Synthesis and Antimicrobial and Toxicological Studies of Amino Acid and Peptide Derivatives of Kanamycin A and Netilmicin

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Amino acid and peptide derivatives of aminoglycosides have been obtained by substitution of the 1-N or 6′-N amino functions of kanamycin A and netilmicin *via* the temporary complexation of vicinal and nonvicinal amino and hydroxy functions by copper ion [1-N kanamycin A derivatives: L-Ala (**6a**), D-Ala (**6b**), Gly (**6c**), L-Asp (**6d**), L-Ala-L-Ala (**6e**). 6′-N kanamycin A derivatives: L-Ala (**3a**), D-Ala (**3b**), Gly (**3c**), L-Ala-L-Ala (**3e**), L-Leu (**3f**). 6′-N netilmicin derivatives: L-Ala (**9a**), D-Ala (**9b**), Gly (**9c**), L-Asp (**9d**), L-Ala-L-Ala (**9e**)]. Characterization was made by FAB-MS, IR, ^{1}H-NMR, and ^{13}C-NMR. All derivatives were essentially inactive. The nephrotoxic potential of the derivatives obtained in sufficient quantities (**3b**,**e** and **9a-e**) was assessed by measuring their inhibitory potential toward the activity of lysosomal phospholipase A_1 acting on phosphatidylcholine embedded in negatively-charged membranes. One compound, 6′-*N*-L-Ala-netilmicin (**9a**), showed a 2-fold decrease of inhibitory potency compared to its parent drug. A conformational analysis revealed that it adopts two equally probable conformations and orientations when interacting with phosphatidylinositol. The first in which the drug lies parallel to the hydrophobic–hydrophilic interface, is similar to that of netilmicin. The second, in which the drug inserts itself in the bilayer across the hydrophilic/hydrophobic interface, is similar to that described for streptomycin, an almost non-nephrotoxic aminoglycoside.

Introduction

Aminocyclitol antibiotics, commonly referred to as aminoglycosides, are highly potent, wide-spectrum agents with many highly desirable chemotherapeutic properties[1–4] that make them often essential in the treatment of life-threatening infections. Yet, their use has always remained limited because of a definite tendency to cause nephro- and ototoxic reactions.[5–8] Only modest progress has been achieved so far to reduce these adverse effects in clinical practice.[1,2] A more recent, and perhaps more intense, cause for concern is the slow but steadily rising emergence of resistance among formerly sensitive pathogens through the production of aminoglycoside-modifying enzymes that may inactivate even semisynthetic derivatives such as amikacin (1-*N*-[(*S*)-2-hydroxy-4-aminobutyryl]kanamycin A[9–11]). The latter drug remains indeed sensitive to 6′-*N*-acetyltransferases[1,2] which are more and more frequently found in resistant strains alone or in combination with other enzymes commonly found in isolates resistant to the other aminoglycosides.[12] Suppressing the sensitivity of the 6′-amino function of aminoglycosides to 6′-*N*-acetyltransferases has been obtained by methylation of this group, such as it occurs naturally for gentamicins (the gentamicin C complex contains a minor amount of 6′-*N*-methylgentamicin C_{12}, named gentamicin C_{2b} or sagamicin) or by human-directed methylation, yielding compounds such as 6′-*N*-methylsisomicin (G52), 6′-*N*-methylamikacin (BB-K 28), and 4′-deoxy-6′-*N*-methylamikacin (BBK 3-11).[13,14] This type of modification, however, usually results in a partial loss of activity with no or little gain in toxicity compared to the parent compounds[13,14] so that none of these derivatives is presently in clinical use, except for sagamicin in some countries. Another approach has been the introduction of an hydroxymethyl substituent in position 1 of gentamicin C_2, yielding a compound (S87351) which has overcome all clinically-relevant types of enzymatically-mediated resistance[15,16] and displays a somewhat reduced toxicity compared to that of gentamicin complex.[17] The same substitution, however, was ineffective in protecting against enzyme modification in kanamycin A (P. Stütz, personnal communication) as well as in kanamycin B.[18]

These observations prompted us to investigate another type of modification in N-6′ or N-1 so far not or only briefly reported, namely the substitution by amino acids. Applied to position N-6′, this approach was made in an attempt to protect the site attacked by the 6′-*N*-acetyltransferases while introducing a free primary amino group at a short distance of its original place, thereby maintaining the antibacterial activity. Two of us, indeed, reported previously that 6′-*N*-aminoacyl derivatives of neamine—which has some antibacterial activity—are as active as neamine itself.[19] Modification in N-1 was attempted somewhat more ambitiously to find a substituent that would not only displace the cationic charge in N-1, as in amikacin, but would play the same protecting role as the hydroxymethyl group in 1-*C*-(hydroxymethyl)gentamicin C2.[15,17]

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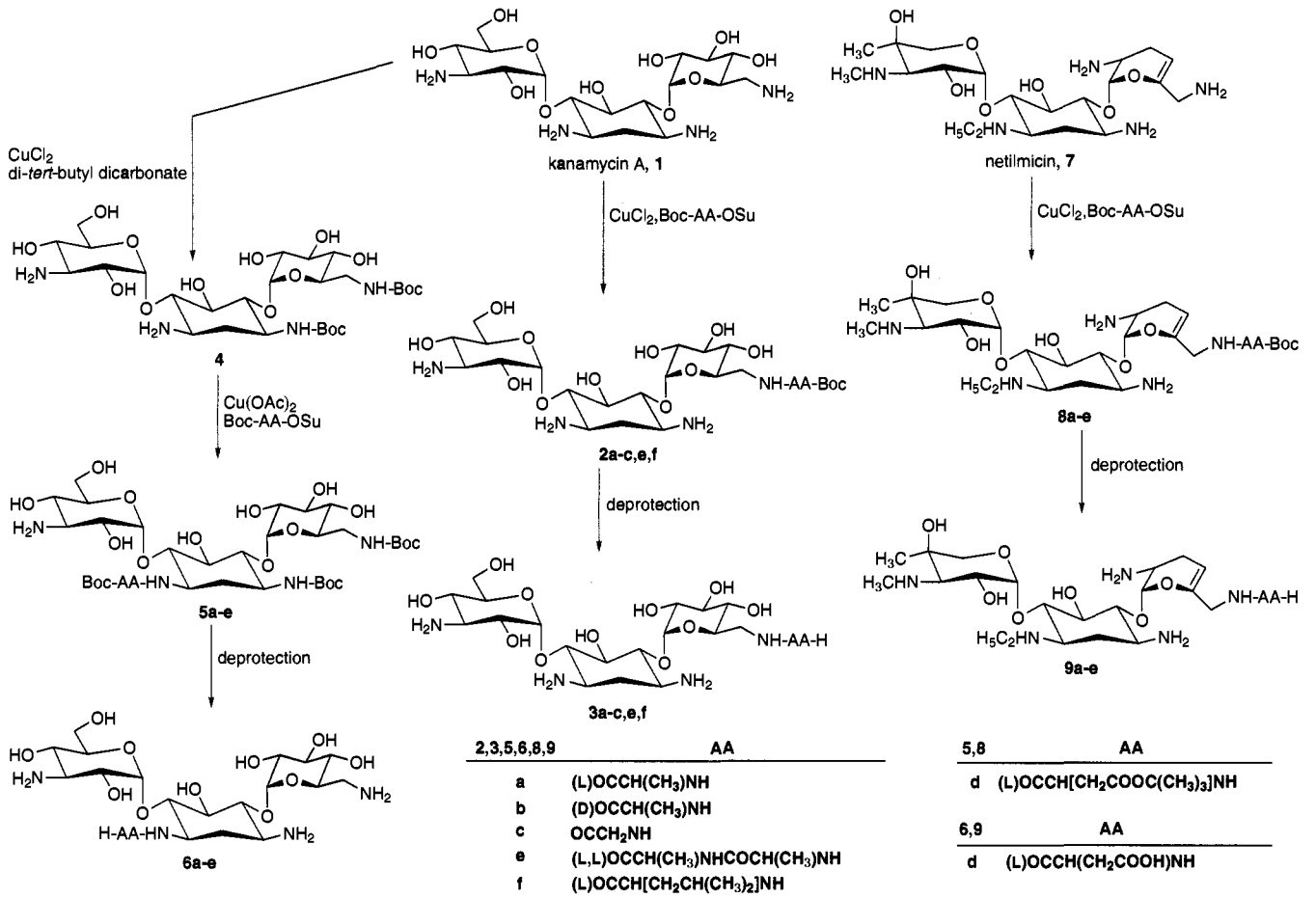
Scheme 1



We present here the syntheses of a series of 6′-N and 1-N aminoacyl derivatives of kanamycin A and another series of 6′-N aminoacyl derivatives of netilmicin, using L-Ala, D-Ala, Gly, L-Asp (OBut), L-Ala-L-Ala-OH, and L-Leu. Antimicrobial activity was tested against organisms sensitive to kanamycin A and netilmicin and their resistant mutants. An *in vitro* toxicological evaluation was performed by examining the inhibitory potency of the derivatives available in sufficient quantity against the hydrolysis of phosphatidylcholine embedded in negatively-charged membranes by lysosomal phospholipase A_1. This *in vitro* model is predictive of the intrinsic toxicological potential of several aminoglycosides.[20,21] For two key compounds, the results were correlated with the data generated by a computer-aided analysis of the interactions of the drugs with phosphatidylinositol.[20,22] For one derivative (6′-*N*-L-Ala-netilmicin, **9a**), this analysis disclosed an unsuspected behavior so far undescribed for aminoglycosides.

Results

Chemical Syntheses and Physicochemical Characterizations. Selective N-acylation of aminoglycosides has been largely made to sites selected for their stereochemical properties[23] or their relative basicities.[24] In this study, we applied the approach of N-acylation to amino acids in high yield *via* the temporary complexation of vicinal and nonvicinal amino and hydroxy functions by copper ion.[25] Because the 6′-N function of kanamycin A and netilmicin is the most basic, treatment of a mixture of kanamycin A or netilmicin and 2 equiv of copper ion with the active hydroxysuccinimide ester of N-protected amino acids or peptides yielded directly the corresponding 6′-N derivatives **2a–c,e,f** and **8a–e** (Scheme 1) after decomposition of the chelate with aqueous ammonia and column chromatography. After N-deprotection, compounds **3a–c,e,f** and **9a–e** were obtained. 1-N derivatives of kanamycin A could be synthesized similarly after the selective protection of the 6′- and the 3-amino function with a *tert*-butoxycarbonyl group. A mixture of kanamycin A and 2 equiv of copper complexing ion was reacted with di-*tert*-butyl dicarbonate and gave in high yield the 6′,3-*N*,*N*-bis(*tert*-butyloxycarbonyl) derivative **4**. An identical compound, as assessed by ^{13}C-NMR spectroscopy, was also obtained in the presence of zinc ion.[26] Compound **4** was coupled with the hydroxysuccinimide ester of *N*-Boc-protected amino acids and peptide, in the presence of 1 equiv of copper ion[27,28] to obtain compounds **5a–e**. These were purified on an Amberlite CG-50 (NH_4^+ form) column to give, after N-deprotection, compounds **6a–e** (Scheme 1).

The physical constants of all the compounds synthesized are given in Table 1. Their structures were assigned on the basis of their ^{13}C-NMR spectra[29] (Tables 3–5). The positional isomers can be distinguished by the upfield chemical shifts for the β-carbon atoms, due to N-acylation or to N-protonation when the spectra are determined at low pH. 6′-N-Acylation of kanamycin A with amino acids (compounds **2a–c,e,f**; Table 3) induced a small upfield shift (1.1–1.5 ppm) of C-6′ and a similar upfield shift (0.9–1.1 ppm) of the β-carbon C-5′ in comparison with the corresponding carbons of kanamycin A. However, in the protonated compounds **3a–c,e,f**,

Table 1. 6′-N and 1-N Derivatives of Kanamycin A

compd	mp, °C	$[\alpha]_D$, deg	formula	FAB-MS	IR (ν_{max}, cm^{-1})	^1H NMR, δ^d
2a	148–150	+61[a]	$C_{26}H_{49}N_5O_{14}$	656 [M + H]	3600–3200 (OH, NH), 1700, 1670 (OCONH, NHCO)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.3 (3H, d, J = 8, $CHCH_3$)
2b	134–136	+25[b]	$C_{26}H_{49}N_5O_{14}$	656 [M + H]	3600–3100 (OH, NH), 1700, 1675 (OCONH, NHCO)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.3 (3H, d, J = 8, $CHCH_3$)
2c	123–125	+90[b]	$C_{25}H_{47}N_5O_{14}$	642 [M + H]	3600–3200 (OH, NH), 1700, 1670 (OCONH, NHCO)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.5 (2H, s, $NHCH_2CO$)
2e	122–124	+22[b]	$C_{29}H_{54}N_6O_{15}$	727 [M + H]	3650–3100 (OH, NH), 1700, 1670 (OCONH, NHCO)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.8 (2H, m, 2 × α-CH), 1.3 (6H, d, J = 8, 2 × $CHCH_3$)
2f	138–140	+10[c]	$C_{29}H_{55}N_5O_{14}$	698 [M + H]	3650–3200 (OH, NH), 1700, 1670 (OCONH, NHCO)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.6 (1H, t, J = 7, α-CH), 3.2 (1H, d, m, $CH(CH_3)_2$), 2.5 (2H, m, $CH_2CH(CH_3)_2$)
3a	150–152	+71[a]	$C_{21}H_{41}N_5O_{12}\cdot 4HCl$	556 [M + H − 4HCl]	3500–2800 (OH, NH_3^+), 1680 (NHCO)	5.6 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.6 (3H, d, J = 8, $CHCH_3$)
3b	141–143	−84[c]	$C_{21}H_{41}N_5O_{12}\cdot 4HCl$	556 [M + H − 4HCl]	3500–2800 (OH, NH_3^+), 1680 (NHCO)	5.5 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.4 (3H, d, J = 8, $CHCH_3$)
3c	138–140	+42[c]	$C_{20}H_{39}N_5O_{12}\cdot 4HCl$	564 [M + Na − 4HCl] 542 [M + H − 4HCl]	3500–2900 (OH, NH_3^+), 1680 (NHCO)	5.5 (1H, d, J = 2, H-1′), 5.1 (1H, d, J = 4, H-1″), 3.5 (2H, s, $NHCH_2CO$)
3e	158–160	+51[c]	$C_{24}H_{46}N_6O_{13}\cdot 4HCl$	627 [M + H − 4HCl]	3550–2800 (OH, NH_3^+), 1680 (NHCO)	5.6 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.8 (2H, m, 2 × α-CH), 1.4 (6H, d, J = 8, 2 × $CHCH_3$)
3f	128–130	+55[b]	$C_{24}H_{47}N_5O_{12}\cdot 4HCl$	620 [M + Na − 4HCl] 598 [M + H^+ − 4HCl]	3500–2900 (OH, NH_3^+), 1685 (NHCO)	5.6 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.6 (1H, m, α-CH), 3.2 [1H, m, $CH(CH_3)_2$], 2.5 (2H, m, $CH_2CH(CH_3)_2$)
4	181–183	+81[b]	$C_{28}H_{52}N_4O_{15}$	685 [M + H]	3600–3200 (OH, NH), 1690 (OCONH)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″)
5a	178–180	+37[a]	$C_{36}H_{65}N_5O_{18}$	857 [M + 2H]	3590–3180 (OH, NH), 1700, 1670 (OCONH, NHCO)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.3 (3H, d, J = 8, $CHCH_3$)
5b	165–167	−112[a]	$C_{36}H_{65}N_5O_{18}$	857 [M + 2H]	3600–3200 (OH, NH), 1700, 1675 (OCONH, NHCO)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.7 (1H, m, α-CH), 1.3 (3H, d, J = 8, $CHCH_3$)
5c	oil	+31[c]	$C_{35}H_{63}N_5O_{18}$	842 [M + H]	3600–3190 (OH, NH), 1695, 1670 (OCONH, NHCO)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.5 (2H, s, $NHCH_2CO$)
5d	125–127	+56[b]	$C_{41}H_{73}N_5O_{20}$	959 [M + 3H]	3600–3200 (OH, NH), 1700, 1670 (OCONH, NHCO)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 4.1 (2H, m, $CHCH_2$), 3.8 (1H, m, α-CH)
5e	118–120	+14[a]	$C_{39}H_{70}N_6O_{19}$	927 [M + H]	3590–3200 (OH, NH), 1700, 1675 (OCONH, NHCO)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.7 (2H, m, 2 × α-CH), 1.3 (6H, d, J = 8, 2 × $CHCH_3$)
6a	144–146	+58[c]	$C_{21}H_{41}N_5O_{12}\cdot 4HCl$	578 [M + Na − 4HCl] 557 [M + 2H − 4HCl]	3500–2830 (OH, NH_3^+), 1680 (NHCO)	5.7 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.6 (3H, d, J = 8, $CHCH_3$)
6b	138–140	−12[c]	$C_{21}H_{41}N_5O_{12}\cdot 4HCl$	578 [M + Na − 4HCl] 557 [M + 2H − 4HCl]	3500–2800 (OH, NH_3^+), 1680 (NHCO)	5.7 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.6 (3H, d, J = 8, $CHCH_3$)
6c	115–117	+27[c]	$C_{20}H_{39}N_5O_{12}\cdot 4HCl$	542 [M + H − 4HCl]	3500–2900 (OH, NH_3^+), 1660 (NHCO)	5.6 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.6 (2H, s, $NHCH_2CO$)
6d	178–180	+84[b]	$C_{22}H_{41}N_5O_{14}\cdot 4CF_3COOH$	600 [M + H − $4CF_3COOH$]	3500–2500 (OH, NH_3^+), 1760 (OCO), 1660 (NHCO)	5.6 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 4.0 (2H, m, $CHCH_2$), 3.8 (1H, t, J = 7, α-CH)
6e	106–108	+22[c]	$C_{24}H_{46}N_6O_{13}\cdot 4HCl$	627 [M + H − 4HCl]	3500–2800 (OH, NH_3^+), 1660 (NHCO)	5.6 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.8 (2H, m, 2 × α-CH), 1.5 (6H, d, J = 8, 2 × $CHCH_3$)

[a] *c* 0.5 in H_2O. [b] *c* 0.5 in DMSO. [c] *c* 0.5 in EtOH–H_2O, 1:1. [d] The spectra of **2a–c,e,f** and **5a–e** were taken in DMSO-d_6 and the spectra of **3a–c,e,f** and **6a–e** in D_2O.

Table 2. 6′-N Amino Acid and Peptide Derivatives of Netilmicin

compd	mp, °C	$[\alpha]_D$, deg	formula	FAB-MS	IR (ν(KBr), cm^{-1})	^1H NMR, δ^c
8a	198–200	+90[a]	$C_{29}H_{54}N_6O_{10}$	648 [M + 2H]	3600–3200 (OH, NH), 1700, 1670 (OCONH, NHCO), 1630 (C=C)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.3 (3H, d, J = 8, $CHCH_3$)
8b	118–120	+99[a]	$C_{29}H_{54}N_6O_{10}$	648 [M + 2H]	3600–3190 (OH, NH), 1690, 1670 (OCONH, NHCO), 1630 (C=C)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.3 (3H, d, J = 8, $CHCH_3$)
8c	106–108	+87[a]	$C_{28}H_{52}N_6O_{10}$	633 [M + H]	3600–3210 (OH, NH), 1700, 1670 (OCONH, NHCO), 1630 (C=C)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.5 (2H, s, $NHCH_2CO$)
8d	194–196	+80[a]	$C_{34}H_{62}N_6O_{12}$	769 [M + Na]	3590–3210 (OH, NH), 1730 (OCO), 1695 (OCONH, NHCO), 1630 (C=C)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 4.1 (2H, m, $CHCH_2$), 3.8 (1H, m, α-CH)
8e	174–176	+75[a]	$C_{32}H_{59}N_7O_{11}$	718 [M + H]	3600–3210 (OH, NH), 1700, 1670 (OCONH, NHCO), 1625 (C=C)	5.3 (1H, d, J = 2, H-1′), 5.0 (1H, d, J = 4, H-1″), 3.8 (2H, m, 2 × α-CH), 1.3 (6H, d, J = 8, 2 × $CHCH_3$)
9a	176–178	+34[a]	$C_{24}H_{46}N_6O_8$·5HCl	592 [M + 2Na − 5HCl]	3500–2850 (OH, NH_3^+), 1660 (NHCO), 1625 (C=C)	5.6 (1H, d, J = 2, H-1′), 5.1 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.3 (3H, d, J = 8, $CHCH_3$)
9b	102–104	+44[b]	$C_{24}H_{46}N_6O_8$·5HCl	592 [M + 2Na − 5HCl]	3500–2850 (OH, NH_3^+), 1665 (NHCO), 1625 (C=C)	5.6 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.8 (1H, m, α-CH), 1.3 (3H, d, J = 8, $CHCH_3$)
9c	98–100	+28[a]	$C_{23}H_{44}N_6O_8$·5HCl	533 [M + H − 5HCl]	3450–2800 (OH, NH_3^+), 1680 (NHCO), 1620 (C=C)	5.6 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.5 (2H, s, $NHCH_2CO$)
9d	173–175	+21[b]	$C_{25}H_{46}N_6O_{10}$·5CF_3COOH	573 [M − NH_3 − 5CF_3COOH]	3500–2500 (OH, NH_3^+), 1760 (OCO), 1680 (NHCO), 1620 (C=C)	5.6 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 4.1 (2H, m, $CHCH_2$), 3.8 (1H, m, α-CH)
9e	188–190	+6[b]	$C_{27}H_{51}N_7O_9$·5HCl	618 [M + H − 5HCl]	3500–2800 (OH, NH_3^+), 1680 (NHCO), 1625 (C=C)	5.6 (1H, d, J = 2, H-1′), 5.2 (1H, d, J = 4, H-1″), 3.9 (2H, m, 2 × α-CH), 1.6 (6H, d, J = 8, 2 × $CHCH_3$)

[a] c 0.5 in DMSO. [b] c 0.5 in EtOH:H_2O, 3:1. [c] The spectra of **8a**–**e** were taken in DMSO-d_6 and the spectra of **9a**–**e** in D_2O.

Table 3. ^{13}C Chemical Shifts for 6′-N Derivatives of Kanamycin A in D_2O Relative to the DMSO-d_6 Peak (40.1 ppm)[a]

	1	**2a**		**2b**		**2c**		**2e**		**2f**		**3a**		**3b**		**3c**		**3e**		**3f**	
	base	base	$\Delta\delta_1$	base	$\Delta\delta_1$	base	$\Delta\delta_1$	base	$\Delta\delta_1$	base	$\Delta\delta_1$	H^+	$\Delta\delta_2$	H^+	$\Delta\delta_2$	H^+	$\Delta\delta_2$	H^+	$\Delta\delta_2$	H^+	$\Delta\delta_2$
C-2	36.1	35.9	−0.2	35.8	−0.3	36.6	0.5	36.6	0.5	36.6	0.5	28.5	−7.4	28.5	−7.3	28.8	−7.8	28.8	−7.8	29.0	−7.6
C-4	87.3	87.5	0.2	87.5	0.2	88.0	−0.7	88.2	−0.9	88.0	−0.7	80.2	−7.3	80.2	−7.3	80.0	−8.0	80.4	−7.8	80.5	−7.5
C-6	88.5	88.0	−0.5	88.0	−0.5	88.2	−0.3	88.4	−0.1	88.9	0.4	84.2	−3.8	84.2	−3.8	84.2	−4.2	84.4	−4.0	85.4	−3.5
C-1′	99.8	100.3	0.5	100.3	0.5	100.2	0.4	100.5	0.7	100.0	0.2	98.9	−1.4	98.9	−1.4	98.5	−1.7	99.0	−1.5	98.1	−1.9
C-5′	72.9	71.8	−1.1	71.8	−1.1	72.0	−0.9	72.0	−0.9	72.0	−0.9	71.2	−0.6	71.2	−0.6	71.2	−0.8	71.2	−0.8	71.1	−0.9
C-6′	42.1	40.6	−1.5	40.6	−1.5	40.8	−1.3	40.9	−1.2	41.0	−1.1	40.0	−0.6	40.0	−0.6	40.1	−0.7	40.2	−0.7	40.3	−0.7
C-2″	72.6	71.9	−0.7	71.8	−0.8	71.9	−0.7	71.5	−1.1	72.5	−0.1	69.0	−2.2	69.0	−2.8	69.2	−2.7	69.0	−2.5	69.6	−2.9
C-4″	70.0	69.4	−0.6	69.4	−0.6	70.1	0.1	69.2	−0.8	70.1	0.1	66.9	−2.5	66.9	−2.5	66.9	−3.2	66.2	−3.0	66.9	−3.2
6′-NHCO		176.2		176.3		172.0		176.3		174.0		174.0	−2.2	174.0	−2.3	170.0	−2.0	174.0	−2.3	172.0	−2.0

[a] $\Delta\delta$ in ppm. Upfield shifts are indicated with negative values and downfield shifts with positive values. $\Delta\delta_1$ = δ(6′-N-protected amino acid derivative) − δ(kanamycin). $\Delta\delta_2$ = δ(6′-N amino acid derivative) − δ(6′-N-protected amino acid derivative).

the presence of the amino acid at the C-6′ position is clearly indicated by the typical upfield shift of β-carbons when their spectra are compared with those of the protected compounds **2a−c,e,f**. Due to N-protonation, there is a large β-shift value of C-2 (7.3−7.8 ppm), C-4 (7.3−8.0 ppm), and C-6 (3.5−4.2 ppm), together with a limited upfield shift of C-2″ (2.2−2.9 ppm) and C-4″ (2.5−3.2 ppm). The lack of any appreciable difference in the chemical shift of C-5′ showed that the amine at C-6′ is protected. 6′-N-Acylation of netilmicin (compounds **8a−e**; Table 4) produced an upfield shift of the carbons C-6′ (2.8−3.0 ppm), C-5′ (4.1−4.4 ppm), and C-4′ (1.0−1.2 ppm) compared to netilmicin. Also, due to protonation, compounds **9a−e** showed, in comparison with **8a−e**, an upfield shift of C-2 (6.3−6.6 ppm), C-4 (5.4−5.8 ppm), C-6 (2.8−3.6 ppm), C-1′ (2.9−3.1 ppm), C-5′ (5.7−8.6 ppm), C-2″ (2.2−3.0 ppm), C-4″ (2.1−2.3 ppm), 3-*N*″-CH_3 (2.0−2.1 ppm), and a downfield shift of C-4′ (4.3−5.1 ppm). 6′-N-Acylation of netilmicin can be easily recognized by the upfield chemical shift of carbon C-5′ (4.1−4.4 ppm, double bonded with carbon C-4′ and in a position β to the acylated amine) in compounds **8a−e**, in comparison with netilmicin. In compounds **5a−e**, the influence of the 1-N-acylation on the α-carbon of kanamycin A shift C-1 (0.8−1.1 ppm upfield) is similar to the C-2 (β-carbon) shift (0.9−1.1 ppm upfield). For the other β-carbon shift, the shift of C-6 is large (7.0−7.3 ppm upfield) in comparison with the corresponding shifts of kanamycin A (Table 5). Similarly the 1-N-acylations of compounds **6a−e** were indicated in comparison with compounds **5a−e** by the upfield shift of C-2 (4.1−4.4 ppm), C-4 (3.6−3.9 ppm), C-5′ (2.0−2.6 ppm), C-2″ (3.9−4.0 ppm), C-4″ (3.9−4.4 ppm), and the unchanged shift of C-6. The chemical shifts of the carbonyls of the amide bond or of the protective groups also allowed for a convenient determination of the acylation sites in kanamycin A, while their signals seem to be characteristically related to the position of the acylated amino function. The amide bond at C-1 appeared generally more downfield than that at C-6′, and the 6′-OCONH carbon also more downfield than the 3-OCONH. Further confirmation of the proposed structures was obtained by FAB mass spectroscopy (Tables 1 and 2).

Microbiological Results. All compounds, converted to sulfate salts, were stable for at least 24 h in distillated water at pH 7.0. Activity was examined against both Gram positive and Gram negative organisms, including strains resistant to selected aminoglycosides. All compounds were virtually inactive, with MIC's higher than 128, except **6b** and **9b** for which a weak activity was occasionally detected (Table 6) in comparison with kanamycin A, netilmicin, gentamicin, amikacin, and isepamicin, the activities of which were in the range of those reported by others.

Toxicological Studies: Inhibition of Lysosomal Phospholipase A_1. Final compounds obtained in sufficient quantities (**9a−e** in the netilmicin series and **3b** and **3e** in the kanamycin A series) were tested for inhibition of the activity of lysosomal phospholipase A_1 toward phosphatidylcholine inserted in phosphatidylinositol-containing liposomes, an *in vitro* test of the potential nephrotoxicity of aminoglycosides.[20,21] This inhibition is related to the ability of aminoglycosides to bind to phosphatidylinositol and thereby to reduce the

Table 4. ^{13}C Chemical Shifts for 6′-N Derivatives of Netilmicin in D_2O Relative to the DMSO-d_6 Peak (40.1 ppm)[a]

	7	8a		8b		8c		8d		8e		9a		9b		9c		9d		9e	
	base	base	$\Delta\delta_3$	base	$\Delta\delta_3$	base	$\Delta\delta_3$	base	$\Delta\delta_3$	base	$\Delta\delta_3$	H^+	$\Delta\delta_4$	H^+	$\Delta\delta_4$	H^+	$\Delta\delta_4$	H^+	$\Delta\delta_4$	H^+	$\Delta\delta_4$
C-2	32.7	33.5	0.8	33.4	0.7	33.4	0.7	33.5	0.8	33.6	0.9	27.1	−6.4	27.1	−6.3	26.9	−6.5	27.2	−6.3	27.0	−6.6
C-4	85.3	85.6	0.3	85.5	0.2	85.5	0.2	85.6	0.3	85.7	0.4	80.0	−5.6	80.0	−5.5	79.8	−5.7	80.2	−5.4	79.9	−5.8
C-6	86.7	86.2	−0.5	86.3	−0.4	86.2	−0.5	86.0	−0.7	86.4	−0.3	83.2	−3.0	83.2	−3.1	83.2	−3.0	83.2	−2.8	82.8	−3.6
C-1′	100.8	100.1	−0.7	100.2	−0.6	100.2	−0.6	100.0	−0.8	100.0	−0.8	97.1	−3.0	97.1	−3.1	97.2	−3.0	97.1	−2.9	97.0	3.0
C-4′	96.7	95.6	−1.1	95.6	−1.1	95.6	−1.1	95.7	−1.0	95.5	−1.2	100.4	4.8	100.4	4.8	100.5	4.9	100.0	4.3	100.6	5.1
C-5′	150.4	146.0	−4.4	146.2	−4.2	146.1	−4.3	146.0	−4.4	146.3	−4.1	139.9	−6.1	139.9	−6.3	139.8	−6.3	140.3	−5.7	137.7	−8.6
C-6′	43.4	40.6	−2.8	40.6	−2.8	40.4	−3.0	40.5	−2.9	40.6	−2.8	39.0	−1.6	39.0	−1.6	38.6	−1.8	38.8	−1.7	38.7	−1.9
C-2″	70.2	69.5	−0.7	69.4	−0.8	69.5	−0.7	69.3	−0.9	69.6	−0.6	67.0	−2.5	67.0	−2.4	66.8	−2.7	67.1	−2.2	66.6	−3.0
C-4″	73.1	72.9	−0.2	72.9	−0.2	72.9	−0.2	72.8	−0.3	72.8	−0.3	70.8	−2.1	70.8	−2.1	70.6	−2.3	70.6	−2.2	70.5	−2.3
6′-NHCO		172.0		172.0		172.0		170.4		172.0		171.6	−0.4	171.0	−1.0	170.0	−1.0	169.8	−0.6	171.0	−1.0
3″-NCH_3	37.7	37.8	0.1	37.8	0.1	37.8	0.1	37.7	0.0	37.7	0.0	35.7	−2.1	35.7	−2.1	35.8	−2.0	35.7	−2.0	35.7	−2.0

[a] $\Delta\delta$ in ppm. Upfield shifts are indicated with negative values and downfield shifts with positive values. $\Delta\delta_3 = \delta$(6′-N-protected amino acid derivative) − δ(netilmicin). $\Delta\delta_4 = \delta$(6′-N amino acid derivative) − δ(6′-N-protected amino acid derivative).

Table 5. ^{13}C Chemical Shifts for 1-N Derivatives of Kanamycin in D_2O Relative to the DMSO-d_6 Peak (40.1 ppm)[a]

	5a		5b		5c		5d		5e		6a		6b		6c		6d		6e	
	base	$\Delta\delta_5$	base	$\Delta\delta_5$	base	$\Delta\delta_5$	base	$\Delta\delta_5$	base	$\Delta\delta_5$	H^+	$\Delta\delta_6$	H^+	$\Delta\delta_6$	H^+	$\Delta\delta_6$	H^+	$\Delta\delta_6$	H^+	$\Delta\delta_6$
C-1	50.4	−0.8	50.4	−0.8	50.1	−1.1	50.3	−0.9	50.3	−0.9	49.6	−0.8	49.6	−0.8	49.5	−0.6	49.6	−0.7	49.6	−0.7
C-2	35.0	−1.1	35.0	−1.1	35.2	−0.9	35.0	−1.1	35.2	−0.9	30.9	−4.1	30.9	−4.1	30.8	−4.4	30.8	−4.2	30.8	−4.4
C-3	49.8	0.1	49.8	0.1	49.6	−0.1	49.8	0.1	49.8	0.1	48.8	−1.0	48.8	−1.0	48.8	−0.8	48.8	−1.0	48.8	−1.0
C-4	83.6	−3.7	83.6	−3.7	83.5	−3.8	83.6	−3.7	83.7	−3.6	79.9	−3.7	79.9	−3.7	79.9	−3.6	79.9	−3.7	79.8	−3.9
C-6	81.4	−7.1	81.4	−7.1	81.2	−7.3	81.5	−7.0	81.4	−7.1	81.5	0.1	81.5	0.1	81.4	0.2	81.4	0.1	81.5	0.1
C-1′	99.4	−0.4	99.4	−0.4	99.3	−0.5	99.1	−0.7	99.3	−0.5	96.3	−3.1	96.3	−3.1	96.2	−3.1	96.2	−2.9	96.3	−3.0
C-4′	71.8	−0.1	71.8	−0.1	71.7	−0.2	71.8	−0.1	71.7	−0.2	71.4	−0.4	71.4	−0.4	71.5	−0.2	71.5	−0.3	71.5	−0.2
C-5′	72.4	−0.5	72.4	−0.5	71.8	−1.1	72.0	−0.9	72.0	−0.9	69.8	−2.6	69.8	−2.6	69.8	−2.0	69.8	−2.2	69.8	−2.2
C-6′	40.9	−1.2	40.9	−1.2	41.0	−1.1	41.2	−0.9	41.3	−0.8	41.3	−0.4	41.3	−0.4	41.0	0.0	41.3	0.1	41.1	−0.2
C-2″	72.8	−0.2	72.8	−0.2	72.8	−0.2	72.8	−0.2	72.8	−0.2	68.8	−4.0	68.8	−4.0	68.8	−4.0	68.9	−3.9	68.9	−3.9
C-4″	70.7	0.7	70.7	0.7	70.6	0.6	70.9	0.9	70.7	0.7	66.4	−4.3	66.4	−4.3	66.7	−3.9	66.5	−4.4	66.6	−4.1
6′-OCONH	159.9		159.9		159.9		159.9		159.9											
3-OCONH	159.1		159.1		159.1		159.1		159.1											
1-NHCO	176.8		176.0		176.8		170.4		176.8		175.7	−1.1	175.7	−0.3	176.0	−0.8	170.0	−0.4	176.0	−0.8

[a] $\Delta\delta$ in ppm. Upfield shifts are indicated with negative values and downfield shifts with positive values. $\Delta\delta_5 = \delta$(6′,3-di-Boc-1-N-protected amino acid derivative) − δ(kanamycin). $\Delta\delta_6 = \delta$(1-N amino acid derivative) − δ(6′,3-di-Boc-1-N-protected amino acid derivative).

density of surface negative charges which are essential for phospholipase A_1 activity to act against phosphatidylcholine embedded in membranes.[30] Results are presented in Table 7, together with those recorded in the present study for kanamycin A, netilmicin, and gentamicin (these values are in the range of those observed by us earlier.[20,21] Substitution of netilmicin in 6′-N by L-Ala (**9a**) caused a 2-fold reduction of its inhibitory potency. Substitution by 6′-N-L-Asp (**9d**) caused only half of this effect, whereas no significant effect was seen with D-Ala (**9b**), Gly (**9c**), or L-Ala-L-Ala (**9e**). Results with substituted kanamycins A are more fragmentary because of lack of sufficient quantities of potentially interesting compounds (in particular, the 6′-L-Ala-kanamycin A could not be tested). No significant difference with their parent drug was observed.

Conformational Analysis. The striking results obtained for the toxicological evaluation of 6′-N-L-Ala-netilmicin prompted us to perform a detailed computer-aided analysis of its interactions with phosphatidylinositol in comparison with its diastereoisomer (**9b**) and with netilmicin (**7**; the analysis of netilmicin was published in ref 31, but the results are given again here for sake of comparison) (Figure 1). As described in refs 32 and 33 and reviewed in refs 20, 34, and 35, both the position and the degree of insertion of an aminoglycoside in a phosphatidylinositol monolayer are likely determinants of its inhibitory potency against lysosomal phospholipase A_1. As shown in Figure 1 (panel A), the most probable conformer of netilmicin (>92% probability) adopts an oblique orientation with respect to the plane of the hydrophobic−hydrophilic interface, with the primed sugar (2′,6′-diamino-2′,3′,4′,6′-tetradeoxy-4′,5′-didehydro-α-D-*erythro*-hexopyranoside) oriented toward the hydrophobic domain, whereas the double-primed sugar (3″-deoxy-3″-(methylamino)-4″-*C*-methyl-β-L-arabinopyranoside) points toward the aqueous phase. A similar orientation of the primed sugar was noticed earlier for gentamicin C_{1a} and for dibekacin (2′,6′-diamino-2′,3′,4′,6′-tetradeoxy-α-D-*erythro*-hexopyranoside in both compounds), but not for kanamycin A (primed sugar: 6′-amino-6′-deoxy-α-D-glucopyranoside.[36] When the molecule of netilmicin is surrounded by phosphatidylinositol (maximum possible: 4; only 3 are represented in the Figure 1 for sake of clarity), it causes a misshaping of the interface already noticed for gentamicin C_{1a}.[37] Thus, even if it appears deeply inserted in the hydrophobic domain, the 6′-amino function of netilmicin may nevertheless establish a close contact with the phosphate head of a phosphatidylinositol molecule (situated on the left in panel A of Figure 1). The same behavior was noticed earlier[20,36] for the primed sugars of gentamicin and dibekacin (which both are deoxygenated in C-3′ and C-4′ like netilmicin; the C-3′−C-4′ bond in netilmicin, however, is unsaturated) but not for tobramycin (which carries an hydroxyl group in C-3′). The energy of interaction of netilmicin with each phospholipid molecule is about 10 kcal. The most probable conformer of 6′-*N*-D-Ala-netilmicin (**9b**; Figure 1, panel B), which has a probability of more than 92%, is oriented almost entirely parallel to the hydrophobic−hydrophilic interface and does not cause the misshaping of the interface observed with netilmicin. The drug could be effectively surrounded by seven phosphatidylinositol molecules, and its calculated energy of interac-

Table 6. Minimum Inhibitory Concentrations (mg/L) of **6b** (6′-*N*-D-Ala-kanamycin A) and **9b** (6′-*N*-D-Ala-netilmicin) in Comparison with Those of Kanamycin A (**K**), Netilmicin (**N**), Amikacin (**A**), Gentamicin (**G**), and Isepamicin (**I**) against Sensitive and Resistant Organisms[a]

Organism/strain[b]	compound						
	K	**6b**	**N**	**9b**	**A**	**G**	**I**
Staphylococcus aureus ATCC 25923	1	16	0.25	32	4	0.5	4
Klebsiella pneumoniae	1	16	0.25	32	2	0.5	1
Escherichia coli ATCC 35218	2	64	0.5	32	2	1	2
Escherichia coli ATCC 25922	8	128	1	128	8	4	8
Citrobacter freundii	4	64	0.25	32	4	1	4
Serratia marcescens	8	128	2	128	16	4	8
Acinetobacter baumanni ATCC 19606	4	128	32	>128	16	32	32
Enterobacter cloacae	4	64	32	>128	4	32	4
Acinetobacter lwoffi ATCC 17986	16	>128	32	128	32	2	16
Enterococcus faecalis ATCC 29212	32	>128	4	128	64	16	64
Pseudomonas *aeruginosa* ATCC 27853	>128	>128	8	>128	32	8	16
Pseudomonas aeruginosa	>128	>128	64	>128	64	64	64
Pseudomonas aeruginosa	>128	>128	>128	>128	64	>128	64
Klebsiella pneumoniae	128	>128	128	>128	32	2	8
Staphylococcus aureus	>128	>128	8	>128	32	>128	2
Escherichia coli	>128	>128	>128	>128	128	16	32
Enterobacter cloacae	>128	>128	>128	>128	32	2	8
Serratia marcescens	64	>128	32	>128	128	4	16
Serratia marcescens	64	>128	>128	>128	64	8	16
Serratia marcescens	>128	>128	128	>128	>128	8	32

[a] MIC values of **3a–c,e** and of **9a,c-e** were >128; **6e** could only be tested at 16 μg/mL and did not show activity. [b] Strains were from clinical origin (Cliniques Universitaires St-Luc and Hôpital Brugmann, Brussels) unless marked with an ATCC (American Tissue Culture Collection) reference no.

Table 7. Inhibitory Potential of Amino Acid and Peptide Derivatives of Kanamycin A and Netilmicin on the Degradation of Labeled Phosphatidylcholine (1-Palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) Included in Negatively-Charged Liposomes (Cholesterol:Phosphatidylcholine:Sphingomyelin: Phosphatidylinositol, Molar Ratio 5.5:4:4:3) Prepared in 40 mM Acetate Buffer pH 5.4 and Exposed to Rat Liver Lysosomal Extracts

compound	IC_{50}[a]
netilmicin (**7**)	55 ± 5
6′-L-Ala-netilmicin (**9a**)	100 ± 2
6′-D-Ala-netilmicin (**9b**)	50 ± 4
6′-Gly-netilmicin (**9c**)	63 ± 15
6′-L-Asp-netilmicin (**9d**)	78 ± 5
6′-L-Ala-L-Ala-netilmicin (**9e**)	63 ± 4
kanamycin A (1)	39 ± 8
6′-D-Ala-kanamycin A (**3b**)	42 ± 6
6′-L-Ala-L-Ala kanamycin A (**3e**)	31 ± 3
gentamicin[b]	45 ± 13

[a] Drug concentration (μg/mL) causing 50% inhibition of lysosomal phospholipase A_1 activity. Each value represent the mean of three determinations ± SD. [b] Commercial mixture of C_1, C_{1a}, and C_2 components.

tion, per phosphatidylinositol, is similar to that reported for the parent compound (11 kcal).

In contrast to all other aminoglycosides examined so far[31–33,36,37] and to 6′-*N*-D-Ala-netilmicin, 6′-*N*-L-Ala-netilmicin (**9a**; Figure 1, panels C and D) could adopt two conformations with an almost equal probability for each of them. The two conformers [referred to hereunder as conformer A (panel C) and conformer B (panel D)] have a different energy of interaction with phosphatidylinositol (11 and 15 kcal/mol for conformer A and conformer B, respectively) and display a markedly different conformation and orientation. Conformer B behaves essentially like 6′-*N*-D-Ala-netilmicin (**9b**). The misshaping of the interface is minimal, and up to 10 molecules of phosphatidylinositol can surround each drug molecule. In contrast, conformer A adopts a highly bent shape with its double-primed sugar and its deoxystreptamine moiety oriented perpendicular to the hydrophobic–hydrophilic interface, whereas its primed sugar displays an orientation parallel to this interface. Careful analysis of the position of the N-6′ and its L-Ala substituent suggests that this bending is due to the interaction of the methylgroup in L-Ala with the hydrophobic domain of the membrane. Insertion of this molecule in the monolayer, which can be surrounded by a maximum of six phosphatidylinositol molecules, results in an important misshaping of the interface, as for netilmicin.

Discussion

The present paper represents a systematic approach to the use of α-amino acids to substitute amino functions of aminoglycoside antibiotics in positions N-1 and N-6 of kanamycin A and netilmicin. Several types of 1-*N*- and 6′-*N*-acyl substitutions in kanamycin A have been reported[10,38] (including one compounds described here (1-*N*-glycylkanamycin A (**6c**)), but mostly using ω-amino acids. Actually these studies revealed that a chain length of 3 to 5 carbons was critical to maintain activity.[38] A potential reason could be that the aminoacyl substituent must fold in a way that brings the ω-NH_2 function close to the N-1 of the deoxystreptamine, restoring a cationic environment in this region of the molecule (see one of the conformer proposed for amikacin in solution in ref 14). Thus, the lack of activity of our α-aminoacyl derivatives could result from an uncorrect displacement of the N-1 amino group. An alternative or additional factor could be the lack of an α-hydroxyl function which was found critical for activity in 1-*N*-aminobutyryl-substituted kanamycins A;[38] note that our compounds (**6c**) (1-*N*-glycylkanamycin A) was already described in ref 38 in a series of ω-aminoalkanoic derivatives of kanamycin A and was found inactive. Because of intrinsic inactivity, we could not explore by microbiological techniques whether the mere presence of a substituent to the N-1 function confers resistence against the bacterial enzymes modifying the

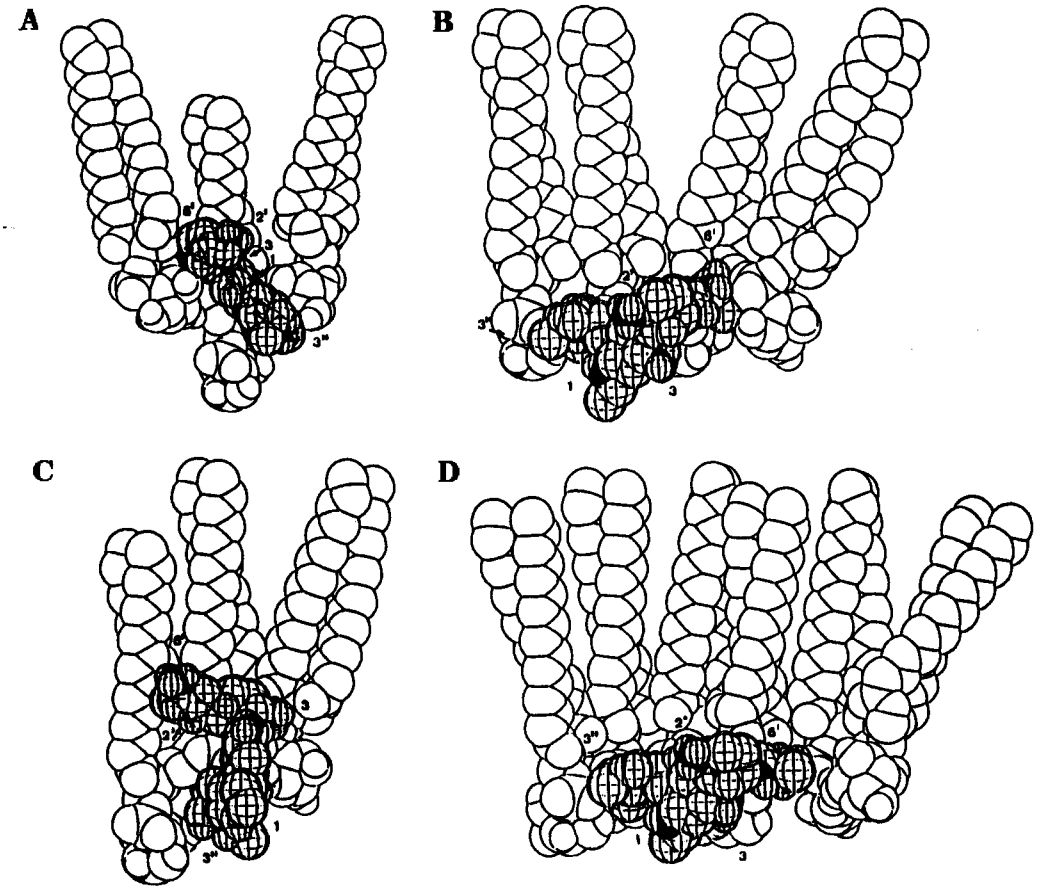


Figure 1. Space-filling views of the mode of assembly of netilmicin (**7**) (panel A) and of compounds **9a** (panels C and D) and **9b** (panel B) (cross-hatched) with phosphatidylinositol. The number of phospholipid molecules surrounding each compound was four for netilmicin (**7**); six for compound **9a** under its conformation A and 10 under its conformation B; and 7 for compound **9b**. The lipid molecules falling in front of the drugs have not been represented for sake of clarity. The arrows figures point to each N atom in netilmicin (**7**) or in the netilmicin moiety of compounds **9a** and **9b** (atom numbering is made according to the convention adopted in ref 25).

positions 2″-OH, N-1, and N-3 which are only at short distance, as is suggested for amikacin.[14] Substitution of the 6′-N function of aminoglycosides with short alkyl group (6′-*N*-methylsisomicin or -amikacin; 6′-*N*-ethyl-, -propyl- and -butylsisomicin, 6′-*N*-methylgentamicin C_{12} (sagamicin)) has already been reported, and these compounds usually show lower but often potentially useful activity.[14]

It was therefore disappointing that our 6′-*N*-aminoacyl or peptidyl derivatives of kanamycin A or netilmicin were all inactive. Probably, not only the presence but also the precise positioning of the amino function in position 6′ is critical. This conclusion would therefore confirm and extend the observation made with the positional isomers of amikacin (**9**) which showed 6′-*N*-[(hydroxyamino)butyryl]kanamycin A to be virtually inactive. Interestingly, the absence of the 6′-N amino group in kanamycins (kanamycin C) still allow for apparent activity. Yet, data obtained with another aminoglycoside devoid of an amino group in that position, namely 3′,4′,6′-trihydroxy-6-deaminogentamicin C_1 (G418, also called geneticin, a compound which inhibits protein synthesis in eucaryotic cells as well as in bacteria) show that activity relates then to another molecular mechanism other than that observed with typical aminoglycosides.[39] As for our 1-N derivatives, we have so far no information about the influence of the aminoacyl substitution of the 6′-N function on resistance of the molecules to 6′-*N*-acetyltransferases.

Compared to the rather disappointing results of the microbiological evaluation, the toxicological evaluation revealed both an unsuspected and potentially interesting result for one netilmicin derivative, namely the 6′-*N*-L-Ala-netilmicin. The 2-fold decrease in the inhibitory potency detected in the biochemical assay of phospholipase activity compared to the parent compound is highly significant in biological terms since it is of the same order of magnitude as that observed between gentamicin C_{1a} and isepamicin, two aminoglycosides with a very large difference in nephrotoxicities and in inhibitory potential toward lysosomal phospholipase A_1 (IC_{50} of ca. 50 and 125 μg/mL, respectively[20]). This is all the more interesting since there is no difference in the number of cationic charges between netilmicin and 6′-*N*-D-Ala-netilmicin. This observation, therefore, reinforces our previous contentions that the inhibitory potency of an aminoglycoside toward the activity of lysosomal phospholipase A_1 is dependent not only on the number of the cationic groups but also on their precise disposition on the molecule.[32] Actually, the conformational analysis suggested that 6′-*N*-L-Ala-netilmicin is in permanent motion between two equally probable conformations, the first one (conformer B, panel D of Figure 1) being very similar to that 6′-*N*-D-Ala-netilmicin (which is as inhibitory as netilmicin itself) and a second one (conformer A, panel C of Figure 1) being highly reminiscent of that of streptomycin,[20,32] which is one of the weakest inhibitor of phospholipase

A_1 among aminoglycosides ($IC_{50} \approx 300$ μg/mL[32]) and which is almost non-nephrotoxic. Its stereospecificity suggests more an effect of the form recognition than of the charge of the molecule. The present study could therefore demonstrate, for the first time, that a dynamic parameter, like an exchange between two positions, could play a critical role in the toxic potential of an aminoglycoside. This may definitely help in the further design of less toxic compounds.

Experimental Section

Chemistry. Melting points were determined on a Buchi micro melting point apparatus and are uncorrected. Specific rotations were determined with a Perkin-Elmer 141 polarimeter using a 10 cm cell. NMR spectra were run on a Bruker AM-200 spectrometer. IR spectra were recorded with a Perkin-Elmer 283B spectrometer. FAB spectra were obtained on a VG Analytical ZAB-SE instrument.

All N-protected amino acids were purchased from Fluka Chemika-Biochemika. *N*-Hydroxysuccinimide carbamate was synthesized as described in the literature.[40] The free bases of kanamycin A and netilmicin were obtained after passage of their sulfate salts over Amberlite IRA 410 (OH^-) resins and lyophilization. All solvents and chemicals were of reagent grade and used without any further purification.

General Procedure for the Preparation of 6′-*N*-Acyl Derivatives of Kanamycin A (2a-c,e,f) and Netilmicin (8a-e). A mixture of **1** or **7** (free base; 1 mmol) and $CuCl_2$ (268 mg, 2 mmol) in DMSO (10 mL) was stirred at room temperature for 24 h. The *N*-hydroxysuccinimide ester of the suitable N-protected amino acid or peptide (**1** mmol) in DMSO (5 mL) was added. The mixture was allowed to stand overnight with stirring at room temperature and absorbed on a column of Amberlite CG-50 (NH_4^+) (50 mL). The column was washed with a mixture of 1,4-dioxane–water (1:1) and thereafter eluted using 1,4-dioxane–water (1:1) containing 0.4% concentrated NH_3 (27%) for the derivatives of kanamycin A and 0.3% concentrated NH_3 for the derivatives of netilminin. The fractions eluted were subjected to lyophilization (yield 70–75%).

6′,3-*N*,*N*-Bis(*tert*-butyloxycarbonyl)kanamycin A (4). A mixture of **1** (free base; 483 mg, 1 mmol) and $CuCl_2$ (268 mg, 2 mmol) in DMSO (10 mL) was stirred at room temperature for 24 h. Di-*tert*-butyl dicarbonate (436 mg, 2 mmol) in DMSO (5 mL) was added. After 5 h of stirring at room temperature, water (15 mL) was added. The mixture was absorbed on a column of Amberlite CG-50 (NH_4^+) (50 mL) washed with 1,4-dioxane–water (1:1). Pure product was obtained by eluting with 1,4-dioxane–water (2:1) containing (0.4%) concentrated NH_3 and evaporation to dryness (491 mg; yield 72%).

General Procedure for the Preparation of 1-*N*-Acyl Derivatives of Kanamycin (5a–e). A mixture of **4** (683 mg, 1 mmol) and $Cu(OAc)_2 \cdot H_2O$ (199 mg, 1 mmol) in DMF (10 mL) was stirred at room temperature for 4 h. The *N*-hydroxysuccinimide ester of the *N*-butyloxycarbonyl-protected amino acid or peptide (1 mmol) in DMF (5 mL) was added, and the mixture was stirred at room temperature for 24 h. The solution was absorbed on a column of Amberlite CG-50 (NH_4^+) (50 mL) washed with 1,4-dioxane–water (1:1). Pure product was obtained by eluting with 1,4-dioxane–water (1:1) containing 0.3% concentrated NH_3 and evaporation to dryness.

General Deblocking Procedure. Method A. Boc-protected compounds (**2a–c,e,f, 5a–c,e, 8a–c,e;** 1 mmol) were dissolved in 4 N HCl/THF (10 mL), and the mixture was stirred for 2 h at room temperature. The solution was then concentrated to dryness and the residue solidified by treatment with anhydrous acetone (yield 85–99%).

Method B: Compounds **5d** or **8d** (0.5 mmol) were dissolved in 99% CF_3COOH (5 mL) and kept at room temperature for 5 min. The reagent was removed by evaporation, and the product was solidified by treatment with anhydrous acetone (yield 80%).

Microbiological Evaluations. Compounds **3a–c,e, 6b,e,** and **9a–e,** dissolved in water and adjusted to pH 9 with concentrated ammonia, were charged onto a packed column of Amberlite CG-50 (100–200 mesh). This column was washed with water and with 0.1 M aqueous ammonia. The fractions obtained after elution with 0.3 M aqueous ammonia were collected and freeze-dried. The free bases of compounds **3a–c,e** and **6b,e** were obtained after purification on a cellulose mikrokristalline column using EtOH:MeOH:H_2O:concentrated NH_3 (5:5:1:2.5). For compounds **9a–e**, the free bases were obtained by column chromatography on a silica gel column using $CHCl_3$:MeOH:concentrated NH_3 (5:5:2) as eluent. The sulfate salts of compounds **3a–c,e, 6b,e,** and **9a–e** were then obtained by preparing 1% aqueous solution of the free bases and adjusting them to pH 5.5 with H_2SO_4. After filtration (0.8μm filter), concentration, and freeze-drying, the solids were triturated with absolute EtOH, dried over P_2O_5, and exposed to the laboratory atmosphere for 24 h before being dissolved in sterile water. The free base content of each of the sulfates was determined by ^1H NMR analysis.[41]

The microbiological activity of compounds **1, 7, 3a–c,e, 6b,e,** and **9a–e** was assessed against Gram positive and Gram negative organisms sensitive to kanamycin A and netilmicin, as well as to organisms resistant to these aminoglycosides but sensitive to commercial gentamicin (mixture of C1, C1a and C2 components), amikacin, or isepamicin (1-*N*-[(*S*)-2-hydroxy-4-aminopropionyl]gentamicin B; Sch 21420[14,20]) as described in ref 42. Defined strains were obtained from the American Tissue Culture Collection, Rockeville, Md. Other strains were obtained from and characterized by the Microbiology Laboratories of the Cliniques Universitaires St-Luc and of the Hôpital Brugmann, Brussels, Belgium. The MIC values reported are the highest dilution at which no visible bacterial growth was apparent and are expressed in mg/L of free base.

In Vitro and Computer-Aided Toxicological Evaluation. The *in vitro* evaluation was performed exactly as described earlier,[43,44] i.e., by determining the inhibitory potential of the derivative under study toward the activity of rat liver lysosomal phospholipase A_1 (measured toward labeled phosphatidylcholine included in negatively-charged liposomes) in comparison with gentamicin (commercial mixture of C1, $C1_a$, and C2 components), kanamycin A, and netilmicin. The conformational analysis of mixed monolayers of phosphatidylinositol and aminoglycosides was performed by a 2-step procedure (calculation of the conformation and orientation of the isolated aminoglycoside molecule at the lipid/water interface, followed by the calculation of the conformation of the drug inserted in a lipid monolayer), as described in detail elsewhere.[22,45] All of these approaches and their rationale are fully described in refs 7, 20, 22, 34, 35, 46.

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