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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 3624–3634
Bioorganic & Medicinal Chemistry

Aminoglycoside antibiotic derivatives: Preparation and evaluation of toxicity on cochlea and vestibular tissues and antimicrobial activity

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Received 13 December 2006; revised 15 March 2007; accepted 18 March 2007
Available online 23 March 2007

Abstract—Aminoglycoside antibiotic derivatives such as neamine, methyl neobiosaminide B, 2-deoxystreptamine, tetra-azidoneamine, tetra-*N*-acetylneamine, tetra-*N*-carboxy-benzylneamine, tetra-*N*-carboxy-methylneamine and tetra-*p*-methoxy-benzyliminoneamine were prepared and evaluated as to their cochlear and vestibular toxicity. Methyl neobiosaminide B, the most promising derivative in the series showed selective, cochlea-dissociated vestibulotoxic activity and was considered to be a potential lead compound for the treatment of Ménière's disease. Antimicrobial properties of the compounds, qualitatively evaluated against a group of pathogenic bacteria, indicated that neomycin B sulfate, neamine as a free base and methyl-neobiosaminide B dihydrochloride show a broader range of activity when compared to the other derivatives.

1. Introduction

Most aminoglycoside antibiotics are produced by *Streptomyces* and *Actinomyces* strains but synthetic approaches also produced several valuable semi-synthetic compounds. They are involved in the treatment of many severe bacterial infections as the ones caused by *Pseudomonas aeruginosa* (gentamicins **1**–**4**, arbekacin **5**) and *Mycobacterium tuberculosis* (streptomycin **6**).[1] Nevertheless, the most effective inhibitor, neomycin (**7**), is used topically in association with bacitracin and polymixin owing to the poor oral bioavailability, cell penetration, and instability.[2]

Some of the aminoglycosides have been also explored as antiviral agents due to their binding affinity to RRE- and TAR-HIV RNA, and disruption of HIV virus replication in cell cultures by competitive inhibition of the natural ligands *Rev* and *Tat* proteins.[3]

There are studies suggesting that aminoglycosides induce conformational structure changes of the A-site, in helix 44, 16S subunit of 30S rRNA, which alter two conformationally flexible adenine residues involved in the selection of cognate aminoacyl-tRNA templates to be added to the mRNA.[4] The induced RNA structural rearrangements may be critical for new interactions leading to base pairing disruption that prevents normal translation, and death of the microorganisms. The aminoglycoside pharmacophore group responsible for translation errors is the aminocyclitol unit present in neamine (**8**), 2-deoxystreptamine (**9**), streptidine (**10**), and spectinamine (**11**). For instance, tests determining minimum inhibitory concentrations (MIC) against *Escherichia coli* indicated values of 1.6 and 50 μM, respectively, for compounds **7** and **8** while compound **9** was inactive.[5]

Different approaches have been used to mimic the pharmacophore amino sugars and aminocyclitol moieties of

Keywords: Aminoglycoside antibiotics; Neamine derivatives; Cochlear and vestibular activity; Ototoxicity; Antimicrobial activity.
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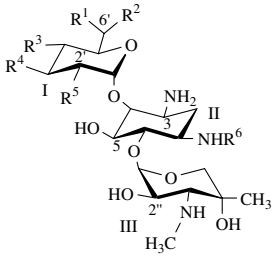
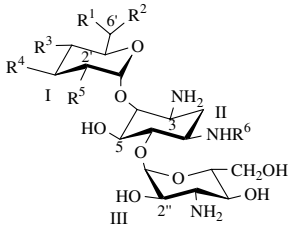
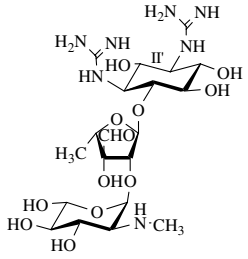
doi:10.1016/j.bmc.2007.03.056



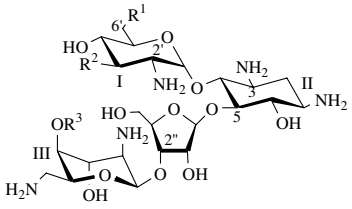
1 Gentamicin B; R^1=H, R^2=NH_2, R^3=OH, R^4=OH, R^5=OH, R^6=H
2 Gentamicin C1; R^1=CH_3, R^2=$NHCH_3$, R^3=H, R^4=H, R^5=NH_2, R^6=H
3 Gentamicin C1A; R^1=H, R^2=NH_2, R^3=H, R^4=H, R^5=NH_2, R^6=H
4 Gentamicin C2; R^1=CH_3, R^2=NH_2, R^3=H, R^4=H, R^5=NH_2, R^6=H
15 Netilmicin*; R^1=H, R^2=NH_2, R^3=H, R^4=H, R^5=NH_2, R^6=CH_2CH_3
*(there is a double bond between C-5' and C-4')



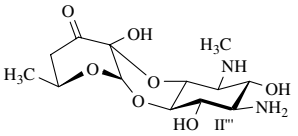
5 Arbekacin: R^1=H, R^2=NH_2, R^3=H, R^4=H, R^5=NH_2, R^6=$COCHOH(CH_2)_2NH_2$
13 Amikacin: R^1=H, R^2=NH_2, R^3=OH, R^4=OH, R^5=OH, R^6=$COCHOH(CH_2)_2NH_2$
16 KanamycinA: R^1=H, R^2=NH_2, R^3=OH, R^4=OH, R^5=OH, R^6=H



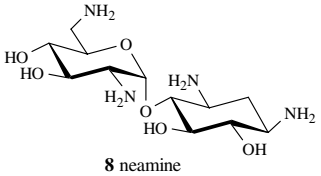
6 Streptomycin



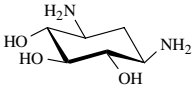
7 Neomycin B: R^1=NH_2, R^2=OH, R^3=H



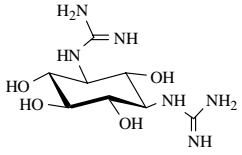
14 Spectinomycin



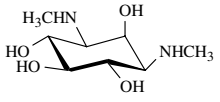
8 neamine



9 2-deoxystreptamine



10 Streptidine



11 Spectinamine

aminoglycoside antibiotics. Recent and relevant results include glycoside dimers and trimers, intercalator conjugates, guanidino and other N-substituted derivatives, peptide and heterocyclic aminoglycosides, along with hybrid heteroconjugates and non-carbohydrate compounds.[6,7]

Aminoglycoside toxicity and resistance development are of great concern despite its selective mechanism of action. Nephro[8] and ototoxicity[9] are most frequently encountered while neuromuscular blockade is not so common. Toxicity mechanisms are not well understood although it is known that the antibiotics accumulate in renal cortical cells, causing structural and functional changes of the plasma membrane, mitochondria and lysosomes ultimately leading to damage of proximal tubules.[10] However, nephrotoxicity is dose-dependent and can be reverted by stopping the treatment. Of more concern is ototoxicity, which according to patient phenotype or extensive use (30 days or more), can induce chronic intoxication in patients, who show irreversible increased deafness and vertigo with equilibrium disturbances due to cochlear dysfunction and vestibular impairments, respectively.[11]

All aminoglycosides have deleterious effects on the cochlear and vestibular functions, although they vary in selectivity. Ototoxic effects of these compounds have been tested in cochlear cell cultures. Neomycin (**7**) produced the highest damage to the cells followed by gentamicins (**1**–**4**), dihydrostreptomycin (**12**) (hydrogenated streptomycin (**1**) derivative), amikacin (**13**), neamine (**8**), and spectinomicin (**14**).[12]

More recently, toxic effects of some aminoglycosides on the cochlea were compared by systemic or transtympanic administration to guinea pigs. Gentamicins (**1**–**4**) were the most toxic followed by amikacin (**13**), streptomycin (**6**) and netilmicin (**15**).[13] A similar study on vestibular damage was conducted using aminoglycosides most commonly employed in clinical practice. In both, systemic and transtympanic administrations in guinea pigs, vestibular histopathological alterations were detected in decreasing order for streptomycin (**6**), gentamicins (**1**–**4**), amikacin (**13**), and netilmicin (**15**).[14]

The three units of kanamycin A (**I**, **II**, and **III**, **16**) were evaluated by Owada[15] as to their cochlear toxicity. The author indicated that unit **III**, related to the 3-aminoglycoside group, was the most toxic producing guinea pig deafness in a period three times shorter than the parent compound. The ototoxic importance of unit **III** was further emphasized in the study by showing the decreased toxicity of *O*-methylated and N-acetylated derivatives in relation to their non-protected counterparts.[15]

The ototoxic action mechanisms of aminoglycoside antibiotics were described by Lesniak et al.[16] as a generation of free radicals due the formation of a complex between the compound and iron. In fact, the use of substances able to chelate iron or scavenge free radicals, attenuate ototoxic symptoms.

Another ototoxicity mechanism may be related to increased activity of the enzyme nitric oxide synthetase (NOS) in the vestibular epithelium, thus locally increasing levels of nitric oxide (NO). Therefore, NOS inhibiting substances would be able to protect auditory cells against apoptosis.[17] An excitotoxic process, modulated by activation of cochlea *N*-methyl-D-aspartate receptors (NMDA) by aminoglycosides, may also be involved in the mechanism responsible for ototoxicity. Recent studies show that NMDA receptor antagonists attenuate aminoglycoside damage to cochlear cells. The positive modulating actions could be due to the presence of several amino groups in the aminosugar and of the aminocyclitol moiety in these antibiotics.[18,19]

Genetic factors have also been described as important determinants of ototoxicity in aminoglycosides therapy, since mutation in position 1555 of the 12S gene in mitochondrial rRNA causes symptoms of hypersensibility. Apparently, an aminoglycoside-induced translation failure of the gene codifying mitochondrial complex I leads to an increased production of mitochondrial superoxide radicals followed by oxidative damage and cell death. More than one third of patients showing aminoglycoside ototoxicity have this mutation.[20]

Ménière disease, an inner ear pathology, is characterized by typical paroxystic dizziness, fluctuating neurosensory hypoacousia, tinnitus and pressure sensation in the ears. Dizziness is usually the most debilitating symptom associated to this disease.[21–23] Vestibulotoxic antibiotics such as gentamicins (**1**–**4**) and streptomycin (**6**) have been used as a chemical labyrinthectomy agents in the sick ear. With the vestibule chemical lesion, dizziness could be controlled improving patient quality of life.[24–26] This technique should be preferable to intracranial surgery, being more secure and having satisfactory results. Ideally, transtympanic application of aminoglycosides should not have adverse effects on hearing quality. However, the compounds are toxic to both vestibule and cochlea although there is some selectivity. As a rule, 90–100% of the patients show dizziness control but 30% suffer hearing losses.[15,23] The impaired hearing function observed during chemical labyrinthectomy, led us to investigate the preparation of simplified bioactive analogues having mostly a neamine core scaffold with selective vestibular activity and devoid of cochlear toxicity to be used in the therapy of Ménière's disease. Since neamine is the smallest structural motif of the highly active neomycin-type antibiotics for selectively binding to the rRNA A-site, we envisaged that chemical modifications on the neamine would produce new lead compounds able to preserve the antibacterial properties. These studies intend to improve the knowledge on structure–activity relationships associated to the hearing function, not forgetting the long-term goal to achieve antimicrobial activity.

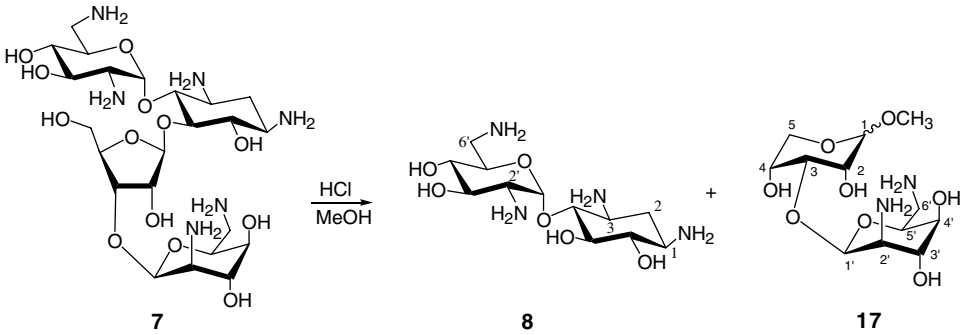
2. Results and discussion

Neomycin B (**7**) antibiotic fragments, such as neamine (**8**), 2-deoxystreptamine (**9**) and methyl neobiosaminide B (**17**), were prepared and evaluated on cochlear and vestibular tissues. Although many studies in aminosugar mimetics are based on neamine analogues, we also investigated the methyl neobiosaminide B (**17**) fragment since its contribution to RNA binding affinity could also be significant to hearing impairment. In fact, K_D values for neomycin B (**7**) and neamine (**8**) were 0.019 and 7.8 μM, respectively, suggesting the importance of the **17** unit to the binding.[27]

In addition neamine derivatives obtained by attaching acetyl (**18**), carboxymethyl (**19**), carboxybenzyl (**20**), and *p*-methoxy-benzylimino (**21**) groups to the amino functions were also prepared. The amino groups were further replaced by azido to produce tetra-azidoneamine (**22**).

Preparation of neamine (**8**) and methyl neobiosaminide B (**17**) proceeded according to classical fragmentation protocols by heating neomycin B sulfate (**7**) (commercially available) in strong acid conditions, as reported earlier and outlined in Scheme 1.[28] Compound **8** was easily separated using the Botto and Coxon[29] procedure involving dropwise addition of ethyl ether to the methanolic solution-containing products in order to precipitate compound **8**. Compound **17** was obtained from the remaining solution after several repeated treatments with ethyl ether. Selective cleavage of the furanose anomeric bond was achieved in methanolysis conditions due to its greater liability as compared to the other stable anomeric positions, hydrogen-bonded to the neighboring nitrogen group. The mechanism proposed for this reaction considers a rearrangement in which a furanose ring is converted into a pyranose unit accompanied by anomeric bond epimerization.[29,30]

Compound **17** was carefully characterized through analysis of the ^{1}H, ^{13}C, ^{1}H–^{1}H COSY, and ^{13}C–^{1}H COSY NMR spectra, since only ^{15}N NMR data are reported and no detailed ^{1}H and ^{13}C NMR data was found in the literature.[29] The ^{1}H NMR spectrum showed a 1:4.5 α/β mixture at C-1 based on the integral values of the methoxyl signals at δ 3.33 and 3.35. Furthermore, the anomeric hydrogens H-1′ and H-1 were observed at δ 5.27 (1H, d, $J = 1.7$) and δ 4.63 (1H, d, $J = 4.5$), respectively, while the methylene group of C-5 shifted



Scheme 1. Chemical fragmentation of neomycin B sulfate (**7**), giving neamine (**8**) and methyl neobiosaminide B (**17**).

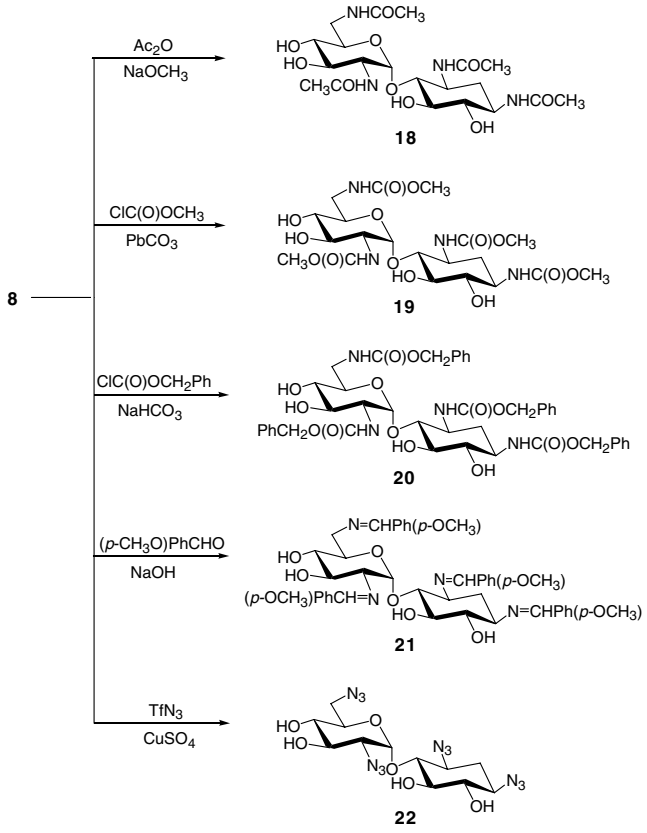
at δ 3.62 and 3.75, and C-6′ at δ 3.20 and 3.28 due to the presence of the amino group.

According to Busscher et al.,[31] 2-deoxystreptamine (**9**) may be synthesized by different procedures but its classical preparation by neamine fragmentation is more reliable, easily done and produces higher yields. Treatment of compound **8** with 48% hydrobromic acid and controlled heating at 130 °C, a stronger acidic condition than the one used for the cleavage of **7**, gave the dihydrobromic salt of **9** in 98% yield.[32] The reaction was monitored by TLC plates using *n*-propanol/triethylamine/H_2O (8:1:4) as eluent.[33] As expected, prolonged heating of the reaction mixture resulted in decomposition of the products.

Regioselective acetylation of **8** with acetic anhydride and catalytic sodium methoxyde produced tetra-*N*-acetylneamine (**18**) as a precipitated pure product in 77% yield.[34,35] Shifting of the hydrogens H-1, H-3, H-2′ and H-6′, bound to C–N groups, from δ 2.66–2.98 to low field (δ 3.09–3.51) and the relative integrals of the methyl groups confirmed the acetylation of all four amino groups (Scheme 2).

Various strategies to introduce the carbamate group were examined to produce different neamine derivatives. For instance, tetra-*N*-carboxy-methylneamine (**19**) was successfully prepared by treating **8** with methyl chloroformate and lead carbonate.[36] The remaining lead salt was removed by Dowex® (OH) resin filtration followed by treatment with hot dioxane, and the product removed by filtration (46% yield). Analysis of the product by ^{1}H NMR revealed the presence of methoxyl groups at δ 3.34, 3.36, 3.37, and 3.39, with the expected integration as previously described.[37] On the other hand, tetra-*N*-carboxy-benzylneamine (**20**) was conveniently prepared by using benzyl chloroformate in the presence of sodium bicarbonate. However, extraction with dioxane lowered the yield of compound **20** to 36%.[38] ^{1}H NMR signals related to aromatic hydrogens (δ 7.17–7.39) and the methylene benzyl groups (δ 4.80–5.11) were characteristic and showed full nitrogen group protection.[39]

Condensation of neamine (**8**) with anisaldehyde in the presence of sodium hydroxide by an adapted procedure



Scheme 2. Chemical transformation of neamine (**8**), giving ester (**18**), carbamates (**19** and **20**), imino (**21**), and azido (**22**) derivatives.

of Myszka et al.[40], produced tetra-*p*-methoxy-benzylimineamine (**21**) in 85% yield. Performing the reaction in an aqueous medium allowed the precipitation of the pure product (Scheme 2).

Following the procedure described by Alper et al.[41], the amino groups of **8** were converted into the corresponding azides using triflic azide and a catalytic amount of $CuSO_4$ to give the tetra-azidoneamine (**22**) in 71% yield. The proposed mechanism may involve a [3 + 2] dipolar reaction resulting in a diazo transfer reaction by Cu^{II}, with retention of the configuration.[42,43] The initial purification was carried out by silica gel filtration and subsequent partitioning in ethyl acetate and 10% EDTA

to remove residual metal and obtain the pure product for biological evaluation.

Cochlear and vestibular activities of compounds **7**–**9** and **17**–**22** were evaluated by brainstem evoked auditory potential (BEAP), distortion product otoacoustic emissions (DPOAE) analysis. To assess the relative damage caused by morphofunctional and histopathological alterations in the cochlear and vestibular tissues, transtympanic administration of the compounds was performed in the right ear of albino guinea pig and keeping the left one as a control. This procedure proved to be more convenient than systemic application since it resembles the treatment protocol, circumvents kidney impairment and local infection. Furthermore, ototoxicity effects were also assessed by scanning electron microscopy (SEM) based on the damage caused in the cochlea hair cells (Corti organ), and vestibule comprising the semicircular channels, utriculus and sacculus.

Samples of compounds tetra-*N*-carboxy-methylneamine (**19**), tetra-*N*-carboxy-benzylneamine (**20**) and tetra-*p*-methoxy-benzyliminoneamine (**21**), were prepared in DMSO due to the poor water solubility. When necessary, sterilization was performed under controlled dry heat, because the traditional sterile membrane filtration is not resistant to DMSO.

The values shown in Table 1 are percentages of disrupted cochlea and vestibule due to the compounds tested. The functional measurements of BEAP confirmed the highest hearing losses at 60 dB thresholds using samples **7**, **8**, **20**, and **21**. In contrast, samples **9**, **17**, and **18** showed normal thresholds (5 dB), while sample **19** showed a light hearing loss threshold (30 dB).

Treatment of guinea pigs with neomycin B sulfate (**7**) or neamine (**8**) as a free base resulted in similar cochlear outer hair cells damage (100%) causing impairment or loss of hearing (Fig. 1).[12] On the other hand, neomycin-treated animals showed only moderate damage to sensitive cells of the otolitic organs and semicircular channels of the vestibule (50%) in contrast to **8** which demonstrated evidence of severe cochlear and vestibular toxicity (100%) (Fig. 2).[44]

Among the hydrolysis products of **7**, neamine (**8**) showed the greatest ototoxicity, while 2-deoxystreptamine (**9**) was non-toxic for both cochlear and vestibular tissues with normal BEAP and DPOAE patterns (Fig. 3). Using in vivo assays, Owada[15] obtained similar results for compound **9**. In this study a more interesting result was obtained with methyl neobiosaminide B dihydrochloride (**17**), which was non-toxic to the cochlea, maintaining a normal functional status on the brainstem evoked auditory potential (5 dB) and normal otoacoustic emission (present) in all the right inner ear at a concentration of 88 mg/mL (0.22 mmol/mL). Its relevant vestibular activity, however, led to utriculus and sacculus damages as outlined in Figure 4. Furthermore, an abundant globular substance present in the semicircular channels could be related to this initial damage. The results suggest that methyl neobiosaminide B (**17**) could be used as a drug for selective chemical labyrinthectomy in patients with Ménière disease, since a selective vestibule chemical lesion could lead to normal auditory function, improving patient quality of life without disrupting the cochlear tissues.[45–47]

Although the low lethal acute toxicity of the *N*-acetyl derivative **18** was described,[15] there is no information concerning its ototoxicity. It was expected that the *N*-acetyl groups would reduce ototoxicity since the positive modulation on NMDA receptors of the cochlea may depend on the free ionizable amino groups. Indeed, compound **18** showed no toxicity according the functional BEAP and DPOAE and morphology SEM analysis.[18]

The animals treated with *N*-carboxy-methylneamine (**19**) displayed normal cochlear and vestibular tissues but altered DPOAE and BEAP patterns owing to the presence of dense secretion in the middle ear. The results observed for tetra-*p*-methoxy-benzyliminoneamina (**21**) pointed out that cochlear outer cells were maintained intact although great damage occurred in the cochlear inner cells. Compound tetra-*N*-carboxy-benzylneamina, (**20**), had the opposite effect damaging the outer hair cells and preserving the normal inner hair cells. As DPOAE does not depend on the inner cells, it was expected that the otoacustic emissions would not be disturbed. However, the hearing loss of animals treated

Table 1. Damage of the cochlear and vestibular tissues induced by neomycin B sulfate and its derivates evaluated by functional and histopathological analysis

Compound*	Cochlear inner cells damage (%)**	Cochlear outer cells damage (%)**	Vestibular damage (%)**	BEAP***	DPOAE****
7	0	100(6)	50(3)	60 dB	Absent
8	0	100(6)	100(6)	60 dB	Absent
9	0	0	0	5 dB	Present
17	0	0	100(6)	5 dB	Present
18	0	0	0	5 dB	Present
19	0	0	0	30 dB	Absent
20	0	100(6)	0	60 dB	Absent
21	100(6)	0	0	60 dB	Absent

* Compounds: neomycin B sulfate (**7**), neamine (**8**), 2-deoxystreptamine (**9**), methyl-neobiosaminide B (**17**), tetra-*N*-acetylneamine (**18**), tetra-*N*-carboxy-methylneamine (**19**), tetra-*N*-carboxy-benzylneamine (**20**) and tetra-*N*-*p*-methoxy-benzyliminoneamine (**21**).

** % of cochlear and/or vestibular damaged in six animals (100%).

*** Thresholds values in dB to induce BEAP responses up to 5, 30 or 60 dB in concentrations of 0.22 mmol/mL.

**** Response to DPOAE.

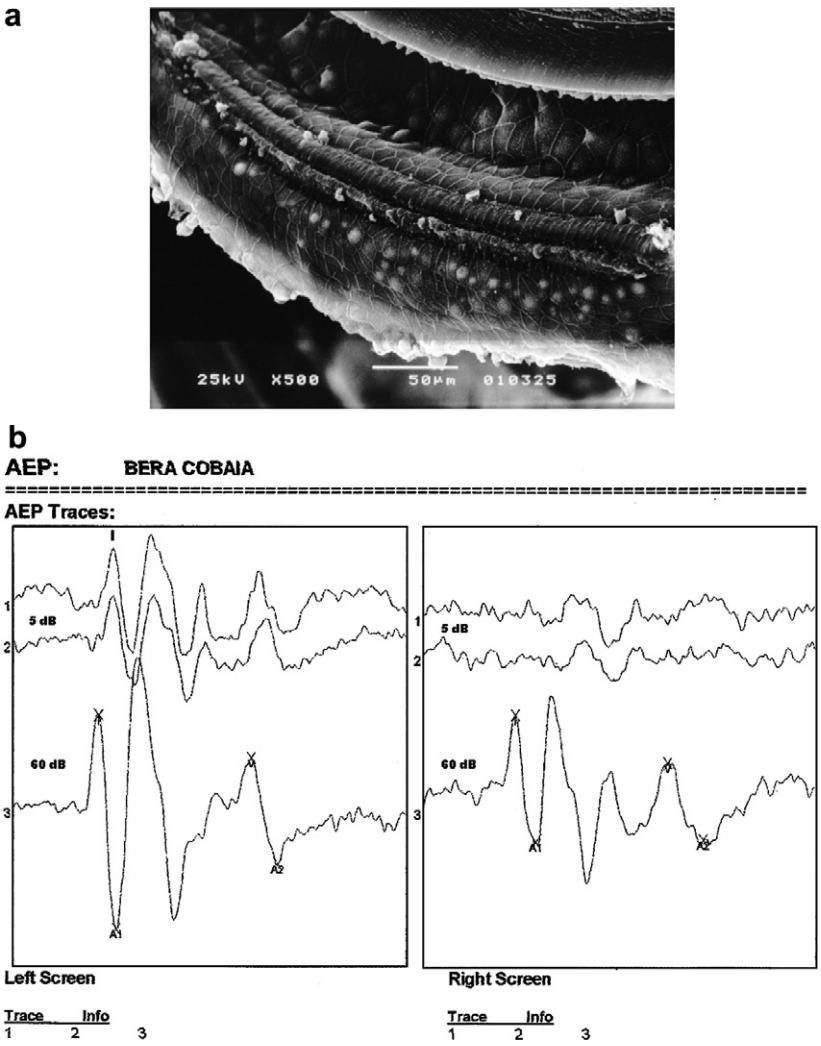


Figure 1. (a) Scanning electron micrograph of the basal cochlear turn of guinea pig treated with neamine (**8**). Note the changes in the outer hair cells, three rows of cilia with destruction of all cilia and ciliary distortion in the inner hair cells (basal turn), 500× magnification. (b) Note the brainstem evoked auditory potential with 60 dB threshold on right ear. Control left ear showed a normal threshold (5 dB).

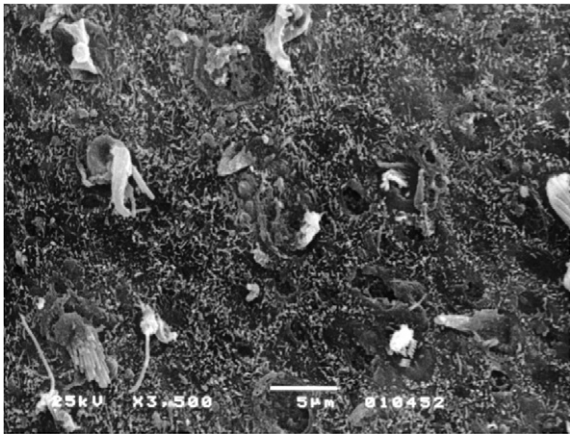


Figure 2. Scanning electron micrograph of the utricular maculae vestibular organ of guinea pig treated with neamine (**8**). Changes in the cilia and disarrangement of the remaining cilia are in evidence, 3500× magnification.

with **21** can be related to the presence of a mucous white substance, probably derived from the precipitation of **21** in the middle ear. Both compounds were not vestibulotoxic at the concentration used in the experiments.

The last compound **22** showed a high degree of systemic toxicity characterized by animal weight losses and mortality during the experiments. Although the isolated intratympanic application of DMSO showed no systemic or ototoxicity the solvent may have contributed to increased absorption of the azido compound and the consequent acute effects.

To investigate a possible correlation between the cochlear and vestibular activities with antibiotic activity of the fragmentation and semi-synthetic products, we carried out a qualitative antibacterial evaluation.

The antimicrobial activity of the compounds was evaluated using micro dilution procedures[48] and standard strains such as *Micrococcus luteus* 9341, *Staphylococcus aureus* 6538, *Staphylococcus epidermidis* 12228, *E. coli*

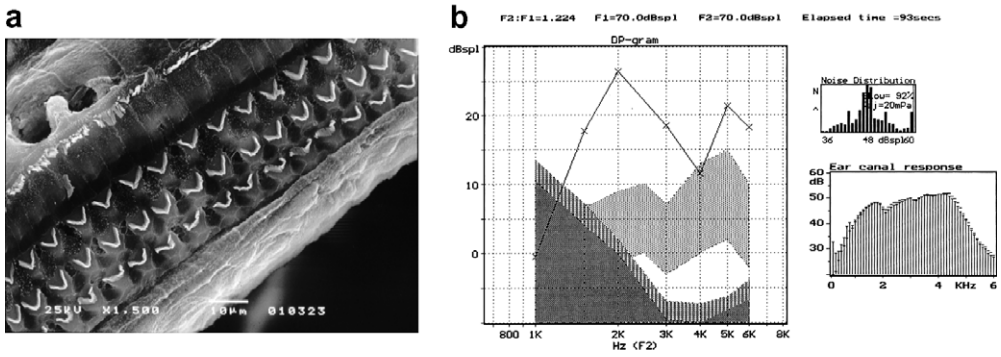


Figure 3. (a) Scanning electron micrograph of the basal cochlear turn of a guinea pig treated with 2-deoxystreptamine (**9**). Outer hair cells are present in all rows, 1500× magnification. (b) Note the response to the normal otoacoustic emission (DPOAE) on guinea pig right ear.

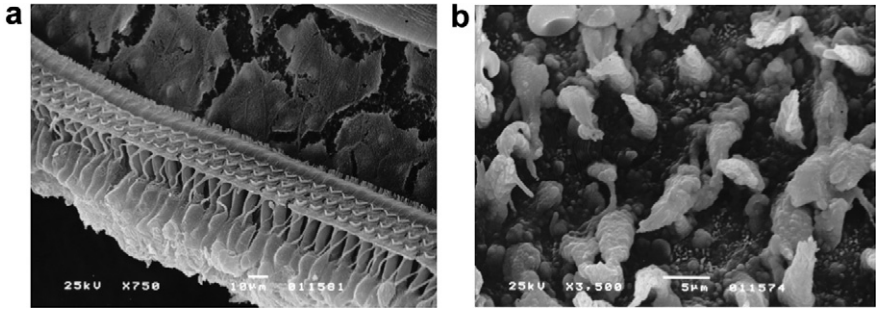


Figure 4. (a) Scanning electron micrograph of the basal cochlear turn of a guinea pig treated with methyl neobiosaminide B hydrochloride (**17**). Note the outer hair cells are present in all rows, 750× magnification. (b) Scanning electron micrograph of the utricular maculae vestibular organ of the guinea pig treated with methyl neobiosaminide B (**17**). Note the changes in the cilia, disarrangement and fusion of the remaining cilia and the presence of globular substances are in evidence. Magnification (3500×).

Table 2. Susceptibility assays using the micro dilution test to assess the qualitative antimicrobial activity of compounds **7**–**9**, **17**, **18**, **22**[48]

Bacterial strain	Compound							
	7	**8**	**17**	**9**	**18**	**22**	**C+**	**C−**
M. luteus 9341	−	−	−	−	−		+	−
S. aureus 6538	−	−	−	+	−	−	+	−
S. epidermidis 12228	−	−	−	+	−	−	+	−
E. coli 25922	−	−	−	+	−	+	+	−
P. aeruginosa 27853	−	−	−	+	+	−	+	−

Compounds were evaluated in duplicates; **C**(+), positive control (inoculated culture medium); **C**(−), negative control (culture medium); +, bacterial growth; −, absence of bacterial growth or inhibition. The compounds were used at concentration of 15 mg/mL.

25922 and *P. aeruginosa* 27853. Neomycin sulfate (**7**) and its fragmentation products, neamine as a free base (**8**) and methyl-neobiosaminide B dihydrochloride (**17**) showed a better pattern of inhibition than compounds **9**, **18**, and **22** against some of the strains as depicted in Table 2. Although compounds **18** and **22** had also displayed antibacterial properties, they were not active against *P. aeruginosa* and *E. coli*, respectively, and the evaluation of **22** against *M. luteus* was not conclusive due its sensitivity to the solvent (DMSO/H_2O).

The hydrophobic derivatives, tetra-*N*-carboxy-benzylneamina (**20**) and tetra-*p*-methoxy-benzyliminoneamina (**21**), precipitated in the culture medium and could not be evaluated even in the presence of 10% DMSO. Solvent interference also prevented the evaluation of compound **19**, soluble only in pure DMSO.

The importance of the aminoglycoside unit for antibacterial activity was evident since the simplest fragment, 2-deoxystreptamine (**9**), showed no activity against the main bacterial strains, although it is fundamental for interaction at the rRNA A-site.[49] Furthermore, the lower activity of compounds **18** and **22**, compared to **7**, **8**, and **17**, suggests the importance of free amino groups and/or a low methodological sensitivity detection.

3. Concluding remarks

Fragmentation of aminoglycoside antibotics and chemical transformations of amino groups were used to prepare amide, carbamate, imine and azido derivatives. It was then demonstrated that starting from a high vestibular and cochlear active aminoglycoside antibiotic

as neomycin sulfate (**7**), it was possible to obtain a fragmentation product, methyl neobiosaminide B (**17**), with the desired vestibular selectivity and lacking cochleotoxicity. Preliminary results showed different patterns of cochlear and vestibular in vivo activities indicating that this may be an interesting approach to develop new drugs. Furthermore, previous studies in this area performed by Owada[15] only explored monomers of neomycin units and did not give information about cochlear and vestibular toxicity of compounds **8** and **17**. In this report we showed the high activity of neamine (**8**) against cochlear and vestibular tissue, the later being higher than neomycin (**7**).

A comparative investigation between the cochlear and vestibular activities and a qualitative antibacterial analysis pointed out that neomycin sulfate (**7**) and its fragmentation products, neamine as a free base (**8**) and methyl-neobiosaminide B dihydrochloride (**17**) showed a broader range of inhibition than compounds **9**, **18**, and **22**. The hydrophobic properties of compounds **19**–**21** prevented their use in microbiological assays due to the low water solubility and the use of DMSO was detrimental since the solvent has displayed some influence on all tested strains.

4. Experimental

^{1}H, ^{13}C, ^{1}H–^{1}H COSY, and ^{13}C–^{1}H COSY NMR spectra were measured on a Bruker DPX-400 (400 and 100 MHz) using $CDCl_3$ (Aldrich) as solvent and TMS as internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (J) in Hz. IR spectra were recorded on a Nicolete Protege FT460 spectrophotometer. ESI-EM spectra data were obtained from ULTROTOF$_Q$ Bruker Daltonics, Billerica. TLC was performed on silica gel (E. Merck) SIL G-25UV254 and detection of the separated compounds was either by viewing with a UV lamp (254 nm), or by spraying with a 10% sulfuric acid, 1.5% molybdic acid, 1% ceric sulfate spray followed by heating to 150 °C. Column chromatography was performed on a Kieselgel 60 (70–230 mm mesh, E. Merck) column. Neomycin sulfate was purchased from GIBCO™. Neamine tetrahydrochloride (**8**),[50,51] 2-deoxyestreptamine (**9**),[52] tetra-*N*-acetylneamine (**18**),[53] tetra-azidoneamine (**22**),[41] tetra-*N*-carboxy-methylneamine (**19**)[36,37] and tetra-*N*-carboxy-benzylneamine (**20**)[38] were prepared in the same manner as previously described.

4.1. Methyl neobisamidine B dihydrochloride (17)[41]

The compound was prepared from neomycin sulfate (**7**) (2 g) as previously described by Ford[28] and Botto,[29] with minor modifications. The remaining mother liquor from the acid methanolysis of neomycin sulfate (**7**) (240 mL) was concentrated in a rotatory evaporator to approximately 50 mL and treated with anhydrous ethyl ether (150 mL). The flocculent white precipitate started to form immediately but the process was completed after refrigeration for 12 h. Filtration under nitrogen pressure through a medium-porosity sintered glass-funnel was used to separate the solid product which was further dried under high vacuum (715 mg). The solution of the solid material in the smallest amount of methanol was titrated with ethyl ether until it became turbid. The insoluble fraction was separated by the same filtration system. The process was repeated until TLC (MeOH/acetone) showed complete removal of impurities in the remaining solution. Thus, the solution was concentrated to dryness in a rotatory evaporator in high vacuum giving compound **17** as a highly hygroscopic white powder (335 mg, 1.03 mmol, 42%) composed of a mixture of 1:4.5 α/β mixture at C-1. The melting point was higher than 300 °C. R_f 0.61 (MeOH/acetone 2:1, v/v), while the main impurity neamine hydrochloride was much slower (R_f, 0.40). Data for the NMR ^{1}H (400 MHz/D_2O) *major isomer*: δ 3.19 (1H, dd, $J_{5',6'a}$ 3.3, $J_{6'a,6'b}$ 13.4, H-6′a); 3.29 (1H, dd, $J_{5',6'b}$ 6.3, $J_{6'a,6'b}$ 13.4, H-6′b); 3.33 (3H, s, OCH_3); 3.48–3.51 (1H, m, H-2′); 3.62 (1H, dd, $J_{4,5a}$ 6.0, $J_{5a,5b}$ 12.1, H-5a); 3.67–3.70 (1H, m, H-4′); 3.72 (1H, t, $J_{2,3}$ 3.8, H-2); 3.75 (1H, dd, $J_{4,5b}$ 3.3, $J_{5a,5b}$ 12.1, H-5b); 3.91 (1H, ddd, $J_{3,4}$ 3.0, $J_{4,5b}$ 3.3, $J_{4,5a}$ 6.0, H-4); 4.11 (1H, dd, $J_{3',4'}$ 3.3, $J_{2',3'}$ 2.5, H-3′), 4.14–4.20 (2H, m, H-5′, H-3); 4.63 (1H, d, J 4.5, H-1); 5.27 (1H, d, J = 1.7, H-1′). NMR ^{13}C (100 MHz/D_2O): δ 40.74 (C-6′), 51.1 (C-2′), 56.24 (O*C*H$_3$), 63.46 (C-5), 67.59 (C-4′), 67.70 (C-4), 68.05 (C-3′), 68.95 (C-2), 70.66 (C-5′), 74.84 (C-3), 95.41 (C-1′), 101.55 (C-1), ESIMS found m/z 325.1725 [M+H$^+$]. Calcd for $C_{12}H_{24}N_2O_8$ 324.1533.

4.2. Tetra-*p*-methoxy-benzyliminoneamine (21)

Neamine tetrahydrochloride (**8**) (93 mg, 0.2 mmol) was sequentially treated with a 1 M NaOH (1 mL) and *p*-anisaldehyde (0.15 mL). The mixture was stirred until the spontaneous precipitation of product **21**. After remaining at 4 °C for 2 h, it was filtered through a medium-porosity sintered glass-funnel. The white solid was washed several times with cold water and portions of ethyl ether/ethanol (1:1, v/v) and then dried under high vacuum to give compound **21** as a pure product (127 mg, 0.17 mmol, 85%). Mp 255 °C; NMR ^{1}H (400 MHz/DMSO-d_6): δ 1.47–1.57 (1H, m, H-2′a), 1.97 (1H, q, J = 13.6, H-2′b), 2.82 (1H, dd, J = 12.6, H-6a), 3.02 (1H, dd, J = 2.5, 9.5, H-3), 3.16–3.25 (1H, m, H-1′ or H-3′), 3.32–3.49 (4H, m, H-1′ or H-3′, H-2′, H-4′, H-4), 3.53–3.67 (4H, m, H-2, H-6b, H-5′, H-6′), 3.70–3.89 (13H, m, H-5, 4 × OCH_3), 4.74 (1H, d, J = 5.0, OH), 4.85–4.90 (2H, m, H-1, OH), 5.05 (1H, d, J = 5.0, OH), 6.34 (1H, s, OH), 7.00 (4H, d, J = 8.8 ArH), 7.05 (4H, d, J = 8.8, ArH), 7.47 (1H, s, =C*H*), 7.63 (2H, d, J = 8.8, H), 7.68 (2H, d, J 8.6, ArH), 7.73 (2H, d, J = 8.6, ArH), 7.77 (2H, d, J = 8.6, ArH), 8.22 (1H, s, =C*H*), 8.24 (1H, s, =C*H*), 8.33 (1H, s, =C*H*); NMR ^{13}C (100 MHz/DMSO-d_6) δ 38.44 (C-2′), 55.65 (O*C*H$_3$), 60.1 (C-6), 70.18, 70.55, 70.89, 71.00, 72.11, 75.03, 76.86, 88.46 (C-1′, C-3′, C-4′, C-5′, C-6′, C-2, C-3, C-4, C-5), 101.79 (C-1), 114.45, 114.32, 114.28, 114.24, 114.87, 129.62, 129.95, 130.25, 130.79 (ArC), 132.18 (ArCq), 160.28 (C=N), 160.42 (C=N), 161.12 (C=N), 164.88 (C=N); IR$_{\sqrt{Max}}$: 3360 (OH), 1641 (C=N).

4.3. Cochlear and vestibular activity

Albino guinea pigs are easily manageable experimental animals for cochlear dissection and manipulation. They have accessible routes of infusion of anesthetics and experimental drugs for transtympanic application in the middle ear, allowing the drug to cross the round window to the inner ear and producing significant cochlear changes. Guinea pigs were kept in the animal facilities of the Experimental Surgery Unit, Department of Surgery, Faculty of Medicine of Ribeirão Preto, USP as recommended by the guidelines for the care and use of laboratory animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, of the US National Research Council and was approved by the Institutional Ethical Committee (protocol No. 070/2005).

The animals weighing between 400 and 600 g were selected at the central animal house, based on the determination of the Preyer reflex. Animals of this size are adequate to tympanic membrane manipulations.

After a hearing rest of 24 h, the animals were re-evaluated by manual otoscopy. Animals presenting signs of outer acute or median otitis were discarded. Only animals presenting a wax plug easily removed were included in the study. The ones showing inflammatory changes of the outer auditory meatus, or an auditory meatus too narrow to accommodate the tube of the otoacoustic emission equipment were discarded. Animals anesthetized with 65 mg/kg ketamine hydrochloride were then submitted to auditory screening using DPOAE and the brainstem evoked auditory potential (BEAP) in an acoustically isolated cabin. The animals selected to be used in the experiments showed a pattern of DPOAE and BEAP up to 5 dB thresholds on both ears. Since this was an experiment using topical drugs, the animal left ear was maintained intact as a control and treated with 0.9% isotonic sodium chloride solution or DMSO. The experiments were performed by transtympanic application of individual samples (0.08–0.10 mL, 0.22 mmol/mL) in 06 albino guinea pigs.

Experimental samples were prepared according to the following protocol: (i) the water soluble ones were heated at 180 °C for about 2 h. The plastic cover and Teflon were treated in 70% ethyl ethanol for 2 h. Using a laminar flow chamber, samples **7**–**9**, **17**, **18** were dissolved in sterile water (Equiplex®), while sample **22** was dissolved in 10% sterile DMSO/water, followed by filtration through 0.22 μm pore MILLEX-GS MILLIPORE®; (ii) samples poorly soluble in water (**19**–**21**) were dissolved in DMSO (1.0 mL) and stored in sterile vials at 180 °C for about 2 h with omission of the filtration step The heating was repeated for 90 min at 90 °C and kept 24 h at room temperature.

4.3.1. Functional measurements. The function of outer cochlear hair cells was determined by DPOAE using an ILO 92 CAE apparatus (system Otodynamics Ltd, United Kingdom). The animals were anesthetized with ketamine hydrochloride and the test performed before treatment and immediately before sacrifice (1st and 31th days for group 1, 1st and 4th days for group 2 and 1st , 31th and 34th days for group 3), according to the frequency relation 2F1–F2 with the ratio F1:F2 = 1.22, and a resolution of two points per octave.

Otoacoustic emissions were considered from 1.5 kHz, since the dimensions of the guinea pig external auditory meatus make it difficult to detect otoacoustic emissions below this frequency, with the occurrence of responses that coincide with noise responses. Thus, by analyzing the 2 kHz frequency, a pure tone a little, above and a little below was offered, in such a way that the ratio between them was 1.22. Thus, a resultant frequency response was obtained automatically according to the 2F1–F2 relation (below the frequency evaluated) and the 2F2–F1 relation (above the resultant frequency). It should also be taken into consideration that the intensities of F2 and F1 can be equal or different. In the present study we used equal intensities of 70 dB spl and frequency range of 1500–8000 Hz. The intensity of the triggering stimulus can vary within the range of 0–70 dB spl and can be measured in the range of 500–8000 Hz. Based on these values, we observed the so-called DPGRAM, that is, the audiocochleogram, in which there is a sound stimulus and a response, which is also a sound that corresponds to the function of the cochlear outer hair cells responsible for the frequencies analyzed.

In this study, the otoacoustic emissions at higher frequencies were considered more important to evaluate the functional state of the outer hair cells of the basal cochlear turn. This evaluation, however, is qualitative. DPOAE were considered to be present or absent.

For functional measurements of BEAP, Compass Meridian 2000 program, Nicolet, LTD was performed using the parameters below:

Tone Burst stimulus, 2000–4000 Hz, 60, 40, 20, 10 and 5 dB thresholds on tip phones; rate, 11 stimulus/seg, 5 μV (200 nV) amplification; 150 and 3.000 Hz low and high pass filters. Wave I was identified and the latency was measured. The wave I was evaluated on different thresholds to detect the hearing loss.

4.3.2. Anatomical evaluation. The anatomy of the organ of Corti and the presence of outer hair cells and their alterations were determined by scanning electron microscopy. After ether anesthesia, the guinea pigs were sacrificed at predetermined times after ip administration of the drugs. The animals were then decapitated and their cochleae were removed from the bullae. After microscopic dissection, the cochlea was perfused with 3% glutaraldehyde at 4 °C for 24 h for fixation. The subsequent steps were carried out in the Electron Microscopy Laboratory of the Department of Cell and Molecular Biology and Pathogenic Bioagents, Faculty of Medicine of Ribeirão Preto, USP. A 3% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4, was injected through the round window for fixation over a period of 4 h at 4 °C. The preparations were washed three times for 5 min with the same buffer,

fixed with 1% osmium tetroxide for 2 h at 4 °C, and then dehydrated at room temperature with an increasing ethanol series (50%, 70%, 90%, and 95%) for 10 min at each concentration, and three times with absolute alcohol for 15 min. After dehydration, the material was dried to the critical point in the presence of CO_2, attached to an appropriate sample holder, sputtered with gold vapor in a vacuum chamber, and examined with a JEOL JSM 5200 scanning electron microscope.

4.4. Antimicrobial activity

The compounds were tested against *M. luteus* (ATCC 9341), *S. aureus* (ATCC 6538), *S. epidermidis* (ATCC 12228), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853). Compounds **7**–**9** and **18** were dissolved in sterile water (15 mg/mL), while compounds **19** and **20**–**22** and were dissolved in DMSO and 10% DMSO/H_2O, respectively.

Micro dilution experiments were carried out with compounds **7**–**9**, **17**, **18**, and **22** using approximately 5×10^4 CFU/mL in each well receiving the sample application. The incubation for 24 h at 35 °C allowed the qualitative observation of medium turbidity which was compared to the inoculated medium as a positive control and to the medium alone as a negative control.[48] For all procedures, the compounds were evaluated in duplicate at concentration of 15 mg/mL.

Acknowledgments

The authors are grateful to Professor Zuleika Rothschild for valuable comments on the article, CAPES and CNPq for financial support and fellowship. The authors also wish to thank Cláudia Castania de Macedo from the Medicinal Chemistry Laboratory, Department of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences of Ribeirão Preto; José Augusto Maulin, Maria Dolores Seabra Ferreira, Maria Teresa Picinoto Maglia from the Electron Microscopy Laboratory, Department of Cellular and Molecular Biology and Pathogenic Bioagents and Sebastião Assis Mazzeto from the Laboratory of Experimental Surgery, Department of Surgery and Anatomy, Faculty of Medicine of Ribeirão Preto, University of São Paulo.

References and notes

1. Chambers, H. F.; Sande, M. A. In *Goodman & Gilman: The Pharmachological Basis of the Therapeutics*; Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W., Gilman, A. G., Eds., 10th ed.; McGrawHill: New York, 1996; pp 1219–1238, Chapter 46.
2. Von Ahsen, U.; Davies, J.; Schreder, R. *Nature* **1991**, *353*, 368.
3. Frankei, A. D.; Young, J. A. T. *Ann. Rev. Biochem.* **1998**, *67*, 1.
4. (a) Fourmy, D.; Recht, M. I.; Blanchard, S. C.; Puglisi, J. D. *Science* **1996**, *274*, 1367; (b) Jana, S.; Deb, J. K. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 140.
5. 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.
6. Silva, J. G.; Carvalho, I. *Curr. Med. Chem.* in press.
7. Ye, X.-S.; Zhang, L.-H. *Curr. Med. Chem.* **2002**, *9*, 929.
8. (a) Rougier, F.; Claude, D.; Maurin, M.; Sedoglavic, A.; Ducher, M.; Corvaisier, S.; Jelliffe, R.; Maire, P. *Antimicrob. Agents Chemother.* **2003**, *47*, 1010; (b) Mingeot-Leclerq, M.-P.; Tulkens, P. M. *Antimicrob. Agents Chemother.* **1999**, *43*, 1003.
9. (a) Dulon, D.; Aran, J. M.; Zajic, G.; Schacht, J.; Jochen, J. *Antimicrob. Agents Chemother.* **1986**, *30*, 96; (b) Brummet, R. E.; Fox, K. E. *Antimicrob. Agents Chemother.* **1989**, *33*, 797.
10. Watanabe, A.; Nagai, J.; Adachi, Y.; Katsube, T.; Kitahara, Y.; Murakami, T.; Takano, M. *J. Controlled Release* **2004**, *95*, 423.
11. Corrado, A. P.; Morais, I. P.; Prado, W. A. *Acta Physiol. Pharmacol. Latinoam.* **1989**, *39*, 419.
12. Kotecha, B.; Richardson, G. P. *Hearing Res.* **1994**, *73*, 173.
13. Kalkandelen, S.; Selimoglu, E.; Erdogan, F.; Ucuncu, H.; Altas, E. *J. Int. Med. Res.* **2002**, *30*, 406.
14. Selimoglu, E.; Kalkandelen, S.; Erdogan, F. *Yonsei Med. J.* **2003**, *44*, 517.
15. Owada, K. *Chemotherapia* **1962**, *5*, 277.
16. Lesniak, W.; Pecoraro, V. L.; Schacht, J. *Chem. Res. Toxicol.* **2005**, *18*, 357.
17. Takumida, M.; Anniko, M. *Acta Otolaryngol.* **2001**, *121*, 346.
18. Darlington, C. L.; Smith, P. F. *Curr. Opin. Invest. Drugs* **2003**, *4*, 841.
19. Segal, J. A.; Skolnik, P. *Eur. J. Pharmacol.* **1998**, *347*, 311.
20. Fischel-Ghodsian, N. *Pharmacogenomics* **2005**, *6*, 27.
21. Paparella, M. M.; Costa, S. S.; Cruz, O. L. M. Doença de Ménière e outras vestibulopatias periféricas. In *Otorrinolaringologia Princípios e Prática*; Costa, S. S., Cruz, O. L. M., Oliveira, J. A. A., Eds., 1st ed.; Artes Médicas Sul: Porto Alegre, 1994; pp 238–248, Chapter 25.
22. Minor, L. B.; Schessel, D. A.; Carey, J. P. *Curr. Opin. Neurol.* **2004**, *17*, 9.
\23. Stokroos, R.; Kingma, H. *Acta Otolaryngol.* **2004**, *124*, 172.
24. Van de Heyning, P. H.; Wuyts, F.; Boudewyns, A. *Curr. Opin. Neurol.* **2005**, *18*, 23.
25. Flanagan, S.; Mukherjee, P.; Tonkin, J. *J. Laryngol. Otol.* **2006**, *120*, 98.
26. Müller, M.; Smolders, J. W. T. *Hearing Res.* **1998**, *120*, 25.
27. Alper, P. B.; Hendrix, M.; Sears, P.; Wong, C.-H. *J. Am. Chem. Soc.* **1998**, *120*, 1965.
28. Ford, J. H.; Bergy, M. E.; Brooks, A. A.; Garrett, E. R.; Alberti, J.; Dyer, J. R.; Carter, H. E. *J. Am. Chem. Soc* **1955**, *77*, 5311.
29. Botto, R. E.; Coxon, B. *J. Am. Chem. Soc.* **1983**, *105*, 1021.
30. Rinehart, K. L.; Argoudelis, A. D.; Culbertson, T. P.; Chilton, W. S.; Streigler, K. *J. Am. Chem. Soc.* **1960**, *82*, 2970.
31. Busscher, G. F.; Rutjes, F. P. J. T.; Van Delf, F. L. *Chem. Rev.* **2005**, *105*, 775.
32. Leach, B. E.; Teeters, C. M. *J. Am. Chem. Soc.* **1952**, *74*, 3187.
33. Molyneux, R. J.; Gardner, D. R.; James, L. F.; Colegate, S. M. *J. Chromathogr. A* **2002**, *967*, 57.
34. Van, T. D.; Khac, D. D.; Fetizon, M.; Tabet, J.-C.; Beyec, Y. L.; Quyen, T. C. *J. Chromathogr.* **1988**, *440*, 71.
35. Kenneth, L.; Rinehart, W.; Chilton, S.; Hichens, M. X. I. *J. Am. Chem. Soc.* **1962**, *84*, 3216.

36. Umezawa, S.; Okazaki, Y.; Tsuchiya, T. *Bull. Chem. Soc. Jpn.* **1972**, *45*, 3619.
37. Jikihara, T.; Tsuchiya, T.; Umezawa, S.; Umezawa, H. *Bull. Chem. Soc. Jpn.* **1973**, *46*, 3507.
38. Kumar, V.; Remers, W. A. *J. Org. Chem.* **1978**, *43*, 3327.
39. Dutcher, J. D.; Donin, M. N. *J. Am. Chem. Soc.* **1952**, *74*, 3420.
40. Myszka, H.; Bednarczyk, D.; Najder, M.; Kaca, W. *Carbohydr. Res.* **2003**, *338*, 133.
41. Alper, P. B.; Hung, S. C.; Wong, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6029.
42. Creary, X.; Wang, Y.-X.; Gill, W. *Tetrahedron Lett.* **1991**, *32*, 729.
43. Nyffeler, P. T.; Liang, C.-H.; Koeller, K. M.; Wong, C.-H. *J. Am. Chem. Soc.* **2002**, *124*, 10773.
44. Moore, R. D.; Smith, C. R.; Lietman, P. S. *J. Infect. Dis.* **1994**, *149*, 23.
45. Nakashima, T.; Teranishi, M.; Hibi, T.; Kobayashi, M.; Umemura, M. *Acta Otolaryngol.* **2000**, *120*, 904.
46. Govaerts, P. J.; Claes, J.; Van de Heyning, P. H.; Jorens, P. G.; Marquet, J.; De Broe, M. E. *Toxicol. Lett.* **1990**, *52*, 227.
47. Akiyoshi, M. Ototoxicity. In *Aminoglycoside*; Ueda, Y., Ed.; Nankohdo: Tokyo, 1985; pp 123–140.
48. Agência Nacional de Vigilância Sanitária (ANVISA); Metodologia dos Testes de Sensibilidade a Agentes Antimicrobianos por Diluição para Bactéria de Crescimento Aeróbico: Norma Aprovada. 6[a] ed., 2003; Vol. 23, Chapter 7, p 14–17.
49. Ban, N.; Nissen, P.; Hansen, J.; Moore, P. B.; Steitz, T. A. *Science* **2000**, *289*, 905.
50. Lima, A. L. C. M.; Marseillan, R. F.; Corrado, A. P. *Symposia of the Giovanni Lorenzini Foundation*. **1981**, *10* (New Trends Antibiot.: Re. Ther.), 274.
51. Bamonte, F.; Barbara, M.; Dionisotti, S.; Ongini, E.; Melone, G.; Takumida, M.; Wersäl, J. *J. Drug Dev.* **1988**, *1*(Suppl. 3), 97.
52. Leach, B. E.; Teeters, C. M. *J. Am. Chem. Soc.* **1951**, *73*, 2794.
53. Suami, T.; Nishiyama, S.; Ishikawa, Y.; Katsura, S. *Carbohydr. Res.* **1978**, *65*, 57.