Bioorganic & Medicinal Chemistry 16 (2008) 8940–8951

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Structure–toxicity relationship of aminoglycosides: Correlation of 2′-amine basicity with acute toxicity in pseudo-disaccharide scaffolds

Lilach Chen [a], Mariana Hainrichson [a], Dmitry Bourdetsky [b], Amram Mor [b], Sima Yaron [b], Timor Baasov [a,*]

[a] The Edith and Joseph Fischer Enzyme Inhibitors Laboratory, Schulich Faculty of Chemistry, Technion—Israel Institute of Technology, Haifa 32000, Israel
[b] Department of Biotechnology and Food Engineering, Technion—Israel Institute of Technology, Haifa 32000, Israel

ARTICLE INFO

Article history:
Received 13 July 2008
Revised 17 August 2008
Accepted 22 August 2008
Available online 27 August 2008

Keywords:
Aminoglycosides basicity
Protein translation inhibition
Resistance to aminoglycosides
Aminoglycosides toxicity
Acute toxicity

ABSTRACT

A new pseudo-disaccharide NB23 with a 3′,4′-methylidene protection was designed and its properties were evaluated in comparison to other two structurally related pseudo-disaccharides. The basicity of the 2′-amine was found to be well correlated to acute toxicity data in mice: the increase in the basicity is associated with the toxicity increase. Based on these data, a new pseudo-trisaccharide NB45 was constructed. NB45 exhibited significant antibacterial activity while at the same time retained low acute toxicity.

1. Introduction

2-Deoxystreptamine (2-DOS) aminoglycosides (Fig. 1) are highly potent, broad-spectrum antibiotics that selectively target the prokaryotic ribosome and bind to the decoding A-site of the 16S ribosomal RNA leading to protein translation inhibition and interference with the translational fidelity.[1–4] These antibiotics have been used in the clinic for almost six decades and because of such prolonged clinical and veterinary use, bacterial resistance to these drugs has become a serious public health problem. The primary resistance mechanism to aminoglycosides is the bacterial acquisition of enzymes which modify the antibiotics by acetyltransferase (AAC), adenyltransferase (ANT), or phosphotransferase (APH) activities.[2,5] Each class of these enzymes performs a regiospecific reaction either on the amine group (AAC) or hydroxyl group (ANT and APH) and the turnover products of these reactions lack antibacterial activity.

In addition to emerged resistance, one of the major limitations in using aminoglycosides as drugs is their high toxicity to mammals through kidney (nephrotoxicity) and ear-associated (ototoxicity) illnesses. The origin of this toxicity is still controversial and probably results from a combination of different factors/mechanisms such as interactions with phospholipids, inhibition of phospholipases and formation of free radicals.[6,7] Furthermore, neuromuscular blockage and related respiratory depression are well known side effects of aminoglycosides therapy.[8] Numerous studies suggested that the interference between aminoglycosides and some steps of calcium-mediated acetylcholine release at the level of presynaptic structures is the main cause of the neuromuscular blockage induced by aminoglycosides.[9,10] Indeed, neuromuscular blocking effects were found to be well correlated to acute toxicity data of aminoglycosides[11,12] and that co-administration of Ca^{2+} antagonized acute toxicity and neuromuscular effects induced by aminoglycosides.[12,13] In light of these observations, it was suggested that both respiratory failure and experimental toxicity follow the neuromuscular blocking effects caused by aminoglycosides.[11]

Much work has been done to understand the mechanisms by which side effects of aminoglycosides develop in patients, but little effort was devoted to understand their structure–toxicity relationship. During the last decades, many examples of synthetic variants of naturally occurring aminoglycosides were reported.[14,15] The majority of these variants, however, were designed with the aim to tackle the problem of resistance to aminoglycosides and it is very rare that the toxicity was either considered in the design or it was determined for the resulting compounds. It is highly noteworthy that, although a new synthetic variant may exhibits favorable antibacterial activity, high toxicity can prevent its clinical application. Therefore, the design of novel variants of aminoglycosides which in addition to resisting to aminoglycoside-modifying enzymes and targeting ribosomal RNA, will also have high probability of reduced toxicity is of high urgency.

For this propose, we sought that it may be possible to separate elements of the aminoglycoside structure that induce toxicity from those that are required for an antibiotic effect. Indeed, earlier study

* Corresponding author. Tel.: +972 4 829 2590; fax: +972 4 829 5703.
E-mail address: chtimor@tx.technion.ac.il (T. Baasov).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.08.053

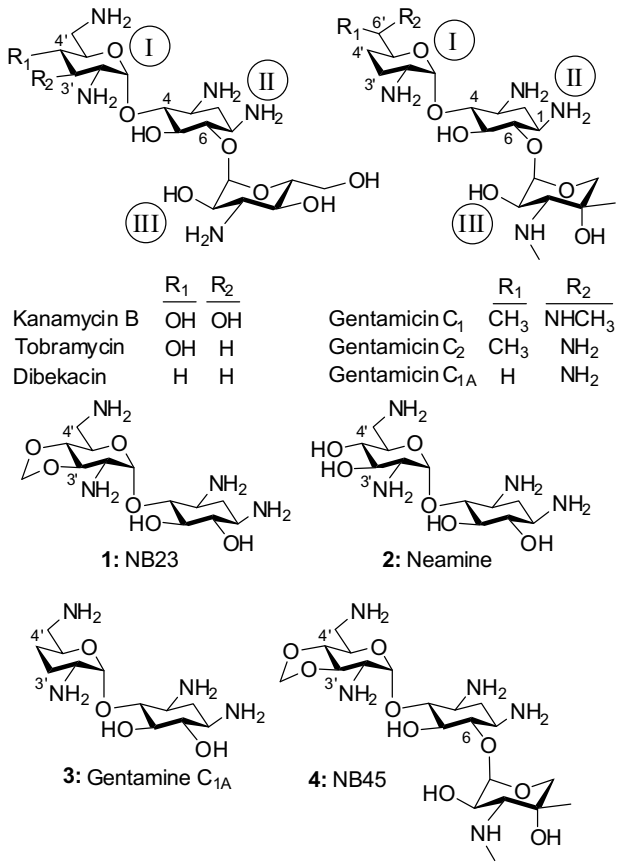


Figure 1. Chemical structures of a series of natural and synthetic 2-deoxystreptamine-derived (ring **II**) aminoglycosides, including compounds **1**–**4** that were investigated in this study.

on aminoglycosides structure–toxicity relationship[16] revealed that one of the factors that significantly affect the acute toxicity of aminoglycosides is the deletion of the ring hydroxyl group(s) (deoxygenation) that is adjacent to the amino group on the same ring. For example, deletion of 3′-OH in kanamycin B (Fig. 1, LD_{50} = 132) gives the significantly more toxic tobramycin (LD_{50} = 79). Further deletion of 4′-OH in tobramycin results in dibekacin (3′,4′-dideoxykanamycin B, LD_{50} = 71) with only a marginal increase in toxicity. The observed increase in the toxicity of the deoxy-analogs could be explained by an increase in the basicity of the 2′-amino group due to the removal of the neighboring electron-withdrawing oxygen atom (3′-OH). Furthermore, the removal of the 3′-OH which is adjacent to the 2′-NH_2, has much more influence than removal of the 4′-OH, which is more distant from this amine. Similar results have been obtained by replacement of the 5-OH with 5-fluorine in kanamycin B and its several clinical derivatives.[17,18] The toxicities of the resulting fluoro analogs were significantly lower than the parent compounds and this phenomenon again was attributed to basicity reduction of the 3-NH_2 group induced by the strongly electron-withdrawing 5-fluorine. Thus, significantly high acute toxicity of the clinical drugs such as tobramycin (3′-deoxy), gentamicin (3′,4′-dideoxy), dibekacin (3′,4′-dideoxy) and arbekacin (3′,4′-dideoxy) could be ascribed to the increased basicity of 2′-NH_2 group (ring I) in these drugs caused mainly because of the lack of 3′-hydroxyl or 3′,4′-hydroxyl groups, respectively.

On the other hand, the increase in basicity of a particular amine group in the aminoglycoside structure may increase its binding affinity to the target ribosomal RNA A site and subsequently increase its antibacterial potency.[19,20] However, bactericidal activity of aminoglycosides was found to be poorly correlated with their A site affinity.[21,22] It is generally accepted that the bactericidal activity of aminoglycosides is determined by their antitranslational impact on prokaryotic protein synthesis.[21]

The purposes of the current study were: (1) to employ the impact of the 2′-NH_2 basicity on the acute toxicity and to design a new pseudo-disaccharide scaffold with potentially low acute toxicity; (2) to compare the basicity of the 2′-NH_2 between the new and structurally related pseudo-disaccharide scaffolds and verify the correlation between the basicity and acute toxicity; (3) to use the newly designed scaffold for the construction of novel aminoglycoside derivatives with potent antibacterial activity and low toxicity.

Towards these ends, here we report the design, synthesis, and comparative analysis of a series of three pseudo-disaccharides **1**–**3** (Fig. 1). The data presented herein verify a relationship between the basicity of the 2′-amine group and the estimated LD_{50} values in mice: the increase in the basicity of the 2′-amino functionality is associated with the acute toxicity increase of an aminoglycoside. Based on the observed data, a new pseudo-trisaccharide **4** (also called NB45) was constructed, which showed significant antibacterial activity and low acute toxicity.

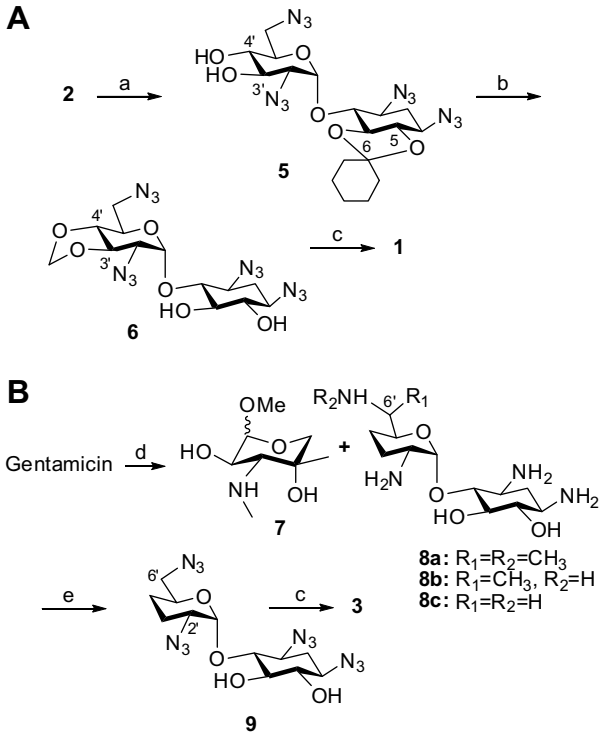
2. Results and discussion

2.1. Design hypotheses and synthesis

Based on the above-discussed structure–toxicity relationship analysis, especially the impact of the 2′-NH_2 basicity on the acute toxicity of the several aminoglycoside antibiotics, we designed the pseudo-disaccharide **1** (also named NB23, Fig. 1) as a minimal structural motif with the following expectations. First, the structure **1** consists of a 3′,4′-methylidene protection as a potential ′defense′ against toxicity; the preservation of 3′,4′-oxygens in **1** should keep the lower basicity of the 2′-NH_2 group and subsequently the lower toxicity than those in the parallel 3′,4′-dideoxy analog **3**. Second, the methylidene group should also protect **1** from the action of various APH(3′) and ANT(4′) resistance enzymes. Third, the 3′,4′-methylidene protection is supposed to be substantially stable under both the acid and base conditions usually used in carbohydrate chemistry, and should easily be constructed from the corresponding 3′,4′-diol (see the synthesis bellow).

To test the validity of our design, we initially synthesized three pseudo-disaccharides, compounds **1**–**3** (Fig. 1), and determined their acute toxicity along with the pK_a values of the individual amino groups in each compound. As a starting material for the synthesis of **1** we used the compound **2** (also named neamine, Fig. 1), which was readily accessible in a large quantity by direct acid hydrolysis of neomycin B according to the previously reported procedure[23] (Scheme 1A). Simultaneous conversion of all the amino groups of **2** into the corresponding azides (TfN_3, $CuSO_4$) was followed by selective protection of the 5,6-hydroxyl groups by cyclohexylidene ketal to afford 3′,4′-diol **5**. Treatment of **5** with two equivalents of CH_2Br_2 under base conditions of NaOH, followed by hydrolysis of cyclohexylidene ketal with aqueous acetic acid, afforded the 3′,4′-methylidene derivative **6**. The Staudinger reaction then generated the desired product **1**.

The third pseudo-disaccharide derivative, compound **3** (also named gentamine C_{1A}, Fig. 1) was most readily accessible from the mixture of commercial gentamicin in three steps (Scheme 1B). Treatment of gentamicin (a mixture of at least five compounds: gentamicin C_{1A}, gentamicin C_1 and gentamicin C_2, while the latter two are a mixture of the corresponding C6′-diastereomers, Scheme 1) with anhydrous HCl (AcCl in MeOH) gave a highly



Scheme 1. Reagents and conditions: (a) i—TfN_3, Et_3N, $CuSO_4$, in CH_2Cl_2/MeOH/H_2O 3:10:3, 90%; (ii) CSA, DMF, cyclohexanonedimethylketal, 65%; (b) i—CH_2Br_2 (2 equiv), THF/aqueous NaOH (10 M) 1:1, nBu_4NBr, 85%; ii—H_2O/1,4-dioxane/HOAc 1:1:2, 85%; (c) PMe_3 (1 M in THF), THF/NaOH (0.1 M) 3:1, 95–99%; (d) AcCl, MeOH, 86%; (e) i—TfN_3, Et_3N, $CuSO_4$, in CH_2Cl_2/MeOH/H_2O 3:10:3, 93%; ii—flash column chromatography. CSA, camphor sulfonic acid.

regioselective hydrolysis between the rings II and III to afford garoseamine derivative **7** (ring III of gentamicin) and a mixture of the corresponding pseudo-disaccharides **8**. Treatment of this mixture (**8**) with TfN_3 converted all the primary amines to the corresponding azides from which the desired component **9** could be easily separated by flash chromatography. The Staudinger reaction on **9** furnished the desired product **3** with excellent purity and isolated yield. The structures of **1–3** were confirmed by a combination of various 1D and 2D NMR techniques, including 2D ^1H–^{13}C HMQC and HMBC, 2D COSY, and 1D selective TOCSY experiments, along with mass spectral analysis.

2.2. Determination of the pK_a values of the individual amine groups in compounds 1–3 by using natural abundance ^{15}N NMR

To evaluate the basicity of individual amine groups, natural-abundance ^{15}N NMR spectra of the free base forms of **1–3** as a function of pH were recorded. Assignments for individual ^{15}N signals in each structure was done by chemical shift comparisons with those previously reported for the neamine part (rings I and II) of neomycin B[24–26] and of other aminoglycosides.[19,27,28] To further secure our assignments, we performed complete set of standard NMR experiments (including ^1H, ^{13}C, ^1H–^{13}C HMQC and HMBC, 2D COSY, and 1D selective TOCSY) along with the 2D ^1H–^{15}N HMBC spectra at the low- and high-pH extremes as well as at several intermediate pHs. While the combination of standard NMR experiments allowed unambiguous assignment of all the hydrogen atoms present in each structure, the 2D ^1H–^{15}N HMBC technique unequivocally secured the assignment of individual ^{15}N signals in each structure (**1–3**) by showing correlations between ^1H and ^{15}N (Fig. 2).

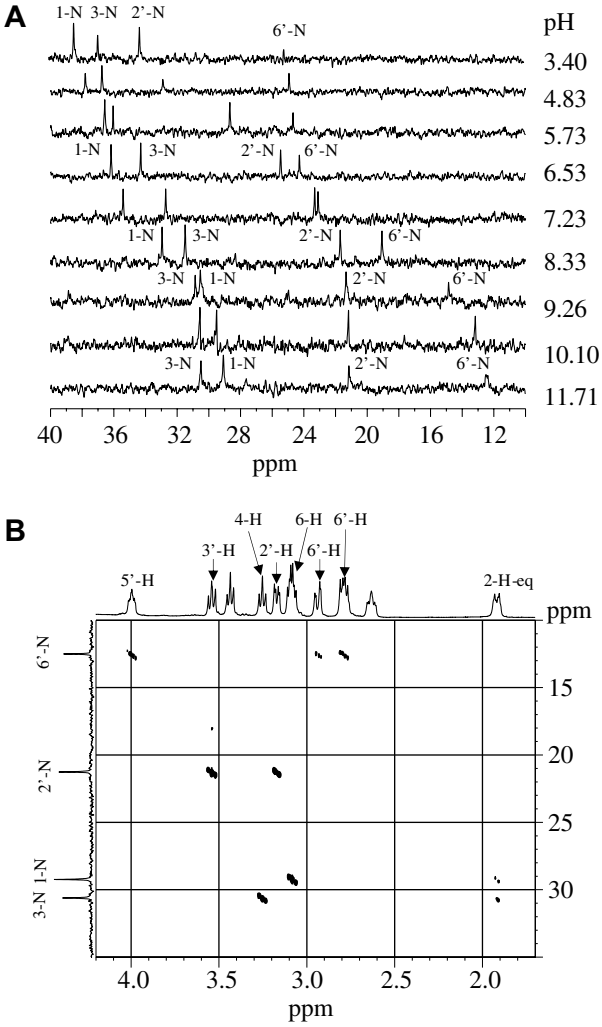


Figure 2. (A) Natural abundance 1 D ^{15}N NMR spectra of compound **1** as a function of pH. The assignments of each nitrogen atom at particular pH are indicated. (B) 2D ^1H–^{15}N HMBC spectrum of compound **1** at pH 13.05. The assignments of the relevant hydrogen and nitrogen atoms are indicated. For the detailed information about the acquisition of both 1D and 2D spectra see Section 4.

As a representative example, Figure 2A illustrates ^{15}N NMR complete titration spectra for the compound **1**. As can be clearly seen from these spectra, the complex dependence of the ^{15}N chemical shifts on pH is observed, especially at intermediate pH values, which made difficult to differentiate between 1-N from 3-N, and 2′-N from 6′-N. Similar problem was noted earlier by Botto and Coxon for neomycin B.[25] To solve this problem, the latter group used ^{15}N spin-relaxation experiments in the presence of Gd[2.2.1] cryptate as a spin-labeling reagent to provide complete assignment of all nitrogen atoms resonances. We used 2D ^1H–^{15}N HMBC technique as the method of choice to confirm the assignments of all ^{15}N resonances at these problematic pH values as well as at low- and high-pH extremes. As a representative example, Figure 2B illustrates the ^1H–^{15}N HMBC spectrum of compound **1** at pH 13. The observed correlations: 6′-N to 5′-H and two 6′-Hs; 2′-N to 3′-H and 2′-H; 1-N to 6-H and 2-Heq; 3-N to 4-H and 2-Heq; unequivocally secured the assignment of all nitrogen atoms of the compound **1** at this particular pH. Plotting of ^{15}N chemical shifts as a function of pH (Fig. 3) gave smooth sigmoid titration curves through the points for each individual nitrogen atoms, which further confirmed the validity of our assignments over the entire pH range.

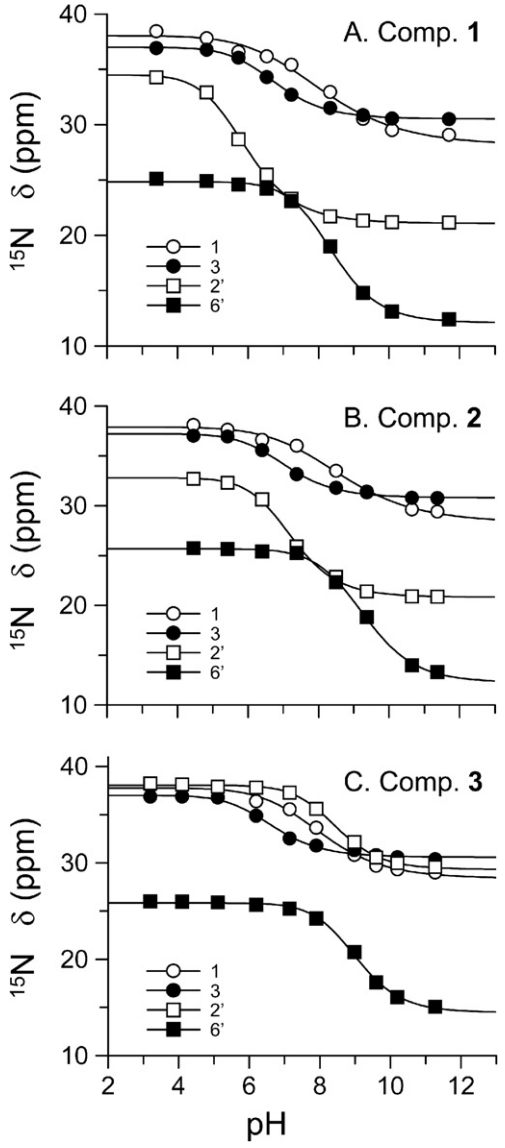


Figure 3. The plots of ^{15}N chemical shifts for individual amino groups versus pH of compound **1** (A), compound **2** (B), and compound **3** (C). The data points reflect the chemical shifts of the indicated amino groups. These chemical shifts are reported according to the IUPAC unified scale[39] using ^{1}H chemical shift referenced to DSS = 0.0 ppm. The continuous lines reflect the calculated fits of the data using Eq. 1.

The pK_a values of individual amine groups in each compound (**1**–**3**) were determined from nonlinear least-squares fits of the pH profiles using Eq. 1.[19]

$$\delta = \frac{(\delta_{NH_3} - \delta_{NH_2})(10^{pH\text{-}pK_a})}{1 + (10^{pH\text{-}pK_a})} + \delta_{NH_3} \quad (1)$$

In this relationship, δ_{NH_3} and δ_{NH_2} are the ^{15}N chemical shifts of the amino nitrogen in their NH_3 and NH_2 states, respectively. The resulted pK_a values are given in Table 1.

As expected, the major difference between the compounds **1**–**3** emerges with respect to the pK_a values of the 2′-amine group. The pK_a value of this amine in compound **3** (pK_a 8.4) is 1.2 pK_a units greater than the corresponding pK_a value in compound **2** (pK_a 7.2). The observed increase in the basicity of 2′-amine is likely due to the absence of the 3′-OH group in compound **3**, while this is present in **2**. The electron withdrawing inductive effect (-*I*) of the neighboring 3′-oxygen causes significant decrease in the electron density on the 2′-amine and subsequently lowers its basicity.

Table 1
^{15}N NMR-derived pK_a values and associated ^{15}N chemical shifts for the individual amine groups of compounds **1**–**3** at 25 °C[a]

Aminoglycoside	pK_a	δ_{NH3} (ppm)[b]	δ_{NH2} (ppm)[b]
*Compound **1***			
1-N	8.13 ± 0.30	38.4 ± 1.1	29.8 ± 0.9
3-N	6.79 ± 0.05	36.9 ± 0.2	30.5 ± 0.1
2′-N	6.02 ± 0.04	34.2 ± 0.3	21.1 ± 0.1
6′-N	8.30 ± 0.05	25.1 ± 0.3	12.4 ± 0.2
*Compound **2***			
1-N	8.64 ± 0.23	38.1 ± 1.0	29.4 ± 0.8
3-N	7.07 ± 0.08	37.0 ± 0.3	30.7 ± 0.1
2′-N	7.20 ± 0.05	32.6 ± 0.3	20.8 ± 0.2
6′-N	9.30 ± 0.07	25.7 ± 0.5	13.2 ± 0.5
*Compound **3***			
1-N	8.04 ± 0.13	37.9 ± 0.5	29.0 ± 0.4
3-N	6.67 ± 0.10	36.8 ± 0.3	30.3 ± 0.2
2′-N	8.40 ± 0.04	38.2 ± 0.2	29.5±0.1
6′-N	9.04 ± 0.06	26.0 ± 0.4	15.0 ± 0.3

[a] The pK_a values and chemical shifts (δ) of protonated (NH_3^+) and deprotonated (NH_2) amine groups were derived from fits of the pH-dependent ^{15}N chemical shift data shown in Figure 2 using Eq. 1. The indicated uncertainties in the values of pK_a and δ reflect the standard deviations of the experimental data from the fitted curves.

[b] ^{15}N chemical shifts were calibrated according to the IUPAC unified scale[39] using ^{1}H chemical shift referenced to internal DSS = 0.0 ppm.

The influence of this effect is further manifested in a significant downfield shift of the 2′-amine resonance in compound **3** than that in **2**, with $\Delta\delta NH_2$ being approximately 8 ppm and $\Delta\delta NH_3$ being about 5 ppm (see Fig. 3). Similar influence of the presence/absence of the 3′-OH group on the estimated pK_a values and ^{15}N chemical shifts of the 2′-amine of several natural aminoglycosides have previously reported.[19,29]

Interestingly, the pK_a value of the 2′-amine in **1** (pK_a 6.02) is even lower then that of the corresponding amine in **2** (pK_a 7.2), and is almost 2.4 pK_a units below the 2′-amine in **3** (pK_a 8.4). To our knowledge, a pK_a value of 6.02 for 2′-amine of the 3′,4′-methylidene derivative **1** is the lowest pK_a value ever estimated to this amine group in any natural or synthetic aminoglycoside reported to date. Two different factors, electronic and steric, are likely operating synergistically to make certain such a low basicity of this amine. In view of the electronic effect, previous measurements of pK_a values of amino sugar derivatives indicate that 3-amino-3-deoxyglycopyranosides are slightly more basic than their 2-OMe derivatives[30] due to a larger inductive effect (-*I*) of the 2-OMe group than that of the 2-OH group. In addition, the higher basicity of the 1-amine to that of the 3-amine of the 2-DOS ring (ring II) in neomycin B has ascribed by a larger inductive effect of the pyranosyloxy group substituent at position 4 than that of the hydroxyl group at position 6.[25] Based on these observations, the methylideneoxy group at 3′-position of **1** may be expected to have a larger inductive effect than the 3′-OH group in **2**. Consequently, 2′-amine in **2** is expected to be more basic than this amine in **1**, as indeed were observed (Table 1). In view of the steric effect, the lower basicity of the 2′-amine in **1** may be further ascribed due to its proximity to the 3′,4′-methylidene five-member ring that hinders proper solvation of the corresponding ammonium anion.[24] This steric effect is likely operational for the 6′-amine too whose pK_a value in compound **1** was estimated to be 1 pK_a unit lower (pK_a 8.3) than that in compound **2** (pK_a 9.3).

2.3. Acute lethal toxicity values (LD_{50}) of 1–3 and structure–toxicity relationship

Having established the impact of the 3′,4′-methylidene protection on the basicity of the 2′-amine in compound **1**, we then attempted to test if the basicity of 2′-amine can correlate with

Table 2
Acute toxicity data in mice, steady-state kinetic parameters for the interaction with APH(3′)-IIb, and antibacterial activity of compounds **1**–**3**.

Compound	LD_{50} (mg/kg)[a]	Kinetic data[b]		Antibacterial activity (μg/mL)[c]
		Substrate activity	K_i (μM)	
1	303 ± 10	—	642 ± 53	384<
2	161 ± 5	K_m = 10. 3 μM k_{cat} = 1.7 s^{-1}	—	96
3	103 ± 8	–	650 ± 61	192

[a] LD_{50} refers to a median lethal dose. The indicated uncertainties in the values of LD_{50} reflect the standard deviations of the experimental data from the fitted dosage-mortality curves using Grafit 5 software.[41]

[b] The substrate (K_m and k_{cat} values) and inhibitory (K_i values) activities were determined with the recombinant APH(3′)-IIb enzyme as outlined in Section 4.

[c] The MIC values were determined against *Escherichia coli* (R477-100) strain by using the double-microdilution method, with 384 μg/mL as starting concentration of each tested compound. All the experiments were performed in duplicates and analogous results were obtained in three different experiments.

acute toxicity of the aminoglycoside. For this purpose the comparative acute intravenous toxicity values of **1**–**3** were determined in mice under the same experimental conditions and the observed data are shown in Table 2. These data suggest a relationship between the 2′-amine basicity and the estimated acute LD_{50} values; the increase in the basicity of 2′-amine is associated with the toxicity increase of the aminoglycoside. With a pK_a value of 6.02 for 2′-amine, compound **1** is considerably less toxic (LD_{50} 303 mg/kg) than neamine (**2**, pK_a 7.2, LD_{50} 160 mg/kg) and has about three times lower toxicity to that of the corresponding 3′,4′-dideoxy derivative (**3**, pK_a 8.4, LD_{50} 103 mg/kg). This result is consistent with the earlier reported structure–toxicity relationship of various fluorinated analogs of kanamycin B.[17,18] Furthermore, since the acute toxicity data of aminoglycosides were found to be well correlated to their neuromuscular blocking effects[11,12], we suggest that the reduction in the pK_a of 2′-amine is linked to the acute toxicity attenuation of aminoglycoside due to the subsequent reduced effects on neuromuscular blocking. This suggestion is supported by earlier observations which demonstrated that total charge of an aminoglycoside correlates with the blocking potency[31,32], and that co-administration of Ca^{2+} antagonizes both acute toxicity and neuromuscular blocking effects induced by aminoglycosides.[12,13]

2.4. Interaction with the resistance enzyme APH(3′)-IIb and antibacterial activity of 1–3

In order to verify the contribution of the 3′,4′-methylidene group in protection of **1** against action of resistance enzymes, compound **1** along with the compounds **2** and **3** were examined for their substrate/inhibitory activities against APH(3′)-IIb[33] enzyme (Table 2). We found that, as per design, while the compound **2** functioned as a substrate (K_m = 10.3 ± 0.5 μM and k_{cat} = 1.7 ± 0.1 s^{-1}), the compounds **1** and **3** exhibited no substrate activity and demonstrated only poor inhibitory activities with the estimated apparent K_i values of 642 ± 52 and 650 ± 62 μM respectively.

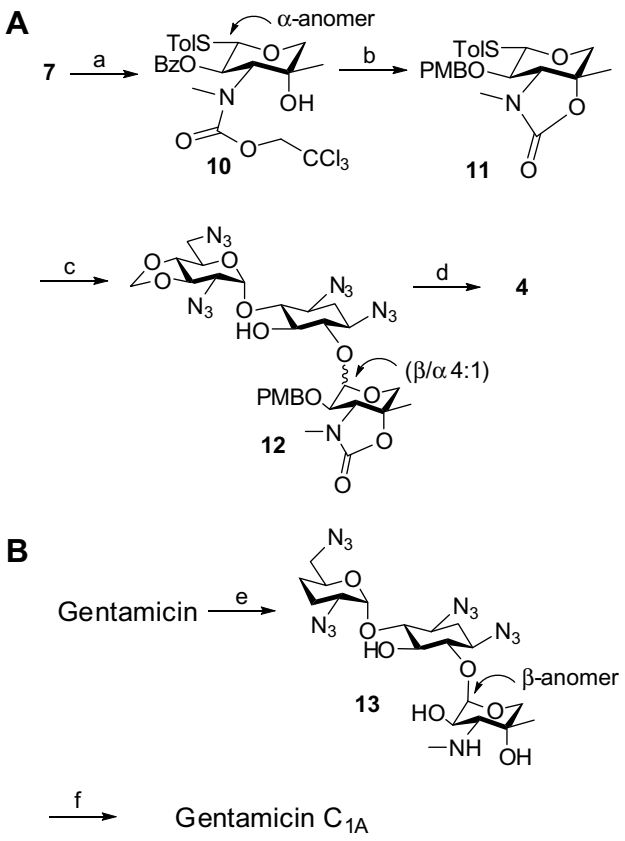
From the earlier studies[21], it is well known that because of its smaller size and the global charge in comparison to those of known aminoglycoside antibiotics, compound **2** exhibits very poor antibacterial activity. Since compound **1** display significantly lower 2′-amine pK_a than that of the compounds **2** and **3**, its binding affinity to the ribosomal RNA and the subsequent antibacterial activity was expected to be even poorer than that of the compounds **2** and **3**. Indeed, from the estimated minimal inhibitory concentration (MIC) values against *Escherichia coli* R477-100 (Table 2) it can be seen that while compound **2** (MIC = 96 μg/mL) exhibited slightly better antibacterial activity than that of compound **3** (MIC = 192 μg/mL), compound **1** (MIC = >384 μg/mL) was lack of antibacterial potency (with the concentration of 384 mg/mL as the maximum starting concentration of each compound tested). Thus, even thogh compound **1** exhibited the lowest acute toxicty in comparison to **2** and **3**, its lack of antibacterial activty was highly concerned. Therefore, we asked the following question: despite of its lack of significant antibacterial potency, whether or not compound **1** can serve as a potential scaffold for the construction of new generation of aminoglycosides with diminished toxicity?

To answer this question, initially we focused our design strategy on the clinically used antibiotic gentamicin. This drug is used as a mixture of several related structures (Fig. 1) and shows potent antibacterial activity against various pathogens. Unfortunately, however, the use of gentamicin in clinic is largely limited because of its high toxicity (LD_{50} values for gentamicin C_1, gentamicin C_2 and gentamicin C_{1A} were reported as 88, 70, and 70 mg/kg, respectively).[16] Our strategy was to mimic the structural features of gentamicin in order to preserve its antibacterial activity and at the same time to reduce its toxicity. For this purpose, since the commercial gentamicin is a mixture of gentamicin C_1, gentamicin C_2 and gentamicin C_{1A} (Fig. 1), initially we constructed the new pseudo-trisaccharide **4** (NB45, Fig. 1) which is a close structural analog of gentamicin C_{1A}. In the following section, the synthesis and comparative antibacterial activities of NB45 and gentamicin C_{1A} are outlined.

2.5. Synthesis and comparative antibacterial activity of NB45 and gentamicin C_{1A}

NB45 was assembled by using compound **6** as an acceptor and the garosamine fragment **7** (Scheme 1) as a precursor of the donor (Scheme 2A). First, the anomeric mixture of **7** was converted to the thioglycoside **10** in three steps; selective protection of the secondary amine with trichloroethyloxycarbonyl (Troc) group was followed by selective benzoylation of the secondary alcohol and by replacement of the anomeric methoxyde with α-thioglycoside. Note that the garosamine ring (ring III) in gentamicin is an unusual L-sugar with the β-L-arabino configuration [3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinose] (Fig. 1 and Scheme 2). Therefore, in order to guarantee the formation of the desired β-L-glycosidic linkage between the garosamine ring (ring III) and the 2-DOS ring (ring II) in NB45, the benzoate protection in **10** was replaced with the *p*-methoxybenzyl ether protection. Treatment of **10** with methylamine (33% $MeNH_2$ in EtOH) resulted in simultaneous removal of the benzoate and the formation of the oxazolidinone ring as confirmed by subsequent structural analysis of the product. Treatment of this product with NaH and *p*-methoxybenzylcloride then furnished the oxazolidinone donor **11**. NIS-AgOTf-promoted glycosidation of acceptor **6** with the donor **11** gave the corresponding pseudo-trisaccharide **12** as a mixture of anomers (β/α 4:1) in 90% isolated yield. Oxidative removal of the *p*-methoxybenzyl ether protection by treatment with CAN was followed by treatment with strong base (aqueous NaOH in EtOH) to open the oxazolidinone ring. Finally, the Staudinger reaction furnished the desired product NB45 as a mixture of anomers (β/α 4:1).

Our attempts to separate the desired gentamicin C_{1A} from the mixture of commercial gentamicin, by using direct column chromatography procedures, were unsuccessful. We solved this problem by subjecting the commercial mixture to two-steps procedure (Scheme 2B). Treatment of gentamicin mixture with TfN_3 converted all the primary amines to the corresponding azides, from which the desired component **13** could be easily separated by flash chromatography. The Staudinger reaction on **13** furnished the desired product gentamicin C_{1A} with excellent purity and isolated yield.



Scheme 2. Reagents and conditions: (a) i—2,2,2-Trichloroethyl chloroformate, $CHCl_3/H_2O$ 5:1, $NaHCO_3$, 93%; ii—BzCl, pyridine, 4-DMAP, 80%; iii—4-methyl benzenethiol, $BF_3{\cdot}Et_2O$, $C_2H_4Cl_2$, 40 °C, 53%; (b) i—33% $MeNH_2$ (33% soln in EtOH), 24 h, 90%; ii—PMBCl, NaH, DMF,nBu_4NBr, HMPA, 82%; (c) i—**6**, NIS, AgOTf, Et_2O/CH_2Cl_2:1, −10 °C, 24 h, 90% (β/α 4:1); (d) i—CAN, CH_3CN, 85%; ii—EtOH/ aqueous NaOH (2 M) 1:1, reflux, 10 h; iii—PMe_3 (1 M in THF), THF/NaOH (0.1 M) 3:1, 75% [for 2 steps (β/α 4:1)]; (e) i—TfN_3, Et_3N, $CuSO_4$, in CH_2Cl_2/MeOH/H_2O 3:10:3, 55%; (ii) flash column chromatography; (f) PMe_3 (1 M in THF), THF/NaOH (0.1 M) 3:1, 93%. 4-DMAP, 4-dimethyl aminopyridine; HMPA, hexamethylphosphoramide, PMBCl, *p*-methoxybenzyl chloride; NIS, *N*-iodosuccinimide, CAN, cerium ammonium nitrate.

The comparative antibacterial activities of NB45 and gentamicin C_{1A} were determined by measuring the minimal inhibitory concentrations (MICs) against both Gram-negative and Gram-positive bacteria, including resistant and pathogenic strains, using the microdilution assay (Table 3). Resistant strains included *E. coli* XL1(pET9d), and *Pseudomonas aeruginosa*. *E. coli* XL1(pET9d) is an antibiotic-sensitive laboratory strain that harbors plasmid pET9d with the cloned aminoglycoside kinase APH(3′)-Ia (Novagen Inc.). *P. aeruginosa* strains possess several different resistance mechanisms to aminoglycosides, including the chromosomal encoded APH(3′)-IIb enzyme[33,34], and the multidrug efflux system MexXY.[35] *P. aeruginosa* is a pathogenic bacterium which is a major cause of mortality among cystic fibrosis patients.[36] In recent years, increased numbers of *P. aeruginosa* clinical isolates were found to be resistant to several antibiotics which prevent its effective treatment. Therefore, in the current study, in addition to the standard *P. aeruginosa* ATCC 27853 strain, we also included clinical isolates of this bacterium, *P. aeruginosa* (584/5) and *P. aeruginosa* (590) (obtained from Rambam Medical Center Hospital, Haifa, Israel).

As seen in Table 3, in general, the observed spectrum of antibacterial activities of NB45 is very similar to that of gentamicin C_{1A}. It is of particular importance to note that the two compounds show good antibacterial activities against the APH(3′) expressing strains (*E. coli* XL1(pET9d), *and strains of P. aeruginosa*) indicating that APH(3′) enzymes are incapable of modifying these compounds because the lack of free 3′-OH group in these structures. We have recently cloned the chromosomal gene *aph(3′)-IIb* that encodes an aminoglycoside 3′-phosphotransferase in *P. aeruginosa*, and overexpressed it in *E. coli*.[33] In the latter study we also have demonstrated that the aminoglycosides tobramicin and gentamicin which lack 3′-OH group, were not substrates to the purified APH(3′)-IIb and that both *P. aeruginosa* and *E. coli* strains harboring APH(3′)-IIb were sensitive to these antibiotics. To further verify that the observed sensitivity of the *P. aeruginosa* strains to NB45 is due to the lack of the free 3′-OH group in this compound, we performed kinetic analysis of NB45 with the purified APH(3′)-IIb enzyme. We found that NB45 has no substrate activity and exhibited only poor inhibitory activity with the estimated apparent K_i value of 420 ± 38 μM.

In general, antibacterial activity (MIC values) of aminoglycosides correlates with their in vitro prokaryotic antitranslational potency (IC_{50} values).[21] Since both NB45 and gentamicin C_{1A} exhibited similar antibicaterial activity against a series of both Gram-negative and Gram-positive bacteria, it was therefore expected that they would also exhibit similar prokaryotic antitranslational potency. To verify this expectation, we used an in vitro luciferase assay[21] and tested NB45 and gentamicin C_{1A} for the inhibition of protein translation in prokaryotic system, and the observed data are shown in Fig. 4 and Table 3. The measured half-maximal inhibitory concentration (IC_{50}) values indicate that NB45 (IC_{50} = 38 nM) is only slightly better inhibitor than gentamicin C_{1A} (IC_{50} = 59 nM). These data are consistent with the antibacterial data obtained for NB45 and gentamicin C_{1A}. Taken together, the observed data suggest that the 3′,4′-methylidene protection in NB45 not just protects it from the deactivation by APH(3′) enzymes, but it also does not perturb its interaction with the ribosomal target or its penetration through the bacterial membrane that could influence its antibacterial activity.

Encouraged by the impacts of the 3′,4′-methylidene scaffold in NB45 on its antibacterial activity and interaction with the APH(3′) resistance enzymes, we were interested whether this scaffold

Table 3
Translation inhibition, antibacterial activity and acute toxicity data of the NB45 and gentamicin C_{1A}[a]

Aminoglycoside	Translation inhibition IC_{50} (μM)[b]	Antibacterial activity MIC (μg/mL)[c]								Acute toxicity LD_{50} (mg/kg)[d]
		Ec-1	*Ec-2*	*Ec-3*	*Se*	*Bs*	*Pa*	*Pa-1*	*Pa-2*	
NB45	0.038 ± 0.003	48	48	12	24	12	24	24	48	226 ± 1
Gentamicin C_{1A}	0.059 ± 0.01	48	96	12	6	6	12	48	384	70[16]

[a] In all biological tests, both NB45 and gentamicin C_{1A} were in their sulfate salt forms.
[b] The half-maximal inhibitory concentration (IC_{50}) values were obtained from concentration response curves (Fig. 3) using Grafit 5 software.[41]
[c] The MIC values were determined by using the double-microdilution method, with 384 μg/mL as starting concentration of the tested compound. All the experiments were performed in duplicates and analogous results were obtained in three different experiments. The bacterial strains used: *Ec*-1, *Escherichia coli* (R477-100); *Ec*-2, *Escherichia coli* (ATCC 25922); *Ec*-3, *E. coli* XL1 blue/ pET9d expressing the APH(3′)-Ia resistance enzyme; *Se*, *Staphylococcus epidermis* (ATCC 12228); *Bs*, *Bacilus subtilis*; *Pa*, *Pseudomonas aeruginosa* ATCC 27853; clinical isolates of *P. aeruginosa*: *Pa*-1, *P. aeruginosa* (584/5); *Pa*-2, *P. aeruginosa* (590).
[d] LD_{50} refers to a median lethal dose. The indicated uncertainty in the LD_{50} value reflects the standard deviation of the experimental data from the fitted dosage-mortality curve using Grafit 5 software.[41]

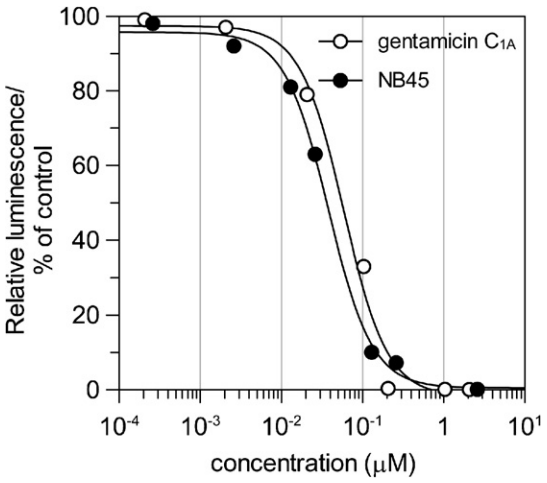


Figure 4. Semilogarithmic plots of in vitro translation inhibition in prokaryotic system of NB45 (●) and gentamicin C_{1A} (○). The translation inhibition was measured in *E. coli* cellular extract and quantified in coupled transcription/translation assays by using active luciferase detection. The percentages of active luciferase are plotted as functions of drug concentration. Each data point represents the average of 2–3 independent experimental results.

could also provoke the low toxicity of NB45. For this purpose, the acute intravenous toxicity value of NB45 was determined and the observed value was compared to that of the gentamicin C_{1A}[16] which was determined earlier under the same experimental conditions (Table 3). The data in Table 3 show that NB45 is about three times less toxic than gentamicin C_{1A} (LD_{50} values of 226 and 70 mg/kg, respectively). Surprisingly, the similar three-fold difference in LD_{50} values was observed between their parent pseudo-disaccharide scaffolds **1** and **3** (LD_{50} values of 303 and 103 mg/kg, respectively; Table 2), suggesting that the presence of 3′,4′-methylidene moiety should be responsible in acute toxicity attenuation of NB45 relative to that of the gentamicin C_{1A}. The observed somewhat higher toxicity of NB45 (LD_{50} 226) to that of its parent pseudo-disaccharide scaffold **1** (LD_{50} 303) can be explained by the difference in the number of amine groups present in these molecules (five in NB45 versus four in compound **1**) and in their size (NB45 consists of three rings versus two rings in compound **1**). Indeed, several earlier studies demonstrated that aminoglycosides with different size and charge interfere differently the steps of calcium-mediated acetylcholine release at the level of presynaptic structures, and that this interaction is the main cause of the neuromuscular blockage induced by aminoglycosides and their subsequent toxicity.[9,11,37]

3. Summary and conclusions

In the current study, the correlation between acute toxicity and the 2′-NH_2 basicity of some aminoglycosides was investigated. For this purpose, we have designed a new pseudo-disaccharide **1** with a 3′,4′-methylidene protection as a potential ′defense′ against toxicity, and its properties were evaluated in comparison to the related pseudo-disaccharides, compounds **2** and **3**. By determining the pK_a values of the individual amino groups in each structure and the acute toxicities of each structure we have demonstrated a relationship between the basicity of the 2′-amine group and the estimated LD_{50} values in mice: the increase in the basicity of the 2′-amino functionality is associated with the toxicity increase of an aminoglycoside with the compound **1** exhibiting the lowest pK_a value for the 2′-amine and being substantially least toxic than the other two derivatives (compounds **2** and **3**).

The pseudo-disaccharide **1** was then used as a scaffold on which the new pseudo-trisaccharide NB45 was constructed, and its properties were compared to the structurally related gentamicin C_{1A}. NB45 exhibited similar antibacterial activity to that of gentamicin C_{1A} against various bacterial strains, including pathogenic and resistant strains, while at the same time retained significantly low toxicity to that of gentamicin C_{1A}. An in vitro prokaryotic antitranslational activity, along with the substrate activity tests with the purified APH(3′)-IIb resistance enzyme, revealed that both NB45 and gentamicn C_{1A} inhibit protein translation process with the similar extent and both lack substrate activity for the APH(3′)-IIb enzyme with NB45 exhibiting poor inhibitory activity. Based on these data it is suggested that the presence of 3′,4′-methylidene protection in compound NB45 provides a ′defense′ against the acute toxicity as well as against the action of APH(3′) resistance enzymes while at the same time it retains significant antibacterial potency.

4. Experimental

4.1. General techniques

^{1}H NMR, ^{13}C NMR, DEPT, COSY, 1D TOCSY, HMQC, HMBC, spectra were recorded on a Bruker Avance™ 500 spectrometer. Chemical shifts reported (in ppm) are relative to internal Me_4Si ($\delta = 0.0$) with $CDCl_3$ as the solvent, and to HOD ($\delta = 4.63$) with D_2O as the solvent. Mass spectral analyses were performed on a Bruker Daltonix Apex 3 mass spectrometer under electron spray ionization (ESI), TSQ-70B mass spectrometer (Finnigan Mat) or under MALDI-TOF on a α-cyano-4-hydroxycinnamic acid matrix on a MALDI Micromass spectrometer. Reactions were monitored by TLC on Silica gel Gel 60 F_{254} (0.25 mm, Merck), and spots were visualized by charring with a yellow solution containing $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (120gr) and $(NH_4)_2Ce(NO_3)_6$ (5gr) in 10% H_2SO_4 (800 mL). Flash column chromatography was performed on Silica gel Gel 60 (70–230 mesh). All reactions were carried out under an argon atmosphere with anhydrous solvents, unless otherwise noted. All chemicals, unless otherwise stated, were obtained from commercial sources. DSS [3-(trimethylsilyl)-1-propanesulfonic acid], neomycin B, and gentamicin were obtained from Sigma. Compound **2** (neamine) was prepared in multigram quantities from neomycin B as previously reported.[23]

4.2. Synthetic part

4.2.1. Compound 5

Neamine (compound **2**) was converted to its perazido derivative, 1,3,2′,6′-tetraazido neamine by using earlier reported[38] azido-transfer reaction. The isolated product (90% isolated yield), after flash chromatography (EtOAc/hexane), has exactly same spectroscopic properties as it was originally reported.[38] The 1,3,2′,6′-tetraazido neamine (2.0 g, 4.70 mmol) was dissolved in DMF (70 mL) and to the resulted mixture were added cyclohaxanone dimethyl ketal (8.0 mL, 51.60 mmol) and catalytic amount of camphor sulfonic acid (CSA). The reaction was heated to 60 °C under argon and the reaction progress was monitored by TLC (EtOAc/hexane 1:1). After 6 h, the mixture was diluted with EtOAc and washed with $NaHCO_3$ and brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (EtOAc/hexane) to give compound **5** (1.5 g, 65%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.61 (bs, 10H, cyclohexane protons), ring **I**: 3.25 (dd, 1H, $J_1 = 3.5$, $J_2 = 10.5$ Hz, H-2′), 3.50–3.64 (m, 3H, H-4′, H-6′, H-6′), 3.94 (t, 1H, $J = 9.5$ Hz, H-3′), 3.99 (m, 1H, H-5′), 5.55 (d, 1H, $J = 3.5$ Hz, H-1′); ring **II**: 1.46 (ddd, 1H, $J_1 = J_2 = J_3 = 12.5$ Hz, H-2ax), 2.31 (bdt, 1H, H-2eq), 3.42 (t, 1H, $J = 9.5$ Hz, H-6), 3.50–3.64 (m, 3H, H-1, H-3, H-4), 3.81 (t, 1H, $J = 9.0$ Hz, H-5). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 23.7 (cyclohexane), 23.8 (cyclohexane), 24.8 (cyclohexane), 33.8 (C-2), 36.0 (cyclohexane), 36.2 (cyclohexane),

51.2 (C-6′), 57.2, 57.3, 60.8, 62.6, 71.2, 71.3, 71.4, 79.3, 79.3, 96.2 (C-1′), 113.8 (cyclohexane). ESI *m/z* 529.2 (M+Na^+, $C_{18}N_{12}O_6H_{26}$ requires 529.2).

4.2.2. Compound 6

To a stirred solution of compound **5** (2.0 g, 3.95 mmol) in THF (80 mL) was added an aqueous solution of 10M NaOH (70 mL) at room temperature. After being stirred for 30 min, nBu_4NBr (3.8 g, 11.8 mmol) and CH_2Br_2 (3.0 mL, 7.9 mmol) were added and the reaction was heated to 60 °C. The reaction progress was monitored by TLC (EtOAc/hexane 1:3). After 40 h, the mixture was diluted with CH_2Cl_2 and washed with $NaHCO_3$ and brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (EtOAc/hexane) to give the corresponding 3′,4′-methylidene product (1.8 g, 85%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.66–1.70 (m, 10H, cyclohexane), ring **I**: 3.19 (t, 1H, J_1 = 9.5 Hz, H-4′), 3.14–3.55 (m, 2H, H-2′, H-6′), 3.60 (dd, 1H, J_1 = 2.5, J_2 = 13.5 Hz, H-6′), 3.86 (t, 1H, J = 9.0 Hz, H-3′), 4.29–4.32 (m, 1H, H-5′), 5.66 (d, 1H, J = 3.5 Hz, H-1′); ring **II**: 1.52 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.33 (dt, 1H, J_1 = 5, J_2 = 13.5 Hz, H-2eq), 3.14–3.55 (m, 3H, H-6, H-1, H-3), 3.66 (m, 1H, H-4), 3.70 (dd, 1H, J_1 = 9.0, J_2 = 11.0 Hz, H-5), 5.15 (s, 1H, CH_2), 5.18 (s, 1H, CH_2'). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 25.5 (C-2′), 25.5 (cyclohexane), 26.7 (cyclohexane), 35.6 (cyclohexane), 37.8 (cyclohexane), 38.0 (cyclohexane), 53.5 (C-6′), 59.0, 62.6, 62.9, 73.6, 78.8, 79.0, 79.1, 81.1, 81.1, 98.4 (CH_2), 98.6 (C-1′) 115.7 (cyclohexane). ESI *m/z* 541.2 (M+Na^+, $C_{19}N_{12}O_6H_{26}$ requires 541.2).

The product from the previous step (600 mg, 1.15 mmol), was dissolved in 1,4-dioxane (7 mL) and water (7 mL). To the resulted mixture was added glacial acetic acid (15 mL), heated at reflux and the reaction progress was monitored by TLC (EtOAc/hexane 7:13). After about 14 h, the mixture was diluted with EtOAc and washed with $NaHCO_3$ and brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (EtOAc/hexane) to yield **6** (450 mg, 85%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: ring **I**: 3.20 (t, 1H, J = 9.0 Hz, H-4′), 3.49 (dd, 1H, J_1 = 5.5, J_2 = 13.5 Hz, H-6′), 3.61 (dd, 1H, J_1 = 2.5, J_2 = 13.5 Hz, H -6′), 3.69 (dd, 1H, J_1 = 4.0, J_2 = 11.0 Hz, H-2′), 3.84 (dd, 1H, J_1 = 9.0, J_2 = 11.0 Hz, H-3′), 4.36–4.40 (m, 1H, H-5′), 5.47 (d, 1H, J = 4.0 Hz, H-1′); ring **II**: 1.53 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.34 (bdt, 1H, H-2eq), 3.35–3.42 (m, 4H, H-4, H-1, H-3, H-6), 3.53 (t, 1H, J_1 = 9.0, J_2 = 13.5 Hz, H-5), 5.16 (s, 1H, CH_2), 5.17 (s, 1H, CH_2'), ^{13}C NMR (125 MHz, $CDCl_3$) δ: 33.8 (C-2), 53.5 (C-6′), 60.7, 61.6, 64.3, 73.6, 78.1, 78.6, 78.8, 79.1, 84.1, 98.5 (CH_2), 100.7 (C-1′). ESI *m/z* 461.1 (M+Na^+, $C_{13}N_{12}O_6H_{18}$ requires 461.1).

4.2.3. Compound 1

To a stirred solution of compound **6** (200 mg, 0.45 mmol) in THF (3.0 mL) was added aqueous solution of NaOH 0.1 M (1.5 mL), followed by addition of PMe_3 (1 M solution in THF, 3.6 mL, 3.60 mmol) at room temperature. The reaction progress was monitored by TLC [CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH) 10:15:6:15], which indicated completion after 2 h. The reaction mixture was directly poured on a short column of silica gel and purified by flash chromatography. The column was washed sequentially with three column volumes of THF, three column volumes of dichloromethane, and three column volumes of MeOH. The product was then eluted with the mixture of 20% $MeNH_2$ solution (33% solution in EtOH) in 80% MeOH. Fractions containing the product were combined and evaporated under vacuum. The residue was re-dissolved in small volume of water and evaporated again (2–3 repeats) to afford the free amine form of **1** (150 mg, 99%). This product was dissolved in water, the pH was adjusted to 6.8 with H_2SO_4 (0.1 N) and lyophilized to afford the sulfate salt of **1** as a white foamy solid. For spectral analysis, the pH of the final product was adjusted to about 3.0 with H_2SO_4 (0.1 N), lyophilized and the resulted solid was re-dissolved in D_2O. ^{1}H NMR (500 MHz, D_2O) δ: ring **I** 3.07–3.15 (m, 2H, H-4′, H-6′), 3.33 (t, 1H, J = 3.0 Hz, H-2′), 3.30–3.31 (m, 1H, H-6′), 3.66 (dd, 1H, J_1 = 9.0, J_2 = 2.0 Hz, H-3′), 4.24 (m, 1H, H-5′), 5.64 (d, 1H, J = 4.0 Hz, H-1′); ring **II** 1.62 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.22 (dt, 1H, J_1 = 12.5, J_2 = 4.0 Hz, H-2eq), 3.07–3.15 (m, 2H, H-1, H-3), 3.43 (t, 1H, J = 6.0 Hz, H-6), 3.53 (t, 1H, J = 9.0 Hz, H-5), 3.59 (t, 1H, J = 9.5 Hz, H-4), 5.02 (s, 1H, CH_2), 5.08 (s, 1H, CH_2')^{13}C NMR (125 MHz, $CDCl_3$) δ: 32.0 (C-2), 42.4 (C-6′), 50.2, 52.0, 55.5 (C-2′), 70.8 (C-5′), 74.5 (C-4), 77.0, 77.1, 78.9 (C-3′), 82.3 (C-6), 98.1 (CH_2), 100.3 (C-1′). ESI *m/z* 357.2 (M+Na^+, $C_{13}N_4O_6H_{26}$ requires 357.2).

4.2.4. Compound 9

To a stirred solution of the commercial gentamicin (5.0 g, 0.01 mol) in MeOH (10 mL) was added AcCl (5 mL) by a dropwise addition at 0 °C. The mixture was heated at reflux for 6 h, cooled and evaporated to dryness. The residue was purified by flash chromatography [CH_2Cl_2/$MeNH_2$ solution (33% solution in EtOH) 15:1]; first eluted the garosamine **7** (2.6 g, 90%) having the R_f value of 0.93, followed by the mixture of **8a–c** (2.5 g, 86%) with R_f value in the range of 0.20–0.46 [CH_2Cl_2/$MeNH_2$ solution (33% solution in EtOH) 3:2]. Data for compound **7**: ^{1}H NMR (500 MHz, $CDCl_3$) α *anomer*: δ 1.06 (s, 3H, CH_3), 2.38 (s, 3H, N-Me), 2.20 (d, J = 10 Hz, 1H, H-3), (s, 3H, OMe), 3.28 (dd, J_1 = 7.5, J_2 = 10.5 Hz, 1H, H-2), (3.40 (d, J = 12 Hz, 1H, H-5), 3.50 (d, J = 12 Hz, 1H, H-5′), 4.16 (d, J = 9.5 Hz, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 20.3 (CH_3), 36.1(N-Me), 54.7 (OMe), 62.6 (C-3), 66.3 (C-4), 66.4 (C-5), 68.3 (C-2), 104.2 (C-1). MALDI-TOFMS calculated *m/z* 192.1 (M+H+, $C_8H_{17}O_4N$ requires 192.1). β *anomer*: δ 1.04 (s,3H, CH_3), 2.36 (s, 3H, N-Me), 2.38 (d, J = 11 Hz, 1H, H-3), 3.21 (s, 3H, OMe), 3.30 (d, J = 12 Hz, 1H, H-5), 3.61 (dd, J_1 = 3, J_2 = 11 Hz, 1H, H-2), 3.65 (d, J = 12 Hz, 1H, H-5′), 4.63 (d, J = 4 Hz, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.7 (CH_3), 36.1(N-Me), 54.7 (OMe), 62.6 (C-3), 66.3 (C-4), 66.4 (C-5), 68.3 (C-2), 98.9 (C-1). MALDI-TOFMS calculated *m/z* 191.1 (M+H+, $C_8H_{17}O_4N$ requires 192.1).

The purified mixture **8a–c** (2.5 g, 8.33 mmol) was dissolved in H_2O (80 mL) to which $CuSO_4$ (0.20 mg, 1.31 mmol), triethylamine (30 mL, 215 mmol), triflic azide solution (0.5 M in CH_2Cl_2 150 mL, 75 mmol) and MeOH (200 mL) were added. The resulted mixture was stirred for 18 h at room temperature and concentrated under reduced pressure. The residue was purified by flash chromatography (EtOAc/hexane) to yield **9** (750 mg, 1.52 mmol) 93% (calculated from the 32% gentamicin C_{1A} present in the commercial gentamicin according to the manufacturer's protocol). ^{1}H NMR (500 MHz, $CDCl_3$) δ: ring **I**: 1.58 (dd, 1H, J_1 = 4.0, J_2 = 12.0 Hz, H-4′), 1.76 (dd, 1H, J_1 = 3.0, J_2 = 13.0 Hz, H-4′), 1.97–2.05 (m, 2H, H-3′, H-3′), 3.26–3.27 (m, 2H, H-6′, H-6′), 3.39–3.41 (m, 1H, H-2′), 4.18–4.21 (m, 1H, H-5′), 5.25 (d, 1H, J = 3.0 Hz, H-1′); ring **2**: 1.22 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 1.48 (dt, 1H, J_1 = 12.5, J_2 = 4.0 Hz, H-2eq), 3.3–3.45 (m, 4H, H-1, H-3, H-5, H-6), 3.51 (t, 1H, J = 9.0 Hz, H-4). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.9 (C-2), 27.0 (C-3′), 32.1 (C-4′), 54.5 (C-6′), 58.6, 59.2, 59.7, 67.9 (C-5′), 75.4, 76.3, 81.6, 98.3 (C-1′). MALDI-TOFMS *m/z* 417.2 (M+Na^+ $C_{12}H_{18}0_4N_{12}$ requires 417.2).

4.2.5. Compound 3

Compound **9** was subjected to a Staudinger reaction as described above for compound **1** [compound **9** (600 mg, 1.52 mmol), THF (7 mL), NaOH (0.1 M, 3.0 mL), PMe_3 (1 M solution in THF, 12 mL, 12.0 mmol)] to yield **3** as a free amine form (420 mg, 95%). The resulted free amine was dissolved in water, the pH was adjusted to 6.9 with H_2SO_4 (0.1 N) and lyophilized to give the sulfate salt as a white foamy solid. For the spectral analysis, the pH of the final product was adjusted to about 3.0 with H_2SO_4 (0.1 N), lyophilized and the resulted solid was re-dissolved in D_2O. ^{1}H NMR (500 MHz, D_2O) δ: ring **I**: 1.25 (dd, 1H, J_1 = 3.5, J_2 = 12.0 Hz,

H-4′), 1.61–1.64 (m, 1H, H-4′), 1.51 (dd, 1H, J_1 = 3.5, J_2 = 12.5 Hz, H-3′), 1.55–1.58 (m, 1H, H-3′), 2.50-2.55 (m, 2H, H-6′, H-6′), 2.71–2.73 (m, 1H, H-2′), 3.70-3.74 (m, 1H, H-5′), 4.99 (d, 1H, J = 3.5 Hz, H-1′); ring **2**: 1.04 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 1.82 (dt, 1H, J_1 = 12.5, J_2 = 4.0 Hz, H-2eq), 2.53–3.57 (m, 1H, H-1), 2.68–2.70 (m, 1H, H-3), 2.98 (t, 1H, J = 9.0 Hz, H-6), 3.12 (t, 1H, J = 9.0 Hz, H-4), 3.31 (t, 1H, J = 9.0 Hz, H-5). ^{13}C NMR (125 MHz, D_2O) δ: 27.4 (C-3′), 29 .0 (C-4′), 37.3 (C-2), 47.2 (C-6′), 51.2, 51.4, 52.0 (C-1), 71.7 (C-5′), 77.8 (C-5), 79.3 (C-6), 89.3 (C-4), 103.2 (C-1′). MALDI-TOFMS *m/z* 329.2 (M+K$^+$ $C_{12}H_{26}0_4N_4$ requires 329.2).

4.2.6. Compound 10

The garosamine fragment **7** from the previous steps (100 mg, 0.52 mmol) was dissolved in $CHCl_3$ (2.5 mL) and H_2O (0.5 mL) 2,2,2-trichloroethyl chloroformate (0.09 mL, 0.68 mmol) and $NaHCO_3$ (80 mg, 1.04 mmol) were added. The reaction was monitored by TLC (EtOAc/hexane 7:1). After 30 min, the mixture was diluted with EtOAc and washed with brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (EtOAc/hexane) to give the selective N-protected product, a mixture of α and β anomers with Troc protection (180 mg, 93%). ^{1}H NMR (500 MHz, $CDCl_3$) α *anomer*: δ 1.17 (s, 3H, CH_3), 3.10 (s, 3H, N-Me), 3.45 (s, 3H, OMe), 3.63 (d, J = 12.5 Hz, 1H, CH_2), 3.73 (d, J = 12.5 Hz, 1H, CH_2), 3.81 (t, J_1 = 5.5, J_2 = 7.5 Hz, 1H, H-2), 4.49 (d, J = 11 Hz, 1H, H-3), 4.53 (d, J = 7.5 Hz, 1H, H-1), 4.60 (d, J = 12 Hz, 1H, H-5), 4.75 (d, J = 12 Hz, 1H, H-5′), ^{13}C NMR (125 MHz, $CDCl_3$) δ: 20.7 (CH_3), 31.5 (N-Me), 56.5 (OMe), 60.4 (C-3), 67.5 (C-2), 73.3 (C-5), 75.3 (CH_2), 105.4 (C-1), 155.1 (carbonyl), 156.7 (carbonyl). MALDI-TOFMS calculated *m/z* 388.0 (M+Na+, $C_{11}H_{18}Cl_3O_6N$ requires 388.1). β *anomer*: δ 1.22 (s, 3H, CH_3), 3.05 (s, 3H, N-Me), 3.40 (s, 3H, OMe), 3.36 (d, J = 12.5 Hz, 1H, CH_2), 3.71 (dd, J_1 = 4, J_2 = 5.5 Hz, 1H, H-2), 3.92 (d, J = 12.5 Hz, 1H, CH_2), 4.68 (d, J = 12 Hz, 1H, H-5), 4.79–4.86 (m, 2H, H-3, H-5′), 5.16 (d, J = 3.5 Hz, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.7 (CH_3), 30.9 (N-Me), 55.5 (OMe), 56.4 (C-3), 67.5 (C-2), 74.3 (C-5), 75.0 (CH_2), 99.2 (C-1), 156.2 (carbonyl), 165.6 (carbonyl). MALDI-TOFMS calculated *m/z* 388.0 (M+Na+, $C_{11}H_{18}Cl_3O_6N$ requires 388.1).

The mixture of anomers from the previous step (1 g, 2.73 mmol) was dissolved in pyridine (15 mL) and BzCl (0.9 mL, 6.82 mmol) and 4DMAP (catalytic amount) were added at room temperature. The reaction was monitored by TLC (EtOAc/hexane 2:3). After 1 h the mixture was diluted with EtOAc and washed with aqueous HCl (2%) and brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (EtOAc/hexane) to give two separate anomers with benzoate protection (1 g, 80%). ^{1}H NMR (500 MHz, $CDCl_3$) α *anomer*: δ 1.20 (s, 3H, CH_3), 3.10 (s, 3H, N-Me), 3.52 (s, 3H, OMe), 3.63 (d, J = 12.5 Hz, 1H, CH_2), 3.73 (d, J = 12.5 Hz, 1H, CH_2), 4.49 (d, J = 11 Hz, 1H, H-3), 4.53 (d, J = 7.5 Hz, 1H, H-1), 4.60 (d, J = 12 Hz, 1H, H-5), 4.75 (d, J = 12 Hz, 1H, H-5′), 5.54 (t, J_1 = 5.5, J_2 = 7.5 Hz, 1H, H-2), 7.42 (t, J = 7 Hz, 2H, *m*-OBz), 7.55–7.58 (m, 1H, *p*-OBz), 7.99 (d, J = 7 Hz, 1H, *o*-OBz). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 20.7 (CH_3), 31.5 (N-Me), 56.5 (OMe), 60.4 (C-3), 67.5 (C-2), 73.3 (C-5), 75.3 (CH_2), 104.1 (C-1), 128.4, 129.3, 129.9, 130.1, 133.4 (aromatic), 156.1 (carbonyl), 165 (carbonyl). MALDI-TOFMS calculated *m/z* 493.0 (M+Na+, $C_{18}H_{22}Cl_3O_7N$ requires 493.0). β *anomer*: δ 1.22 (s, 3H, CH_3), 3.05 (s, 3H, N-Me), 3.40 (s, 3H, OMe), 3.36 (d, J = 12.5 Hz, 1H, CH_2), 3.92 (d, J812.5 Hz, 1H, CH_2), 4.68 (d, J=12 Hz, 1H, H-5), 4.79–4.86 (m, 2H, H-3, H-5′), 5.16 (d, J=3.5 Hz, 1H, H-1), 5.51 (dd, J_1=4, J_2=5.5 Hz, 1H, H-2), 7.42 (t, J=7 Hz, 2H, *m*-OBz), 7.54–7.57 (m, 1H, *p*-OBz), 7.99 (d, J=7 Hz, 1H, *o*-OBz). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.7 (CH_3), 30.9 (N-Me), 55.5 (OMe), 56.4 (C-3), 67.5 (C-2), 74.3 (C-5), 75.0 (CH_2), 97.4 (C-1), 128.4, 129.2, 129.9, 130.1, 133.4 (aromatic), 156.2 (carbonyl), 165 (carbonyl). MALDI-TOFMS calculated *m/z* 493.0 (M+Na+, $C_{18}H_{22}Cl_3O_7N$ observed 493.0).

To a mixture of anomers from the previous step (400 mg, 0.85 mmol) in dichloroethane (6 mL) were added 4-methyl benzenethiol (200 mg, 1.7 mmol) and boron trifluoride diethyl etherate (0.2 mL, 1.7 mmol). The reaction was heated to 40 °C and the reaction progress was monitored by TLC (EtOAc/hexane 3:7, two runs). After 1 h the mixture was diluted with EtOAc and washed with $NaHCO_3$ and brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (EtOAc/hexane) to give compound **10** as a single anomer, anomer α (250 mg, 53%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.20 (s, 3H, CH_3), 2.31 (s, 3H, CH_3-STol), 3.08 (s, 3H, N-Me), 3.63 (d, J = 12 Hz, 1H, CH_2), 3.77 (d, J = 12 Hz, 1H, CH_2), 4.52 (d, J = 11 Hz, 1H, H-3), 4.57 (d, J = 12 Hz, 1H, H-5), 4.73 (d, J = 12 Hz, 1H, H-5′), 4.85 (d, J = 9.5 Hz, 1H, H-1), 5.57 (t, J_1 = 9.5, J_2 = 11 Hz, 1H, H-2), 7.10 (d, J = 6.5 Hz, 2H, aromatic), 7.35–7.46 (m, 4H, atomatic), 7.59 (t, J = 7 Hz, 1H, *p*-OBz), 8.04 (d, J = 11 Hz, 2H, aromatic). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 19.4 (CH_3), 20.7 (N-Me), 30.3 (Me-STol), 60.4 (C-3), 66.6 (C-2), 73.3 (C-5), 77.3 (CH_2), 88.7 (C-1), 128.4, 128.5, 129.2, 129.7, 129.8, 129.9, 130, 133.3, 133.4 (aromatic), 156.1 (carbonyl), 165 (carbonyl) MALDI-TOFMS calculated *m/z* 584.1 (M+Na$^+$, $C_{24}H_{26}Cl_3O_6NS$ requires 584.1).

4.2.7. Compound 11

Compound **10** (1 g, 1.78 mmol) was dissolved in 33% methylamine solution in EtOH and the resulted mixture was stirred overnight at room temperature. The reaction progress was monitored by TLC (EtOAc/hexane 1:1). After about 24 h, the reagent and the solvent were removed by evaporation and the crude product was purified by flash chromatography (EtOAc/hexane) to yield the corresponding oxazolidinone product (500 mg, 90%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.38 (s, 3 H, CH_3), 2.29 (s, 3H, CH_3-STol), 2.95 (s, 3H, N-Me), 3.33 (d, J = 6 Hz, 1H, H-3), 3.53 (d, J = 12.5 Hz, 1H, H-5), 3.73 (t, J_1 = 6.5, J_2 = 13.5 Hz, 1H, H-2), 4.06 (d, J = 12.5 Hz, 1H, H-5′), 4.74 (d, J = 7.5 Hz, 1H, 1-H), 7.09 (d, J = 7.5 Hz, 2H, aromatic), 7.36 (d, J = 7.5 Hz, 2H, aromatic). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.0 (CH_3- STol), 23.7 (CH_3-C-5), 30.4 (N-Me), 65.8 (C-3), 68.2 (C-5), 70.4 (C-2), 88.3 (C-1), aromatic carbons: (128.4, 129.8, 132.8, 132.8), 157.4 (carbonyl).

The product from the previous step (70 mg, 0.22 mmol) was dissolved in DMF (3 mL), and to the resulted mixture were added *p*-methoxybenzylchloride (0.04 mL, 0.28 mmol), tetra-*n*-butylammonium iodide (104 mg, 0.28 mmol) and hexamethylphosphoramide (0.5 mL) at room temperature. The reaction was then cooled to −10 °C (ice-water–salt bath) and NaH (8.1 mg, 0.35 mmol) was added. The reaction was brought to room temperature and the reaction progress was monitored by TLC (EtOAc/hexane 2:3). After about 1 h the reaction was diluted with EtOAc, washed with brine, dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography (EtOAc/hexane) to yield **11** (80 mg, 82%): ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.50 (s, 3H, CH_3), 2.34 (s, 3H, CH_3-STol), 2.86 (s, 3H, N-Me), 3.41 (d, J = 4 Hz, 1H, H-3), 3.61 (d, J = 12 Hz, 1H, H-5), 3.80 (t, J_1 = 6, J_2 = 1.5 Hz, 1H, H-2), 3.80 (s, 3H, OMe), 4.15 (d, J = 12 Hz, 1H, H-5′), 4.49 (d, J = 11.5 Hz, 1H, CH_2-PMB), 4.77 (d, J = 11.5 Hz, 1H, CH_2-PMB), 5.24 (d, J = 3 Hz, 1H, 1-H), 6.88 (d, J = 9 Hz, 2H, OPMB-aromatic), 7.14 (d, J = 7.5 Hz, 2H, STol-aromatic), 7.25 (d, J = 9 Hz, 2H, OPMB-aromatic), 7.37 (d, J = 7.5 Hz, 2H, STol-aromatic). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 21.0 (CH_3-STol), 23.9 (CH_3-C-5), 30.2 (N-Me), 55.2 (OMe), 64.0 (C-3), 66.2 (C-5), 72.5 (CH_2-PMB), 75.0 (C-2), 85.9 (C-1), aromatic carbons: (113.9, 113.9, 128.8, 129.4, 129.8, 130.2, 132.0, 137.8), carbonyl carbon (157.4). MALDI-TOFMS calculated *m/z* 452.2 (M+Na$^+$, $C_{23}H_{27}0_5NS$ observed 452.1).

4.2.8. Compound 12

To powdered, flame dried 4 Å molecular sieves (300 mg) was added a mixture of diethyl ether (2.5 mL) and dichloromethane

(0.5 mL), followed by the addition of acceptor **6** (100 mg, 0.22 mmol) and donor **11** (127 mg, 0.29 mmol). After being stirred 10 min at room temperature, the mixture was treated with NIS (72.4 mg, 0.29 mmol). After additional 5 min at room temperature, the mixture was cooled to −40 °C and AgOTf (76 mg, 0.29 mmol) was added. The reaction was brought to −10 °C and was left at this temperature until completion (about 14 h) while the reaction progress was monitored by TLC (EtOAc/hexane 3:7, three runs). The reaction was diluted with EtOAc, filtered through celite, and the celite was washed thoroughly with EtOAc. The combined EtOAc fraction was extracted with saturated $NaHCO_3$, brine, dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography (EtOAc/hexane) to yield the corresponding pseudo-trisaccharide product **12** (150 mg, 90%) as a mixture of anomers (β/α 4:1). Following is given the spectral data for the β-**12** anomer. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.38 (s, 3 H, CH_3), 2.80 (s, 3H, N-Me), 3.79 (s, 3H, OMe). Ring **1**: 3.19 (t, J = 9 Hz, 1H, H-4′) 3.47–3.57 (m, 3H, H-2, H-6′, H-6′), 3.85 (t, J = 9 Hz, 1H, H-3′), 4.40-4.44 (m, 1H, H-5′), 5.84 (d, J = 3.5 Hz, 1H, H-1′). Ring **2**: 1.15 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, 2-ax), 2.36 (bdt, 1H, H-2eq), 3.33–3.45 (m, 2H, H-1, H-3,), 3.52–3.58 (m, 2H, H-4, H-6,), 3.72 (t, J = 9.5, 1H, H-5). Ring **3**: 3.42–3.42 (m, 1H, H-3″), 3.97–3.99 (m, 1H, H-2″), 4.51 (d, J = 11.5 Hz, 1H, H-5″), 4.82 (d, J = 11.5 Hz, 1H, H-5″), 4.96 (d, J = 3.5 Hz, 1H, H-1″). 3.81 (d, J = 11 Hz, 1H, CH_2OBn), 4.14 (d, J = 11 Hz, 1H, CH_2OBn), 5.17 (s, 1H, CH_2), 5.24 (s, 1H, CH'_2), 6.90 (d, J = 6.5 Hz, 2H, aromatic), 7.25 (d, J = 6.5 Hz, 2H, aromatic). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 22.9 (CH_3), 30.0 (N-Me), 31.4 (C-2), 51.1 (C-6′), 55.2, 58.8, 59.4, 61.4, 62.2, 62.8, 67.1 (C-5″), 71.1, 72.1 (CH_2-benzyl), 74.2, 75.1, 76.6, 76.8, 79.3, 83.9, 92.6, 96.0 (CH_2-methylidene), 96.7 (C-1′), 97.6 (C-1″), aromatic carbons: 113.3, 113.4, 128.9, 129.2. MALDI-TOFMS m/z 766.3 (M+Na$^+$, $C_{29}H_{37}O_{11}N_{13}$ requires 766.0).

4.2.9. Compound 4 (NB45)

To a stirred solution of **12** (50 mg, 0.06 mmol) in CH_3CN (5 mL) was added a solution of CAN (110 mg, 0.20 mmol) in minimum volume of water at −10 °C. The reaction was then brought to room temperature and the reaction progress was monitored by TLC (EtOAc/hexane 1:1). After 3 h, the reaction was diluted with EtOAc, washed with $NaHCO_3$ and brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (EtOAc/hexane) to give the desired de-benzylated product (36 mg, 85%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.43 (s, 3H, CH_3), 2.91 (s, 3H, N-Me). Ring **1**: 3.20 (dd, J_1 = 8.5, J_2 = 10 Hz, 1H, H-4′) 3.48–3.58 (m, 3H, H-2′, H-6′, H-6′), 3.84 (dd, J_1 = 9, J_2 = 10.5 Hz, 1H, H-3′), 4.40–4.44 (m, 1H, H-5′), 5.82 (d, J = 4 Hz, 1H, H-1′). Ring **2**: 1.15 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.36 (dt,, J_1 = 4, J_2 = 13 Hz, 1H, H-2eq), 3.30-3.59 (m, 4H, H-1, H-3, H-4, H-6), 3.65 (t, J = 9, 1H, H-5). Ring **3**: 3.68 (d, J = 3.5 Hz, 1H, H-3″), 3.76 (d, J = 11.5 Hz, 1H, H-5″), 4.10 (d, J = 11.5 Hz, 1H, H-5″), 4.26 (t, J = 3.5 Hz, 1H, H-2″), 4.90 (d, J = 3 Hz, 1H, H-1″). 5.13 (s, 1H, CH_2), 5.15 (s, 1H, CH'_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.0 (CH_3), 30.2 (N-Me), 31.8 (C-2), 51.7 (C-6′), 58.8, 59.1, 60.4, 61.5, 62.9, 65.0, 67.0 (C-5″), 71.8, 73.8, 75.1, 75.5, 76.0, 83.5, 95.6, 96.5 (CH_2), 96.6 (C-1′), 99.2 (C-1″) 157.3 (carbonyl). MALDI-TOFMS m/z 646.2 (M+Na$^+$, $C_{21}H_{29}O_{10}N_{13}$ requires 646.0).

The purified product from the previous step (60 mg, 0.09 mmol) was dissolved in EtOH (12 mL) and aqueous solution of NaOH (2 M, 12 mL) was added. The reaction was heated to 90 °C and the reaction progress was monitored by TLC [CH_2Cl_2/$MeNH_2$ solution (33% solution in EtOH) 20:1]. After being stirred 10 h at 90 °C, the reaction was diluted with EtOAc and washed with brine. The combined organic layer was dried over $MgSO_4$ and evaporated. The residue (60 mg, 0.09 mmol) was then subjected to a Staudinger reaction as described above for the compound **6** with the following quantities: THF (2 mL), NaOH 0.1 M (1.5 mL), PMe_3 (0.77 mL) to yield free amine form of compound **4** (NB45) as a mixture of anomers (β/α 4:1) (35 mg, 75% for two steps). The free amine was dissolved in water, the pH was adjusted to 6.8 with H_2SO_4 (0.01 M) and lyophilized to give the sulfate salt of the product. For the spectral analysis of the product, the pH of the free amine was adjusted to about 3.0, lyophilized, and re-dissolved in D_2O. Data for β-**4**: ^{1}H NMR (500 MHz, D_2O) δ 1.42 (s, 3H, CH_3), 2.80 (s, 3H, N-Me). Ring **1**: 3.25 (t, J = 10 Hz, 1H, H-4′) 3.40–3.41 (m, 2H, H-6′, H-6′), 3.79–3.81 (m, 1H, H-2′), 3.96 (t, J = 9 Hz, 1H, H-3′), 4.37–4.40 (m, 1H, H-5′), 6.03 (d, J = 4 Hz, 1H, H-1′). Ring **2**: 1.95 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.43 (bdt, 1H, H-2eq), 3.46–3.48 (m, 2H, H-1, H-3), 3.77–3.81 (m, 2H, H-4, H-6), 4.05–4.07 (m, 1H, H-5). Ring **3**: 3.40–3.42 (m, 1H, H-3″), 3.70–3.77 (m, 1H, H-5″), 3.94–3.96 (m, 1H, H-5″), 4.11–4.14 (m, 1H, H-2″), 5.02 (d, J = 3.5 Hz, 1H, H-1″), 5.08 (s, 1H, CH_2), 5.09 (s, 1H, CH'_2). ^{13}C NMR (125 MHz, D_2O) δ: 20.2 (CH_3), 23.8 (N-Me), 27.0 (C-2), 39.9 (C-6′), 47.7, 49.0, 51.9, 62.6, 65.6, 65.7, 67.1 (C-5″), 68.9, 73.0, 74.4, 75.7, 76.1, 82.7, 85.2, 97.3 (C-1′), 98.8 (CH_2), 102.9 (C-1″). MALDI-TOFMS m/z 532.3 (M+K$^+$, $C_{20}H_{39}O_9N_5$ requires 532.0).

Data for α-**4**: ^{1}H NMR (500 MHz, D_2O) δ 1.24 (s, 3H, CH_3), 3.10 (s, 3H, N-Me) Ring **1**: 3.21 (dd, J_1 = 10, J_2 = 8.5 Hz, 1H, H-4′), 3.50 (dd, J_1 = 14, J_2 = 5 Hz, 1H, H-6′), 3.59 (dd, J_1 = 14, J_2 = 5 Hz, 1H, H-6′), 3.64–3.69 (m, 1H, H-2′), 3.82 (t, J = 10.5 Hz, 1H, H-3′), 4.35–4.40 (m, 1H, H-5′), 5.49 (d, J = 3.5 Hz, 1H, H-1′). Ring **2**: 1.48 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, 2-ax), 2.27 (dt, J_1 = 4, J_2 = 13 Hz, 1H, H-2eq), 3.31–3.48 (m, 4H, H-1,H-3, H-4, H-6), 3.70-3.68 (m, 1H, H-5). Ring **3**: 3.28 (d, J = 8 Hz, 1H, H-3″), 3.42 (d, J = 12.5 Hz, 1H, H-5″), 3.84–3.89 (m, 1H, H-2″), 4.01 (d, J = 12.5 Hz, 1H, H-5″), 4.72 (d, J = 7 Hz, 1H, H-1″), 5.11 (s, 1H, CH_2), 5.13 (s, 1H, CH'_2). ^{13}C NMR (125 MHz, D_2O) δ: 14.1 (CH_3), 20.7 (N-Me), 30.7 (C-2), 51.5 (C-6′), 58.7, 58.9, 62.2, 65.3, 67.0, 71.7 (C-5″), 73.5, 75.2, 76.1, 77.3, 77.8, 81.6, 83.5, 96.6 (CH_2), 98.9 (C-1′), 104.1 (C-1″), 157.4 (cabonil). MALDI-TOFMS m/z 532.3 (M+K+, $C_{20}H_{39}O_9N_5$ requires 532.0).

4.2.10. Compound 13

Commercial gentamicin (15 g, 0.05 mol) was dissolved in H_2O (25 mL) to which $CuSO_4$ (0.58 g, 9 mmol), triethylamine (40 mL, 280 mmol), TfN_3 solution (0.5 M, in 30 mL CH_2Cl_2, 50 mmol) and MeOH (100 mL) were added. The reaction was monitored by TLC (EtOAc/hexane 1:1). The resulted mixture was stirred for 18 h at room temperature, after which concentrated under reduced pressure. The residue was purified by flash chromatography (silica, EtOAc/hexane) to yield **13** (2.5 g, 55%), (calculated from the 32% gentamicin C_{1A} present in the commercial gentamicin according to the manufacturer's protocol). ^{1}H NMR (500 MHz, $CDCl_3$) δ Ring **1**: 1.56 (dd, 1H, J_1 = 4, J_2 = 12 Hz, H-4′), 1.75 (dd, 1H, J_1 = 3, J_2 = 13 Hz, H-4′), 1.90–1.93 (m, 1H, H-3′), 2.07 (dd, 1H, J_1 = 3.5, J_2 = 12.5 Hz, H-3′), 3.22–3.26 (m, 3H, H-6′, H-6′, H-2′), 4.20–4.24 (m, 1H, H-5′), 5.52 (d, 1H, J = 3 Hz, H-1′). Ring **2**: 1.22 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 1.48 (dt, 1H, $J_1 = J_2$ = 4 Hz, H-2eq), 3.3–3.45 (m, 4H, H-1, H-3, H-5, H-6), 3.51 (t, 1H, J = 9 Hz, H-4). Ring **3**: 2.18 (d, 1H, J = 6 Hz, H-3″), 3.72 (d, 1H, J = 12.5 Hz, H-5″), 3.94 (d, 1H, J = 12.5 Hz, H-5″), 4.12(d, 1H, J = 2 Hz, H-2″), 5.05 (d, 1H, J = 3.5 Hz, H-1″). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.9 (C-2), 27.0 (C-3′), 31.7 (C-4′), 43.7, 54.5 (C-6′), 54.7, 58.6, 58.9, 59.7, 65.0, 66.6, 67.9 (C-5′), 75.4, 76.3, 79.6, 81.6, 84.9, 97.8 (C-1′), 100.8 (C-1″). MALDI-TOFMS m/z 594.0 (M+K+ $C_{19}H_{31}O_7N_{13}$ requires 594.4).

4.2.11. Gentamicin C_{1A}

Compound **13** was subjected to a Staudinger reaction as described above for compound **1** under following conditions: compound **13** (600 mg, 1.08 mmol), THF (7 mL), NaOH (0.1 M, 3.0 mL), PMe_3 (1 M solution in THF, 12 mL, 12.0 mmol) to yield gentamicin C_{1A} as a free amine form (290 mg, 93%). The resulted free amine was dissolved in water, the pH was adjusted to 6.9 with H_2SO_4

(0.1 N) and lyophilized to give the sulfate salt a white foamy solid. For the spectral analysis, the pH of the free amine form was adjusted to about 3.0, lyophilized and re-dissolved in D_2O. ^1H NMR (500 MHz, D_2O) δ Ring **1**: 1.45–1.49 (m, 1H, H-4′), 1.75–1.78 (m, 1H, H-4′), 1.88–1.94 (m, 2H, H-3′, H-3′), 2.95–2.98 (m, 1H, H-6′), 3.14 (dd, 1H, J_1 = 3, J_2 = 13.5 Hz, H-6′), 3.46–3.49 (m, 1H, H-2′), 4.0–4.04 (m, 1H, H-5′), 5.76 (d, 1H, J = 3.0 Hz, H-1′); Ring **2**: 1.21 (ddd, 1H, $J_1 = J_2 = J_3$ = 12.5 Hz, H-2ax), 2.43 (dt, 1H, $J_1 = J_2$ = 4 Hz, H-2eq), 2.35–3.46 (m, 3H, H-1, H-3, H-4), 3.72 (t, 1H, J = 9 Hz, H-6), 3.96 (t, 1H, J = 9 Hz, H-5). Ring **3**: 3.35 (d, 1H, J = 12.5 Hz, H-5″), 3.66 (d, 1H, J = 6 Hz, H-3″), 3.89 (d, 1H, J = 12.5 Hz, H-5″), 4.43 (dd, 1H, J_1 = 3.5, J_2 = 11 Hz, H-2″), 5.07 (d, 1H, J = 3.5 Hz, H-1″). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 22.6 (C-2), 27.4 (C-3′), 29 (C-4′), 37.3, 47.2 (C-6′), 50.3, 51.2, 51.4, 52 (C-1), 65.1, 67.8, 69.6, 71.7 (C-5′), 77.8 (C-5), 79.3 (C-6), 85.4, 89.3 (C-4), 102.1 (C-1″), 103.6 (C-1′). MALDI-TOFMS m/z 488.3 (M+K+ $C_{19}H_{39}O_7N_5$ requires 488.0).

4.3. ^{15}N NMR spectroscopy and pK_a measurements

^{15}N NMR data were acquired in a 11.7 T field on a Bruker Avance 500 spectrometer using a dual channel broad-band probe with z-gradients and ^2H lock at 25 °C. Aqueous solutions (85% H_2O/ 15% ^2H_2O) of an aminglycoside free base form (at concentration of about 270–300 mg/mL) at natural abundance ^{15}N were placed into 5.0 mm o.d. glass NMR tubes (Wilmad). 1D ^1H NMR spectra (500.13 MHz resonance frequency) were typically acquired in a single shot using a 41.6 μs dwell time and 64k points then Fourier transformed. Assignments of the proton spectra were straightforward based on chemical shifts, J-couplings and 1D selective TOCSY experiments. For the 2-DOS ring, standard 2D ^1H–^{13}C HMQC and HMBC experiments ascertained the assignment.

1D ^{15}N NMR spectra (50.7 MHz resonance frequency, 18.9 kHz B_1 field) were recorded with ^1H-decoupling (waltz-16) during the acquisition period (inverse gated decoupling). The FIDs were acquired with a dwell time of 66 μs with 16k digitization points. The optimal repetition delay was experimentally determined to be 6s. For good signal-to-noise ratios 1200–2600 transients were averaged with two dummy scans. Data were processed with zero filling to 32k using an exponential window function of 2 Hz line broadening. The spectra were calibrated according to the ratio Ξ of the resonance frequencies between ^{15}N and ^1H nuclei (Ξ equal to 0.10136767)[39], where the ^1H signal was referenced to internal DSS = 0 ppm. Assignment of the ^{15}N spectra was based on the assigned proton NMR spectra via 2D ^1H–^{15}N HMBC. Spectra were acquired with pulsed field gradients and a low-pass J-filter with no decoupling during acquisition. Inverse detection of the ^{15}N in the 2D experiment averaged at least 32 transients with 16 dummy scans. At least 2048 × 32 acquisition points were typically acquired with spectral widths of 3 kHz (^1H) and 20–30 kHz (^{15}N). 2D data sets were Fourier transformed in magnitude mode with zero filling to 1024 × 512 points with a sine window function applied in both dimensions.

The pH of the NMR samples was adjusted by addition of either HCl or KOH in 85% H_2O/15% ^2H_2O. All pH measurements of NMR samples were acquired using a sensoreX semi micro electrode pH meter (SensoreX). The pK_a values for compounds **1–3** amino group were measured by monitoring the ^{15}NMR spectra of each compound at different pH values. Plots of the observed ^{15}N chemical shifts as a function of pH resulted in well-defined titration curves (Fig. 2) from which the pK_a values were estimated by fitting the observed data to the Eq. 1[19,27]:

4.4. Acute toxicity tests

Male ICR mice 22 days of age (18−20 g) purchased from Harlan Laboratories Limited (Jerusalem, Israel) were used to evaluate the LD_{50} of the aminoglycosides. Mice were maintained on a 12 h light/dark cycle (light from 07:00 to 19:00) at 20−22 °C, with relative humidity of 50−55%, and were given food and water ad libitum in an animal care facility. Eight animals were housed in each cage. The aminoglycosides tested were administered in single intravenous (i.v.) doses of 0, 50, 100, 150, 200, 250, 300, 350, 400 and 500 mg/kg, each concentration was applied to 6–8 animals. The aminoglycosides were dissolved in PBS, and 150 μL of final solution (pH 7) were injected into the tail vein at the injection speed of 150 μL/7 s. Changes in the appearance and behavior, and mortality in mice were monitored for 4 days after the administration. The LD_{50} values were determined according to the Reed and Muench method.[40]

4.5. Biochemical assays

Prokaryotic in vitro translation inhibition by the different aminoglycosides was quantified in coupled transcription/translation assays[21] by using *E. coli* S30 extract for circular DNA with the pBESTluc plasmid (Promega), according to the manufacturer's protocol. Translation reactions (25 μL) that contained variable concentrations of the tested aminoglycoside were incubated at 37 °C for 60 min, cooled on ice for 5 min, and diluted with a dilution reagent (tris-phosphate buffer (25 mM, pH 7.8), DTT (2 mM), 1,2-diaminocyclohexanetetraacetate (2 mM), glycerol (10 %), Triton X-100 (1 %) and BSA (1 mg mL^{-1})) into 96-well plates. The luminescence was measured immediately after the addition of the Luciferase Assay Reagent (50 μL; Promega), and the light emission was recorded with a Victor3™ Plate Reader (Perkin-Elmer). The concentration of half-maximal inhibition (IC_{50}) was obtained from fitting concentration-response curves to the data of at least two independent experiments by using Grafit 5 software[41]

4.6. Antibacterial activity

The MIC values were determined using the double-microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS)[42] with starting concentration of 384 μg/mL of the tested compound. All the experiments were performed in triplicates and analogous results were obtained in three different experiments. The clinical isolates of *P. aeruginosa* used in this study were obtained from Rambam Medical Center, Haifa, Israel. The existence of the chromosomal *aph(3′)-IIb* gene in these strains was confirmed by PCR analysis, using the same oligonucleotide primers as we used earlier for the cloning of this gene in *E. coli*[33] (T. Baasov, unpublished data).

4.7. Kinetic analysis with the purified APH(3′)-IIb

The APH(3′)-IIb enzyme was overexpressed and purified using neomycin B affinity column as previously described.[33] The phosphoryl transfer activity was monitored by the pyruvate kinase/lactate dehydrogenase coupled assay. The assay measures the rate of NADH absorbance decrease at 340 nm, which is proportional to the rate of steady-state ATP consumption. The oxidation of NADH was followed by continuously monitoring the absorbance at 340 nm using Ultrospec 2100 pro UV/visible spectrophotometer (Amersham Biosciences). A typical assay mixture contained Tris–HCl buffer (50 mM, pH 7.5), KCl (40 mM), $MgCl_2$ (10 mM), NADH (0.11 mg/mL), PEP (2.5 mM), ATP (1 mM), pyruvate kinase (20 u/mL), lactate dehydrogenase (20 u/mL), varied concentrations of the substrate neamine (comp. **2**) (1–100 μM) and varied concentrations of the inhibitor (0–1000 μM). The mixture was preincubated at 37 °C for 5 min and the reaction was initiated by addition of purified APH(3′)-IIb (10 μL of 0.05 U/mL stock solution). Values of K_i were determined by nonlinear regression using the program GraFit 5.0.[41]

Acknowledgments

We thank Dr. Yael Balazs for her guidance and help in recording ^{15}N NMR spectra. This research was partly supported by the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities (Grant No. 515/07), by the US–Israel Binatinal Science Foundation (Grant No. 2006/301), and by the Technion V.P.R. fund.

References and notes

1. Davis, B. D. *Microbiol. Rev.* **1987**, *51*, 341.
2. Magnet, S.; Blanchard, J. S. *Chem. Rev.* **2005**, *105*, 477.
3. Ogle, J. M.; Brodersen, D. E.; Clemons, W. M., Jr.; Tarry, M. J.; Carter, A. P.; Ramakrishnan, V. *Science* **2001**, *292*, 897.
4. Vicens, Q.; Westhof, E. *Biopolymers* **2003**, *70*, 42.
5. Jana, S.; Deb, J. K. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 140.
6. Forge, A.; Schacht, J. *Audiol. Neurootol.* **2000**, *5*, 3.
7. Nagai, J.; Takano, M. *Drug Metab. Pharmacokinet.* **2004**, *19*, 159.
8. *Antimicrobial Agent: The Aminoglycosides*; Chambers, H. F., Sande, M. A., Eds.; McGraw-Hill: New York, 1996.
9. Vital Brazil, O.; Prado-Franceschi, J. *Arch. Int. Pharmacodyn.* **1969**, *179*, 65.
10. Wright, J. M.; Collier, B. *J. Pharmacol. Exp. Ther.* **1977**, *200*, 576.
11. Albiero, L.; Bamonte, F.; Ongini, E.; Parravicini, L. *Arch. Int. Pharmacodyn.* **1978**, *233*, 343.
12. Barnett, A.; Ackermann, E. *Arch. Int. Pharmacodyn.* **1969**, *181*, 109.
13. Paradelis, A. G.; Triantaphyllidis, C.; Markomichelakis, J. M.; Logaras, G. *Arzneimittel-Forschung* **1977**, *27*, 141.
14. Li, J.; Chang, C. W. *Anti-Infect. Agents Med. Chem.* **2006**, *5*, 255.
15. Silva, J. G.; Carvalho, I. *Curr. Med. Chem.* **2007**, *14*, 1101.
16. Fujisawa, K.; Hoshiya, T.; Kawaguchi, H. *J. Antibiot. (Tokyo)* **1974**, *27*, 677.
17. Shitara, T.; Kobayashi, Y.; Tsuchiya, T.; Umezawa, S. *Carbohydr. Res.* **1992**, *232*, 273.
18. Shitara, T.; Umemura, E.; Tsuchiya, T.; Matsuno, T. *Carbohydr. Res.* **1995**, *276*, 75.
19. Kaul, M.; Barbieri, C. M.; Kerrigan, J. E.; Pilch, D. S. *J. Mol. Biol.* **2003**, *326*, 1373.
20. Barbieri, C. M.; Kaul, M.; Bozza-Hingos, M.; Zhao, F.; Tor, Y.; Hermann, T.; Pilch, D. S. *Antimicrob. Agents Chemother.* **2007**, *51*, 1760.
21. Greenberg, W. A.; Priestley, E. S.; Sears, P. S.; Alper, P. B.; Rosenbohm, C.; Hendrix, M.; Hung, S. C.; Wong, C. H. *J. Am. Chem. Soc.* **1999**, *121*, 6527.
22. Kaul, M.; Barbieri, C. M.; Pilch, D. S. *J. Am. Chem. Soc.* **2006**, *128*, 1261.
23. Park, W. K. C.; Auer, M.; Jaksche, H.; Wong, C. H. *J. Am. Chem. Soc* **1996**, *118*, 10150.
24. Blount, K. F.; Zhao, F.; Hermann, T.; Tor, Y. *J. Am. Chem. Soc.* **2005**, *127*, 9818.
25. Botto, R. E.; Coxon, B. *J. Am. Chem. Soc.* **1983**, *105*, 7.
26. Kaul, M.; Barbieri, C. M.; Srinivasan, A. R.; Pilch, D. S. *J. Mol. Biol.* **2007**, *369*, 142.
27. Barbieri, C. M.; Pilch, D. S. *Biophys. J.* **2006**, *90*, 1338.
28. Ozen, C.; Serpersu, E. H. *Biochemistry* **2004**, *43*, 14667.
29. Dorman, D. E.; Paschal, J. W.; Merkel, K. E. *J. Am. Chem. Soc.* **1976**, *98*, 6885.
30. Barlow, C. B.; Bishop, E. O.; Carey, P. R.; Guthrie, R. D. *Carbohydr. Res.* **1969**, *9*, 99.
31. Rothlin, C. V.; Katz, E.; Verbitsky, M.; Vetter, D. E.; Heinemann, S. F.; Elgoyhen, A. B. *Neuropharmacology* **2000**, *39*, 2525.
32. Talbot, P. A. *J. Pharmacol. exp. Ther.* **1987**, *241*, 686.
33. Hainrichson, M.; Yaniv, O.; Cherniavsky, M.; Nudelman, I.; Shallom-Shezifi, D.; Yaron, S.; baasov, T. *Antimicrob. Agents Chemother.* **2007**, *51*, 3.
34. Hachler, H.; Santanam, P.; Kayser, F. H. *Antimicrob. Agents Chemother.* **1996**, *40*, 1254.
35. Masuda, N.; Sakagawa, E.; Ohya, S.; Gotoh, N.; Tsujimoto, H.; Nishino, T. *Antimicrob. Agents Chemother.* **2000**, *44*, 2242.
36. Emerson, J.; Rosenfeld, M.; McNamara, S.; Ramsey, B.; Gibson, R. L. *Pediatr. Pulmonol.* **2002**, *34*, 91.
37. Amici, M.; Eusebi, F.; Miledi, R. *Neuropharmacology* **2005**, *49*, 627.
38. Alper, P. B.; Hung, S. C.; Wong, C. H. *Tetrahedron Lett.* **1996**, *37*, 6029.
39. Kahovec, J.; Harris, R. K. *Chemicke Listy* **2001**, *95*, 111.
40. Reed, L. J.; Muench, H. *Am. J. Hyg.* **1938**, *27*, 493.
41. Leatherbarrow, R. J. *GraFit 5*; Erithacus Software Ltd.: Horley, U.K., 2001.
42. NCCLS. *National Committee for Clinical Laboratory Standards, Performance standards for antimicrobial susceptibility testing. Fifth information supplement: Approved Standard M100-S5*; NCCLS: Villanova, PA, 1994.