inactivation at remote susceptible groups, such as *N*-6'-amine and *O*-4'/3'-hydroxyl functionalities catalyzed by AAC(6'), ANT(4') and APH(3') modifying enzymes (Figure 1), while still preserving most of the antibiotic activity of the parent compound against susceptible strains.

According to these results, we hypothesized that selective substitution of one or two amines respectively could provide valuable guidelines to characterize the level of tolerance for substitution at those positions that could be further elaborated for the design of new antibiotics.

To test this idea, we have focused on a fairly simple chemical modification: the *N*-guanidinylation of **1**. This choice is fully justified by the current knowledge on RNA ligands, which frequently include several guanidine groups in their structures that contribute substantially to their biological activities.[8] Additionally, it has been demonstrated that the substitution of an amino group in biologically active compounds by the strongly basic guanido moiety often results in a significant increase in potency and/or selectivity (Fig. S1).[9] This was further encouraged by the fact that the guanido groups of the aminoglycoside streptomycin are not susceptible to attack by any aminoglycoside modifying enzymes known to date (Fig. S1).

Herein, we studied modified amine prevalence and distribution about the aminoglycoside, and their effects on the resultant antibiotic activities.

Table 1 Structures of kanamycin A (**1**) and guanidino-kanamycin derivatives **3**-**7**.



	R^1	R^2	R^3	R^4
1	H	H	H	H
3	H	$(C=NH)NH_2$	$(C=NH)NH_2$	H
4	$(C=NH)NH_2$	H	H	$(C=NH)NH_2$
5	$(C=NH)NH_2$	H	H	H
6	H	$(C=NH)NH_2$	H	H
7	H	H	H	$(C=NH)NH_2$

With this objective in mind, we synthesized a series of kanamycin A derivatives presenting two (compounds **3** and **4**) or one (compounds **5**, **6** and **7**) *N*-modifications, in lieu of the respective amines (Table 1). It should be pointed out that the effect of modifications at the *N*-1 amino group (R^3, Table 1) in the aminoglycosides has been widely explored (Fig. S2).[10] For this reason, modification of this position has not been considered in this work. Finally, we studied the influence of these amino-modifications on aminoglycoside inactivation by bacterial defense proteins, employing three relevant resistant enzymes: ANT(4'), AAC(6'), and APH(3').

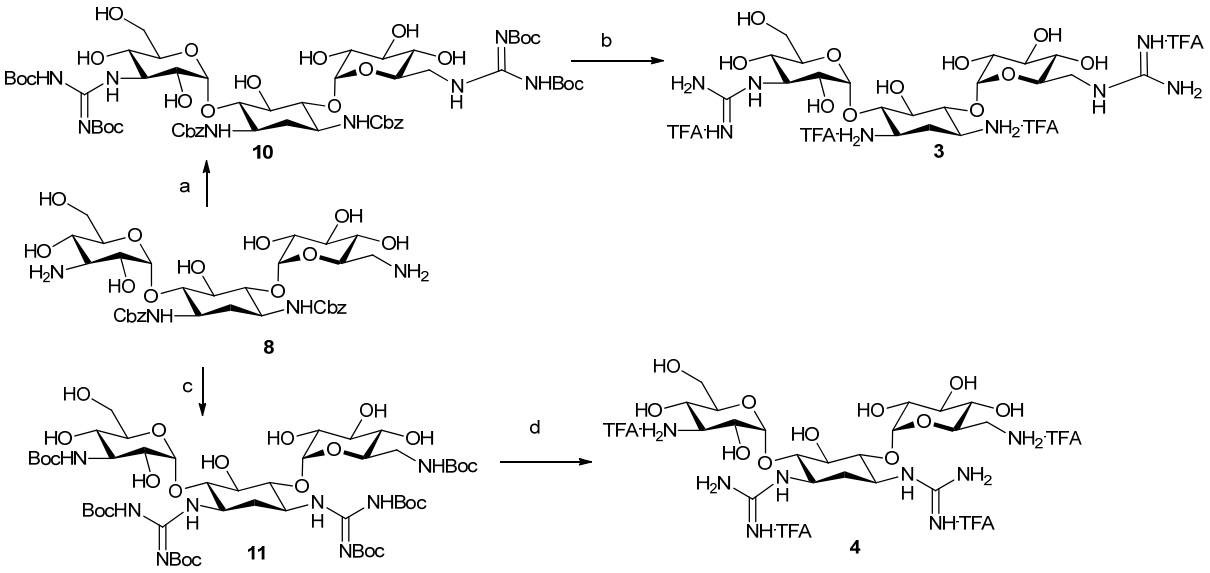
Results and discussion

Synthesis of *N*-kanamycin A derivatives (3-7).

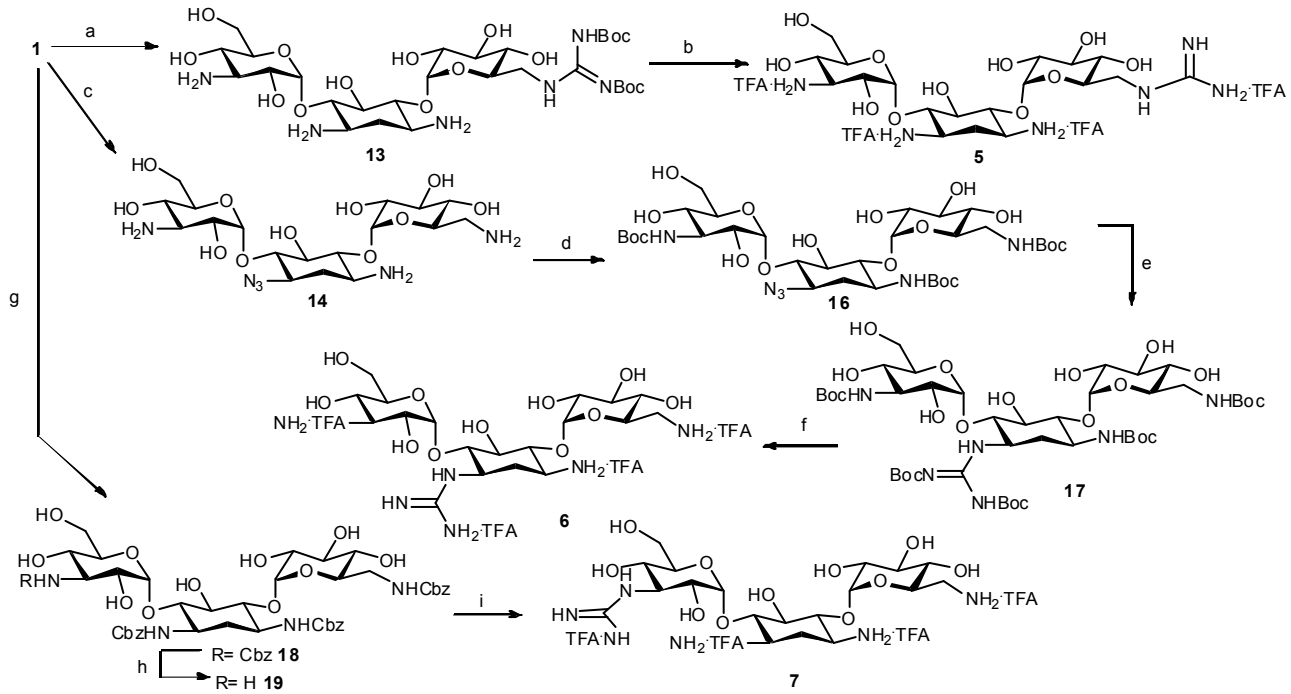
Di-guanidinylated compounds **3** and **4** were prepared from the common intermediate 1,3-$(Cbz)_2$-kanamycin A (**8**) (Cbz: benzyloxycarbonyl), whose synthesis had been previously described in two steps from **1**.[11] Conversion of 6',3''-amino groups of this compound to the corresponding guanidine derivatives was achieved by treatment of **8** with 1,3-$(Boc)_2$-2-(trifluoromethylsulfonyl) guanidine (**9**) (Boc: *tert*-butoxycarbonyl) and Et_3N, obtaining 1,3-$(Cbz)_2$-6',3''-di-guanidino$(Boc)_4$-kanamycin A (**10**) in 63% yield. Catalytic hydrogenolysis of the Cbz protecting groups, followed by acidic removal of the Boc groups, afforded compound **3** in 57% yield as its TFA salt (scheme 1).

For the synthesis of compound **4**, 6'- and 3''-amines of **8** were protected as Boc-carbamates, obtaining 1,3-$(Cbz)_2$-6',3''-$(Boc)_2$-kanamycin A. Catalytic hydrogenolysis of the Cbz groups afforded 6',3''-$(Boc)_2$-kanamycin A, which after treatment with **9** in the presence of Et_3N gave the fully Boc-protected 1,3-di-guanidino$(Boc)_4$-6',3''-$(Boc)_2$-kanamycin A (**11**) in 44% yield. Acidic deprotection of the amines using a mixture of TFA in dichloromethane afforded the desired 1,3-di-guanidinokanamycin A (**4**) in 85% yield as its TFA salt (Scheme 1).

The synthesis of mono-guanidinylated compounds **5**-**7** is shown in Scheme 2. For the synthesis of **5**, **1** was treated with one equivalent of *N*,*N*'-$(Boc)_2$-1*H*-pyrazole-1-carboxamidine (**12**) and DIPEA in a mixture of 1,4-dioxane and H_2O, providing 6'-guanidino$(Boc)_2$-kanamycin A (**13**) in 78% yield. Next, acidic removal of the Boc protecting groups afforded 6'-guanidinokanamycin A (**5**) in 74% yield as its TFA salt. The synthesis of compound **6** started with 3-azido-kanamycin A (**14**). Very recently, imidazole-1-sulfonyl azide (**15**) was successfully employed as a diazo-transfer reagent for the regioselective introduction of a 3-azide in neamine-based antibiotics.[12] Treatment of **1** with a large excess of **15** in H_2O provided 3-azido-kanamycin A (**14**) in 60% yield. In the next step, the 1,6',3''-amines were protected as Boc-carbamates, obtaining 3-azido-1,6',3''-$(Boc)_3$-kanamycin A (**16**) in 75% yield. Reduction of the azide employing the Staudinger reaction, and subsequent guanidinylation furnished the fully Boc-protected 3-guanidino$(Boc)_2$-1,6',3''-$(Boc)_3$-kanamycin A (**17**) in 48% yield. Acidic deprotection of the Boc groups afforded 3-guanidinokanamycin A (**6**) in 92% yield as its TFA salt.



Scheme 1. Reagents and conditions: (a) **9**, Et_3N, 1,4-dioxane, 50 °C, 24 h, 63%; (b) i. H_2, Pd/C, MeOH/AcOH (3:1), r.t., 18h; ii. TFA/CH_2Cl_2 (1:4), r.t., 24 h, 91% (two steps); (c) i. Boc_2O, Et_3N, DMF, 50 °C, 18 h; ii. H_2, Pd/C, MeOH/AcOH (3:1), r.t., 18h; iii. **9**, Et_3N, 1,4-dioxane, 50 °C, 62 h, 44% (three steps); (d) TFA/CH_2Cl_2 (1:4), r.t., 24 h, 85%.



Scheme 2. Reagents and conditions: (a) 12, DIPEA, 1,4-dioxane/H_2O (2:3), r.t., 5 h, 78%; (b) CH_2Cl_2/TFA, r.t., 5h, 95%; (c) 15 (Imidazole-1-sulfonyl azide), H_2O, r.t., 3h, 60%; (d) Boc_2O, Et_3N, DMF, 50 °C, 18 h, 75%; (e) i. PMe_3 (0.1M in THF), THF, aq. NaOH (0.5M), 55 °C, 12 h; ii. 9, Et_3N, 1,4-dioxane, 50 °C, 24 h, 48% (two steps); (f) CH_2Cl_2/TFA, r.t., 5h, 92%; (g) CbzCl, Na_2CO_3 (sat.), acetone, r.t., 18 h, 84%; (h) NaOH (1.5M), H_2O/1,4-dioxane (1:1), 4 °C, 12 h,78%; (i) i. **9**, Et_3N, 1,4-dioxane, 50 °C, 24 h, 72%; ii. H_2, Pd/C, MeOH/AcOH (3:1), r.t., 18h; iii. TFA/CH_2Cl_2 (1:4), r.t., 24 h, 35% (two steps).

Lastly, a slightly different approach was employed for the synthesis of 3''-guanidino kanamycin A (**7**). When 1,3,6',3''-$(Cbz)_4$-kanamycin A (**18**)[11] was reacted with a 1.5 M aqueous NaOH solution in a 1,4-dioxane and H_2O mixture at 4 °C, the carbamate at position *N*-3'' was selectively removed, affording 1,3,6'-$(Cbz)_3$-kanamycin A (**19**) in 62% yield. Treatment of this compound with **9** according to the guanidinylation procedure described above, furnished 1,3,6'-$(Cbz)_3$-3''-guanidino$(Boc)_2$-kanamycin A. Catalytic hydrogenolysis of this derivative for Cbz removal followed by acidic treatment with TFA afforded *N*-3''-guanidinokanamycin A (**7**) in a moderate yield of 25% as its TFA salt (Scheme 2). From a structural point of view, compounds **8**, **13**, **14** and **19** are the key intermediates for the subsequent selective structural modification. Compounds **8** and **19** had been described previously by Cheng and coworkers.[11] For compounds **13** and **14**, the structures were established by 2D NMR analysis (Fig. S3). The purity of the final compounds (**3**-**7**) was determined by ^{1}H-NMR titration using DMSO as the internal standard (See supporting information).

Effect of the *N*-modification on the Antibiotic Activity.

To assess the effect that amino-modification of kanamycin A (**1**) has on the antibiotic activity, minimum inhibitory concentration (MIC) values of the natural aminoglycoside **1** and its modified antibiotics (**3**-**7**), were determined against a wide variety of bacterial strains (Table 2). According to the obtained data, the number and position of these modifications has a determining effect on the antibiotic activity. In fact the presence of more than one guanidine group (**3** or **4**) resulted in very poor antibiotic activity (MIC >50 μg/mL). When analyzing the antibiotic activity with only one amine modification (derivatives **5**-**7**), we noticed that the position bearing the substitution was a key factor. In fact, *N*-3-kanamycin A derivative **6** showed a dramatic loss of efficacy (MIC >50 μg/mL) in comparison with its parent antibiotic **1** (MIC= 4-6 μg/mL). On the other hand, the *N*-6'-kanamycin A derivative **5** exhibited moderate efficacy against some bacterial strains, while the *N*-3''-kanamycin A derivative **7** provided the best activity profile against a wide variety of bacterial strains (Table 2).

Therefore, compound **7** was tested against hospital-isolated strains (entries 9-12, table 2). In the case of the E. *coli* strain 2323 and *S. aureus* strain 2638, compound **7** showed a potency (MICs 2 and 6 μg/mL) comparable to that of the parent compound **1** (MIC= 2 μg/mL for both strains). Remarkably, derivative **7** showed marked improvement against the E. *coli* strain 2501 and the *K. pneumonia* strain 2556 compared to kanamycin A (**1**), reducing the MIC value from 24 μg/mL to 2 μg/mL in both cases.

It is well established that the distribution of amino-groups within the aminoglycoside antibiotic plays a key role in its molecular recognition by RNA.[13] Based on the obtained data, it can be concluded that the introduction of one or more modifications at positions *N*-1, *N*-3 or *N*-6' causes a loss of biological activity. A speculative but plausible explanation for these results could be that those modifications are too close to the receptor binding pocket. In contrast, derivative **7** retains its antibiotic activity after the *N*-3'' modification probably due to its remote position in the A-site in the RNA. These results are consistent with previous studies carried out in our group demonstrating that the A-site shows a clear preference for modification at the *N*-3'' position of the aminoglycosides (Fig. S4a).[14] Additionally, this result is in agreement with the natural occurrence of several *N*-3''-substituted aminoglycosides, thus validating our results (Fig. S4b).

Notwithstanding that the *N*-3''-amine is the least involved in the RNA recognition event, according to its outwards orientation in the 3D structure of the rRNA-kanamycin A complex (Fig. 2a),[15] both *O*-2'' and *N*-3'' functionalities provide key contacts with the G1405 residue, which have been shown to be essential for both aminoglycoside affinity and specificity (Fig. 2a-b and Fig. S5).[16]

Table 2. MICs (μg/mL) of kanamycin A (**1**) and modified compounds (**3**-**7**) against non-resistant strains.[a] Entries 9-12 correspond to hospital strains with a phenotype of resistance enzymes (Experimental details)

Entry	Organism (strain)	Compounds					
		1	**3**	**4**	**5**	**6**	**7**
1	*E. coli* (ATCC25922)	4	>50	>50	**50**	>50	**4**
2	*E. coli* (BL21)	6	>50	>50	**25**	>50	**15**
3	*E. faecalis* (ATCC29212)	16	>50	>50	>50	>50	50
4	*S. gallolyticus* (ATCC49147)	16	>50	>50	>50	>50	**20-30**
5	*B. circulas* (CECT10)	5	>50	>50	**50**	>50	**5**
6	*P. eruginosa*(ATCC15692)	25	>50	>50	>50	>50	50
7	*S. aureus* (ATCC29213)	4	>50	>50	>50	>50	**2**
8	*D. radiodurans* (ATCC13939)	<25	>50	>50	>50	>50	<50
9	*E. coli* (2501)[a]	24	nd	nd	nd	nd	**2**
10	*E. coli* (2323)[a]	2	nd	nd	nd	nd	**2**
11	*K. pneumonia* (2556)[a]	24	nd	nd	nd	nd	**2**
12	*S. aureus* (2638)[a]	2	nd	nd	nd	nd	**6**

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Therefore, the replacement of the amine functionality of position *N*-3'' by a more basic and a more effective hydrogen-bond donor/ acceptor, operates synergistically to further increase the biological activity. Besides, the formation of more than one interaction between the drug and the target could also improve the binding affinity. To provide insight into the proposed hypothesis, molecular docking studies were performed to explore the binding interactions of compound **7** with rRNA, using the crystallographic structure of kanamycin A (**1**) itself as the positive control (Fig. 2a).[15] In the calculated rRNA-compound **7** complex, the aminoglycoside is positioned in an analogous manner to kanamycin A (**1**) and the *N*-3'' group can be accommodated within the receptor binding pocket without any apparent hindrance. Interestingly, despite the fact that the *N*-3'' guanidine group of **7** retains the same exposed character as the *N*-3'' amine of **1** (Fig. 2b), clear differences between the amine and the guanidine contacts with the target are evident (Fig. 2a and 2b). Indeed, the guanidine group presents a stronger interaction with G1405 residue relative to the amine in kanamycin A (**1**). All these observations are in agreement with the experimental results previously described and suggest that the punctual modification of the *N*-3''-amine should be carefully considered in the design of new antibiotics with improved activity.

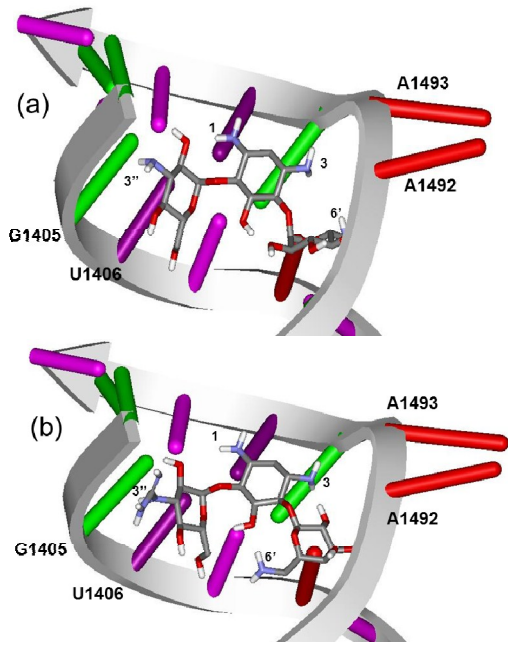

Fig. 2. (a) Structure of the **1**/rRNA complex according to X-ray data.[15] (b) Modeling of the compound **7**/rRNA A-site complex.

Effect of *N*-modification of kanamycin A on the Inactivation by bacterial aminoglycoside modifying enzymes.

In order to test the effect of *N*-modification on the activity of individual aminoglycoside modifying enzymes, the most prevalent method of resistance amongst aminoglycoside-resistant bacterial strains, enzymes ANT(4'), APH(3') and AAC(6') were studied. Kanamycin A (**1**) and compound **7** were tested as ligands of the aforementioned enzymes by means of the thermal melting experiments (Table S1). Ligand binding affinity has been estimated from the change of unfolding transition temperature (ΔT_m), obtained both in the presence and absence (control) of the ligands.[17] These compounds proved to be appropriate ligands for these enzymes, producing clear thermal stabilization of the enzymes (ΔT_m= 5-6 ^{o}C).

When compound **7** was subjected to kinetic analysis, different trends were observed for each of the tested enzymes. For APH(3'), the rate of phosphorylation was barely affected [V_{rel}(**7**)/V_{rel}(**1**)= 0.8], whereas in the cases of ANT(4') and AAC(6'), adenylation and acetylation of **7** was less efficiently achieved [V_{rel}(**7**)/V_{rel}(**1**)= 0.5]‡. This data indicates that compound **7** has a significantly slower inactivation rate relative to that of kanamycin A (**1**).

In the case of ANT(4'), a speculative but plausible explanation for this behavior is that, according to the 3D structure of the enzyme in complex with kanamycin A (**1**) (Fig. 6Sa), the close proximity of the guanidine structural motifs at *N*-3''-position could prevent optimal adaptation of the ligands in the receptor binding pocket. For AAC(6'), it has been recently demonstrated that the modification of a remote position from *N*-6', specifically position 2'' of kanamycin A (**1**), decreased the activity of AAC(6').[5] Docking studies carried out in our group support the idea that the incorporation of *N*-3'' amine modifications may disrupt the proper orientation of the antibiotic in the binding site (Fig. 6Sb-d), which could account for the observed decrease in the enzyme activity.

In order to correlate these results with the effect of *N*-3'' amine modification in bacterial systems, we selected *E. coli* (BL21) strains expressing each one of the modifying enzymes previously employed as resistant strains for antibiotic activity determination (Experimental details). Kanamycin A (**1**) is a powerful antibiotic with a MIC value of 4 μg/mL for Gram + (*B. circulans)* or Gram – (*E. coli*) strains, respectively (entries 2 and 5, Table 2). However, when the aminoglycoside modifying enzymes are being expressed, kanamycin A (**1**) completely loses its antibiotic activity (MIC>100 μg/mL). According to the MIC values

measured for the *N*-3''-guanidine **7** (entries 1-3, table 3), while considering that a total loss of activity for the parent antibiotic **1** was observed, it seems that this singular modification exerts a degree of protection against aminoglycoside inactivation performed by ANT(4'), APH(3') and AAC(6'). In a similar way, compound **7** showed a two-fold improvement over **1** against two hospital-isolated MRSA strains (entries 4 and 5, Table 3).

Table 3. MICs of **1** and **7** against resistant strains (Experimental details).

Entry	Organism (strain)	MIC [a]	
		1	**7**
1	*E. coli* (pET-ANT(4'))	>100	50
2	*E. coli* (pET-APH(3'))	>100	75
3	*E. coli* (pET-AAC(6')-Ib)	>100	50
4	*MRSA* (ATCC1228)	>100	>50
5	*MRSA* (ATCC33591)	20	10

[a]MIC: Minimum Inhibitory Concentration, values (μg/mL).

Taking into consideration the kinetic parameters previously presented, as well as the MIC values, compound **7** maintains its bactericidal activity in resistant strains, while ANT(4') and AAC(6') enzymes present a lower catalytic efficiency for this substrate. These results strongly suggest that a punctual modification of *N*-3'' position of 2-deoxystreptamine 4,6-disubstituted aminoglycosides should be carefully considered in the design of new antibiotics with improved activity against resistant strains.

Conclusions

In summary, we have designed a novel series of kanamycin A (**1**) derivatives by means of a selective amine modification. Six new compounds were synthesized and their antibiotic performance was tested through the determination of the corresponding MIC values, from which *N*-3''-guanidine **7** stood out as a potential lead in further optimization studies. To investigate the reasons of this behavior, compound **7** was submitted to further analysis against three clinically-relevant aminoglycoside modifying enzymes (ANT(4') from *S. aureus*, APH(3') from *E.coli* and ACC(6') from *S. aureus*), showing that the reaction rates were significantly lower than for the natural antibiotic **1**. These results were corroborated by the incubation of **7** with bacterial strains expressing the same inactivating enzymes, showing promising *in vivo* behavior. The protecting effect of the remote moiety at the 3''-position was rationalized in terms of a binding hindrance with the respective inactivating enzymes that presumably does not take place with the rRNA A-site. In fact, docking calculations suggest that compound **7** may be able to establish optimal interactions with the G1405 residue of the A-site, showing once more the potential of position *N*-3'' for the development of improved antibiotics that are not susceptible to deleterious enzymatic inactivation. To conclude, we have demonstrated that the *N*-3'' amine modification is a promising direction to search for novel aminoglycosides with potency against resistant bacterial strains

Experimental details

Kanamycin A (**1**) was purchased from Sigma-Aldrich. All the reactions that needed dry conditions were carried out under argon atmosphere and the solvents were appropriately dried before use by standard techniques. Commercial reagents were used as received. All reactions were magnetically stirred and monitored by TLC in precoated Kiesel gel 60 F254 (Merck). Flash column chromatography (FC) was carried out on Silica Gel 60 (32-63 μm). Detection was first carried out by UV light (254 nm) and then charring with a solution of sulfuric acid, ninhydrin or with Monstain. NMR experiments were recorded on a Varian Unity 400 MHz at 313K. The spectra of aminoglycosides **3**-**7** were assigned employing a combination of 2D TOCSY, COSY and HSQC experiments. Low and High resolution mass were provided by the Mass Spectrometry Facilities, CSIC, Madrid. The bacterial strains utilized in this study were obtained from various sources: clinical isolates of *E. coli*, *K. pneumoniae* and *S. aureus* were obtained from the Hospital de Fuenlabrada (Madrid). The rest of the strains were obtained from the ATCC (American Type Culture Collection) or CECT (Spanish Type Culture Collection). The genes for ANT(4') and APH(3') were obtained from an *S. aureus* strain and the pACYC177 plasmid, respectively. They were cloned in a pET28b plasmid, and expressed in *E coli* BL21 (DE3) in our lab. The plasmid of pET28b-AAC-6' was a gift from Prof. Sylvie Garneau-Tsodikova (University of Michigan). The ATP, acetyl-CoA, kanamycin A, NADH, sypro orange, DTP, pyruvate kinase/lactate dehydrogenase enzymes and all organic compounds were purchased from Sigma–Aldrich. Analysis by UV-Vis assays were carried out on a SpectraMax plus reader using 96-well plates (Molecular Devices). Analysis by HPLC Jasco PU-2089 plus used a Vydac 30I column at 260 nm. The mobile phase was aqueous TFA at pH 3.0, and the flow was 1.0 mL min^{-1}. The T_m values of the proteins were measured by an iCycler iQ PCR plate.

MIC Determination.

A solution of the selected bacterial strain was grown in 1 mL of Mueller–Hinton broth to an optical density (OD_{600}) of 0.5 units. The desired concentrations of antibiotic and guanidino-glycosides (**3**-**7**) were added from stock solutions. After incubation at 37 ºC for 24 h, the OD_{600} of each sample was read. In both cases, the MIC was taken as the lowest antibiotic concentration inhibiting bacterial growth by greater than 90%. All compounds were converted to their free-base by exposure to a strong basic anion exchange resin prior to their evaluation. In the case of the resistance strains expressing AAC(6'Ib), ANT(4') or APH-(3'), *E. coli (BL21)* containing the pET28b-AAC-(6'I), pET28b-ANT-(4'), and pET28b-APH(3')

plasmids, respectively, were induced with IPTG prior to addition of the antibiotics. The resistance enzyme phenotype of the strains from hospital isolates is as follows: *E. coli* (2501) - AAC(6'); *E. coli* (2323) - AAC(6')-AAC(3)-ANT(2''); *K. pneumonae* - AAC(6'); *S. aureus* - APH(3')-III.

Enzymatic Activity.

The formation of AMP-kanamycin or AMP-compound **7** was followed by HPLC. Assay mixtures contained 20 mM sodium phosphate, pH 7.5, 5mM $MgCl_2$, 3 mM ATP, and variable concentrations of kanamycin A or *N*-3'' derivative (**1**, **7**) (0.03–3 mM). The reaction was started by the addition of 0.5 µM of ANT-4' enzyme. The formation of the reaction product was also confirmed by mass spectrometry of the final reaction mixtures: MS-API-ES (AMP-**1**): 814.7 $[M+H]^+$; MS-API-ES (AMP-**7**): 856.7 $[M+H]^+$. The phosphorylation of kanamycin A (**1**) and compound **7** was determined by a spectrophotometric assay. The assay mixture contained the following components in a volume of 1 mL: 50 mM hepes (pH 7.7), 40 mM KCl, 10 mM $MgCl_2$, 247 µM NADH, 2.5 mM phosphoenolpyruvate, 1 mM ATP, various concentrations of aminoglycosides (5 µM to 1.25 mM for aminoglycoside), 5 µL pyruvate kinase/lactate dehydrogenase enzyme solution, and 10 µL of APH-3'IIa. The mixtures without APH-3'IIa were incubated at 37 °C for 5 min, and the reactions were initiated by adding 10 µL of the partially purified protein. Negative controls were prepared by omitting aminoglycosides, and the readings were subtracted from the test samples. The acetylation of kanamycin A and compound **7** was followed by a spectrophotometric assay. Briefly, reactions (500mL) containing Tris buffer pH 7.6 (50 mM), DTP (2 mM), Acetyl-CoA (40 µM), 1mM EDTA, and enzyme (10µL at 5mg/mL) were initiated by addition of the aminoglycoside (0.1mM-1mM kanamycin or derivative **7**). The reactions were incubated at 37 °C and were monitored for CoASH production by taking readings at 324 nm every 30 s for 30 min.

Thermal shift assay.

To monitor the protein unfolding, the fluorescent dye Sypro orange was used.[17] The thermal shift assay was carried out in the iQ5 Real Time Detection System (Bio-Rad, CA). Final solutions of 100µL contained 90µL of 10mM Tris buffer pH 8.0, 0.2 mM NaCl, 2.8X Sypro Orange, and 4.4µM of protein (ANT-4' and APH-3'-IIa, and AAC-6'-Ib), plus 10µL of kanamycin A or the corresponding derivative **7** at 10mM. The plate was heated from 25º to 70ºC with a heating rate of 0.5 ºC/min. The fluorescence intensity was measure with Ex/Em 490/530.

Molecular docking studies.

The Crystal structure of the complex between kanamycin A and the decoding A site, APH(3'-IIa), ANT(4'), and AAC(6'-Ib) were obtained from the pdb (ID: 2esi, 1nd4, 1kny, 2bue). The molecular studies with compound **7** were done by docking calculations. The receptor was prepared using Wizard (Schrödinger). The initial conformation of kanamycin A (**1**) was taken from the crystal structure of the antibiotic bound to an oligonucleotide containing the bacterial ribosome A-site or to the aminoglycoside modifying enzymes. AMPPNP or Acetyl-CoA and the magnesium ions from the aminoglycoside ternary complex were included in the compound **1** model during the simulation. Next, it was prepared using LigPrep by modifying the torsions of the ligand and assigning the appropriate protonation state/s. In Glide, 32 stereochemical structures were generated per compound with possible protonation states at target pH 7.0 ± 2.0 using Ionizer, then tautomerized, desalted and optimized by producing low-energy 3D structures for the ligand under the OPLS 2005 force field while retaining the specified chiralities of the input Maestro file. Then, the receptor grid was calculated for the prepared receptor such that various ligand poses bind within the predicted site during docking. A cubic box of specific dimensions (15 Å x 15 Å x 15 Å) centred on the aminoglycoside was generated for the receptor. After that, SP flexible ligand docking was carried out in Glide of Schrödinger-Maestro v9.2.7. Final scoring was performed on energy-minimized poses and displayed as Glide score. The best docked pose was recorded for the ligand.

N-6', 3''-Di-guanidinokanamycin A (**3**).

To a solution of **8**[1] (200 mg, 0.266 mmol) in 1,4-dioxane (13.5 mL, 50 mL/mmol) was added 1,3-(Boc)2-2-(trifluoromethylsulfonyl) guanidine **9** (312 mg, 0.798 mmol, 3 eq) and triethylamine (0.22 mL, 1.60 mmol, 6 eq) and the reaction mixture was allowed to stir at r.t. for 5 days, and then stopped by evaporating the solvent. The residue was purified through a silica column chromatography (AcOEt/MeOH 0% to 10%) to yield the desired compound **10** (207 mg, 63%) as a white foam. ^{1}H-NMR (MeOD-d4, 300 MHz): δ 7.35 (10H, m), 5.23-4.75 (6H, m), 4.25 (1H, dd, J=9.6, 9.6 Hz), 4.15 (1H, m), 3.85-3.40 (16H, m), 3.15 (1H, dd, J=9.3, 9.3 Hz), 2.15-2.05 (1H, m), 1.60-1.35 (37H, m). MS-API-ES: 1237 $(M+H)^+$. To a solution of **10** (200 mg, 0.167 mmol) in methanol (3.4 mL, 20 mL/mmol) was added palladium on carbon 20% (40 mg, 20% w/w) and acetic acid (1 mL). The reaction flask was purged three times and the mixture was stirred under H_2 atmosphere overnight, then filtered over a Celite pad, washed with methanol and concentrated to dryness. The crude residue was used in the following reaction without further purification. MS-API-ES: 970 $(M+H)^+$. Finally, this material was submitted to deprotection under acidic conditions by dissolving it in DCM/TFA (2.5 mL, 4:1 v/v). The reaction mixture was stirred at r.t. for 5 h, then evaporated to dryness and co-evaporated twice with toluene. The residue thus obtained was dissolved in distilled water, and the clear supernatant was taken and freeze-dried to yield a white fluffy powder **3** (155 mg, 91% two steps) as its corresponding TFA salt. ^{1}H-NMR (D_2O, 400 MHz): δ 5.50 (1H, d, J = 3.9 Hz), 5.04 (1H, d, J = 3.6 Hz), 3.96-3.45 (18H, m), 3.35 (1H, dd, J = 9.6, 9.6 Hz), 2.49 (1H, ddd, J = 12.3, 4.0, 4.0 Hz), 1.86 (1H, ddd, J = 12.6, 12.6, 12.3 Hz). ^{13}C-NMR (D2O, 100 MHz): δ 158.3, 157.9,

101.0, 97.6, 83.9, 79.0, 72.9, 72.9, 72.3, 71.4, 71.0, 70.3, 69.7, 67.6, 60.2, 57.6, 49.9, 48.2, 41.8, 27.7. MS-API-ES: 569 $(M+H)^+$. HRMS (ES): calcd (%) for $C_{20}H_{40}N_8O_{11}$ $[M+H]^+$: 569.5860; found: 569.2859.

N-1,3-Di-guanidinokanamycin A (**4**).

To a solution of **8** (100 mg, 0.133 mmol) in anhydrous DMF (6.6 mL, 50 mL/mmol) was added dropwise a solution of Boc_2O (116 mg, 0.532 mmol, 2 eq/amine) in DMF (2 mL) and triethylamine (0.11 mL, 0.80 mmol, 3 eq/amine). The mixture was stirred under Ar atmosphere at 50 °C overnight. The reaction was stopped by evaporating the solvent. The residue was dissolved in AcOEt (20 mL), washed with 1M HCl and brine, dried over anhydrous Na_2SO_4 and evaporated. The crude was filtered through a short silica column (DCM/MeOH, 0 to 20%) to yield 6',3''-(Boc)2-1,3-$(Cbz)_2$-kanamycin A as an amorphous solid that was immediately used in the following reaction. MS-API-ES: 953 $(M+H)^+$. To a solution of this compound in methanol (2.7 mL, 20 mL/mmol) was added palladium on carbon 20% (20 mg, 20% w/w) and acetic acid (1mL). The reaction flask was purged three times and the mixture was stirred under H_2 atmosphere overnight, then filtered over a Celite pad, washed with metanol and concentrated to dryness. The crude product 6',3''-$(Boc)_2$-Kanamycin A was used in the next step without further purification. MS-API-ES: 684 $(M+H)^+$. To a solution of the crude (45 mg, 0.064 mmol) in 1,4-dioxane (3.2 mL, 50 mL/mmol) was added 1,3-(Boc)2-2-(trifluoromethylsulfonyl)guanidine **9** (151 mg, 0.386 mmol, 3 eq/amine) and triethylamine (54 µL, 0.386 mmol, 3 eq/amine) and the reaction mixture was allowed to stir at r.t. for 5 days, and then stopped by evaporating the solvent. The residue was purified through a silica column chromatography (AcOEt/MeOH 0% to 5%) to yield 6',3''-(Boc)2-1,3-$(Boc)_2$-guanidino-Kanamycin A **11** (61 mg, 44% three steps) as a white foam. ^{1}H-NMR (DMSO-d6, 300 MHz): δ 5.35 (1H, d, J=3.4 Hz), 5.17 (1H, d, J=3.3 Hz), 4.43-4.22 (2H, m), 4.10-4.01 (1H, m), 3.85-3.48 (7H, m), 3.43-3.30 (2H, m), 3.18 (1H, dd, J=9.3, 9.3 Hz), 3.10-2.95 (1H, m), 2.25-2.15 (1H, m), 1.60-1.35 (55H, m). MS-API-ES: 1053 $(M+H)^+$. This compound (55 mg, 0.057 mmol) was submitted to deprotection under acidic conditions by dissolving it in DCM/TFA (5 mL, 4:1 v/v). The reaction mixture was stirred at r.t. for 5 h, then evaporated to dryness and co-evaporated twice with toluene. The residue thus obtained was dissolved in distilled water, and the clear supernatant was taken and freeze-dried to yield a white fluffy powder **4** (50 mg, 85%) as its corresponding TFA salt. ^{1}H-NMR (D_2O, 500 MHz): δ 5.57 (1H, d, J = 3.4 Hz), 5.17 (1H, d, J = 3.7 Hz), 4.05 (1H, ddd, J = 9.9 Hz, 3.1, 3.1), 3.86-3.78 (4H, m), 3.77-3.57 (7H, m), 3.46-3.38 (2H, m), 3.35 (1H, dd, J = 13.7, 4.5 Hz), 3.31 (1H, dd, J = 13.7, 4.0, Hz), 2.26 (1H, ddd, J = 6.4, 3.4, 3.4 Hz), 1.69 (1H, ddd, J= 12.6, 12.6, 6.4Hz). ^{13}C-NMR (D_2O, 125 MHz): δ 156.4, 99.0, 98.0, 80.8, 80.4, 74.8, 72.3, 72.0, 70.9, 69.6, 68.1, 67.7, 65.3, 59.4, 54.9, 51.3, 50.1, 39.4, 32.6. MS-API-ES: 569 $(M+H)^+$. HRMS (ES): calcd (%) for $C_{20}H_{40}N_8O_{11}$ $[M+H]^+$: 569.5860; found: 569.2886.

N-6'-Guanidinokanamycin A (**5**).

To a solution of kanamycin A **1** (100 mg, 0.2 mmol) in water (6 mL) was added a solution of *N*,*N*'-di-Boc-1*H*-pyrazole-1-carboxamidine **12** (62 mg, 0.2 mmol) in 1,4-dioxane (4 mL) and diisopropyl-ethyl-amine (DIPEA) (38 µL, 0.22 mmol). The reaction mixture was vigorously stirred at r.t. for 5 h and stopped by evaporating the solvent under vacuum. The crude residue was purified through a silica column chromatography (NH_4OH:BuOH:EtOH:Tol, 4:3:5:3), obtaining **13** as a white solid (113 mg, 78%). MS-API-ES: 727 $(M+H)^+$. Finally, this compound was deprotected using the general procedure with TFA/CH_2Cl_2, obtaining **5** as its TFA salt (%). ^{1}H-NMR (D_2O, 400 MHz): δ 5.38 (1H, d, J = 3.9 Hz), 4.96 (1H, d, J = 3.6 Hz), 3.83-3.37 (15H, m), 3.34 (1H, dd, J = 10.5, 10.5 Hz), 3.24 (1H, dd, J = 9.6, 9.6 Hz), 2.39 (1H, ddd, J = 12.3 4.1, 4.1 Hz), 1.76 (1H, ddd, J= 12.6 Hz, 12.3, 12.3,). ^{13}C-NMR (D_2O, 100 MHz): δ 157.9, 100.7, 97.7, 83.9, 79.0, 73.1, 72.9, 72.3, 71.4, 71.1, 69.7, 68.3, 65.4, 59.9, 55.1, 49.8, 48.1, 41.8, 27.8. MS-API-ES: 526 $(M+H)^+$. HRMS (ES): calcd (%) for $C_{19}H_{38}N_6O_{11}$ $[M+H]^+$: 527.2671; found: 527.2688.

N-3-Guanidinokanamycin A (**6**).

To a solution of kanamcyin A **2** (128 mg, 0.22 mmol) in water (10 mL) was added a solution of imidazole-1-sulfonyl azide hydrochloride **15** (737 mg, 3.52 mmol, 16 equiv) in water (10 mL). The reaction mixture was adjusted to pH 6.6 by addition of aq. 2M NaOH solution and stirred at room temperature for 12 h. The mixture was washed twice with dichloromethane (20 mL). The aqueous solution was concentrated under reduced pressure to remove dichloromethane residues and freeze-dried. The crude residue was purified through a silica column chromatography (NH_4OH:BuOH:EtOH:CH_2Cl_2, 4:2:2.5:1), obtaining **14** as a white solid (87 mg, 78%). ^{1}H-NMR (D_2O, 400 MHz): δ 5.33 (d, J= 3.4 Hz, 1H), 5.04 (d, J= 3.9 Hz, 1H), 4.02 (d, J= 9.4 Hz, 1H), 3.90-3.81 (m, 2H), 3.77-3.50 (m, 6H), 3.52-3.36 (m, 3H), 3.31 (d, J= 13.3 Hz, 1H), 3.25 (t, J= 9.5 Hz, 1H), 3.07 (dd, J= 12.6, 8.1 Hz, 1H), 2.38 (dt, J= 12.6, 4.2 Hz, 1H), 1.66 (q, J= 12.6 Hz, 1H) ^{13}C-NMR (D2O, 100 MHz): δ 99.9, 98.0, 87.2, 84.4, 73.9, 72.3, 72.0, 71.3, 71.0, 70.9, 69.7, 68.5, 60.1, 54.2, 50.0, 48.6, 40.6, 34.3. MS-API-ES: 511$(M+H)^+$. To a solution of **14** (87 mg, 0.17 mmol) in anhydrous DMF (8.5 mL) was added dropwise a solution of Boc_2O (222 mg, 1.02 mmol, 2eq/amine) in DMF (4 mL) and triethylamine (0.23 mL, 1.53 mmol, 3eq/amine). The mixture was stirred under Ar atmosphere at 50 °C overnight. The reaction was stopped by evaporating the solvent. The residue was dissolved in AcOEt (25 mL), washed with 1M HCl and brine, dried over anhydrous Na_2SO_4 and evaporated. The crude was filtered through a short silica column chromatography ($CHCl_3$:MeOH:NH_4OH, 6:2:0.2) to yield compound **16** (103 mg, 75%) as a white solid. ^{1}H-NMR (MeOD-d_4, 400 MHz): δ 5.23 (d, J= 4.3 Hz, 1H), 5.06 (d, J= 3.8 Hz, 1H), 4.03 (ddd, J= 9.7, 4.7, 2.4 Hz, 1H), 3.89 (ddd, J= 9.7, 6.5, 2.7 Hz, 1H), 3.78 (dd, J= 11.7, 2.5 Hz, 1H), 3.72-3.60 (m, 3H), 3.59-3.49 (m, 1H), 3.47 (dd, J= 10.0, 4.5 Hz, 1H), 3.45-3.41

(m, 2H), 3.42 (d, J= 4.0 Hz, 1H), 3.41-3.32 (m, 4H), 3.25 (dt, J= 13.7, 5.7 Hz, 1H), 3.15 (t, J= 9.5 Hz, 1H), 2.27-2.16 (m, 1H), 1.73-1.30 (m, 28H) ^{13}C-NMR (MeOD-d_4, 100 MHz): δ 157.9, 157.3, 156.6, 100.7, 98.6, 83.4, 80.7, 80.2, 79.2, 78.9, 78.7, 78.4, 78.3, 75.0, 73.2, 73.0, 72.6, 71.4, 71.3, 70.7, 68.4, 60.8, 59.7, 32.8, 27.4. MS-API-ES: 811 $(M+H)^+$. Compound **16** (130 mg, 0.16 mmol) was dissolved in THF (6.8 mL) in a reaction vial equipped with a reflux condenser, and 0.1M NaOH(aq) (258 µL) and PMe3 (1M in THF, 224 µL) were added. The reaction mixture was stirred at 50 °C overnight. After completion of the reaction, it was cooled to room temperature and loaded into a short silica column ($CHCl_3$:MeOH:NH_4OH, 10:2:0.2). The product 6',3''-$(Boc)_2$-Kanamycin A was used in the next step without further purification. MS-API-ES: 785 $(M+H)^+$. To a solution of the crude (125 mg, 0.16 mmol) in 1,4-dioxane (8 mL) was added 1,3-$(Boc)_2$-2-(trifluoromethylsulfonyl)guanidine **9** (187 mg, 0.48 mmol) and triethylamine (67 µL, 0.48 mmol) and the reaction mixture was allowed to stir at r.t. for 5 days, and then stopped by evaporating the solvent. The residue was purified through a silica column chromatography (AcOEt:MeOH, 8:2) to yield 6',1,3''-$(Boc)_3$-3-$(Boc)_2$-guanidino-Kanamycin A **17** (79 mg, 48% two steps) as a white foam. ^{1}H-NMR (MeOD-d_4, 400 MHz): δ 5.15 (d, J= 3.9 Hz, 1H), 5.07 (d, J= 3.9 Hz, 1H), 4.33 (dd, J= 10.6, 1.9 Hz, 1H), 4.15 (dd, J= 10.6, 6.9 Hz, 1H), 3.78 (dd, J= 11.7, 2.5 Hz, 1H), 3.74 (dd, J= 3.4, 1.7 Hz, 1H), 3.68 (dt, J= 4.0, 2.5 Hz, 1H), 3.66 (dt, J= 2.9, 1.6 Hz, 1H), 3.61 (dd, J= 4.3, 2.8 Hz, 1H), 3.60-3.57 (m, 4H), 3.56-3.50 (m, 5H), 3.44 (dt, J= 3.2, 1.6 Hz, 1H), 2.14-2.05 (m, 1H), 1.56-1.48 (m, 46H) ^{13}C-NMR (MeOD-d_4, 100 MHz): δ 159.2, 104.5, 101.3, 83.9, 83.6, 83.4, 82.6, 80.7, 79.1, 73.0, 72.7, 71.4, 71.0, 70.8, 70.7, 70.6, 70.4, 70.0, 69.9, 66.7, 60.9, 53.7, 49.1, 29.3, 26.9. MS-API-ES: 1028 $(M+H)^+$. Finally, this compound was deprotected using a mixture of TFA/CH_2Cl_2 (1:4), obtaining compound **6** as its TFA salt (%).^{1}H-NMR (D_2O, 400 MHz): δ 5.31 (d, J= 3.9 Hz, 1H), 4.88 (d, J= 3.2 Hz, 1H), 3.76 (ddd, J= 10.9, 8.3, 3.1 Hz, 1H), 3.75-3.70 (m, 2H), 3.64 (t, J= 2.9 Hz, 1H), 3.59-3.57 (m, 2H), 3.56 (dd, J= 4.4, 2.4 Hz, 1H), 3.55-3.51 (m, 3H), 3.50 (d, J= 1.9 Hz, 1H), 3.46-3.43 (m, 1H), 3.42 (dd, J= 5.7, 4.0 Hz, 1H), 3.39 (dd, J= 6.7, 3.8 Hz, 1H), 3.33-3.31 (m, 2H), 3.26 (t, J= 10.5 Hz, 1H), 3.20 (dd, J= 13.5, 3.2 Hz, 1H), 3.16-3.10 (m, 1H), 2.93 (dd, J= 13.5, 8.2 Hz, 1H), 2.31 (dt, J= 12.5, 4.2 Hz, 1H), 1.70 (q, J= 12.5 Hz, 1H). ^{13}C-NMR (D_2O, 100 MHz): δ 157.1, 107.1, 100.4, 95.5, 83.8, 78.6, 78.0, 72.8, 72.0, 71.9, 70.7, 70.5, 69.4, 68.5, 59.7, 54.7, 49.6, 47.8, 41.5, 27.4. MS-API-ES: 526 $(M+H)^+$.

N-3''-Guanidinokanamycin A (**7**).

Compound **19** was obtained from **18** according to the procedure described by Chen and coworkers.[11] To a solution of **19** (235 mg, 0.266 mmol) in 1,4-dioxane (13.5 mL, 50 mL/mmol) was added **8** (156 mg, 0.399 mmol, 1.5 eq) and triethylamine (0.11 mL, 0.8 mmol, 3 eq) and the reaction mixture was allowed to stir at r.t. for 5 days, and then stopped by evaporating the solvent. The residue was purified through a silica column chromatography (AcOEt/MeOH 0% to 10%) to yield 1,3,6'-$(Cbz)_3$-3''-$(Boc)_2$-guanidine-kanamycin A (216 mg, 72%) as a white foam. ^{1}H-NMR (MeOD-d_4, 300 MHz): δ 7.41-7.23 (15H, m), 5.20 (1H, d, J= 3.6 Hz), 5.14-4.97 (12H, m), 4.95 (1H, d, J= 3.6 Hz), 4.09 (1H, d, J= 9.6 Hz), 3.84-3.54 (12H, m), 3.52(1H, d, J= 4.5 Hz), 3.50-3.38 (7H, m), 2.07 (1H, ddd, J= 12.9, 12.6, 12.6 Hz), 1.47 (9H, m). MS-API-ES: 1129 $(M+H)^+$. To a solution of this compound (216 mg, 0.192 mmol) in methanol (3.8 mL, 20 mL/mmol) was added palladium on carbon 20% (43 mg, 20% w/w) and acetic acid (1.1 mL). The reaction flask was purged three times and the mixture was stirred under H_2 atmosphere overnight, then filtered over a Celite pad, washed with methanol and concentrated to dryness. The crude residue was used in the following reaction without further purification. MS-API-ES: 729 $(M+3H)^+$. This material was subsequently submitted to deprotection under acidic conditions by dissolving it in DCM/TFA (2.9 mL, 4:1 v/v). The reaction mixture was stirred at r.t. for 5 h, then evaporated to dryness and co-evaporated twice with toluene. The residue thus obtained was dissolved in distilled water, and the clear supernatant was taken and freeze-dried to yield a white fluffy powder **7** (43 mg, 35% two steps) as its corresponding TFA salt. ^{1}H-NMR (D_2O, 400 MHz): δ 5.38(d, J = 3.6 Hz, 1H), 5.04-4.92 (s, 1H), 3.83-3.75 (m, 3H), 3.72 (dd, J = 19.1, 10.4 Hz, 2H), 3.83-3.75 (m, 3H), 3.72 (dd, J = 19.1, 10.4 Hz, 2H), 3.67-3.53 (m, 3H), 3.49 (dd, J = 10.0, 3.8 Hz, 1H), 3.43 (d, J = 9.7 Hz, 1H), 3.36 (t, J = 10.5 Hz, 1H), 3.24 (t, J = 9.7 Hz, 1H), 2.40 (dd, J = 12.3, 4.1 Hz, 1H), 1.77 (ddd, J = 12.6, 12.3, 12.3 Hz, 1H). ^{13}C-NMR (D_2O, 100 MHz): δ 157.5, 100.3, 97.7, 83.6, 78.6, 72.8, 72.6, 71.9, 71.1, 70.7, 69.4, 67.9, 65.7, 59.5, 54.8, 49.5, 47.8, 41.5, 27.4. MS-API-ES: 527 $(M+H)^+$. HRMS (ES): calcd (%) for $C_{19}H_{38}N_6O_{11}$ $[M+H]^+$: 527.2671; found: 527.2697.

Acknowledgments

This investigation was supported by a research grant from the Spanish Dirección General de Investigación, "CTQ2013-45538-P". The authors thank Romain Garbage and Hanna Katarzyna for their help in the synthesis of the derivatives.

Notes and References

‡ The V_{rel}(**7**)/V_{rel}(**1**) was determined instead of K_m and K_{cat} because of the very low reactivity.

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