Effects of the 1-*N*-(4-Amino-2*S*-hydroxybutyryl) and 6′-*N*-(2-Hydroxyethyl) Substituents on Ribosomal Selectivity, Cochleotoxicity and Antibacterial Activity in the Sisomicin Class of Aminoglycoside Antibiotics

Amr Sonousi[a], **Vikram A. Sarpe**[a], **Margarita Brilkova**[b], **Jochen Schacht**[c], **Andrea Vasella**[d], **Erik C. Böttger**[b], and **David Crich**[a,*]

[a]Department of Chemistry, Wayne State University, 5101 Cass Avenue, Detroit, MI 48202, USA
[b]Institut für Medizinische Mikrobiologie, Universität Zürich, Gloriastrasse 28/30, 8006 Zürich, Switzerland [c]Kresge Hearing Research Institute, Department of Otolaryngology, 1150 West Medical Center Dr, University of Michigan, Ann Arbor, MI 48109, USA [d]Laboratorium für Organische Chemie, ETH Zürich, Vladimir-Prelog-Weg 1-5/10, 8093 Zürich, Switzerland

Abstract

Syntheses of the 6′-*N*-(2-hydroxyethyl) and 1-*N*-(4-amino-2*S*-hydroxybutyryl) derivatives of the 4,6-aminoglycoside sisomicin and that of the doubly modified 1-*N*-(4-amino-2*S*-hydroxybutyryl)-6′-*N*-(2-hydroxyethyl) derivative known as plazomicin are reported together with their antibacterial and antiribosomal activities and selectivities. The 6′-*N*-(2-hydroxyethyl) modification results in a moderate increase in prokaryotic/eukaryotic ribosomal selectivity, the 1-*N*-(4-amino-2*S*-hydroxybutyryl) modification has the opposite effect. When combined in plazomicin the effects of the two groups on ribosomal selectivity cancel each other out leading to the prediction that plazomicin will exhibit comparable ototoxicity to the parent and to the current clinical aminoglycoside antibiotics gentamicin and tobramycin, as borne out by ex-vivo studies with mouse cochlear ex-plants. The 6′-*N*-(2-hydroxyethyl) modification restores antibacterial activity in the presence of the AAC(6′) aminoglycoside-modifying enzymes, while the 1-*N*-(4-amino-2*S*-hydroxybutyryl) modification overcomes resistance to the AAC(2′) class, but is still affected to some extent by the AAC(3) class. Neither modification is able to circumvent the ArmA ribosomal methyltransferase-induced aminoglycoside resistance. The use of phenyltriazenyl protection for the secondary amino group of sisomicin facilitates synthesis of each derivative and their characterization through the provision of sharp NMR spectra for all intermediates.

Graphical Abstract
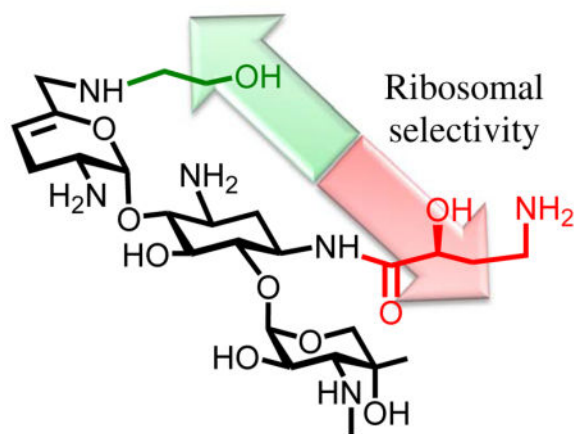
Keywords

Aminoglycosides; Structure-Activity Relationship; Aminoglycoside modifying enzymes; ribosomal methyltransferases; cell-free translation assays
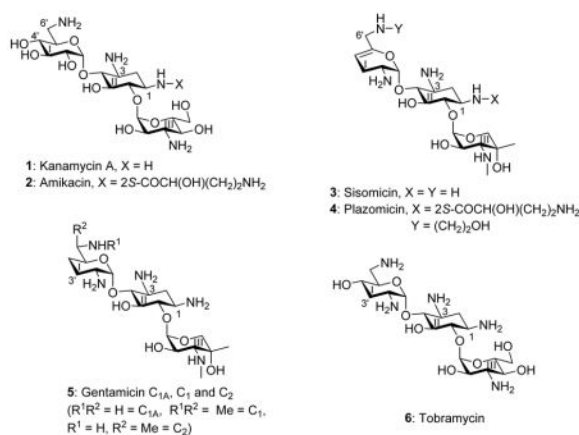
The growing threat to public health posed by the spread of multidrug-resistant infectious diseases necessitates the development of new antibiotic substances.[1–7] This may be achieved either by the discovery of new classes of antibiotic molecules or by further development of existing antibiotic classes. The aminoglycoside antibiotics (AGAs) are an attractive starting point for the latter avenue as, with more than fifty years of development and clinical application, an extensive knowledge of their mechanism of action, pharmacology, and chemistry provides a strong foundation for rational development.[8–23] The decades of clinical use have also given rise to extensive resistance to AGAs, which is mostly due to the aminoglycoside-modifying enzymes (AMEs) or the ribosomal methyltransferases (RMTases).[24–29] AMEs can be thwarted by AGA redesign to prevent modification without compromising antibacterial activity. Indeed, this has long been a strategy in the pharmaceutical industry and led to a series of semisynthetic AGAs, of which the kanamycin A **1** derivative amikacin **2** was the last to enter the clinic in the early 1990s,[19–22,30,31] and which continues with the sisomicin **3** derivative plazomicin **4** that is currently in phase III clinical trials.[32–34] Plazomicin was designed to overcome the action of most AMEs, but it is still a target for the RMTases and most notably the G1405 methyl transferases.[35] AGA modification can also impact selectivity by reducing affinity for the eukaryotic ribosomes, particularly the mitochondrial ribosome for which drug affinity underlies the major side effect of ototoxicity.[36–38] Unfortunately, little is known about the manner in which AGA modifications affect affinity for and thus selectivity among the bacterial and eukaryotic ribosomes. In our laboratories, using cell-free translation assays with bacterial wild type ribosomes and hybrid bacterial ribosomes carrying humanized decoding A sites,[39] we have begun to address these issues with particular emphasis on modification of ring I in the 2-deoxystreptamine class of AGAs.[40–48]

Continuing our studies of the relation between structure, activity, and selectivity of the AGAs, we report expedient methods for the synthesis of sisomicin derivatives modified at either or both of the *N*1 or the *N*6′-positions. These syntheses incorporate phenyltriazene protection of the secondary amino group in ring III of the parent system facilitating spectral characterization of intermediates, a notable advantage over the classical carbamate-based protecting schemes. The consequent effective availability of plazomicin **4**, a semisynthetic AGA possessing two previously known individual structural modifications (the 6′-*N* (2-hydroxyethyl and the 1-*N*-(4-amino-2*S*-hydroxybutyryl) substituents) of the parent drug, and of the two individual modifications **10** and **11** enable us to report on the contribution of these modifications, alone and in combination, to antibacterial activity and ribosomal selectivity. We further report on ex-vivo studies with mouse cochlear explants leading to the conclusion that the cochleotoxicity of plazomicin is comparable to that of typical AGAs including sisomicin **3**, gentamicin **5**, and tobramycin **6**.

1: Kanamycin A, X = H
2: Amikacin, X = 2*S*-COCH(OH)(CH₂)₂NH₂

3: Sisomicin, X = Y = H
4: Plazomicin, X = 2*S*-COCH(OH)(CH₂)₂NH₂
Y = (CH₂)₂OH

5: Gentamicin C₁ₐ, C₁ and C₂
(R¹R² = H = C₁ₐ, R¹R² = Me = C₁,
R¹ = H, R² = Me = C₂)

6: Tobramycin

Results and Discussion

Synthesis

Adopting the use of the *N*-phenyl triazenes described earlier for the selective protection of secondary amines in the presence of their primary counterparts, we oxidized the known sisomicin derivative **7**[49–51] with selenium dioxide in pyridine to afford the α,β-unsaturated aldehyde **8** in 77% yield.[52] Reductive amination of **8** with ethanolamine then afforded derivative **9** in 63% yield, from which the known sisomicin derivative **10**[51] was obtained by Staudinger reduction of the azides,[53] cleavage of the triazene with trifluoroacetic acid,[49] and purification by ion exchange chromatography over Sephadex (Scheme 1).

The preparation of the 1-*N*-(4-amino-2(*S*)-hydroxybutyryl) sisomicin **11**, a compound previously described in the patent literature[54,55] and characterized as an impurity in plazomicin from the Achaogen synthesis,[56] began with the selective protection of the 6′-NH₂ group of sisomicin **3** using 4-nitrobenzyl *N*-hydroxysuccinimidyl carbonate[33,57] and zinc acetate giving 6′-*N*-(4-nitrobenzyloxycarbonyl)sisomicin **12** in 75% yield. The amino substituents at positions 2′ and 3 were then selectively protected as the Boc derivatives by temporary protection of the other amines in the form of zinc chelates, resulting in the formation of **13** in 52% yield.[58] Reaction of **13** with a 10 mol % excess of phenyldiazonium

tetrafluoroborate in acetonitrile in the presence of potassium carbonate gave the desired triazene **14** in 88% yield. The remaining amino group was then coupled to *N*-Boc-4-amino-2(*S*)-hydroxy-butyric acid (LHABA)[59] under standard carbodiimide conditions in the presence of *N*-hydroxy-5-norbornene-2,3-dicarboximide (HONB),[60] and afforded the amide **15** in 89% yield. The 4-nitrobenzyl carbamate group was then cleaved with aqueous sodium hydroxide in dioxane to yield 70% of the amine **16**, which on treatment with TFA followed by ion exchange chromatography over Sephadex afforded the target 1-LHABA sisomicin **11**. Alternatively, reductive amination of *tert*-butyldimethylsilyloxy acetaldehyde with **16** and sodium triacetoxyborohydride[61] gave **17** in 52% yield. This reaction was conducted in the presence of Hünig's base[62] so as to avoid premature cleavage of the triazene group. Finally, removal of the Boc, silyl, and triazene groups from **17** was achieved with 50% trifluoroacetic acid in dichloromethane and gave plazomicin **4** in 40% yield after purification by chromatography over Sephadex and lyophilisation (Scheme 2).

The syntheses of **4**, **10**, and **11** all feature the use of the phenyltriazene group for the protection of secondary amines. In addition to the convenient protection of secondary amines in the presence of their primary counterparts, and because of the absence of the rotamer problems associated with the more common carbamates, the use of this protecting group affords sharp, well-resolved NMR spectra which facilitate structural elucidation. We also note that this synthesis affords the opportunity for full characterization of plazomicin, as the published synthesis provides neither experimental details, nor characterization data,[32] and the patent literature[33] reveals only high resolution mass spectrometric measurements.

Antibacterial Screening

Compounds **10**, **11** sisomicin **3** and plazomicin **4** were screened for activity against two methicillin-resistant clinical isolates of the Gram-positive *Staphylococcus aureus* (*MRSA*) and against two clinical isolates of the Gram negative *Escherichia coli* (*E. coli*) from the Diagnostic Division of the Institute of Medical Microbiology (Table 1). Gentamicin C **5**, tobramycin **6** and the structurally unusual apramycin **18**, characterized by activity in the presence of most common resistance determinants and low levels of ototoxicity,[40,63,64] were also screened against the same panel for comparison. For both the Gram-positive and the Gram-negative organisms the installation of the 6′-*N*-(2-hydroxyethyl) substituent on sisomicin as in compound **10** results in a modest 2–4 fold reduction in antibacterial activity, consistent with our earlier observations on the influence of the same substituent as applied to the 4,5-aminoglycoside neomycin B.[47,65] Similarly, the 1-*N*-LHABA sisomicin derivative **11** exhibits a small 2–4-fold decrease in activity against the same clinical strains of *MRSA* and *E. coli* (Table 1). Not surprisingly, therefore, the combination of both substituents into the single compound plazomicin **4** also results in a modest decrease in antibacterial activity against wild type *MRSA* and *E. coli* (Table 1). The moderately reduced antibacterial activity of plazomicin, as compared to the parent sisomicin, is consistent with the literature data.[32] Broad antibacterial screening was not conducted as the activity of plazomicin is widely reported in the literature,[32,66–68] nevertheless all compounds were screened against a small panel of ESKAPE pathogens (Table 2).

18: apramycin
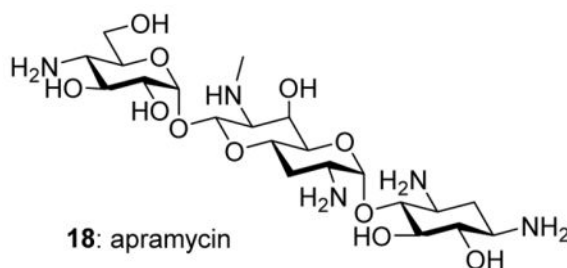
All compounds were screened for antibacterial activity against a panel of wild-type, recombinant, and clinical strains of *E. coli* carrying specific resistance determinants in order to distinguish the influence of the individual modifications (Table 3). Noteworthy is the fact that the 6′-*N*-(2-hydroxyethyl) modification restores activity to compound **10** in the presence of the aminoglycoside-modifying enzyme AAC(6′)-1 that causes a very significant drop in the activity of the parent, and of the clinical AGA tobramycin. This result is consistent with the early indications from Mallams and coworkers,[51] and with application of the same modification to neomycin B and to related 4,6-AGAs.[69,70] Modification of sisomicin with the 1-LHABA group **11** largely restores activity in the presence of AAC(3)-I, and is also effective in protecting against the action of AAC(2′) and AAC(6′)-I (Table 3). Consistent with these observations, and those of the Achaogen group,[32] plazomicin retains most of its activity in the face of the AAC(2′), AAC(3)-I, and AAC(6′)-IAMEs. Most importantly, however, as members of the 4,6-class of AGAs, all of these compounds including plazomicin succumb to resistance arising from modification of the decoding A site of helix 44 of bacterial 16S ribosomal RNA by the RMTase ArmA acting on N5 of G1405.[24,35,71–73] Apramycin **18** on the other hand, despite its moderately lower activity against wild-type bacteria (Tables 1 and 2), retains full activity in the presence of most resistance determinants including ArmA (Table 3).

Antiribosomal Activity and Selectivity

In a series of cell-free translation assays, 6′-*N*-(2-hydroxyethyl)sisomicin **10**, 1-*N*-LHABA sisomicin **11**, plazomicin **4**, sisomicin **3**, and the comparators gentamicin **5**, tobramycin **6**, and apramycin **18** were screened for inhibition of luciferase production by the bacterial ribosome. Inhibitory activities against hybrid bacterial ribosomes carrying either the complete mitochondrial decoding A site (Wild-type mitohybrid) or its A1555G mutant (Mutant deafness mitohybrid) (Figure 1), that confers hypersusceptibility to drug-induced hearing loss, were also determined (Table 4).[36,37,39] Selectivities for the bacterial decoding A site over the wild-type and mutant mitochondrial were then calculated (Table 4).

Compound **10**, the 6′-(*N*-2-hydroxyethyl) derivative of sisomicin, displays largely comparable selectivity to **3** over the wild type mitochondrial ribosomes and a two fold increase in selectivity over the A1555G mutant mitochondrial ribosomes, suggestive of modest reduction in ototoxicity and largely consistent with the influence exerted by this modification in the 4,5-aminoglycoside neomycin B.[47] The sisomicin derivative **11** carrying the 1-*N*-LHABA modification, on the other hand exhibits reduced selectivity over both the wild type and mutant mitochondrial ribosomes. Thus, at least for sisomicin the 1-*N*-LHABA modification used to overcome the action of AAC(2′) and AAC(3) AMEs attenuates

ribosomal selectivity. The opposing influences of the two modifications on ribosomal selectivity are canceled by their combination in plazomicin **4**, resulting overall in comparable selectivity to that exhibited by the parent, and suggesting that plazomicin and sisomicin will display comparable cochleotoxicity. As reported previously,[40] apramycin **18** exhibits excellent selectivity for the bacterial ribosome over both the human mitochondrial and mutant mitochondrial ribosomes (Table 4).

Cochleotoxicity

The cochleotoxicity of plazomicin **4**, and of comparators tobramycin **5**, gentamicin **6**, and apramycin **18**, was examined using the mouse cochlear explant model, as described previously for apramycin,[40] in 24 and 72 h exposures, enabling the measure of μM EC_{50} values (Table 5).

The difference in EC_{50} values for the 24 and 72 h exposures clearly indicates the importance of exposure time in these studies. While 24 h exposure requires suprapharmacological drug concentrations, the longer 72 h period better models the clinical conditions, requiring at least an order of magnitude less drug to kill 50% of the sensory cells in the explant (EC_{50}). Regardless of exposure time the data demonstrate that plazomicin has cochleotoxicity largely comparable to the clinical AGA tobramycin. The ototoxicity of tobramycin is similar to that of amikacin and sisomicin in an in-vivo guinea pig model,[74] and moderately lower than that of gentamicin. This observation is consistent with a preliminary report by Achaogen suggesting that plazomicin may be less ototoxic than gentamicin in the guinea pig model,[75] albeit only a single relatively low concentration of 80 mg/kg^{-1} was employed that does not allow for firm conclusions in this regard. It is clear from Table 5 that plazomicin is significantly more cochleotoxic than apramycin, a compound that is known to exhibit very low levels of ototoxicity in the guinea pig model.[40]

Conclusion

The 6′-*N*-(2-hydroxyethyl) and 1-*N*-(4-amino-2*S*-hydroxybutyryl) modifications of the 4,6-aminoglycoside sisomicin have been prepared and characterized, and exhibit contrasting influences on the ribosomal selectivity of the parent. Combination of the two modifications in a single derivative, the experimental drug plazomicin, results in effective cancellation of their effects on ribosomal selectivity and the prediction that plazomicin will exhibit comparable ototoxicity to the parent sisomicin and other AGAs in current clinical practice. This conclusion is clearly borne out ex-vivo studies with mouse cochlear ex-plants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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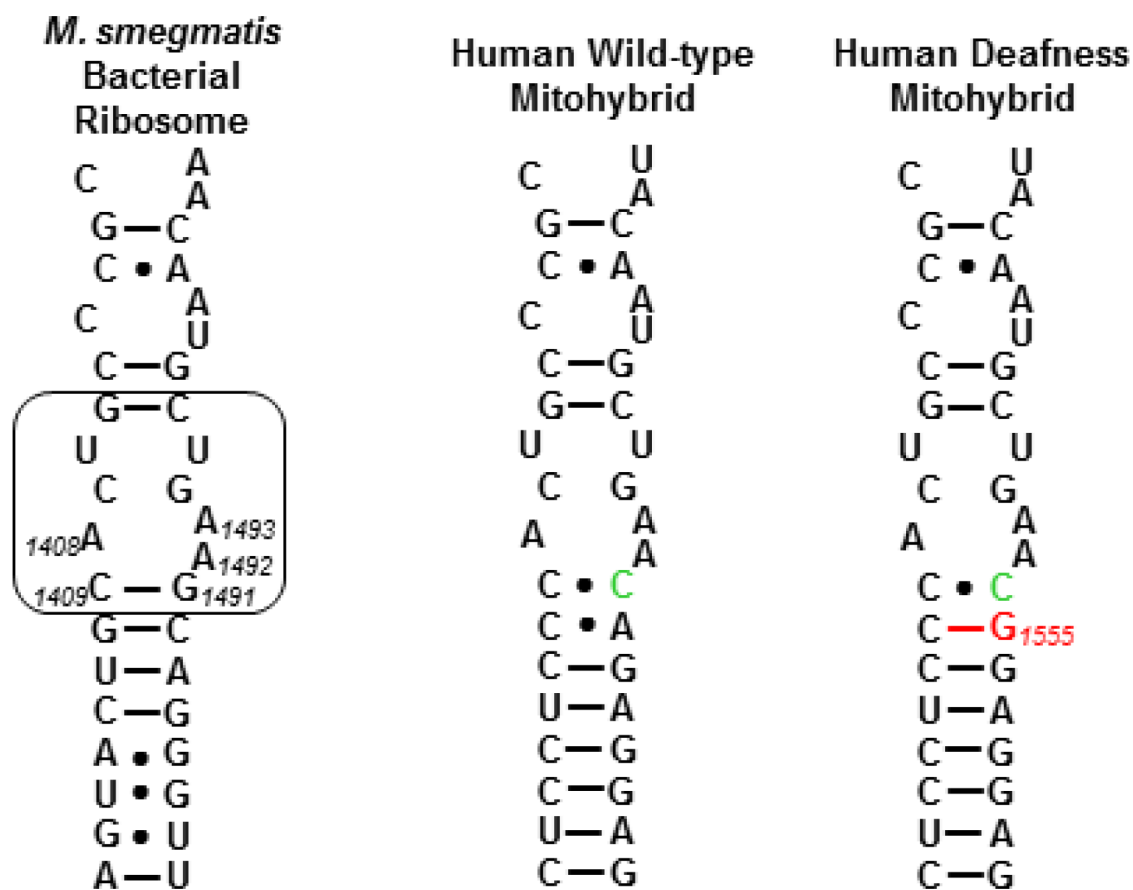
Figure 1.
Decoding A sites of bacterial, human wild-type mitohybrid, and human deafness mitohybrid ribosomes. The AGA binding pocket is boxed. The bacterial numbering scheme is illustrated for the AGA binding pocket. Changes from the bacterial ribosome binding pocket are coloured green. The A1555G mutant conferring hypersusceptibility to AGA ototoxicity is coloured red.
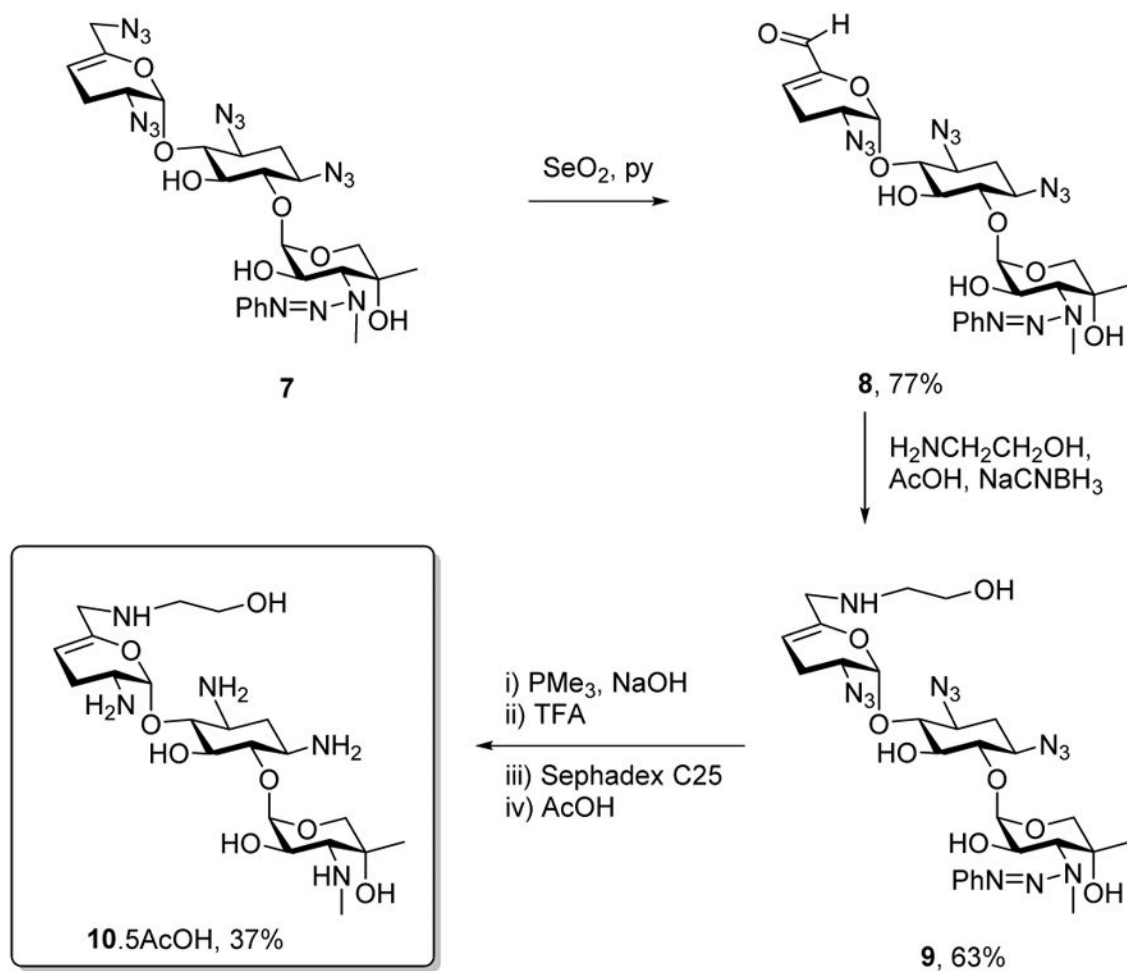
Scheme 1.
Synthesis of 6′-*N*-(2-hydroxyethyl)sisomicin **10**
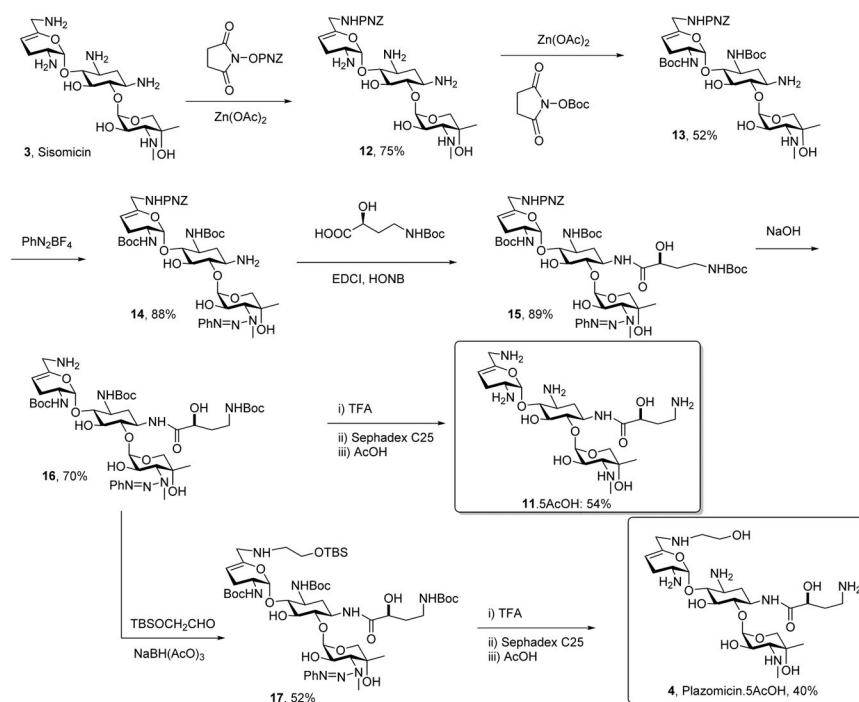
Scheme 2.
Synthesis of 1-*N*-LHABA sisomicin **11** and of plazomicin **4**

Table 1

Antibacterial Activities (MIC, µg/mL)[a]

| | MRSA | | E. coli | |
	AG038	AG039	AG001	AG055
Sisomicin **3**	0.25	0.25	0.5	0.25
6′-(2-Hydroxyethyl)sisomicin **10**	1	1	1–2	1
1-LHABA sisomicin **11**	0.5	1	2	0.5
Plazomicin **4**	2	2	2	2
Gentamicin **5**	0.25	0.25	1–2	0.5
Tobramycin **6**	0.5–1	>128	4–8	2
Apramycin **18**	4	4	4	4

[a]All values were determined in duplicate using twofold dilution series.

Table 2

Activity Against ESKAPE Pathogens (MIC, μg/mL)[a]

	E. coli (AG212)	K. pneu (AG215)	A. baum (AG225)	E. cloacae (AG290)	P. aerug (AG220)
Sisomicin **3**	0.25–0.5	0.125	0.5	0.25–0.5	0.25–0.5
6′-(2-Hydroxyethyl)sisomicin **10**	1	0.5	8–16	0.5–1	2–4
1-LHABA sisomicin **11**	0.25–0.5	0.125	1	0.5	1
Plazomicin **4**	1	0.25–0.5	2–4	0.5–1	0.5–1
Gentamicin **5**	0.5–1	0.25	0.5–1	0.25	1
Tobramycin **6**	1	0.25–0.5	1	0.5	0.5
Apramycin **18**	4	1–2	4	2–4	4

[a]All values were determined in duplicate using twofold dilution series.

Table 3

Antimicrobial Data Against Wild Type, Engineered Strains, and Clinical Strains of *E. coli* Carrying Specific Resistance Determinants (MIC, μg/mL)[a]

Strain	AG006[b]	AG007[b]	AG105[b]	AG036[b]	AG037[b]	AG103[b]	ATCC25922[c]	AG175[c]
Resistance Mechanism	WT	AAC(3)-I,	AAC(2′)	ANT(4′,4″)	APH(3′)-IIIa	ArmA	WT	AAC(6′)-I
Sisomicin **3**	0.25	16	>64	0.125	0.125	>64	0.5	16–32
6′-(2-Hydroxyethyl)sisomicin **10**	0.5	>64	>64	0.125	0.25	>64	1	1–2
1-LHABA sisomicin **11**	0.25	1	0.25–0.5	0.125	0.125	>64	0.5	1–2
Plazomicin **4**	0.5	2–4	1	0.25	0.25	>64	1	1–2
Gentamicin **5**	0.25	32	64	0.125	0.125	>64	0.5–1	1
Tobramycin **6**	0.5	2	2	8	0.5	>64	1	64
Apramycin **18**	4	4–8	4	4	4	2	4–8	nd

[a] All values were determined in duplicate using twofold dilution series.

[b] Genetically engineered strain from Pasteur Institute.

[c] Clinical isolates

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Table 4

Antiribosomal Activities (IC$_{50}$ μg/mL)[a] and Selectivities

	Antiribosomal Activity[a] IC$_{50}$ μg/mL			Selectivity[b]	
	Bacterial	Wild-type Mitohybrid	Mutant Deafness Mitohybrid	Wild-type Mitohybrid	Mutant Deafness Mitohybrid
Sisomicin **3**	0.01	9.8	0.6	980	60
6′-(2-Hydroxyethyl)sisomicin **10**	0.06	59	6.6	983	110
L-HABA Sisomicin **11**	0.01	4.7	0.25	470	25
Plazomicin **4**	0.06	50	2.8	833	47
Gentamicin **5**	0.015	9	0.5	600	33
Tobramycin **6**	0.02	15	0.5	750	25
Apramycin **18**	0.08	68	38	850	475

[a] All values were determined in duplicate using twofold dilution series.

[b] Selectivity = eukaryotic/prokaryotic antiribosomal activity

Table 5

Ex-vivo Ototoxicity

	EC$_{50}$ µM	
	24 h	**72 h**
Plazomicin **4**	75–150	16.4
Tobramycin **5**	125–250	9.3
Gentamicin **6**	150	4.5
Apramycin **18**	3500	85.5