New Derivatives of Kanamycin B Obtained by Combined Modifications in Positions 1 and 6″. Synthesis, Microbiological Properties, and in Vitro and Computer-Aided Toxicological Evaluation

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Substitution of the C-1 atom in the 2-deoxystreptamine moiety of gentamicin C_2, a broad-spectrum aminoglycoside antibiotic, by an axial hydroxymethyl group has been reported to confer protection against most clinically important bacterial enzymes inactivating aminoglycosides, while simultaneously reducing the nephrotoxic potential of this drug. We report here on a similar modification of kanamycin B. Microbiological evaluation, however, revealed no useful protection, as established by the almost complete lack of activity of 1-*C*-(hydroxymethyl)kanamycin B against an array of organisms producing defined types of aminoglycoside-inactivating enzymes and against which 1-*C*-(hydroxymethyl)gentamicin C_2 and amikacin (1-*N*-[(*S*)-2-hydroxy-4-aminobutyryl]kanamycin A) are active. Moreover, toxicological evaluation, based on the in vitro measurement of the drug inhibitory potential toward lysosomal phospholipases, a predictive test of the intrinsic nephrotoxic potential of aminoglycosides, showed not decreased but rather increased toxicity. Comparative conformational analysis of the interactions of the drug with a phosphatidylinositol monolayer explained the lack of protective effect, since no significant change of the mode of insertion of the derivative in this monolayer was detected compared to that of kanamycin B. Combination of a 1-*C*-(hydroxymethyl) substituent with a 6″-chloro, 6″-azido, or 6″-acetamido substituent resulted in a partial improvement of the toxicological behavior with no loss of activity for the 6″-chloro and the 6″-azido derivatives, but not to the extent of obtaining better derivatives than kanamycin B itself. We, therefore, suggest that the advantages of an axial hydroxymethyl substituent at C-1 are probably restricted to the gentamicin family and do not extend to kanamycins. It might be concluded that the structural differences between gentamicins and kanamycins play an important, still undescribed role both in their effective recognition by aminoglycoside-inactivating enzymes, which are responsible for most of the clinically important cases of resistance to aminoglycosides, and also in the interactions with phospholipids, which in turn cause nephrotoxicity.

Introduction

Kanamycins and gentamicins are broad-spectrum antibiotics belonging to the group of 4,6-disubstituted 2-deoxystreptamine aminoglycosides. Many organisms, however, produce enzymes capable of adenylating, acetylating, and phosphorylating amino and hydroxyl functions essential for activity in naturally occurring molecules of these families.[1] A useful approach to circumvent this problem is the chemical modification of the drug molecule. The most success in this direction so far has been obtained by acylation of the N-1 function by a (*S*)-2-hydroxy-4-aminobutyryl- or (*S*)-2-hydroxy-3-aminopropionyl moiety, yielding amikacin or isepamicin, respectively.[2] These modifications were also found to reduce the nephrotoxicity of the corresponding derivative,[3] which is one of the major side effects of most natural aminoglycosides.[4] More recently, the introduction of a hydroxymethyl substituent in position 1 of gentamicin C_2 (*O*-3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1 → 6)-*O*-[2,6-diamino-2,3,4,6-tetradeoxy-6-*C*-methyl-α-D-*erythro*-hexopyranosyl-(1 → 4)]-2-deoxy-D-streptamine (see Figure 1 for structure of this compound) was reported to overcome all clinically relevant types of enzyme-mediated resistance toward gentamicin.[5,6] 1-*C*-(Hydroxymethyl)gentamicin C_2 also showed an improved tolerance with respect to nephrotoxicity in rats, associated with a decreased inhibitory potency toward lysosomal phospholipases in vitro.[7] Measurement of this inhibition has been proposed as a predictive test for the intrinsic nephrotoxicity of aminoglycosides.[3,4,8,9] We have synthesized and evaluated the antibacterial activity and intrinsic toxicity of new derivatives of kanamycin B with hydrophobic substituents in position C-6″.[10,11] We now report on the introduction of

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∥ Laboratoire de Chimie Physique des Macromolécules aux Interfaces.

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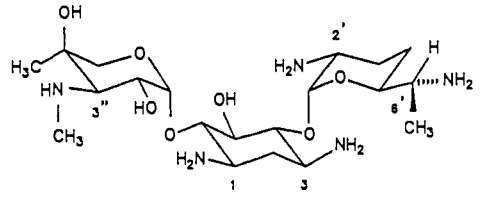
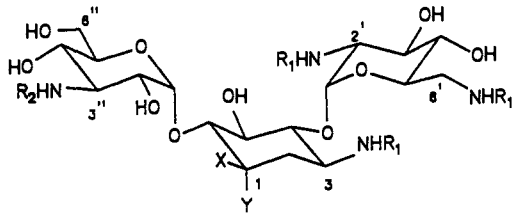


Figure 1. Structure of gentamicin C_2.



1 X = NH_2, Y = H, R_1 = R_2 = H

2 X = NH_2, Y = H, R_1 = BOC, R_2 = H

3 X = NH_2, Y = H, R_1 = BOC, R_2 = TFA

4 X = NHZ, Y = H, R_1 = BOC, R_2 = TFA

5 X = NHZ, Y = H, R_1 = BOC, R_2 = H

6 X = NHZ, Y = H, R_1 = R_2 = BOC

7 X = NH_2, Y = H, R_1 = R_2 = BOC

8 X = NO_2, Y = H, R_1 = R_2 = BOC

9 X = NO_2, Y = CH_2OH, R_1 = R_2 = BOC

10 X = NH_2, Y = CH_2OH, R_1 = R_2 = BOC

11 X = NH_2, Y = CH_2OH, R_1 = R_2 = H

12 X = CH_2OH, Y = NH_2, R_1 = R_2 = H

13 X = NHBOC, Y = H, R_1 = R_2 = BOC

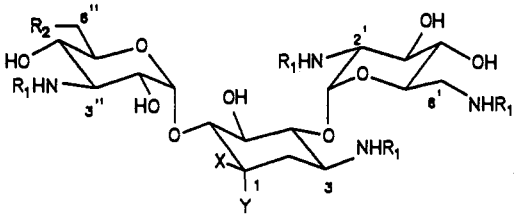
Figure 2. Structure of kanamycin B (1), 1-*C*-(hydroxymethyl)kanamycin B (11), and intermediates used for its synthesis. BOC, *tert*-butoxycarbonyl; TFA, trifluoroacetyl; Z, (benzyloxy)carbonyl.

a 1-*C*-hydroxymethyl function in kanamycin B (*O*-3-amino-3-deoxy-α-D-glucopyranosyl-(1 → 6)-*O*-[2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl-(1 → 4)]-2-deoxy-D-streptamine) and on the combination of this modification with 6″-substitutions (6″-chloro, 6″-azido, and 6″-acetamido) which modulate the inhibitory potency of kanamycin B toward lysosomal phospholipase A_1[11] without affecting to any great extent its antibacterial activity.[10]

Results

Chemical Syntheses. Figures 2 and 3 show the structural formulae of kanamycin B (1) and of all compounds synthesized during our studies. The structural formula of gentamicin C_2 is shown in Figure 1, and the structures of all other aminoglycosides mentioned in this paper are shown in refs 3, 5, and 6.

A first objective was to obtain 1-*C*-(hydroxymethyl)kanamycin B (11), the synthesis of which can be divided into two parts, namely the preparation of 7, through selective N-protection of kanamycin B (1), and the introduction of the hydroxymethyl function. To obtain the selectively protected compound 7, all of the amino groups of kanamycin B had to be protected except for the 1-amino group, which is the second in order of reactivity in acylation reactions.[12] Two different routes were followed to obtain



14 X = NHZ; Y = H; R_1 = BOC; R_2 = OTIBS

15 X = NH_2; Y = H; R_1 = BOC; R_2 = OTIBS

16 X = NO_2; Y = H; R_1 = BOC; R_2 = OTIBS

17 X = NO_2; Y = CH_2OH; R_1 = BOC; R_2 = OTIBS

BOC-18 X = NHBOC; Y = CH_2OH; R_1 = BOC; R_2 = OTIBS

BOC-19 X = NHBOC; Y = CH_2OH; R_1 = BOC; R_2 = Cl

19 X = NH_2; Y = CH_2OH; R_1 = H; R_2 = Cl

BOC-20 X = NHBOC; Y = CH_2OH; R_1 = BOC; R_2 = N_3

20 X = NH_2; Y = CH_2OH; R_1 = H; R_2 = N_3

BOC-21 X = NHBOC; Y = CH_2OH; R_1 = BOC; R_2 = NHCOMe

21 X = NH_2; Y = CH_2OH; R_1 = H; R_2 = NHCOMe

Figure 3. 6″-Modified-1-*C*-(hydroxymethyl)kanamycin B derivatives and intermediates used in their synthesis. BOC, *tert*-butoxycarbonyl; TIBS, (2,4,6-triisopropylphenyl)sulfonyl; Z, (benzyloxy)carbonyl. Numbers preceded by BOC refer to penta-*N*-BOC protected compounds.

7, using, respectively, six and four steps starting from kanamycin B, with a global yield of 24% in both cases. Both sequences started with the selective protection of the 3-, 2′-, and 6′-amino groups by *tert*-butoxycarbonylation of kanamycin B in the presence of Zn^{2+} ions.[13] The resulting tris-*tert*-butoxycarbonyl (BOC) derivative (2) was then further transformed by regiospecific trifluoroacetylation[13] of the 3″-amino group (3), N-benzyloxycarbonylation of the remaining amino function in position 1 (4), removal of the trifluoroacetyl group (5), *tert*-butoxycarbonylation of the free 3″-amino function (6), and, finally, catalytic deprotection of the amino at position 1. An alternative and more attractive sequence for the synthesis of 7 consisted of the conversion of tri-BOC derivative 2 into 5 by selective introduction of a (benzyloxy)carbonyl group at the 1-amino function by performing the reaction in the presence of Cu^{2+} ions. The known preferential complexation of vicinal hydroxy amino groups by Cu^{2+} would provide an intermediate protection of the 3″-amino function.[14,15] This was followed by *tert*-butoxycarbonylation of the 3″-amino function and deprotection of the 1-amino group. Application of the *N*-[[(benzyloxy)carbonyl]oxy]phthalimide reagent in the absence of copper complexing, which has been used[16] for the synthesis of the analogous 3,2′,6′,3″-tetra-*N*-BOC-gentamicin C_2, was unsuccessful in the case of kanamycin B. The reaction sequence used for the introduction of the hydroxymethyl group used the intermediate activation of the 1-hydrogen by transformation of the 1-amino into a 1-nitro function.[5] Thus, 7 was oxidized to the nitro derivative 8 in 45% yield

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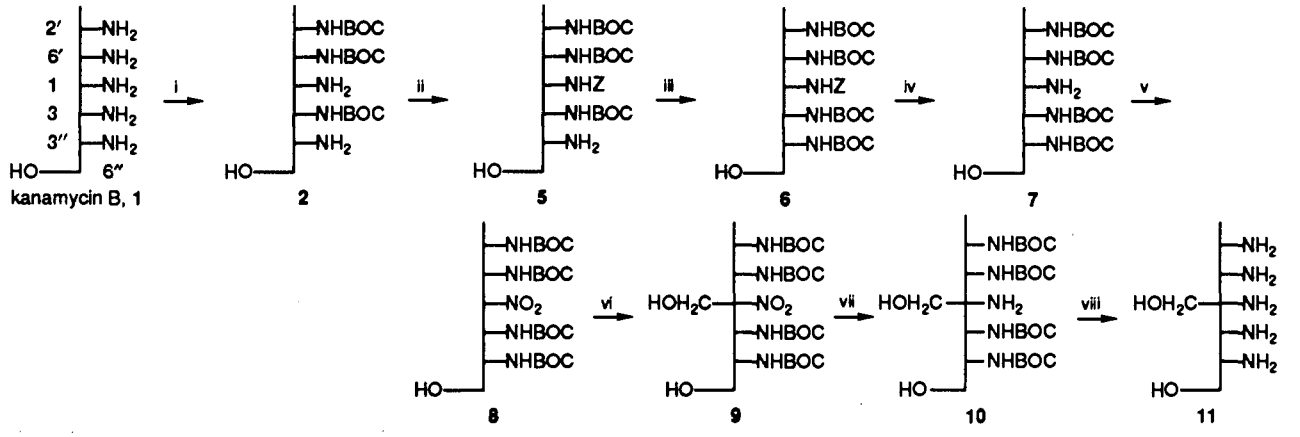
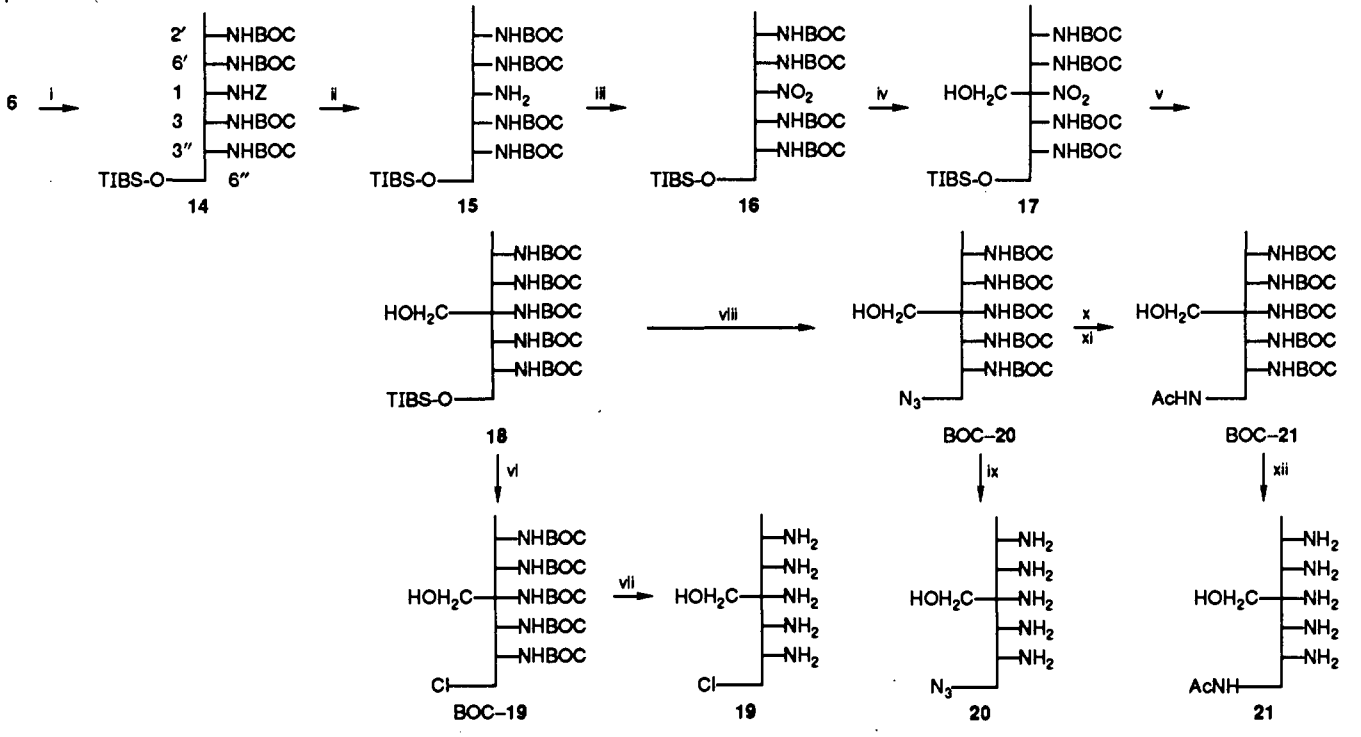
Scheme I:[a] Reaction Scheme Used for the Synthesis of 1-*C*-(Hydroxymethyl)kanamycin B (Part A) and 6''-Substituted 1-*C*-(Hydroxymethyl)kanamycin B End Products (Part B)

part A



(i) $(BOC)_2O$, $Zn(OAc)_2$; (ii) Z-*O*-succinimide, $Cu(OAc)_2$; (iii) $(BOC)_2O$; (iv) Pd/H_2; (v) *m*-chloroperbenzoic acid; (vi) CH_2O, Et_3N; (vii) Ni/H_2; (viii) CF_3COOH.

part B



(i) TIBS–Cl; (ii) Pd/H_2; (iii) *m*-chloroperbenzoic acid; (iv) CH_2O, Et_3N; (v) Ni/H_2, $(BOC)_2O$; (vi) LiCl; (vii) CF_3COOH; (viii) LiN_3; (ix) CF_3COOH; (x) $(C_6H_5)_3P$, NH_4OH; (xi) Ac_2O; (xii) CF_3COOH.

[a] BOC, *tert*-butoxycarbonyl; TIBS, (2,4,6-triisopropylphenyl)sulfonyl; Z, (benzyloxy)carbonyl.

with *m*-chloroperbenzoic acid in 1,2-dichloroethane at 90 °C followed by chromatographic purification. Introduction of a hydroxymethyl by reaction of **8** with paraformaldehyde and triethylamine gave **9** in an 80% yield. The compound was reduced catalytically (Raney nickel W_2) and the resulting **10** was deprotected with TFA, affording 1-*C*-(hydroxymethyl)kanamycin B (**11**). The order of addition of paraformaldehyde and triethylamine was very critical. Indeed, when triethylamine was added before the paraformaldehyde, an additional compound (ratio to **11** ca. 1:4) was formed which, however, was detected only after reduction and deprotection. This compound could be partially separated from **11** by ion-exclusion chromatography[17] on a Bio-Rad AG 1-X2 resin in the OH^- form using water as eluent, and analysis of the ^{13}C NMR of the enriched mixture showed that it was a C-1 epimer of **11** (compound **12**).

The presence of an additional CH_2OH group in **11** was evidenced by FAB mass spectrometry. Since the position of this newly introduced hydroxymethyl group is defined by that of the free amino group before oxidation, careful examination of the structure of the tetra-BOC-protected starting derivative **7** is of primary importance. Correct location of the different protective groups was anticipated on the basis of literature data on similar conversions of related aminoglycosides and further confirmed here by the fact that the same intermediate **5** was obtained in two clearly different and independent ways. Conclusive evidence for the proposed structures came from comparison of the ^{13}C NMR spectra of derivatives **2** and **5**–**7**, the chemical shift values of which are summarized in Table

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Table I. ^{13}C Chemical Shifts and Peak Assignments[a] for Compounds **2**, **5**–**7**, **13**[b]

carbon	2	5	6	7	13
1	51.0	51.0	50.9	50.9	50.7
2	38.0	35.4	35.4	37.9	35.4
3	50.4	50.2	50.2	50.4	50.1
4	82.8	82.5	82.8	82.8	82.7
5	76.0	75.9	75.5	75.9	75.7
6	89.6	84.3	83.9	89.2	83.8
1′	100.4	100.6	100.7	100.5	100.6
2′	56.7	56.7	56.8	56.6	56.6
3′	72.0	72.0	72.0	71.9	71.8
4′	72.4	72.5	72.5	72.4	72.5
5′	72.7	72.5	72.5	72.5	72.5
6′	41.9	41.9	41.9	41.8	41.8
1″	100.9	99.6	99.3	100.8	99.3
2″	73.6	73.5	71.6	71.7	71.5
3″	56.7	56.5	57.6	57.8	57.5
4″	71.1	70.9	69.1	69.7	69.1
5″	74.6	74.6	74.8	74.9	74.7
6″	62.0	61.9	61.7	61.9	61.6
3″-NHCO			157.8	157.9	157.8
6′-NHCO	157.6	157.2	157.3	157.5	157.2
3-NHCO	157.2	157.0	157.1	157.2	157.0
1-NHCO		156.9	156.8		156.3
2′-NHCO	155.8	155.7	155.7	155.7	155.6

[a] Assignments for close-lying lines are tentative and may be interchanged. [b] In pyridine-d_5 solution and expressed in ppm downfield from tetramethylsilane (TMS); peak positions were measured relative to the center of the solvent multiplet, set at 135.3 ppm vs TMS.

I. ^{13}C NMR has already been used before on several occasions to determine the exact position of protective groups,[18,19] or the site of metal complexation[20] in kanamycins, and since this method is fast and nondestructive, it is preferred over degradation studies often used in literature.[12,13,18,21,22] Location of the (benzyloxy)carbonyl group on the 1-amino function in intermediate **5** or **6** is clearly indicated by the typical upfield shift (β-substituent effect) of the β-carbons C-6 and C-2 in going from **2** to **5**, or by the analogous downfield shift of these carbons upon debenzyloxycarbonylation in going from **6** to **7**. Comparison of the spectrum of **7** with that of the penta-*N*-BOC derivative **13** gives additional confirmation of the presence of a free 1-amino function in **7**. Similarly, the upfield shift of carbons 2″ and 4″, when the spectrum of **5** is compared with that of **6**, indicates that the last BOC protective group was introduced on the 3″-amino function. At the same time then this conclusion fixes the structure of **2** as the 3,2′,6′-tri-BOC derivative. Our results are also consistent with the shifts observed for selectively *N*-formyl-protected aminoglycosides.[18] Because of the insolubility of **7** in D_2O, it was impossible to determine the β-carbon shift upon protonation,[23] which would have provided additional proof of the position of the free amino group in compound **7**. The chemical shifts of the carbonyls of the protective groups, indicated in Table I, allowed a convenient determination of the acylation site in kanamycin B derivatives. Indeed, the shift of these carbonyl signals seems to be characteristically related to the position of the acylated amino function, at least in pyridine solution. Regardless of the nature and number of the acyl residues (Z or BOC), the observed δ values remain almost constant and always in the same relative, descending order, i.e. 3″, 6′, 3, 1, 2′. The carbonyl shift sequence proposed here, interestingly also holds for the amide carbonyls of the different positional isomers of amikacin.[24] It seems that for such closely related substances, this may be a very useful diagnostic tool to define the exact position of the acyl group in partly acylated compounds. Chemical shift values for compounds **7**–**12** are summarized in Table II. The shift changes observed for the deoxystreptamine carbons in the spectra of **8** and **9** were all in close agreement with the α- or β-effects normally expected upon oxidation of the 1-amino function or upon introduction of a CH_2OH group. The small shift difference observed for C-1″ offered additional support for the modification being introduced at the C-1 position, since both centers are spatially proximate and may easily influence each other. Finally, from a comparison of the spectrum of the end product **11** with that of its C-1 epimer **12**, it was easily deduced that the new CH_2OH substituent in **11** is axially oriented and thus that the amino group on C-1 still has the same spatial position as before the modification. This important conclusion was based on the fact that an axially oriented substituent always resonates at a higher field than its equatorial counterpart (64.3 vs 68.0 ppm).

The second objective of this study was then the synthesis of compounds in which a 6″-modification is combined with a 1-*C*-(hydroxymethyl) substituent in the deoxystreptamine part of the molecule. Since some of the desired 6″-substituents probably would not have survived the reaction conditions to be used for the modification at C-1, it was decided to introduce the 1-*C*-(hydroxymethyl) group before the 6″-substituent. However, as it was found that the leaving group on the 6″-position used in previous work[10] (*O*-[(2,4,6-triisopropylphenyl)sulfonyl], O-TIBS) could not be introduced selectively in the presence of the 1-*C*-(hydroxymethyl) group, the 6″-OH was sulfonylated prior to the C-1 branching. The key intermediate for the synthesis of these doubly modified compounds is thus 1-*C*-(hydroxymethyl)-6″-*O*-[(2,4,6-triisopropylphenyl)-sulfonyl]penta-*N*-BOC-kanamycin B (BOC-18). For its preparation, the following sequence of reactions was applied starting from kanamycin B (**1**), namely a three-step selective protection of the amino groups, leading to **6** (with a (benzyloxy)carbonyl group on the 1-nitrogen and BOC protection on the other amino functions), 6″-O-sulfonylation with TIBS-Cl (**14**), deprotection of the amine at C-1 (**15**) and oxidation into a nitro function (**16**), and introduction of a hydroxymethyl group (**17**), followed by restoration of the amine at C-1 and its protection with a BOC group. The latter two reactions were combined in a single step to avoid intra- or intermolecular attack of the otherwise free amine on the O-TIBS function during workup, and, therefore, the catalytic reduction of the nitro group of **17** was conducted in the presence of an N-acylating agent such as $(BOC)_2O$ (di-*tert*-butyl dicarbonate). Nucleophilic displacement on the key intermediate BOC-**18**, as in the synthesis of 6″-substituted kanamycin B derivatives,[10] readily afforded the chloro and azido derivatives BOC-**19** and BOC-**20**. Reduction of the azide function of the latter, followed by acetylation, gave acetylamino analogue BOC-**21**. Numbers preceded by BOC refer to penta-*N*-BOC protected compounds. On account

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Table II. ^{13}C Chemical Shifts and Peak Assignments[a] for Protected Compounds 7–10 and Unprotected Compounds 11, 12, 1, and 19

carbon	7[b]	8[b]	9[b]	10[b]	11[c]	12[c,d]	1[c]	19[c]
1	50.9	84.6	93.8	58.7	56.8 (−4.2)	56.6 (−3.9)	51.3 (0.6)	56.5 (−4.1)
2	37.9	34.1	35.5	33.0	37.7 (7.8)	37.5 (6.2)	36.4 (7.6)	37.6 (8.1)
3	50.4	49.7	48.9	48.2	48.6 (0.4)	48.0 (0.1)	50.2 (0.7)	48.2 (0.4)
4	82.8	81.9	82.8	81.3	88.2 (10.3)	88.0 (9.9)	87.7 (9.9)	88.2 (10.7)
5	75.9	74.2	75.4	74.9	74.1 (0.1)	id.	75.3 (0.0)	73.8 (0.9)
6	89.2	81.9	84.5	85.8	90.5 (4.7)	84.3 (2.0)	88.8 (4.2)	89.9 (4.4)
1′	100.5	100.9	100.8	99.6	101.3 (5.2)	id.	101.2 (4.9)	101.0 (5.2)
2′	56.6	56.9	56.9	56.2	56.4 (1.7)	id.	56.2 (1.6)	56.0 (1.8)
3′	71.9	72.1	72.2	71.4	74.6 (4.3)	id.	74.5 (4.2)	74.3 (4.4)
4′	72.4	72.5	72.7	72.1	72.4 (0.4)	id.	72.2 (0.2)	72.1 (0.5)
5′	72.5	72.5	72.7	72.1	74.1 (4.8)	id.	74.0 (4.8)	73.5 (4.7)
6′	41.8	42.0	42.1	41.4	42.7 (1.2)	id.	42.5 (1.0)	42.3 (1.3)
1″	100.8	98.9	100.8	102.6	102.0 (−0.4)	100.6 (−0.8)	100.7 (−0.8)	101.8 (−0.3)
2″	71.7	71.1	71.3	71.4	73.2 (3.9)	id.	72.7 (3.5)	72.7 (3.9)
3″	57.8	57.3	57.4	57.0	55.2 (−0.9)	id.	55.1 (−0.9)	54.6 (−0.7)
4″	69.7	69.2	69.2	68.4	70.4 (3.9)	id.	70.2 (3.7)	70.7 (3.9)
5″	74.9	74.9	75.1	73.7	73.2 (−0.3)	id.	72.9 (−1.0)	71.7 (−0.7)
6″	61.9	61.7	61.8	61.4	61.3 (0.3)	id.	61.2 (0.2)	45.2 (0.7)
CH_2OH			60.7	60.8	64.3 (4.8)	68.0 (3.6)		64.0 (5.0)

[a,b] See notes *a* and *b* in Table I. [c] In D_2O solution; peak positions were measured relative to external DMSO, set at 40.1 ppm vs TMS; protonation shifts, given in parentheses, are calculated as $\Delta\delta = \delta(\text{pD} > 10) - \delta(\text{pD} < 1.5)$. [d] Id. = identical with the values of 11.

Table III. Minimum Inhibitory Concentrations (MIC, mg/L) of 1-*C*-(Hydroxymethyl)kanamycin B and of Kanamycin B Derivatives with Combined Substitutions in C-6″ and in C-1 against Selected Target Organisms in Comparison with Kanamycin B, Gentamicin, and Amikacin

organism and strain no.	kanamycin B[c] (6″-OH, 1-H)	11 (6″-OH, 1-CH_2OH)	19 (6″-Cl, 1-CH_2OH)	20 (6″-N_3, 1-CH_2OH)	21 (6″-$NHCOCH_3$, 1-CH_2OH)	genta-micin	ami-kacin
1. Kanamycin B Sensitive Organisms							
Gram-positive							
Staphylococcus aureus ATCC 25923	0.5	0.25	0.5	0.5	1	1	1
Staphylococcus epidermidis ATCC 12228	0.25	0.25	0.5	1.0	1	1	
Bacillus subtilis ATCC 6633	0.5	0.5	1.0	2.0	8		
Gram-negative[a]							
Escherichia coli ATCC 25922	1.0	0.5	1.0	1.0	4	1	4
Klebsiella pneumoniae ATCC 13883	0.25	0.25	0.5	0.5	2	1	
Salmonella typhimurium ATCC 14028	1.0	1.0	2.0	1.0	8	1	
Enterobacter cloacae ATCC 23355	0.25	0.25	0.25	0.25	0.25	1	1
Serratia marcescens ATCC 8100	1	1	2.0	2.0	16	1	
2. Kanamycin B Resistant Organisms							
P. aeruginosa ATCC 27853	>128	>128	>128	>128	>128	1	
S. marcescens S 69	>64	>64	>64	>64	>64	128	2
S. marcescens S20[b]	>64	>64	>64	>64	>64	128	8

[a] Selected organisms. A pattern similar to that of *K. penumoniae* and *E. coli* was observed for *Enterobacter aerogenes, Citrobacter freundii, S. marcescens, Shigella sonnei, Shigella flexneri,* and *Proteus vulgaris.* [b] Strain producing a combination of AAC-(3)-V + AAC-(6′)-I aminoglycoside modifying enzymes (see ref 1 for nomenclature). [c] Information in parentheses represents 6″-C and 1-C substituents.

of the large number of steps involved, and especially because of the low yield of the oxidation step, the overall yields were very low, usually below 5%. N-deprotection of BOC-**19**–BOC-**21** was successfully carried out with CF_3COOH at room temperature, and the compounds were then converted into their sulfate salts. Thin-layer chromatographic analysis demonstrated at least 99% purity for all compounds synthesized. Confirmation of the structures proposed for **19**–**21** was supported by FAB mass spectra and by ^{13}C NMR data. The latter are shown for compound **19** in Table II. For compounds **20** and **21**, diagnostically important signals are given in the Experimental Section. The same shift changes as for **11** were found for compounds **19**–**21**, clearly indicating the presence of a hydroxymethyl group at the expected C-1 position with retention of the natural configuration of this center. As is explained in more detail in the first paper of this series, we found shift changes for C-5″ and C-6″ with respect to intermediate **11** which were entirely analogous to those of corresponding monomodified compounds.

Microbiological Results. All unprotected end compounds were of sufficient stability to withstand incubation at 37 °C for up to 24 h, and therefore could be used for microbiological investigations. Table III shows the minimum inhibitory concentrations (MIC) of each derivative determined against selected kanamycin B sensitive and kanamycin B resistant organisms. The activities of 1-*C*-(hydroxymethyl)kanamycin B (**11**) and of the 6″-deoxy-6″-chloro and 6″-deoxy-6″-azido derivatives of 1-*C*-(hydroxymethyl)kanamycin B (**19**, **20**) were essentially similar, or only slightly lower than that of kanamycin B (**1**). Activity dropped for 6″-deoxy-6″-acetamido-1-*C*-(hydroxymethyl)kanamycin B (**21**), as described for the analogous derivative of kanamycin B.[10] In contrast, none of the compounds synthesized showed significant activity against kanamycin B resistant organisms. This included not only *Pseudomonas aeruginosa*, a bacteria which is normally sensitive to gentamicin but poorly acted upon by kanamycin B, but also two strains of gentamicin and kanamycin B resistant *Serratia marcescens* producing aminoglycoside acetyltransferase (3) V and aminoglycoside acetyltransferase (6′) I. In order to better analyze the spectrum of activity of kanamycins B substituted at C-1 toward aminoglycoside-resistant bacteria, 1-*C*-(hydroxymethyl)kanamycin B (**11**) and 6″-deoxy-6″-chloro-1-*C*-(hydroxymethyl)kanamycin B (**19**) were subjected to a systematic screening using a total of 110 different strains of a series of Gram-positive and Gram-negative organisms

Table IV. Typical MIC's (mg/L) of Compounds 11 and 19, in Comparison with Those of 1-*C*-(Hydroxymethyl)gentamicin C_2 (1-C-G_2), Amikacin (AK), and Gentamicin Complex (GM) toward Bacteria Producing Defined Aminoglycoside (AG) Modifying Enzymes[a]

AG modifying enzyme[b]	organism and strain no.	11	19	1-C-G_2	AK	GM
AAD-(2″)	E. coli OLA 290R5	8	8		2	64
	E. coli 0000JR66	>128	>256	8	2/4	32/128
	K. pneumoniae 00GT3020	>128	>256		2	32
	K. pneumoniae 75012101	4	16	2	0.5/1	64/32
AAC-(3)-I	E. coli 72091801	>128	>256		8	2
	E. coli 76070703			2	4	128
	K. pneumoniae 760070702	1	1	1	0.5/2	16/64
AAC-(3)-II	K. pneumoniae 7608271	>128	>256	1	8/2	256
AAC-(2′)	P. stuartii 00000164	>128	256		16	256
	P. stuartii 72052305	16	32		1	32
	P. stuartii 75082808			8	16	64
APH-(3′)	E. coli 001574-1	4	2		2	1
	E. coli 00000589	>128	>128		8	2
AAD-(4′)	E. coli 71120101	>128	128		4	2
	S. aureus 76080401	>128	256	0.5	8/2	<0.25/0.5
AAC-(6′)-I	E. coli 73110901	>128	128	8	256	4
AAC-(6′)-III	S. marcescens 75022704	4	8		1	0.5
AAD-(2″)+AAC-(6′)	S. marcescens 75012711	>128	>256	2	2	32

[a] AAD, adenyltransferase; AAC, acetyltransferase; APH, phosphotransferase; see refs 1, 28, and 32 for details of nomenclature). Data kindly supplied by R. Hare and G. Miller. [b] Type of aminoglycoside-modifying enzyme determined by resistance-pattern analysis.[32]

Table V. Effect of Kanamycin B, 1-*C*-(Hydroxymethyl)-kanamycin B, and Derivatives of Kanamycin B Combining Substitutions in C-6″ and in C-1 on the Degradation of Labeled Phosphatidylcholine (1-Palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) Included in Negatively Charged Liposomes by Lysosomal Phospholipase A_1

compound name	compound no.[a]	IC_{50},[b] μM
kanamycin B	1	110 ± 11 (10)
1-*C*-(hydroxymethyl)kanamycin B	11	79 ± 12* (2)
1-*C*-(hydroxymethyl)-6″-deoxy-6″-chlorokanamycin B	19	105 ± 8 (2)
1-*C*-(hydroxymethyl)-6″-deoxy-6″-azidokanamycin B	20	95 ± 11* (1)
1-*C*-(hydroxymethyl)-6″-deoxy-6″-acetamidokanamycin B	21	119 ± 20 (3)

[a] See Figures 1 and 2. [b] Drug concentration causing 50% inhibition of the release of labeled lysophosphatidylcholine; values are means (±SD) of independent experiments (number shown in parentheses), each using triplicate determinations. *Significantly different from kanamycin B by Student's *t* test ($p < 0.05$).

producing defined aminoglycoside-modifying enzymes. Typical results, in comparison to those of gentamicin, amikacin, and 1-*C*-(hydroxymethyl)gentamicin C_2, are shown in Table IV. It clearly appears that 1-*C*-(hydroxymethyl)gentamicin C_2 is active against bacteria producing enzymes modifying aminoglycosides in the different parts of the molecule, namely the ″sugar moiety (2″-OH), the deoxystreptamine (N-3), and the ′sugar moiety, including the N-6′ position. Only the latter function was not as well protected for amikacin, as found by others.[1] In contrast, most of these bacteria were insensitive to compounds 11 and 19. In particular, no or unconstant protection was afforded toward bacteria modifying the 2″-OH or 3-NH_2 functions which, nevertheless, are close to the 1-*C*-(hydroxymethyl) group.

Toxicological Studies. Biochemical Studies. The intrinsic toxicity of the new derivatives was examined in comparison to that of kanamycin B on the basis of their capacity to inhibit lysosomal phospholipases in an in vitro model consisting of negatively charged liposomes and lysosomal extracts.[8] Dose–response curves were obtained as previously described,[11] and the IC_{50} of each derivative (i.e. the concentration causing 50% inhibition of activity) was determined by intrapolation. Results are shown for phospholipase A_1 in Table V. The introduction of an hydroxymethyl group at C-1 significantly decreased the

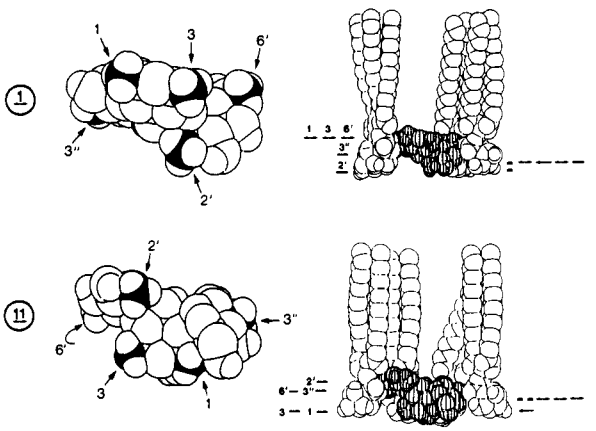

Figure 4. Left: full atomic views of the most probable conformers of kanamycin B (1) and 1-*C*-(hydroxymethyl)kanamycin B (11) equilibrated at an hydrophobic–hydrophilic interface running horizontally through the molecule, with the hydrophobic phase at the top of the figure (the numbered arrows point to the corresponding amino groups of each drug, following the numbering convention used in Figure 2); right: full atomic views of mixed monolayers of phosphatidylinositol and 1 or 11 (the molecules of phosphatidylinositol located in front of the drugs have not been represented in order for these to be seen in full. The numbered arrows at the left of each model refer to the level of the corresponding amino groups of each drug; the nonnumbered arrows at the right of each model refer to the position of the inositol moieties of each phosphatidylinositol molecule surrounding the drugs, with the full arrows pointing to the molecules displayed on the figure, whereas the arrowheads refer to the molecules not represented on the figure).

IC_{50} of kanamycin B. Simultaneous addition of hydrophobic moieties in position C-6″ allowed for a partial return to the original value of kanamycin B. A similar trend was observed for lysosomal phospholipase A_2 and for β-lysophospholipase (data not shown).

Conformational Analysis. The principles of the method used have been reviewed by Brasseur[25,26] as well as their application to the study of the interactions between aminoglycosides and phosphatidylinositol,[3,27] as performed

(25) Brasseur, R. In *Molecular Description of Biological Membrane Components by Computer-Aided Conformational Analysis*; Brasseur, R., Ed.; CRC Press: Boca Raton, FL, 1990; pp 203–219.

(26) Brasseur, R.; Cornet, B.; Burny, A.; Vandenbranden, M.; Ruysschaert, J. M. *AIDS Res. Hum. Retroviruses* **1988**, *4*, 83.

Table VI. Characteristics of the Isolated Conformers of Kanamycin B (1) and 1-C-(Hydroxymethyl)kanamycin B (11) at an Hydrophobic–Hydrophilic Interface and Inserted in a Phosphatidylinositol Monolayer[a]

drug	torsional angles,[b] deg θ_1	θ_2	θ_3	θ_4	energy, kJ/mol	relative position[c]	orientation[d]
kanamycin B	228	215	204	133	−48.5	↓	
						−—P−− *	=
1-C-(hydroxymethyl)-kanamycin B	184	92	218	126	−60.6	↓	
						−—P−− *	=

[a] Data need to be compared to those previously published for gentamicin, amikacin, and kanamycin B derivatives.[4,11,27] [b] The torsional angles relevant to this analysis are those of the C–O–C bonds between the amino sugars and the 2-deoxystreptamine (see refs 9, 11, and 27). [c] The P indicates the phospho group and the * the inositol moiety of the lipid; the arrow indicates the position of the aminoglycoside atom the most oriented toward the aqueous phase. [d] =, parallel, i.e. the greatest axis of the drug oriented in parallel with the hydrophobic–hydrophilic interface; see refs 3, 9, and 11 for examples of perpendicular orientation.

here and in the study of derivatives of kanamycin B carrying only a substitution in 6''.[11] Full atomic views of the most probable conformers of kanamycin B (1) and of 1-C-(hydroxymethyl)kanamycin B (11) at a hydrophilic/hydrophobic interface, along with their mode of insertion in a phosphatidylinositol monolayer, are shown in Figure 4 (data of kanamycin B have already been presented in ref 11 but are shown here again for sake of comparison). Both molecules adopt a rather "flat" configuration with their greatest axis extending almost parallel to the hydrophobic–hydrophilic interface. Yet, the introduction of the hydroxymethyl at C-1 caused the molecule of kanamycin B to rotate along its axis, so that the zones of the molecule exposed to the hydrophilic and the hydrophobic phases were the reverse of those of kanamycin B. This rotation, however, did not modify the degree of insertion of the drug in the phosphatidylinositol monolayer, neither did it visibly affect its position with respect to the plane of the phosphorus atoms or of the fatty acid chains (note that the phospholipid molecules located in front of the drugs have not been represented in order for these to be seen almost in full). Table VI shows quantitative parameters characterizing the conformation of 1 and 11 and their interaction with the phosphatidylinositol monolayer.

Discussion

Substitution of aminoglycosides in C-1 is an original approach to the development of new, semisynthetic aminoglycosides which, in our knowledge, has only been published for gentamicins.[5] The present work shows that a similar approach can be applied to kanamycin B, and probably to other kanamycins. Moreover, this substitution can be made simultaneously with another modification, so far not often applied, namely in position C-6''. The results of the microbiological studies reveal that as for gentamicin C_2, introduction of a hydroxymethyl function in position C-1 does not cause a decrease of activity, further suggesting that the steric environment of the equatorial 1-amino function is not highly critical. Additional modifications at C-6'' only modulate the activity as expected from the behavior of the corresponding derivatives of kanamycin B without a 1-C-(hydroxymethyl) group.[10] Thus, 6''-deoxy-6''-chloro- and 6''-deoxy-6''-azido-1-C-(hydroxymethyl)kanamycin B showed an activity similar to that of kanamycin B itself and of 1-C-(hydroxymethyl)kanamycin B, whereas the activity of 6''-deoxy-6''-acetamido-1-C-(hydroxymethyl)kanamycin B was similar to that found for 6''-deoxy-6''-acetamidokanamycin B.[10] It was reported that addition of a hydroxymethyl group at C-1 of gentamicin C_2 provides resistance against most aminoglycoside-modifying enzymes,[6] and the results shown here largely confirm these claims (see Table IV). No explanation, however, has been given so far for that observation which appears remarkable since protection is afforded without displacement of the charge carried by the N-1 atom of the molecule, although such a displacement is considered to be critical for wide protection such as that endowed to aminoglycosides acylated at N-1 with an (S)-2-hydroxy-4-aminobutyryl or an (S)-2-hydroxy-3-aminopropionyl moiety (viz., amikacin or isepamicin, e.g.).[2,28,29] Substitution of the 1-amino function without charge displacement confers protection usually only to spatially close functions [viz., 1-N-ethylsisomicin (netilmicin), e.g.].[28,29] The present data, however, clearly show that the introduction of a 1-C-(hydroxymethyl) substituent in kanamycin B actually completely failed to afford significant protection even against enzymes acting very closely to that position. While this work was in progress, we also learned that 1-C-(hydroxymethyl)kanamycin A shows surprisingly little activity against kanamycin A resistant strains (P. Stütz, personal communication). Thus, it can be concluded that a hydroxymethyl substitution at C-1 may only have applications in the gentamicin series for protection against aminoglycoside-inactivating enzymes. These findings could also imply that in addition to the recognition of the 1- and 3-amino functions of aminoglycosides,[28] these enzymes also are influenced by other parts of the molecule. These could include positions 3'', 4'', 5'', 3', and 4', which all differ between gentamicins and kanamycins, or position 6'' which is absent in gentamicins. It is interesting to note, in this connection, that a hydroxyaminobutyryl or hydroxyaminopropionyl side chain in N-1 confers only wide protection to kanamycins (A and B) or to gentamicin B, but not to gentamicins C (G. Miller, personal communication), which differ from gentamicin B by a lack of hydroxylation in positions 3' and 4', again pointing to an unsuspected role of a part of the molecule quite distinct from the putative enzyme-anchoring sites N-1 and N-3.[28]

Toxicological studies in vitro also revealed an unexpected behavior of 1-C-(hydroxymethyl)kanamycin B. Previous in vitro studies and computer-aided conformational analysis had revealed that the introduction of a CH_2OH group in position 1 of gentamicin C_2 reduces the binding, the energy of interaction toward negatively charged lipids, and the inhibitory potency toward lysosomal phospholipases.[7,30] These changes were ascribed to a destabilizing effect of the hydroxymethyl substituent on the equilibrium position of the molecule at an hydrophobic–hydrophilic interface,[30,31] allowing a partial change in

(27) Mingeot-Leclercq, M. P.; Schanck, A. N.; Ronvaux-Dupal, M. F.; Deleers, M.; Brasseur, R.; Ruysschaert, J. M.; Laurent, G.; Tulkens, P. M. *Biochem. Pharmacol.* **1989**, *38*, 729.

(28) Nagabhushan, T. L.; Miller, G. H.; Weinstein, M. J. In *The Aminoglycosides: Microbiology, Clinical Use and Toxicology*; Whelton, A., and Neu, H. C., Eds.; Marcel Dekker: New York, 1982; pp 3–27.

(29) Leitner, F.; Price, K. E. In *The Aminoglycosides: Microbiology, Clinical Use and Toxicology*; Whelton, A., Neu, H. C., Eds.; Marcel Dekker, Inc.: New York, 1982; pp 29–63.

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(31) Brasseur, R.; Tulkens, P. M., unpublished results.

the mode of insertion of the molecule in the monolayer from a parallel to a slightly angled orientation with respect to that interface. In parallel, in vivo studies at low, clinically relevant doses revealed a 2–3-fold decrease of nephrotoxicity of 1-*C*-(hydroxymethyl)gentamicin C_2 in rats compared to gentamicin C complex.[7] For 1-*C*-(hydroxymethyl)kanamycin B, we actually observed an increased inhibitory potency toward lysosomal phospholipases, which would suggest that this compound is intrinsically more toxic. The conformational analysis confirmed that an hydroxymethyl group in C-1 destabilizes the buoyant position of the molecule at the interface since it allows for its complete rotation, with the hydroxymethyl group pointing toward the aqueous phase. However, this did not result in any significant change in the positioning of the molecule with respect to the plane of the phosphorus atoms, which we showed to be critical in the modulation of the inhibitory activity of aminoglycosides and of their nephrotoxicity.[3,4] Interestingly enough, however, the energy of interaction of 1-*C*-(hydroxymethyl)kanamycin B with a phosphatidylinositol monolayer was considerably larger than that of kanamycin B (Table VI), as well as that of gentamicin.[3,27] This, however, is probably not the only explanation for the potentially increased toxicity of 1-*C*-(hydroxymethyl)kanamycin B compared to kanamycin B, since we know of compounds, at least in the streptomycin series, with high energy of interaction and low inhibitory potency.[3] As could be anticipated from the results obtained with the 6″-substituted derivatives of kanamycin B,[11] the combination of a 6″-chloro substitution with the C-1 substitution by the hydroxymethyl group allowed for a decrease in inhibitory potency (increase in IC_{50}), but unfortunately not to the point of providing better derivatives than kanamycin B itself.

In conclusion, 1-*C*-(hydroxymethyl) derivatives of kanamycin B did not prove superior to kanamycin B with respect to either activity against sensitive and kanamycin B resistant organisms or toxicological behavior, even when carrying also potentially favorable substitutions at C-6″. Other approaches need to be followed if improvement of the activity and tolerance of aminoglycoside antibiotics of the kanamycin family is being sought.

Experimental Section

Chemistry. Kanamycin B was obtained from Meiji Seika Kaisha, Ltd. (Tokyo 104, Japan), and all other chemicals from Janssen Chimica, Beerse, Belgium. Precoated Merck silica gel F254 plates were used for TLC with the following mobile phases: A, $CHCl_3$–MeOH–33% NH_3 (70:25:4); B, lower layer of $CHCl_3$–MeOH–20% HOAc (5:4:5); C, $CHCl_3$–MeOH–33% NH_3 (10:20:16); D, $CHCl_3$–MeOH–33% NH_3 (70:10:2). Revelation of the spots was done with ninhydrin–acetic acid. Column chromatography was performed on silica gel (Merck, 0.040–0.063 mm). Infrared (IR) spectra were taken on a Perkin-Elmer 257 spectrophotometer. ^{13}C NMR spectra were run on a JEOL FX90Q spectrometer in 5-mm tubes using sample concentrations of 10–15% and at the operating temperature of the spectrometer. Fast atom bombardment (FAB) spectra were obtained on a VG-70SEQ mass spectrometer at 8 kV accelerating voltage. An Ion Tech saddle-field atom gun was used with Xe gas at 8 kV voltage and 1.0 mA current. The compounds were dissolved in thioglycerol acidified with TFA or methanesulfonic acid. $Zn(OAc)_2 \cdot 2H_2O$ was washed[13] with THF before use to remove acetic acid. 3-Chloroperbenzoic acid and paraformaldehyde were dried overnight in vacuo over phosphorpentoxide at room temperature. 1,2-Dichloroethane was refluxed overnight over phosphorpentoxide and distilled.

3,2′,6′-Tris-*N*-(*tert*-butoxycarbonyl)kanamycin B (2). Kanamycin B free base (2.0 g, 4.14 mmol) and $Zn(OAc)_2 \cdot 2H_2O$ (3.72 g, 16.9 mmol) in DMSO (150 mL) were stirred overnight at room temperature. Di-*tert*-butyl dicarbonate (3.1 g, 13.7 mmol) was added and the mixture was stirred for 5 h. After this period 450 mL of water was added and the mixture was adsorbed on a column of 400 mL of Amberlite CG-50 resin (100–200 mesh) in the NH_4^+ form. The resin bed was washed with water to remove DMSO, and the product was then eluted with 1 M NH_3 in MeOH–H_2O (6:5), affording 2.37 g (73%) of **2** which was used as such in the next step. An analytical sample of **2** was obtained by chromatography on a silica gel column using $CHCl_3$–MeOH–33% NH_3 (70:25:7) as eluent: ^{13}C NMR; R_f (system A) 0.15.

3,2′,6′-Tris-*N*-(*tert*-butoxycarbonyl)-3″-*N*-(trifluoroacetyl)kanamycin B (3). Ethyl trifluoroacetate (0.714 g, 4.97 mmol) and **2** (3.0 g, 3.8 mmol) in DMSO (7.5 mL) were kept for 1.5 h at room temperature. Anhydrous Et_2O (75 mL) was added and the precipitated material was filtered off, affording 3.14 g (98% yield) of **3**, which was used as such in the next step: R_f (system A) 0.37.

1-*N*-[(Benzyloxy)carbonyl]-3,2′,6′-tris-*N*-(*tert*-butoxycarbonyl)-3″-*N*-(trifluoroacetyl)kanamycin B (4). A solution of **3** (3.211 g, 3.8 mmol) and [[(benzyloxy)carbonyl]oxy]succinimide (1.46 g, 5.74 mmol) in DMF (20 mL) was kept for 15 h at room temperature. The reaction mixture was evaporated, water was added, and the precipitate of **4** (2.94 g, 79%) was used without further purification: R_f (system A) 0.56.

1-*N*-[(Benzyloxy)carbonyl]-3,2′,6′-tris-*N*-(*tert*-butoxycarbonyl)kanamycin B (5). Starting from 4. A solution of **4** (130 mg, 0.134 mmol) in tetrahydrofuran–water (2:1) (30 mL) was treated with 33% aqueous NH_3 (4.0 mL) for 39 h at room temperature. The reaction mixture was evaporated to dryness and dried by coevaporation with absolute ethanol. Trituration of the residue with anhydrous ether afforded 125 mg of crude **5** (96%). An analytical sample could be obtained by column chromatography over silica gel, using the lower layer of $CHCl_3$–MeOH–33% NH_3 (70:11:9) as eluent.

Starting from 2. To a mixture of **2** (3.0 g, 3.8 mmol) and copper(II) acetate monohydrate (7.8 g, 3.9 mmol) in DMF (90 mL) was added [[(benzyloxy)carbonyl]oxy]succinimide (4.87 g, 19.5 mmol). After 1.5 h the reaction was quenched by addition of 33% aqueous NH_3 (100 mL). The mixture was diluted by addition of distilled water (100 mL) and then extracted with $CHCl_3$ (4 × 50 mL). Evaporation of the combined organic layers and chromatographic purification of the residue on a silica gel column, using the lower layer of $CHCl_3$–MeOH–33% NH_3 (70:11:9) as eluent, afforded 2.57 g (73% yield) of pure **5**: ^{13}C NMR; R_f (system A) 0.36.

1-*N*-[(Benzyloxy)carbonyl]-3,2′,6′,3″-tetrakis-*N*-(*tert*-butoxycarbonyl)kanamycin B (6). Di-*tert*-butyl dicarbonate (1.93 g, 8.5 mmol) was added to a solution of **5** (1.09 g, 1.19 mmol) in dioxane–MeOH (20:1) (95 mL). After heating at 40 °C for 0.5 h, the reaction mixture was cooled to room temperature and quenched with 33% aqueous NH_3 (10.9 mL). The solvent was evaporated under reduced pressure and the residue triturated with dry ether (2 × 20 mL), affording 0.86 g (71%) of crude **6** as an amorphous powder. The compound can be further purified by chromatography on a silica gel column using the lower layer of $CHCl_3$–MeOH–33% NH_3 (70:10:9) as eluent: ^{13}C NMR; R_f (system A) 0.72.

3,2′,6′,3″-Tetrakis-*N*-(*tert*-butoxycarbonyl)kanamycin B (7). A solution of **6** (1.23 g, 1.2 mmol) in methanol–water (3:1) (67 mL) was reduced with hydrogen at 3 atm, in the presence of 10% Pd/C (400 mg) as catalyst. After 3 h the catalyst was removed by filtration and the solvent evaporated under reduced pressure. Chromatography over a silica gel column using the lower layer of $CHCl_3$–MeOH–33% NH_3 (70:10:9) as eluent yielded 0.66 g of pure **7** (62%): ^{13}C NMR; R_f (system A) 0.58.

1-Deamino-1-nitro-3,2′,6′,3″-tetrakis-*N*-(*tert*-butoxycarbonyl)kanamycin B (8). A mixture of 3-chloroperbenzoic acid (85%, 2.75 g, 13.5 mmol) and **7** (1.116 g, 1.26 mmol) in 1,2-dichloroethane (125 mL), to which was added the radical inhibitor 3-*tert*-butyl-5-methyl-4-hydroxyphenyl sulfide (315 mg, 0.88 mmol), was heated at 90 °C for 45 min. The solution was then concentrated under reduced pressure and the residue washed (20% $Na_2S_2O_5$, saturated $NaHCO_3$ solution and H_2O), dried, and chromatographed over silica gel with $CHCl_3$–MeOH (96:4) as eluent, yielding 0.519 g (45%) of **8**: IR (KBr) ν_{max} 1555 cm^{-1} (NO_2); ^{13}C NMR; R_f (system B) 0.64.

1-*C*-(Hydroxymethyl)-1-deamino-1-nitro-3,2′,6′,3″-tetrakis-*N*-(*tert*-butoxycarbonyl)kanamycin B (9). Paraform-

aldehyde (27.5 g) was suspended in a solution of 8 (2.216 g, 2.4 mmol) in $CHCl_3$ (370 mL). Triethylamine (2.7 mL, 19.2 mmol) was added and the reaction mixture was refluxed for 30 min. The suspension was filtered and the filtrate evaporated. Chromatographic purification on a silica gel column with $CHCl_3$–MeOH (96:4) as eluent yielded 1.855 g (81%) of pure **9**: ^{13}C NMR; R_f (system B) 0.59.

1-*C*-(Hydroxymethyl)-3,2',6',3''-tetrakis-*N*-(*tert*-butoxycarbonyl)kanamycin B (10). A solution of **9** (1.855 g, 1.97 mmol) in MeOH (500 mL) was reduced with hydrogen at 4 atm in the presence of a Raney nickel W_2 catalyst (5 teaspoons). After 3 h the catalyst was removed by filtration and the filtrate evaporated. The residue was chromatographed over silica gel with $CHCl_3$–MeOH (94:6) as eluent, yielding 1.446 g of pure **10** (80.5%): ^{13}C NMR; R_f (system B) 0.28.

1-*C*-(Hydroxymethyl)-6''-*O*-[(2,4,6-triisopropylphenyl)-sulfonyl]-1,3,2',6',3''-penta-*N*-BOC-kanamycin B (BOC-18). Compound **6** (11.0 g, 10.77 mmol) dried by coevaporation with anhydrous pyridine under reduced pressure, was dissolved in anhydrous pyridine (140 mL) and 2,4,6-triisopropylbenzenesulfonyl chloride (97%; 16.9 g, 54.0 mmol) was added. After 64 h an aqueous solution of $NaHCO_3$ (55 mL) was added. The mixture was stirred for 1 h and then evaporated. The residue was triturated with $CHCl_3$ (275 mL); the solid material was filtered off and washed with $CHCl_3$. The combined $CHCl_3$ filtrates were evaporated, and the residue was purified on a silica gel column using a stepwise gradient ($CHCl_3$–MeOH (99:1) to $CHCl_3$–MeOH (98:2)) affording 9.02 g of pure **14** (65%); ^{13}C NMR; R_f (system D) 0.64.

A solution of **14** (8.58 g, 6.68 mmol) in MeOH (360 mL) was reduced with H_2 (3 atm) in the presence of 10% Pd/C (2.25 g). The catalyst was carefully removed and the solvent was evaporated. The residue was purified by column chromatography (eluent: $CHCl_3$–MeOH (97:3)), yielding 5.84 g (76%) of **15**: ^{13}C NMR; R_f (system D) 0.54. A solution of **15** (11.06 g, 9.6 mmol) in 1,2-dichloroethane (650 mL) containing 3-*tert*-butyl-5-methyl-4-hydroxyphenyl sulfide (1.66 g, 4.6 mmol) and *m*-chloroperbenzoic acid (97.5 mmol) was refluxed for 30 min. The solvent was evaporated and the residue was washed with 20% $Na_2S_2O_5$, saturated $NaHCO_3$, and distilled water. Purification by column chromatography (eluent: $CHCl_3$–MeOH (99:1)) afforded 4.48 g (40%) of **16**: ^{13}C NMR; R_f (system D) 0.58. Et_3N (3.9 mL, 27.6 mmol) was added to a suspension of paraformaldehyde (38.24 g) in $CHCl_3$ (450 mL) containing **17** (3.76 g, 3.2 mmol). The stirred mixture was refluxed for 1.5 h. The suspension was filtered, the filtrate was washed with dilute HCl and evaporated, and the residue was purified by column chromatography (eluent: $CHCl_3$–MeOH (98:2)), yielding 2.78 g (72%) of **17**: ^{13}C NMR; R_f (system D) 0.48. Nitro derivative **17** (2.67 g, 2.2 mmol) was dissolved in MeOH (400 mL) and catalytically (Raney nickel W_2) reduced with H_2 (4 atm) in the presence of $(BOC)_2O$ (1.5 g, 6.8 mmol). After 4 h the catalyst was filtered off and repeatedly washed with DMF, and the combined filtrates were evaporated. The residue was reacted further for 20 h with $(BOC)_2O$ (0.95 g, 4.4 mmol) in MeOH (200 mL). The solvent was evaporated and the residue was purified by column chromatography (eluent: $CHCl_3$–MeOH (99:1)), yielding 2.27 g (81%) of BOC-18: ^{13}C NMR; R_f (system D) 0.53.

1-*C*-(Hydroxymethyl)-6''-deoxy-6''-chloro-1,3,2',6',3''-pentakis-*N*-(*tert*-butoxycarbonyl)kanamycin B (BOC-19). Lithium chloride (103 mg, 2.43 mmol) was added to a solution of BOC-18 (518 mg, 0.40 mmol) in anhydrous DMF (3.4 mL). The solution was heated at 90 °C for 7 h and the solvent was removed under reduced pressure. Purification of the reaction mixture by chromatography on a silica gel column using $CHCl_3$–MeOH (98.5:1.5) afforded 370 mg of pure BOC-19 (88.5% yield): ^{13}C NMR; R_f (system D) 0.48.

1-*C*-(Hydroxymethyl)-6''-deoxy-6''-azido-1,3,2',6',3''-pentakis-*N*-(*tert*-butoxycarbonyl)kanamycin B (BOC-20). A mixture of BOC-18 (480 mg, 0.37 mmol) and LiN_3 (148 mg, 3.0 mmol) in anhydrous DMF (10 mL) was heated at 65 °C for 23 h. After evaporation of the solvent, the residue was treated with $CHCl_3$ (25 mL) and the insoluble material was filtered off and washed with $CHCl_3$ (2 × 25 mL). The combined $CHCl_3$ filtrates were evaporated and purified by chromatography over silica gel with $CHCl_3$–MeOH (99:1) as eluent, affording 370 mg of pure BOC-20 (95% yield): IR (KBr) ν_{max} 2120 cm^{-1} (N_3); ^{13}C NMR; R_f (system D) 0.44.

1-*C*-(Hydroxymethyl)-6''-deoxy-6''-acetamido-1,3,2',6',3''-pentakis-*N*-(*tert*-butoxycarbonyl)kanamycin B (BOC-21). Triphenylphosphine (0.28 g, 1.05 mmol) was added to a solution of 6''-deoxy-6''-azido derivative BOC-**20** (0.52 g, 0.5 mmol) in anhydrous pyridine (5 mL) and the reaction mixture was stirred for 2 h at room temperature. Aqueous 33% NH_3 (0.5 mL) was added, the reaction mixture was stirred for 24 h and evaporated. Pyridine was removed by coevaporation with toluene, and the residue was purified by column chromatography (eluent: $CHCl_3$–MeOH (95:5)), yielding 0.38 g (76%) of 6''-amino derivative: ^{13}C NMR; R_f (system D) 0.17. Ac_2O (0.09 mL, 0.95 mmol) was added to a solution of the latter (321 mg, 0.32 mmol) in EtOH (17 mL). After 1.5 h at room temperature, the reaction mixture was stirred with a saturated $NaHCO_3$ solution (2.3 mL) for 0.5 h and evaporated. Purification by chromatography over silica gel with $CHCl_3$–MeOH (98.5:1.5) afforded 245 mg of pure BOC-21 (73%): ^{13}C NMR; R_f (system D) 0.36.

Deprotection of *N*-BOC Derivatives—General Procedure. This was performed as described for the C-6'' substituted kanamycin B derivatives in the first paper of this series.[10] For each compound, TLC (system C) R_f value, FAB MS (m/z for the $(M + H)^+$ ion), and ^{13}C NMR chemical shifts for diagnostic signals are given (at pH <1.5 and in the following order: 4'', 5'', 6''; 1-C-CH_2OH; α, β of the 6''-N-Ac group).

1-*C*-(hydroxymethyl)kanamycin B (**11**): R_f 0.31; FAB m/z 514.3; ^{13}C NMR 66.5, 73.5, 61.0; 59.5 (CH_2OH) ppm.

1-*C*-(hydroxymethyl)-6''-deoxy-6''-chlorokanamycin B (**19**): R_f 0.58; FAB m/z 532; ^{13}C NMR 66.8, 72.4, 44.5; 59.0 (CH_2OH) ppm.

1-*C*-(hydroxymethyl)-6''-deoxy-6''-azidokanamycin B (**20**): R_f 0.64; FAB m/z 539; ^{13}C NMR 66.9, 72.3, 51.2; 59.1 (CH_2OH) ppm.

1-*C*-(hydroxymethyl)-6''-deoxy-6''-acetamidokanamycin B (**21**): R_f 0.58; FAB m/z 555: ^{13}C NMR 67.4, 71.6, 40.1; 59.0 (CH_2OH); 175.4, 22.5 (N-Ac) ppm.

Microbiology. Activity of the N-deprotected compounds, prepared as their sulfate salts, was first examined against gentamicin and kanamycin B sensitive strains as described earlier[10] with the same types of organisms. In addition, we also used strains of *S. marcescens* with resistance to aminoglycosides, obtained from clinical isolates at the Cliniques Universitaires Saint-Luc, Brussels, Belgium. Resistance mechanisms were established by pattern analysis[32] by R. Hare and G. Miller at Schering-Plough Research, Bloomfield, NJ. Activity of 1-*C*-(hydroxymethyl)kanamycin B (**11**) and 6''-deoxy-6''-chloro-1-*C*-(hydroxymethyl)kanamycin B (**19**) in comparison to gentamicin (commercial mixture of C_1, C_{1a}, and C_2 components) and amikacin toward bacteria producing defined types of aminoglycoside-inactivating enzymes was also determined by R. Hare and G. Miller (who were kept unaware of the structure of the compounds submitted to their analysis). The same investigators had tested previously on the same or similar organisms 1-*C*-(hydroxymethyl)gentamicin C_2, which had been synthesized at Sandoz Forschungsinstitut, Vienna, Austria,[5] and these results have therefore been included in the present report for comparison purposes. All methods of assay used both agar and microtiter (broth) dilution techniques,[33] and incubations were performed in Mueller-Hinton medium.

In Vitro and Computer-Aided Toxicological Evaluation. This was performed exactly as described earlier,[11] i.e. using the inhibition of lysosomal phospholipases (measured toward labeled phosphatidylcholine included in negatively charged liposomes) by the drugs in comparison with kanamycin B, and by conformational analysis of drug–phosphatidylinositol mixed monolayers. These approaches and their rationale are fully described in refs 3, 8, 9, 25, and 27.

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having performed the evaluation of compounds 11 and 19 in their screening assay systems using bacteria with known aminoglycoside-resistance mechanisms and for allowing us to use their results obtained previously with this approach for 1-C-(hydroxymethyl)gentamicin C_2, Dr. P. Stütz, from the Sandoz Forschungsinstitut, Vienna, Austria, for communication of unpublished data concerning 1-C-(hydroxymethyl)kanamycin A, and Dr. M. Delmée (Université Catholique de Louvain) for advice in the microbiological studies. Mrs. F. Renoird-Andries and Mr. F. Van Rompay provided skillful technical assistance, and Mrs. N. Amat and L. Palmaerts dedicated secretarial help. A. Van Schepdael, M. P. Mingeot and R. Brasseur are, respectively, Aspirant, Chargé de Recherches, and Chercheur Qualifié and P. M. Tulkens was Maître de Recherches of the National Fund for Scientific Research (N.F.W.O./F.N.R.S.). This work was supported by the Belgian Fund for Medical Scientific Research (grant no. 3.4553.88/F) and Services de la Programmation et de la Politique Scientifique (grant no. 7 bis/PAI), and with a grant-in-aid from the Bristol Myers Co., Wallingford, CN.

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Quinazolineacetic Acids and Related Analogues as Aldose Reductase Inhibitors

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A variety of 2,4-dioxoquinazolineacetic acids (10, 11) were synthesized as hybrids of the known aldose reductase inhibitors alrestatin (8), ICI-105,552 (9), and ICI-128,436 (2) and evaluated for their ability to inhibit partially purified bovine lens aldose reductase (in vitro) and their effectiveness to decrease galactitol accumulation in the 4-day galactosemic rat model (in vivo). In support to SAR studies, related analogues pyrimidinediones (12), dihydroquinazolones (13), and indazolidinones (14, 15) were synthesized and tested in the in vitro and in vivo assays. All prepared compounds (10–15) have shown a high level of in vitro activity ($IC_{50} \sim 10^{-6}$ to 4×10^{-8} M). However, only the 2,4-quinazolinedione analogues 10 and 11, with similar *N*-aralkyl substitution found in 2 and 9, have exhibited good oral potency. The remaining compounds were either inactive or had only a marginal in vivo activity. The structure–activity data support the presence of a secondary hydrophobic pocket in the vicinity of the primary lipophilic region of the enzyme.

Introduction

Although the discovery of insulin has had a dramatic effect on prolonging the lives of diabetics, diabetes-related, long term complications such as neuropathy, nephropathy, and retinopathy that can lead to amputations, blindness, and death still frequently occur. These complications originate in tissues that do not require insulin for glucose transport, i.e. nerve, lens, retina, and kidney, and are therefore exposed to the ambient blood glucose level, which may increase dramatically even in well-controlled diabetic patients. During such hyperglycemic episodes, excess glucose in nerve, ocular, and renal tissues enters the sorbitol (polyol) pathway and is reduced by aldose reductase (AR) to sorbitol. Compelling evidence from animal studies has implicated the increased flux of glucose through the polyol pathway in the development of a number of the secondary complications of diabetes. In these studies compounds which inhibit aldose reductase prevented the formation of cataracts, the loss of neural function, the increased excretion of albumin in the urine, and retinal deterioration in diabetic or galactosemic animals.[1e-j]

While a wide variety of compounds have been identified as potent aldose reductase inhibitors (ARI) over the past decade, only a small fraction of these compounds have expressed this activity very well in vivo and have been proven effective in delaying or even preventing pathologies associated with chronic diabetes in animals and humans.[1b,2] These in vivo active compounds are limited, for the most part, to two distinct structural classes, i.e. the carboxylic acids and the cyclic imides. Representative compounds of these two types include[3] tolrestat (1, AY-27,773, currently marketed under the tradename Alredase) and drugs undergoing clinical trials ponalrestat (2, ICI-128,436), epalrestat (3, ONO-2235), FR74366 (4, Fujisawa), sorbinil (5, CP-45,634, Pfizer), imirestat (6, AL-1576, Alcon), and M79175 (7, Eisai) (Chart I).

As part of our studies to identify new aldose reductase inhibitors, we focused on the structural similarities between

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