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Synthesis of neamine-based pseudodisaccharides as potential vestibulotoxic agents to treat vertigo in Ménière's disease

Flávio Roberto Pinsetta [a], Daniel Fábio Kawano [a,b], Marcelo Rodrigues de Carvalho [a], José Antônio A. de Oliveira [c], Alexandre P. Corrado [c], Miguel Ângelo Hyppolito [c], Ivone Carvalho [a,*]

[a] Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café s/n, 14040-903 Ribeirão Preto-SP, Brazil
[b] Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, 90610-000 Porto Alegre-RS, Brazil
[c] Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. dos Bandeirantes 3900, 14049-900 Ribeirão Preto-SP, Brazil

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ABSTRACT

Ménière's disease (MD) is a progressive disease of the inner ear characterized by recurring attacks of disabling vertigo, hearing loss and tinnitus. Patients who do not respond to vestibular sedatives or steroids may require an intratympanic application of aminoglycoside antibiotics, which destroys the vestibular function of the affected ear in order to avoid the debilitating vertigo attacks. Although effective, this procedure causes hearing loss in almost one third of the patients due to the aminoglycosides cochlear toxicity. Here we describe the synthesis of two pseudodisaccharides structurally related to neamime aiming to mimic the aminoglycosides pharmacophore core by replacing their toxic amine by azide and hydroxyl groups. Products **1** and **2** selectively promoted 'in vivo' damage to vestibular tissues without causing hearing loss or cochlear toxicity. Therefore, these pseudodisaccharides stand as promising lead compounds for the development of a safer and more effective therapeutic procedure to manage the symptoms of MD severe dizziness.

1. Introduction

Ménière's disease (MD) is a progressive inner ear disease which is characterized by vertigo, tinnitus, hearing loss and ear pressure sensation.[1] Patients with MD usually experience recurrent attacks of vertigo with nausea and vomiting, drop attacks (which consist of sudden falls without loss of consciousness) and a transient or permanent tinnitus, fluctuating hearing loss and an intermittent sensation of fullness within the impaired ear.[2] There is considerable disagreement in the literature about the incidence of MD and, depending on the study, it may range from 10 to 1000 per 100,000 population.[2] Nevertheless, due to its low incidence, MD can be considered an orphan disease which, in the absence of government incentives to guarantee a minimal commercial return on investment, generally attracts little or no interest from pharmaceutical companies.[3]

Currently available treatments for MD cannot reverse the underlying causes of the disorder but aims to relieve the symptoms and hearing preservation; it may include diet, vestibular exercises for disequilibrium, diuretics and vestibular suppressant medications.[4] Some patients may still require surgical procedures to destroy the vestibular function of the affected ear in order to suppress the debilitating vertigo attacks.[5] Among the surgical procedures, the intratympanic application of vestibulotoxic aminoglycoside antibiotics, such as gentamicin and streptomycin, is regarded as safer and more effective process than the intracranial surgery, even though it may still cause hearing loss in almost one third of the patients due to the aminoglycoside cochlear toxicity.[1] These naturally occurring antibiotics contain a pseudoglycoside commonly known as neamine, in which the 2-deoxystreptamine portion (II) is glycosylated with an amino-substituted glucopyranoside moiety (I and IV) (Fig. 1).[6] In the neomycin class, the amino sugar moieties I and IV are connected to 2-deoxystreptamine at C-4 (Fig. 1, II) and C-5 positions (intercalated by a furanosyl moiety III), while in kanamycin, gentamicin and amikacin classes the glycosidic bonds are at C-4 and C-6. Regarding the pharmacophore core, neamine (I + II) is the minimal antibiotic scaffold that can disrupt the binding to the rRNA A-site and promote the antimicrobial activity, while the amino sugar IV is not essential for drug–RNA interactions.[1] On the other hand, the 3-amino sugar of kanamycin, comparable to unit IV of neomycin, is a cochlear toxic agent, which produces guinea pig deafness in a higher rate than the parent aminoglycoside antibiotic.[7] Neamine itself is practically non-toxic to the cochlear system when tested as separate units (I or II) in guinea pigs by transtympanic administration.[7,8] Therefore, neamine can be considered a potent lead compound for the development of safer vestibulotoxic aminoglycoside antibiotics.

* Corresponding author. Tel.: +55 16 3602 4709; fax: +55 16 3602 4178.
E-mail address: carronal@usp.br (I. Carvalho).

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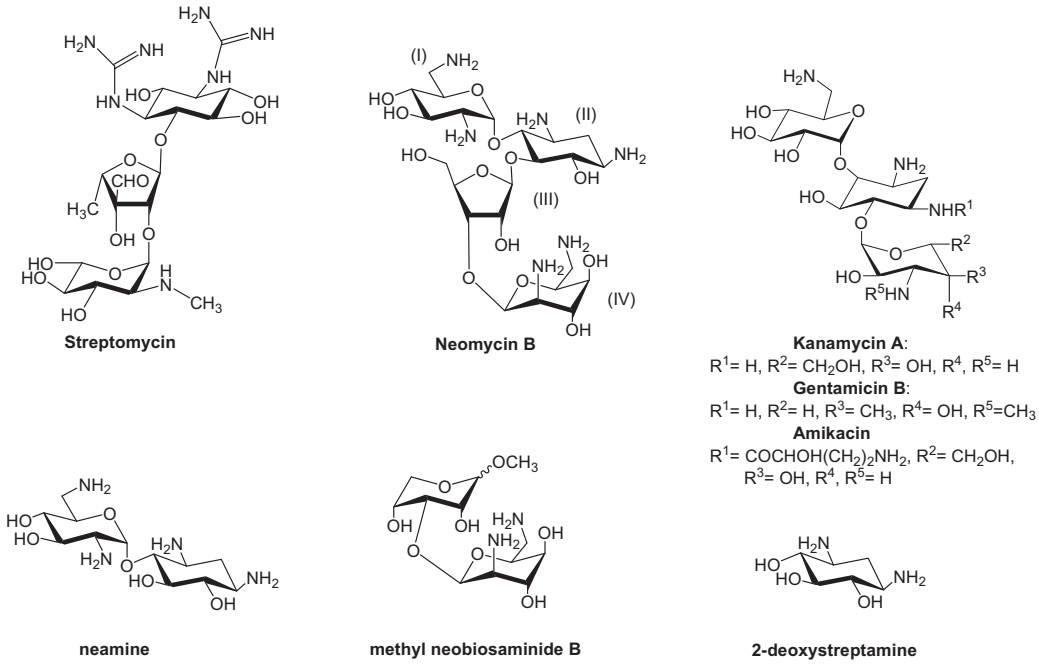


Figure 1. Chemical structures of aminoglycoside antibiotics related to streptomycin, neomycin B, kanamycin A, gentamicin B and amikacin, and the methanolysis products (fragments) of neomycin B such as neamine, methyl neobiosaminide B and 2-deoxystreptamine.

All aminoglycoside antibiotics used in clinical practice have toxic effects on cochlear and vestibular functions but they vary in selectivity. Comparisons regarding the aminoglycosides toxicity profiles must be made in view of their antibacterial potency, since nontoxic compounds may also be less active against a certain bacterial strain. Despite the relative potency against *Staphylococcus aureus* being: gentamicins > neomycin = amikacin > neamine,[9,10] the potential of these antibiotics to cause vestibular plus cochlear toxicity is: neomycin > gentamicins > amikacin > neamine.[11]

Early studies on structure–toxicity relationship pointed out that ionisable amino groups of aminoglycoside antibiotics are responsible for the cochlear toxicity[8] and, further on, for the generation of free radicals from aminoglycosides with iron complexes.[12] According to this hypothesis, complexation of aminoglycosides with iron increases iron-catalyzed oxidations and, consequently, the formation of free radicals and reactive oxygen species that will conduce to apoptotic cell death.[12,13] Using copper(II)–gentamicin complexes as a model, it was demonstrated that metal binding occurs via two of the amine nitrogens of a sugar moiety and a deprotonated oxygen of the hydroxyl group of the 2-deoxystreptamine unit.[14] Alternative ototoxic mechanisms related to the activation of the *N*-methyl-D-aspartate receptors (NMDA) in the cochlea might also be ascribed to the presence of several basic amino groups on aminoglycoside and aminocyclitol antibiotic units.[15]

In this regard, we have been studying the damage of cochlear and vestibular tissues by neomycin B intratympanic applications and its corresponding fragments, such as neamine, 2-deoxystreptamine and methyl neobiosaminide B. The results revealed that both neomycin B and neamine were able to disrupt the normal BEAP and DPOAE patterns, whereas for the vestibulotoxicity a ratio of 50 and 100%, was observed, respectively.[16] In contrast, 2-deoxystreptamine, as the smallest antibiotic fragment, was non-toxic, preserving intact the saccular and utricular sensory cells and ampullary sensory cells, while methyl neobiosaminide B led to selective vestibular activity related to utriculus and sacculus damages, maintaining a normal cochlear functional status on the brainstem evoked auditory potential (5 dB) and normal otoacoustic emission (present) at a concentration of 88 mg/mL (0.22 mol L^{-1}). In addition, the influence of the ionizable amino groups of neamine on the cochlear over vestibular toxicities was also pursued by testing the corresponding tetra-azide analogue, but in this case, the results were not conclusive due to its high local absorption probably increased by the presence of DMSO, which leads to systemic toxicity.[16]

From these findings, we speculated if simpler structures than the larger antibiotics, such as pseudodisaccharides lacking the toxic amino groups, could mimic the neamine core scaffold and promote selective damage to vestibular tissues without causing hearing loss or cochlear toxicity in animal models. Thus, the four basic amino groups of neamine (Fig. 1), represented by the 4-O-linked 1,3-diaminocyclitol (2-deoxystreptamine, unit II) to the 2,6-diamino-2,6-dideoxy-D-glucopyranosyl moiety (unit I), were replaced by azide and hydroxyl groups in order to produce 4-O-linked 1,3-diazidocyclitol to a D-glucopyranosyl portion, compound **1**. In addition, the importance of the pseudodisaccharide glycosyl moiety was also investigated by linking the 1,3-diazidocyclitol to a D-galactopyranosyl unit, compound **2**. These compounds were tested for brainstem evoked auditory potential (BEAP) and distortion product otoacoustic emissions (DPOAEs), besides scanning electron microscopy (SEM) to assess the damage caused in the cochlear and vestibular hair cells. Based on these analyses, we found that both pseudodissaccharides **1** and **2** showed similar results highlighted by their potent vestibulotoxicity and concomitant maintenance of the cochlear tissue and hearing function.

2. Results and discussion

Early attempts to treat vertigo attacks associated to MD were accomplished with local anesthetic (e.g., lidocaine), albeit no longer used in clinical practice.[17] Currently, the intratympanic application of aminoglycoside antibiotics, mainly gentamicin, and steroids (dexamethasone and methylprednisolone) has become increasingly popular in treating MD hearing loss, in spite of the fact that more detailed studies are still required to confirm the efficacy of

the treatment and elucidate their exact mechanism of action.[18,19] Alternatively, the intratympanic application of the antiviral ganciclovir to inhibit a neurotropic virus, probably involved in the internal auditory MD polyganglionitis, gave no conclusive results since both treated and control group patients showed similar improvements of vertigo symptoms.

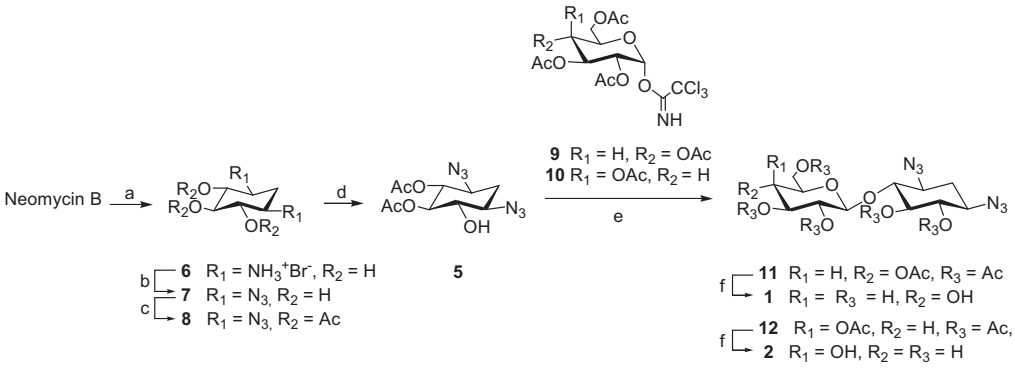
The outcomes of this scenario with the clear need for new drug candidates to replace the current therapy,[20] besides the vestibulotoxic profile shown by neamine and methyl neobiosaminide B,[16] led us to synthesize pseudodisaccharides 4-*O*-(β-D-glucopyranosyl)-1,3-diazido-1,2,3-trideoxy-*myo*-inositol (**1**) and 4-*O*-(β-D-galactopyranosyl)-1,3-diazido-1,2,3-trideoxy-*myo*-inositol (**2**) by selective glycosylation of the partially protected 1,3-diazido-1,2,3-trideoxy-*myo*-inositol with the glucose and galactose trichloroacetimidate donors **3** and **4**, respectively (Scheme 1). Accordingly, we started the preparation of the intermediate 5,6-di-*O*-acetyl-1,3-diazido-1,2,3-trideoxy-*myo*-inositol (**5**) through neomycin B fragmentation using 48% hydrobromic acid at 100 °C to produce the *meso* 2-deoxystreptamine bromhydrate (**6**) in 59% yield.[21] The amino groups of **6** were then converted into azides using triflic azide and a catalytic amount of $CuSO_4$[22] to give the corresponding 1,3-diazido-1,2,3-trideoxy-*myo*-inositol (**7**) in 55% yield. Subsequent acetylation of hydroxyl groups, using acetic anhydride and DMAP in pyridine, afforded compound **8** (72% yield). Despite the two known strategies for the enantioselective mono-deacetylation of **8**,[23,24] we used the resin-immobilized lipase from *Candida antarctica*, namely Novozym 435, since the absolute stereochemistry of **8** was already established by its conversion into paromamine (obtained from the glycosylation of **8** with 2-azido-2-deoxy-3,4,6-tri-*O*-benzyl-1-phenylthio-α-D-glucopyranoside, followed by reduction/deprotection reactions) and its comparison with spectra data with the same product obtained from the natural source,[23] whereas the Ley and co-workers procedure,[24] involving dispiroketal protection/desymmetrization chemistry proved not to be convenient for the further glycosylation reaction due to the bulk steric influence of the dispiroketal protecting group.[23] Therefore, the enantiomerically pure 5,6-di-*O*-acetyl-1,3-diazido-1,2,3-trideoxy-*myo*-inositol (**5**) was obtained in 63% yield, after purification by chromatographic column.

The subsequent glycosylation of **5** with either α-trichloroacetimidates of glucose **9** or galactose donors **10**, prepared as previously described,[25–28] in the presence of trimethylsilyl trifflate in anhydrous dichloromethane gave the intermediates **11** and **12** in 89% and 86% yields, respectively, which were quantitatively deprotected[29] to afford the desired pseudodisaccharides **1** and **2**.

Cochlear and vestibular activities of the pseudodisaccharides **1** and **2** were evaluated by brainstem evoked auditory potential (BEAP) and distortion product otoacoustic emissions (DPOAEs) analysis through the Intelligent Hearing Systems (Miami, FL, USA). To assess the relative damage caused by morphofunctional and histopathological alterations in the cochlear and vestibular tissues, transtympanic administration of the compound was performed in the right ear (target ear) of the albino guinea pig (*Cavia porcellus*) and the left one was kept as control. This procedure proved to be more convenient than the systemic application since it resembles the Ménière Disease 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 cochlear hair cells (Corti organ), and vestibular hair cells in semicircular channels, utriculus and sacculus, using JEOL scanning microscope JSM 5200.[30]

Based on the analysis of the data from BEAP and DPOAEs (Table 1), the transtympanic administration of **1** and **2** gave similar results and revealed that they did not cause damages in the cochlear hair cells, thus, preserving the animals' normal hearing function. Therefore, the difference of the threshold values necessary to induce BEAP before and after treatment with both products, which ranged between 5 and 15 dB, showed the audition maintenance and cochlear nucleus integrity. This observation was also supported by the SEM analysis that was implied in a normal morphology in the cochlear hair cells, whereas vestibular hair cells were destroyed after treatment with pseudodisaccharides **1** and **2** (Fig. 2A–D). Comparatively, altered BEAP and DPOAE responses (60–99 dB), and cochlear and vestibular hair cells morphologies were achieved when the animals were treated with neamine and neomycin B, albeit the number of animals with impaired vestibular hair cells which received neamine was greater (100%) than neomycin B (50%).

In spite of the scarce data concerning the aminoglycosides structure–toxicity relationship and the little information about the factors that drive selectivity between cochlear and vestibular toxicity, our results suggest a direct correlation between the toxicity of an aminoglycoside and the basic strength of its amino groups, which can be quantitatively expressed by the pK_a of their conjugate acids.[31] Therefore, the absence of cochlear toxicity, noticed when the four amino groups of neamine were replaced by two 'harmless' hydroxyl groups in the 2,6-diamino-2,6-deoxy-glucopyranoside moiety and two azide groups in the 2-deoxystreptamine central core of pseudodisaccharides 1 and 2, can be associated with pKa values of the azide group (4.75), which is considerably lower than amine groups in the 2-deoxystreptamine moiety (pK_aN_1 = 7.77; pK_aN_3 = 6.44).[32] Additionally, the replacement of amino by azide groups still preserves the ability to produce chelated metal complexes,[33] considered as an essential process to generate reactive oxygen species and, consequently, to induce the desired vestibular



Scheme 1. Reagents, conditions and yields: (a) HBr 48%, 100 °C, 5 days, 59%; (b) Tf_2O, NaN_3, $CuSO_4$, $H_2O/CH_3OH/CH_2Cl_2$, rt, 18 h, 55%; (c) Ac_2O, DMAP, Py, rt, 3 days, 72%; (d) Novozym 435, toluene/phosphate buffer pH 6.2, rt, 72 h, 63%; (e) TMSOTf, CH_2Cl_2, rt, 1 h, 86%; (f) 1 mol L^{-1} CH_3ONa MeOH solution, pH 10, CH_3OH, rt, 0.5 h, qtt.

Table 1
Electrophysiological and functional audiologic assessment by BEAP and DPOAEs in albino guinea pigs after and before transtympanic injection of the pseudodisaccharides **1** and **2** and their cytotoxic effect on the cochlear and vestibular hair cells assessed by SEM

Compound	Thresholds values to induce BEAP		Response to DPOAEs		Morphology of cochlear hair cells in the target ear (right) (SEM)[b]	Morphology of vestibular hair cells in the target ear (right) (SEM)[b]
	Δ Control ear (left)[a] (dB)	Δ Target ear (right)[a] (dB)	Target ear (right) (before injection)	Target ear (right) (after injection)		
1	5–10	10–15	Positive (present)	Positive (present)	Normal (100%)	Altered (100%)
2	5–10	5–10	Positive (present)	Positive (present)	Normal (100%)	Altered (100%)
Neamine	5–15	60–99	Positive (present)	Negative (absent)	Altered (100%)	Altered (100%)
Neomycin B	5–15	60–90	Positive (present)	Negative (absent)	Altered (100%)	Altered (50%)

BEAP = brainstem evoked auditory potential, 5–15 (normal thresholds values to induce BEAP, that represents normal auditory response); DPOAEs = distortion product otoacoustic emissions; SEM = scanning electron microscopy.

[a] Variation in the thresholds values necessary to induce BEAP stands for the difference between the values measured after and before the transtympanic administrations of pseudodisaccharides **1** and **2**, neamine and neomycin B in the right ear of seven, eight, six and six animals, respectively.

[b] The morphology of cochlear hair cells in the target right ear demonstrated a normal status for all animals treated with compounds **1** and **2** and an altered status for all animals treated with neamine and neomycin B. Regarding the vestibular system SEM analysis, the four tested compounds produced hair cells alterations in all animals, albeit neomycin B caused this effect only in three of the six animals.

toxicity.[34] Regarding the influence of the glucose and galactose portions of these new neamine mimic derivatives, no differences were achieved in any of the experiments, being both compounds, probably, involved in target interactions in the inner ear along with the modified cyclitol.

3. Experimental section

All chemicals were purchased as reagent grade and used without further purification. Novozym 435 (*Candida antarctica* lipase immobilized on a macroporous acrylic resin) was purchased from Novo Nordisk. Solvents were dried according to standard methods.[35] Chromatography was performed on a silica gel column (0.040–0.063 mm). Nuclear magnetic resonance spectra were recorded on Bruker Advance DRX 300 (300 MHz), DPX 400 (400 MHz) or DPX 500 (500 MHz) spectrometers. Chemical shifts (d) are given in parts per million downfield from tetramethylsilane. Assignments were made with the aid of HMQC, HMBC and COSY experiments. Accurate mass electrospray ionization mass spectra (ESI-HRMS) were obtained using positive ionization mode on a

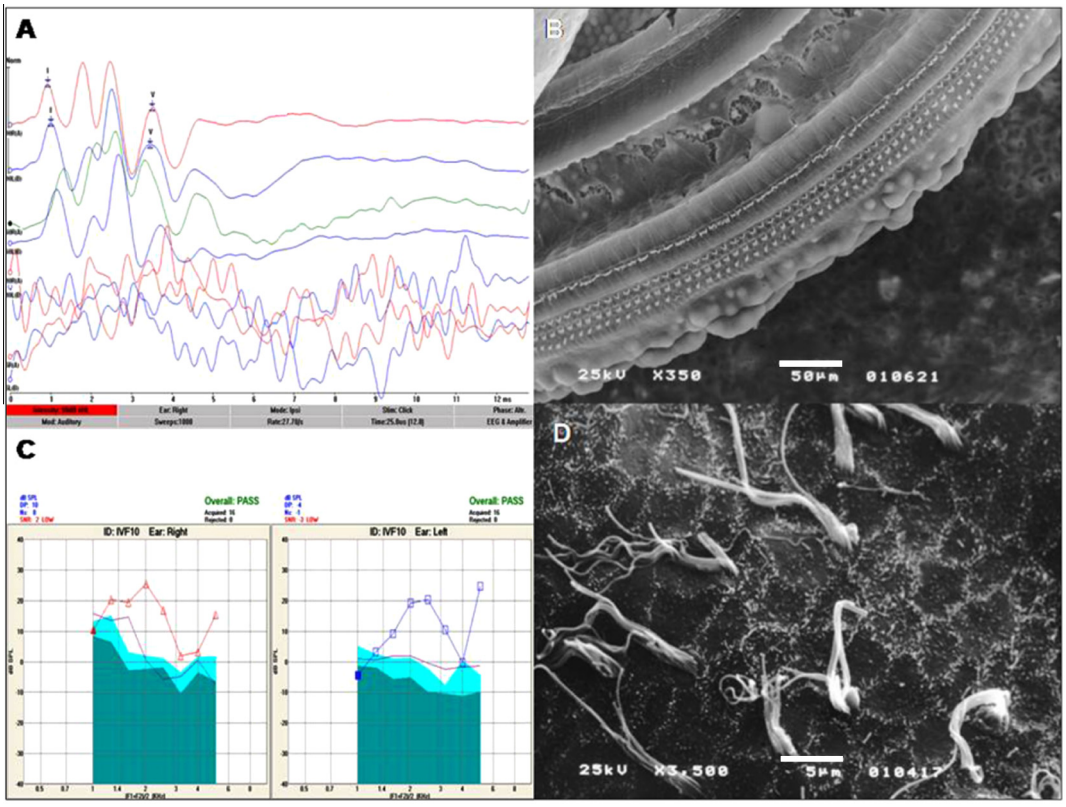


Figure 2. Brainstem evoked auditory potential (A) recorded in response to a 10 dB threshold and the distortion-product otoacoustic emissions (C) on the right ear (after treatment with **1** or **2**, highlighted in red) and left ear (control, highlighted in blue) are consistent with normal hearing and intact cochlear status. Scanning electron microscopic (SEM) of the basal cochlear turn (B) of the right ear of a guinea pig treated with compounds **1** or **2** that exhibits a normal morphology of the internal and external hair cells whereas the SEM of the utricular maculae vestibular organ (D) of the same ear of the guinea pig makes evident the extensive ciliary destruction and the disarrangement and diffusion of the remaining cilia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Bruker Daltonics UltrOTOF-Q-ESI-TOF mass spectrometer. Optical rotation measurements were performed in a JASCO P-2000 digital polarimeter.

3.1. 4-*O*-(β-D-Glucopyranosyl)- and (1) and 4-*O*-(β-D-galactopyranosyl)-1,3-diazido-1,2,3-trideoxy-*myo*-inositol (2)

General procedure: Compounds **5** (48 mg, 0.16 mmol) and **9** (or **10**) (150 mg, 0.30 mmol) were dissolved in anhydrous dichloromethane under N_2 at 0 °C, treated with a trimethylsilyl triflate solution (1.78 mg, 1.5 μmol) in dichloromethane (1 mL) and the mixture stirred for 40 min. After neutralization with Et_3N, the solution was concentrated and purified by flash chromatography (ethyl acetate–hexane 2:8 v/v) to give hexa-acetylated pseudodisaccharides **11** or **12** in 89 and 86% yields, respectively (approx. 87 mg, 0.14 mmol). This compound was dissolved in methanol and completely deprotected (30 min, rt) by the addition of sodium methoxide methanolic solution (1 mol L^{-1}, pH 9–10). The solution was neutralized using Dowex® 50WX8 (H^+), filtered and concentrated to quantitatively yield the desired pseudodisaccharides **1** and **2**, which was used in the otoxicity assays without further chromatographic purification.

3.1.1. 4-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-5,6-di-*O*-acetyl-1,3-diazido-1,2,3-trideoxy-*myo*-inositol (11)

^{1}H NMR (500 MHz/$CDCl_3$): 5.22 (1H, t_{app}, *J* 9.4 Hz, H-3′), 5.08 (1H, t_{app}, *J* 9.7 Hz; H-4′), 4.91–4.99 (2H, m, H-5, H-6), 4.94 (1H, dd, $J_{2,3}$ 9.3 Hz, $J_{1',2'}$ 8.0 Hz, H-2′), 4.80 (1H, d, $J_{1',2'}$ 8.0 Hz, H-1′), 4.40 (1H, dd, $J_{5',6'a}$ 4.3 Hz; $J_{6'a,6'b}$ 12.5 Hz, H-6′a), 4.05 (1H, dd, $J_{5',6'b}$ 1.8 Hz; $J_{6'a,6'b}$ 12.5 Hz, H-6′b), 3.70 (1H, ddd, $J_{5',6'a}$ 4.3 Hz; $J_{5',6'b}$ 1.8 Hz; $J_{4,5}$ 9.6 Hz H-5′), 3.59–3.46 (3H, m, H-1, H-3, H-4), 2.37 (1H, dt, $J_{1,2eq}$ 4.4 Hz, $J_{2eq,3}$ 4.1 Hz; $J_{2eq,2ax}$ 12.9 Hz, H-2 eq), 2.10–2.01 (18H, 6s, 6 COC*H*$_3$), 1.55 (1H, q, *J* 12.9 Hz, H-2ax). ^{13}C NMR (125 MHz/$CDCl_3$) δ 169.9, 169.7 (CO), 101.0 (C-1′), 80.4 (C-4), 73.9, 73.5 (C-2′, C-5′), 72.2, 71.9, 71.5 (C-3′, C-5, C-6) 68.3 (C-4′), 62.0 (C-6′), 61.2, 58.2 (C-1, C-3), 32.6 (C-2), 21.0, 20.9 (CO*C*H$_3$).

3.1.2. 4-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-5,6-di-*O*-acetyl-1,3-diazido-1,2,3-trideoxy-*myo*-inositol (12)

^{1}H NMR (500 MHz/$CDCl_3$): 5.36 (1H, d, $J_{3',4'}$ 3.3 Hz, H-4′), 5.10 (1H, dd, $J_{1',2'}$ 7.9 Hz; $J_{2'3'}$ 10.4 Hz, H-2′), 5.02 (1H, dd, $J_{3',4'}$ 3.3 Hz; $J_{2',3'}$ 10.4 Hz, H-3′), 5.00–4.94 (2H, m, H-5, H-6), 4.74 (1H, d, $J_{1',2'}$ 7.9 Hz, H-1′), 4.16 (1H, dd, $J_{5',6'a}$ 6.6 Hz; $J_{6'a,6'b}$ 11.4 Hz, H-6′a), 4.10 (1H, dd, $J_{5',6'b}$ 7.1 Hz, $J_{6'a,6'b}$ 11.4 Hz, H-6′b), 3.90 (1H, dt, $J_{5',6'a}$ 6.6 Hz; $J_{5',6'b}$ 7.1 Hz, H-5′), 3.59–3.47 (3H, m, H-1, H-3, H-4), 2.34 (1H, dt, $J_{1,2eq} = J_{2eq,3}$ 4.3 Hz; $J_{2eq,2ax}$ 12.7 Hz, H-2 eq), 2.14–1.97 (18H, 6s, 6 COC*H*$_3$), 1.51 (1H, q, *J* 12.7 Hz, H-2ax). ^{13}C NMR (125 MHz/$CDCl_3$): δ_C 169.5 (CO), 101.4 (C-1′), 80.0 (C-4), 73.9, 71.7, 71.4, 71.0 (C-6, C-5, C-3′, C-5′), 69.5 (C-2′), 67.2 (C-4′), 61.3 (C-6′), 61.0 (C-3), 58.3 (C-1), 32.7 (C-2), 21.1 (CO*C*H$_3$), 21.0 (CO*C*H$_3$), 21.0 (CO*C*H$_3$), 20.9 (2 CO*C*H$_3$), 20.9 (CO*C*H$_3$).

3.1.3. 4-*O*-(β-D-glucopyranosyl)-1,3-diazido-1,2,3-trideoxy-*myo*-inositol (1)

$[\alpha]_D^{25}$ −4.9 (*c* 1.0 MeOH); ^{1}H NMR (300 MHz/D_2O): δ 4.58 (1H, d, $J_{1',2'}$ 7.8 Hz, H-1′), 3.79 (1H, dd, $J_{5',6'a}$ 1.5 Hz, $J_{6'a,6'b}$ 12.5 Hz; H-6′a), 3.61 (1H, dd, $J_{5',6'b}$ 5.3 Hz, $J_{6'a,6'b}$ 12.5 Hz, H-6′b), 3.54 (1H, $J_{1,2eq}$ or $J_{2eq,3}$ 4.4 Hz, $J_{1,2ax}$ or $J_{2ax,3}$ 12.1 Hz, H-1 or H-3), 3.57–3.25 (7H, m, H-4′, H-3′, H-5, H-4, H-5′, H-6, H-1 or H-3), 3.20 (1H, dd, $J_{1',2'}$ 7.8 Hz, $J_{2',3'}$ 9.3 Hz, H-2′), 2.27 (1H, dt, $J_{1,2eq} = J_{2eq,3}$ 4.4 Hz, $J_{2eq,2ax}$ 12.8 Hz, H-2 eq), 1.48 (1H, q_{app}, *J* 12.4 Hz; H-2ax). ^{13}C NMR (75 MHz/D_2O) δ 102.6 (C-1′), 82.3 (C-4), 75.8, 75.4, 75.1, 73.1, 73.0, 69.3 (C-4′, C-5, C-6, C-2′, C-3′, C-5′), 60.5 (C-6′), 60.1, 60.0 (C-3, C-1), 31.2 (C-2). ESI HRMS: *m*/*z* 399.12406 [M+Na^+]. Calcd for $C_{12}H_{20}N_6O_8$ 399.1241

3.1.4. 4-*O*-(β-D-Galactopyranosyl)-1,3-diazido-1,2,3-trideoxy-*myo*-inositol (2)

$[\alpha]_D^{25}$ +10.2 (*c* 1.0 MeOH); ^{1}H NMR (300 MHz/D_2O): δ 4.62 (1H, d, $J_{1',2'}$ 7.8 Hz, H-1′), 3.91 (1H, d, $J_{3',4'}$ 3.4 Hz, H-4′), 3.78 (1H, dd, $J_{5',6'a}$ 8.1 Hz; $J_{6'a,6'b}$ 11.5 Hz, H-6′a), 3.71 (1H, dd, $J_{5',6'b}$ 3.7 Hz; $J_{6'a,6'b}$ 11.5 Hz, H-6′b), 3.69–3.35 (8H, m, H-1, H-3′, H-5′, H-3, H-4, H-5, H-6, H-2′), 2.36 (1H, dt, $J_{1,2eq} = J_{2eq,3}$ 4.3 Hz; $J_{2eq,2ax}$ 12.8 Hz, H-2 eq), 1.48 (1H, q, *J* 12.8 Hz, H-2ax). ^{13}C NMR (75 MHz/D_2O): δ 102.3 (C-1′), 81.5 (C-4), 74.5, 74.4, 72.6, 71.8, 70.2 (C-3′, C-6, C-5, C-5′, C-2′), 68.0 (C-4′), 60.5 (C-6′), 59.6, 59.4 (C-1, C-3), 30.6 (C-2). ESI HRMS: *m*/*z* 399.1235 [M+Na^+]. Calcd for $C_{12}H_{20}N_6O_8$ 399.1241.

3.2. Ototoxicity assays

Albino guinea pigs were handled according to 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. The animals were selected from the central animal house of the University of São Paulo (USP), Campus at Ribeirão Preto, on the basis of the determination of the Preyer reflex.[36] After a hearing rest for 24 h, they were reevaluated using manual otoscopy. Albino guinea pigs showing signs of otitis external or acute otitis media, wax of difficult removal, inflammatory changes of the external auditory meatus, or a meatus too narrow to adequately accommodate the probe of the otoacoustic emission equipment were discarded.

The selected animals were then submitted to auditory screening using distortion product otoacoustic emissions (DPOAEs) and brainstem evoked auditory potential (BEAP) in an acoustically isolated cabin under anesthesia with 65 mg/kg ketamine hydrochloride and 6.5 mg/kg xylazine. Seven albino guinea pigs that showed the presence of DPOAEs in both ears and thresholds values to induce BEAP responses of 5 dB were then chosen for the transtympanic application of the pseudodisaccharides **1** or **2** in the right ear, while the left ear was used as control (distilled water).

A 0.222 mol L^{-1} aqueous solution of the pseudodisaccharides were prepared, filtered through a 0.22 μm membrane filter and an aliquot of 0.1 mL administered transtympanically in the right ears of the seven albino guinea pigs immediately after otoscopic and audiological examinations. The animals were kept in the supine position with the head tilted 45° to the healthy side for 3 h in order to prevent the leaking of the inoculated substance from the ear cleft to the Eustachian tube. Otoacoustic emissions and BEAP were determined before treatment and after 11 days, when animal were sacrificed for the electron microscopy study.

The function of outer cochlear hair cells was determined by DPOAEs using the Intelligent Hearing Systems (Miami, FL, USA). For the test, the animals were anesthetized with ketamine hydrochloride and xylazine. Before the recording of evoked otoacoustic emissions (EOAEs), the animals were submitted to manual otoscopy for the evaluation of the auditory meatus and the tympanic membrane. None of the seven albino guinea pigs showed signs of otitis or wax of difficult removal, which could cause them to be excluded from the test.

The DPOAE test was performed before treatment and immediately before sacrifice, following the relation of the 2F1–F2 frequency, with an F1:F2 ratio = 1.22, with a resolution of two points per octave. We considered otoacoustic emissions starting from 1.5 kHz, as the dimensions of the external auditory meatus of the guinea pig make it difficult to detect otoacoustic emissions below this frequency, with the occurrence of responses that coincide with noise responses, which were maintained at 10 dB for

these frequencies. In this study, we used equal intensities of 70 dB sound pressure level (SPL).

The intensity of the triggering stimulus can vary within the range 0–70 dB SPL and can be measured in the range 500–8000 Hz.[37] Resulting otoemissions usually are about 55 dB less intense than the triggering stimulus. With a stimulus of 70 dB SPL, we would probably have a DPOAE with variability of about 10 or 15 SPL, a value that varies from an individual to another. Thus, we observed the so-called DPGRAM, the audiocochleogram, in which the stimulus is a sound and the response also is a sound, and which expresses the function of the cochlear outer hair cells responsible for the frequencies analyzed. DPOAEs were scored as present or absent.

We performed the functional measurements of BEAP using the Intelligent Hearing Systems (Miami, FL, USA) according to the following parameters: (1) Click stimulus: 2000–4000 Hz, 60, 40, 20, 10 and 5 dB thresholds on tip phones; (2) Stimulus rate: 11 stimulus/seg; (3) Amplification: 5 lV (200 nV) and (4) Low and high pass filters: 150 and 3.000 Hz. Wave I was identified and the latency was measured. The wave I was evaluated on different thresholds to detect the hearing loss.[16]

The microanatomical damage to the outer hair cells was evaluated by scanning electron microscopy (SEM) using a JEOL Scanning Microscope, JSM 5200. The guinea pigs were sacrificed at scheduled times after transtympanic drug administration. Subsequently to administration of high doses of ketamine and xylazine, the animals were decapitated and the cochleae were removed from the bullae. After microscopic dissection, the cochleae were perfused with 3% glutaraldehyde solution for fixation at 4 °C for 24 h. 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 scanning electron microscope JSM 5200.[30] The results obtained by SEM were photographed and analyzed. The numbers of outer hair cells of the basal turn of the cochlea were counted in a determined photographic field, with ten cells being counted as present or absent.

4. Conclusions

In summary, we have synthesized new neamine-derived pseudodisaccharides that selectively induced vestibular but not cochlear damage 'in vivo'. Considering that the current clinical intratympanic application of vestibulotoxic antibiotics used to manage vertigo in MD still causes hearing loss in almost one third of the patients due to the cochlear toxicity,[1] pseudodisaccharides **1** and **2** stand as promising lead compounds for the development of a safer and effective therapeutic procedure to manage the symptoms of severe dizziness in Menière's disease.

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Supplementary data

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