magnitude of the experimental value; the agreement is good when considering the severe approximations made in a multiple substituent effect analysis of this type. This demonstrates that the rate of reaction of **1** and **3a** can be accounted for by the electronegativity of the substituent(s) without involving covalent adduct formation as proposed by Hemmerich[50] or Hamilton.[49] Most hydride transfers exhibit modest deuterium kinetic isotope effects in the 2-5 range, which is less than that calculated from the difference in zero-point stretching vibrations.[52,53] The isotope effect for the reaction of **1** and **3a** agrees well for a direct hydride transfer mechanism, especially when coupled with the knowledge that a $2e^-$ mechanism (without covalent adduct formation) is favored.

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Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy of Neomycin B and Related Aminoglycosides

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Abstract: Natural-abundance ^{15}N NMR spectra of four neomycin B derivatives and their structurally related components are reported. The data suggest that substituents at locations more remote than the γ position have little influence on the ^{15}N chemical shifts, and, therefore, a set of substituent effect parameters could be derived. Assignments for individual ^{15}N signals of the antibiotic derivatives are based on chemical shift comparisons with model aminosaccharides, where possible. Specific assignments of the N-1 and N-3 resonances are based on ^{15}N spin-relaxation experiments in which Gd[2.2.1] cryptate was employed as a spin-labeling reagent for neomycin B in aqueous and dimethyl sulfoxide solutions. For all derivatives of neomycin B studied, the ^{15}N chemical shift data suggest that the 2,6-diamino-2,6-dideoxy-L-idopyranosyl portion (ring D) adopts the $^4C_1$ conformation (**1b**). Complete ^{15}N chemical shift titration data for the antibiotic are used to compute pK_a values for the individual nitrogen functions to within ± 0.04 pK_a unit and also to determine the extent and sites of protonation in commercial neomycin sulfate preparations. In general, ^{15}N protonation shifts are found to be downfield (6.4-14.2 ppm) and have been correlated with nitrogen structural types.

Nuclear magnetic resonance (NMR) spectroscopy has enjoyed an increasingly important role in the structural elucidation of aminoglycoside antibiotics during the past two decades. Initially, ^1H NMR studies[1] at low field provided a direct method for assignment of the configuration of anomeric linkages between specific sugar residues in a number of antibiotic substances. Since the advent of pulse Fourier transform methods, ^{13}C NMR spectroscopy has been applied successfully in studies of the gross structures of many antibiotics.[2]

More recently, natural-abundance, ^{15}N NMR spectroscopy has proved to be a promising method for the structural, conformational, and quantitative analysis of a wide variety of nitrogen containing compounds,[3] including amino sugar derivatives[4-7] and the aminoglycoside antibiotics.[8,9] However, full realization of the potential of this technique must depend on the development of a wider range of spectral assignment methods and correlations of ^{15}N NMR parameters with structure and conformation than is available at the present time. The parameters of particular interest are ^{15}N chemical shifts, coupling constants, spin-lattice relaxation times (T_1), nuclear Overhauser effects (NOE), protonation shifts, substituent effects, and pK_a values. Measurement of ^{15}N relaxation times at natural abundance and on reasonably small samples has become feasible only recently, owing to the development of high-field, superconducting spectrometers having improved sensitivity.

The use of ^{15}N NMR rather than ^{13}C NMR to probe the structures of complex aminoglycosides offers advantages because of the generally simpler and more readily interpretable ^{15}N spectra, the greater sensitivity (by a factor of 2^{3b}) of ^{15}N chemical shifts to structure, and the opportunity to examine pendant amino groups directly, for which conformational effects would be expected to be maximal. However, the ^{15}N method also presents some hazards because of the possibility of nulled resonances due to a partial negative NOE, the absence of well-defined substituent and geometric shift parameters in systems as complex as aminosaccharides, and the uncertain effects of solvents on chemical shifts and of trace paramagnetic metal ions on ^{15}N relaxation phenomena. Also, in contrast to most $^{13}C-^1H$ coupling constants, the measurement of $^{15}N-^1H$ coupling constants is usually complicated by the presence of rapid chemical exchange of NH protons.

In the hope of gaining additional insight into correlation of ^{15}N NMR parameters with the molecular structure and stereochem-

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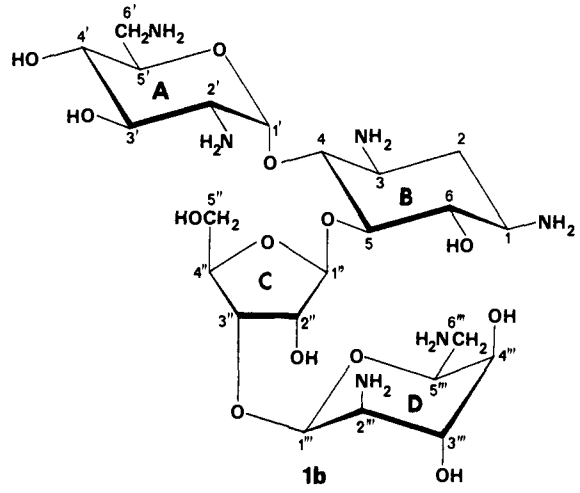
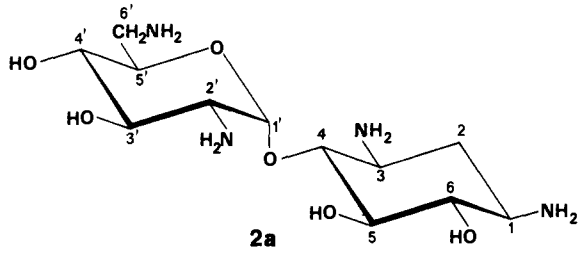
Table I. ^{15}N Chemical Shifts[a] of Neomycin B Derivatives in Aqueous Solution

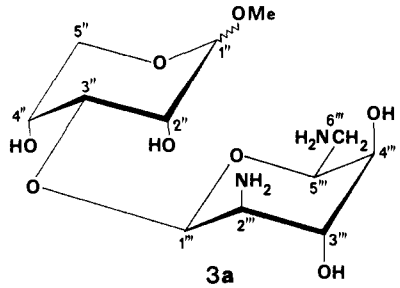
derivative	N-1	N-3	N-2′	N-6′	N-2‴	N-6‴
sulfate[b] (**1a**)	−338.6	−340.4	−343.4	−350.0	−347.8	−349.6
free base (**1b**)	−345.8	−343.5	−353.9	−361.8	−360.7	−359.8
hexahydrochloride (**1c**)	−337.9	−336.3	−342.1	−349.3	−346.5	−348.9
hexa-*N*-acetyl (**1d**)	−246.3	−244.5	−253.4	−259.8	−257.3	−257.7

[a] For solutions in 85:15 (v/v) H_2O:D_2O, in ppm upfield from an external capillary of 1.0 M $NH_4{}^{15}NO_3$ in H_2O; negative values denote upfield shifts. [b] Native pH 6.7 of commercial preparation purchased from Sigma Chemical Co.

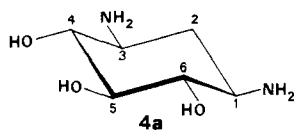
istry of aminoglycosides, the ^{15}N chemical shifts of four derivatives of neomycin B (**1a–d**) have been measured and compared with

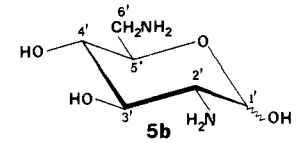


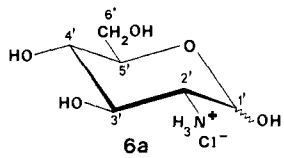


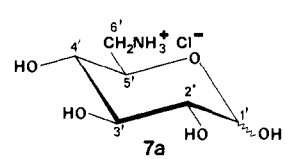


those of its structurally related hydrolytic products, including derivatives of neamine (**2a**), methyl neobiosaminide B (**3a**), 2-deoxystreptamine (**4a**), 2,6-diamino-2,6-dideoxy-D-glucose (**5b**), and also 2-amino-2-deoxy-D-glucose and 6-amino-6-deoxy-D-glucose hydrochlorides (**6a** and **7a**, respectively). Measurements









of pK_a values, ^{15}N relaxation studies, and spin-labeling experiments based on steric accessibility factors were designed to confirm the spectral assignments.

Results and Discussion

The ^{15}N NMR spectrum of an aqueous solution of a commercial preparation[10] of neomycin sulfate[11] displays eight well-resolved resonances with chemical shifts ranging from −338.6 to −350.3 ppm. The six more intense resonances are assigned to the ^{15}N nuclei in neomycin B sulfate. The two less intense signals at −344.5 and −350.3 ppm are assumed to be the resonances of N-2‴ and N-6‴, respectively, in the second 2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl group (ring D) of neomycin C. The remaining four resonances of neomycin C not resolved in this spectrum must coincide with those of neomycin B. The neomycin B/C ratio (9:1) was determined by integration of the ^{15}N signals and was found to be in good agreement with results from ^1H NMR at 400 MHz.[12] Because of the complex behavior of the ^{15}N shifts at intermediate pH values, a more detailed analysis of the ^{15}N spectrum will be presented later in this paper.

The ^{15}N chemical shifts of four derivatives of neomycin B are presented in Table I for comparison with the ^{15}N data of the structurally related pseudo- and monosaccharides reported in Table II. Thus, the resonances in the spectrum of neomycin B hexahydrochloride (**1c**) were assigned by correlation with the ^{15}N shifts of hydrochloride salts of compounds **2–7** in the following manner. The N-2′ and N-6′ resonances for the α anomer of 2,6-diamino-2,6-dideoxy-D-glucose dihydrochloride (**5a**) were assigned by analogy with the ^{15}N shifts found for the 2-amino (**6a**[13]) and 6-amino (**7a**) derivatives and were then used to determine the N-2′ and N-6′ shifts of neamine tetrahydrochloride (**2b**) and the analogous shifts for neomycin B (**1c**). Furthermore, the position of the N-2′ resonance for the α anomer of **5a** is the same in the spectrum of **2b** and nearly unchanged (within 0.1 ppm) in that of **1c**. This suggests that substituents at locations more remote than the γ position have little influence on the ^{15}N shifts and do not complicate the assignments. The same is true for compounds that contain an aminomethyl ($-CH_2NH_2$) group; this type of nitrogen exhibits a fairly narrow range of chemical shifts, −348.9 to −349.8 ppm. Consequently, it is possible to assign ^{15}N shifts for N-6′ and N-6‴ in **1c** on the basis of the relative resonance positions of the aminomethyl ^{15}N nuclei in neamine tetrahydrochloride (**2b**) and methyl neobiosaminide B dihydrochloride (**3b**), and to assign the resonance at −346.5 ppm as N-2‴, by analogy with the unassigned resonance in the spectrum of **3b**. The remaining two resonances at −336.3 and −337.9 ppm were assigned to N-1 and N-3, on the basis of the ^{15}N shift of 2-deoxystreptamine dihydrochloride (**4b**). The latter resonances have been assigned specifically on the basis of spin-labeling experiments using a paramagnetic relaxation reagent (vide infra).

Similar arguments were used to classify nitrogens in the remaining neomycin B derivatives according to their general structural type. Because of the complex dependence of the ^{15}N shifts on pH, specific assignments for neomycin B (**1b**) and its sulfate (**1a**) were based on data obtained from ^{15}N chemical shift

(10) The identification of commercial products in this publication does not constitute endorsement by the National Bureau of Standards.

(11) A mixture containing ~90% neomycin B and ~10% neomycin C, purchased from the Sigma Chemical Co.

(12) Digital integration of well-resolved signals in the H-1 region of the 400-MHz ^1H NMR spectrum of the commercial preparation established a neomycin B:C ratio of 89:11.

(13) The ^{15}N NMR spectrum of this compound has been analyzed in detail previously but was remeasured to conform with the experimental conditions used for the other compounds in this study; see ref 5 and 6.

Table II. ^{15}N Chemical Shifts[a] of Neomycin B Family Aminosaccharides

derivative	N-1	N-3	N-2'	N-6'	N-2'''	N-6'''
neamine						
free base (**2a**)	−345.2	−343.7	−353.8	−361.1		
tetrahydrochloride (**2b**)	−338.0	−336.9	−342.2	−349.8		
tetra-*N*-acetyl (**2c**)	−246.1	−244.3	−253.1	−260.2		
methyl Neobiosaminide B						
free base (**3a**)					−359.8	−359.6
dihydrochloride (**3b**)					−346.4	−349.3
di-*N*-acetyl (**3c**)					−256.7	−257.6
2-deoxystreptamine						
free base (**4a**)	−344.9	−344.9				
dihydrochloride (**4b**)	−337.7	−337.7				
dihydrobromide (**4c**)	−337.6	−337.6				
di-*N*-acetyl (**4d**)	−245.7	−245.7				
2,6-diamino-2,6-dideoxyglucose						
dihydrochloride (**5a**)			α, −342.2	−349.3		
(α:β = 71:29)			β, −343.9	−349.1		
free base (**5b**)			α, −352.6	−361.2		
(α:β = 75:25)			β, −353.3	−361.5		
2-amino-2-deoxyglucose						
hydrochloride (**6a**)			α, −341.7			
			β, −343.2			
N-acetyl (**6b**)			α, −253.6			
			β, −254.4			
6-amino-6-deoxyglucose						
hydrochloride (**7a**)				α, −349.6		
				β, −349.6		
N-acetyl (**7b**)				α, −258.6		
				β, −258.6		

[a] In ppm upfield from an external capillary of 1.0 M $NH_4{}^{15}NO_3$ in H_2O; negative values denote upfield shifts.

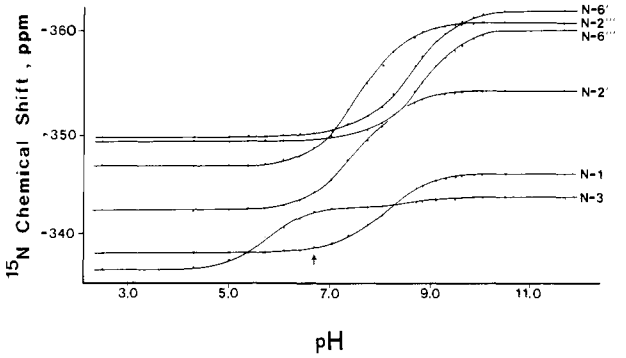
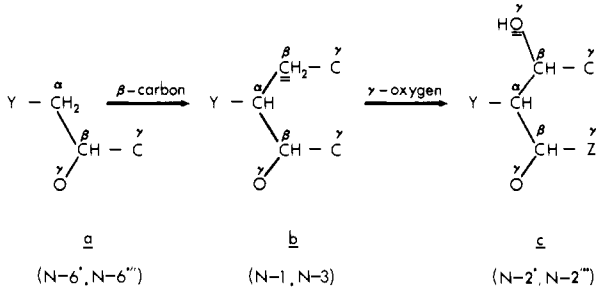


Figure 1. ^{15}N chemical shift titration curves for neomycin B (**1b**).



Y	β-carbon, ppm	γ-oxygen, ppm
NH_2	+16.0	-9.2
$NH_3^+Cl^-$	+12.2	-4.7
NHAc	+13.7	-8.1

Figure 2. Substituent effects on ^{15}N chemical shifts of aminoglycosides.

titration curves of individual nitrogen nuclei (see Figure 1). Thus, the resonances at −360.7 and −359.8 ppm in the spectrum of **1b** were assigned to N-2''' and N-6''', respectively. Assignments for neomycin B sulfate (**1a**) were made by comparing values of the chemical shifts obtained from titration curves at pH 6.7.

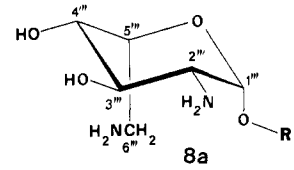
In general, the chemical shifts of the N-acetylated compounds studied are 99.0–103.4 ppm downfield of the corresponding shifts found for their free bases. The magnitude of ^{15}N shift associated with the change $-\ddot{N}H_2 \rightarrow -\ddot{N}HCOCH_3$ is, to some extent, dependent on the structural type (see Figure 2) of nitrogen involved: ~99 ppm for N-1 and N-3 (b) in deoxystreptaminyl groups and ~103 ppm for N-2''' (c) in 2,6-diamino-2,6-dideoxyidosyl groups, with intermediate values found for the remaining nitrogens of the antibiotic. The assignments of the N-2''' and N-6''' resonances in the spectrum of hexa-*N*-acetylneomycin B (**1d**) are based on those made by selective heteronuclear decoupling experiments for methyl di-*N*-acetylneobiosaminide (**3c**). The proton chemical shifts of NH protons in acetamido groups were determined for a solution of **3c** that was identical with that used for measurement of the proton-decoupled ^{15}N NMR spectra. Proton assignments were based on the observed multiplicity of the resonances produced by coupling of the NH proton with either vicinal methylene or methine protons, depending on the type of acetamido group. Continuous wave irradiation employing low-power levels (~0.1 W) at the frequency of the $-NHCOCH_3$ doublet, δ 7.87 (^3J = 8.6 Hz), resulted in a ^{15}N spectrum with an intense resonance at −256.7 ppm and a weaker, partially collapsed doublet (splitting ~30 Hz) centered at −257.6 ppm; irradiation of the $-NHCOCH_3$ triplet at δ 8.19 (^3J = 6.7 Hz) produced the opposite result. Unfortunately, overlapping of resonances in the $-NHCOCH_3$ region of the proton spectrum of **1d** precluded direct analysis of the antibiotic by this method.

From comparisons of the ^{15}N shifts of model compounds, it is possible to make assignments for the various antibiotic derivatives with a reasonable degree of certainty, with the exception of N-1 and N-3 in the deoxystreptaminyl residue. More striking, however, is the regularity of the shifts for the different nitrogen groups within a specific structural class, in compounds that differ widely in their complexity, i.e., number of sugar units. The small variations in chemical shifts may result from changes in solvation

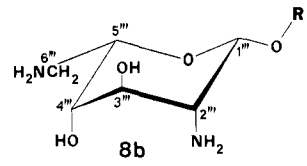
around nitrogen sites with increasing complexity of the aminoglycoside. One observation that has not yet been addressed is the 4–6 ppm shift to higher field found for N-2‴ with respect to the shifts of N-2′ in either the neomycin B derivatives **1a–d** or in compounds **2a–c** and **3a–c**. If the chemical shift correlations are as good as they appear to be, then these differences are significant and may well be a consequence of some "unusual" conformational preference of the idopyranosyl residue. Furthermore, nitrogen chemical shifts calculated from the substituent effect parameters of the compounds studied here may allow distinction of the conformational forms of gluco- and idopyranosyl moieties in neomycin B.

Substituent Effects. Well-defined substituent effect parameters have been established for the ^{15}N chemical shifts of aliphatic and acyclic amines and their hydrochlorides.[14] In general, the α shifts are small and downfield. The β shifts for primary amines and their hydrochlorides (in methanol) are also downfield, but are considerably larger, averaging 21.3 and 15.2 ppm, respectively, for introduction of the first β-carbon substituent. Decreasingly smaller β shifts have been found for the second and third β substituents; a corrected β parameter of −5.0 and −3.6 ppm was introduced to account for each additional branching at the α-carbon in the amines and their hydrochloride salts, respectively.[14b] The γ effects are generally small and upfield, although the magnitude of the shift appears to be dependent upon the electronegativity of the atom at the γ position. Thus, the effect of a γ-carbon atom for primary amines and their hydrochlorides was found to be −3.4 and −2.2 ppm, respectively,[14b] while an oxygen atom at this position causes an upfield shift of ~9 ppm in amines.[14a]

Substituent effects derived for the aminoglycosides studied are shown in Figure 2. The structure–chemical shift correlations indicated are in excellent agreement with those derived for the simple aliphatic amino compounds[14] and for β-carbon effects in piperidines[15] and *trans*-decahydroquinolines.[16] On this basis the empirical calculation of nitrogen chemical shifts for molecules as complex as aminoglycosides may be feasible. It has been noted previously[14b] that the experimental ^{15}N shifts of amino compounds having cyclic substituents do not correlate well with substituent effect parameters, where the shift effects produced by γ-carbons with a completely anti orientation appear to be negligible. No shift effect at all was found for equatorial γ-methyl groups in piperidines.[15] Our independent findings on the ^{15}N shifts of simple aminosaccharides led to similar conclusions concerning the effects of γ-oxygen substituents disposed anti to the nitrogen group,[5,6] although the fact that equatorial ^{15}N nuclei of the common 2-amino-2-deoxy-D-hexopyranoses resonate at lower field than the corresponding axial ^{15}N nuclei can be interpreted in an alternative way in terms of deshielding of the equatorial ^{15}N produced by the anisotropy of a larger number of anti C–C and C–O bonds.[6] A qualitative examination of all of the data leads to a single conclusion: *for amino compounds containing cyclic substituents having a relatively rigid framework, negligible γ effects on shifts are produced when bonding orbitals of γ substituents, independent of whether they are carbon or oxygen, are antiperiplanar to the C–N bond of the amino group.* Using this modified approach to the general method of Duthaler and Roberts,[17,18] we attempted to recalculate the nitrogen shifts for the alicyclic amino compounds studied previously. Ignoring substituent effect parameters for anti γ-carbons led to calculated shifts that were in excellent agreement with the experimental values. Again with our modification, we calculated shifts expected for the various nitrogen structural types found in aminoglycosides (see Figure 2), with the results shown in Table III. Here again, a very good correspondence is found between calculated and experimental ^{15}N chemical shifts, except for the N-2‴ shifts, which are to higher field. On the basis of the substituent parameters, the N-2‴ shifts of L-idopyranosides in the $^4C_1$ conformation (**8a**) are expected to be similar to those



8a



8b

of 2-amino groups in D-glucopyranosides, but this is not the case. In the earlier studies,[5,6] the ^{15}N shifts of α-D-mannosamine derivatives, the structures of which are analogous to that of **8b**,[19] were found to be upfield of their respective α-D-*gluco* derivatives by an increment similar to those found here. The observed differences can be explained on the basis of conformer **8b**, although the possibility of a "twist" conformer cannot be ruled out. It should be pointed out that the N-2‴ shifts calculated for **8b** ($-NH_2$ = −344.2 and $-NH_3^+Cl^-$ = −357.0 ppm) and for α-D-mannosamine hydrochloride are consistently to low field of the experimental values. Thus, the axial–equatorial differences observed for aminodeoxyhexopyranoses cannot be explained solely on the basis of the γ effect associated with the gauche interaction of C-4 with the nitrogen nucleus in structures such as **8b**. Results obtained from studies of aminosaccharide hydrochlorides suggest that the γ effect of a ring oxygen can be as large as −10.7 ppm,[5] presumably as a consequence of the spatial orientation of the lone pairs of electrons on the ring oxygen.[20]

pH Dependence of ^{15}N Chemical Shifts. As mentioned before, pH has a substantial effect on the chemical shifts of these basic nitrogens, as would be expected when protonation occurs directly at the nucleus undergoing the NMR transition. As indicated by the data in Tables I–IV, protonation of the free bases leads to downfield shifts ranging from 6.4 to 14.2 ppm. The magnitude of the protonation shift ($\Delta\delta_N$) depends on the structural type as defined previously. Thus, aminomethyl groups (structural type a) exhibit large values of $\Delta\delta_N$, in the range 10.3–12.5 ppm, but the introduction of a β-carbon atom to give structural type b diminishes $\Delta\delta_N$ to 6.4–7.9 ppm, in agreement with results reported for simple aliphatic amines[14b] and piperidines.[21]

For nitrogen types in the last category (c), the situation is somewhat more complex. Although the insertion of a γ-oxygen atom (b → c) increases $\Delta\delta_N$ once more, the $\Delta\delta_N$ values for 2-amino

Table III. Calculated and (Experimental) ^{15}N Chemical Shifts of Structural Types a–c[a]

chemical formula	a (N-6′, N-6‴)	b (N-1, N-3)	c (N-2′, N-2‴)
$-NH_3^+Cl^-$	−349.7 (−349.4)[b]	−335.9 (−337.5)	−341.6 (−342.0, −346.5)
$-NH_2$	−363.7 (−361.2)	−344.0 (−344.9)	−353.2 (−353.5, −360.3)

[a] Calculated values (in ppm) derived by using additive substituent effect parameters for aliphatic amines in methanol as in ref 17 and γ-oxygen values in Figure 2 with modification; see text for discussion. [b] Chemical shifts (ppm) in ref 17 measured with respect to "1 M HNO_3", conversion constant = −1.4 ppm.[18] Average experimental shifts determined for amino groups of each structural type in compounds **1–7**.

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(17) The method is described in detail in ref 14b; however, we chose not to use the "cor γ" parameter that was introduced tentatively by the authors and, by their own admission, was at the limit of statistical relevance.
(18) The authors assigned positive values to shifts upfield of their reference, which is opposite to the current ASTM convention.
(19) Both pyranose structures contain an axial 2-amino function with *one* trans coplanar hydroxyl group, albeit different ones.
(20) One of the lone pairs on the oxygen atom must assume a 1,3-diaxial relationship with the nitrogen atom. Orientation of lone pairs of electrons on neighboring nuclei has been invoked to explain unusually large differences in nitrogen shielding in other systems; see: Westerman, P. W.; Botto, R. E.; Roberts, J. D. *J. Org. Chem.* **1978**, *43*, 2590–2596 and references therein.
(21) Duthaler, R. O.; Roberts, J. D. *J. Am. Chem. Soc.* **1978**, *100*, 3882–3889.

Table IV. pK_a Values, Protonation Shifts[a] ($\Delta\delta_N$), and Chemical Shifts[b] ($\delta_{NH_3^+}$) Computed for Protonated ^{15}N Functions in Neomycin B

parameter	N-1[c]	N-3[c]	N-2'	N-6'	N-2'''	N-6'''
pK_a	8.04 ± 0.03[d]	5.74 ± 0.04	7.55 ± 0.04	8.60 ± 0.02	7.60 ± 0.02	8.80 ± 0.01
$\Delta\delta_N$	7.5 ± 0.1	6.4 ± 0.1	11.3 ± 0.3	12.0 ± 0.1	13.8 ± 0.2	10.8 ± 0.1
$\delta_{NH_3^+}$	−338.1 ± 0.1	−336.2 ± 0.1	−342.4 ± 0.2	−349.7 ± 0.1	−346.7 ± 0.1	−349.1 ± 0.1

[a] In ppm, for solutions in 85:15 (v/v) $H_2O:D_2O$. [b] In ppm from an external capillary of 1.0 M $NH_4{}^{15}NO_3$ in H_2O; negative values denote upfield shifts. [c] Assignments based on spin-labeling experiments; see text for discussion. [d] ± standard error computed by curve-fitting procedure.

groups in D-glucopyranoses and -pyranosides range from 9.4 to 11.8 ppm, while those for 2-amino groups in the L-idopyranosides are considerably larger (mean, 13.8 ppm). The behavior of the shifts on changing pH undoubtedly reflects the different nitrogen environments in D-gluco- and L-idopyranosides. Indeed, there should be substantial differences in solvation and hydrogen-bonding properties for axial and equatorial amino groups in acidic and basic solutions.[22]

Complete titration data for neomycin B are presented in Figure 1. The complex dependence of the ^{15}N shifts on pH is apparent, especially at intermediate pH values, where problems with peak overlap and relative order of the resonances are most severe. Once resonance assignments have been confirmed at the low-pH extremes by comparing the ^{15}N shifts of an isolated sample of neomycin B hexahydrochloride, the construction of smooth sigmoid curves through the points for each individual nitrogen facilitates assignments over the entire pH range. In those regions where there is some ambiguity, the curves as constructed conform with the values expected for $\Delta\delta_N$. It may also be seen from the curves that all amino groups in the antibiotic are essentially fully protonated at pH <4.5 and fully unprotonated at pH >10.5, as has been found for apramycin and tobramycin.[8,9]

As alluded to previously, the assignments of individual resonances in the spectrum of neomycin B sulfate are based on the titration curves in Figure 1. ^{15}N chemical shift values extracted from the curves at pH 6.7 agree within ±0.1 ppm with those determined for the solution of native neomycin sulfate at the same pH. The close correspondence of the ^{15}N shifts for different salts of the antibiotic suggests that effects from the counterion are minimal in these aqueous solutions. A similar result was obtained for aqueous solutions of hydrochloride and hydrobromide salts of 2-deoxystreptamine (see Table II). It is also apparent from the curves that one of the deoxystreptaminyl nitrogen atoms in neomycin B is essentially unprotonated in solutions of neomycin sulfate preparations at the native pH. This observation is consistent with elemental analysis of the solid salt, which indicated 2.5 sulfur atoms per neomycin molecule.

The pK_a values, $\Delta\delta_N$ values, and ^{15}N chemical shifts for the individual protonated nitrogen functions shown in Table IV were computed from the data in Figure 1 by use of a general curve fitting program,[23] a procedure which assumes that chemical shifts are a linear function of concentration. The pK_a values fall into distinct categories on the basis of the type of nitrogen and have been used to confirm the spectral assignments. Aminomethyl groups (a) are clearly the most basic sites in the molecule, with pK_a values of about 8.7. 2-Amino groups (c) fall into a second category with values ~7.6. On the other hand, the pK_a values for N-1 and N-3 in the deoxystreptaminyl group (b) are very different. Whereas N-1 seems to titrate quite normally with a pK_a value intermediate between those of the other nitrogen types, protonation of N-3 appears to be inhibited (pK_a ~ 5.7) by the repulsive forces established between two positively charged ammonium groups in close proximity. The computed values of $\Delta\delta_N$ (Table IV) are in good agreement with those reflected by the ^{15}N chemical shifts measured (Table I) for the discrete free base (**1b**) and hexahydrochloride salt (**1c**).

Table V. ^{15}N Chemical Shifts, T_1, and NOE Data for 0.24 M Aqueous Neomycin B Solutions at pH 7.6 in the Presence or Absence of 2.4×10^{-3} M $[Gd(2.2.1)]^{3+}$ Cryptate

assignment[a]	δ_N, ppm[b]	T_1(dia), s[c]	NOE(dia)[c]	T_1(para), s[c]	$T_1{}^e$, s[d]	$T_1{}^e$(norm), s[e]
N-1	−340.3	1.5	−4.3	1.1	4.1	1.1
N-3	−342.5	1.7	−3.6	1.2	4.1	4.0
N-2'	−348.2	1.0	−4.4	0.9	9.0	4.8
N-6'	−349.9	3.7	−4.0	2.5	7.7	0.7
N-2'''	−353.6	2.5	−4.6	1.9	7.9	4.0
N-6'''	−351.0	1.8	−4.6	1.4	6.3	0.4

[a] Based on $T_1{}^e$(norm) values and ^{15}N chemical shift titration curves. [b] Negative values denote upfield shifts from an external capillary of 1.0 M $NH_4{}^{15}NO_3$ for diamagnetic solutions. [c] Estimated accuracy ±5–10%. [d] Derived from $1/T_1{}^e = 1/T_1$(para)$-1/T_1$(dia). [e] Normalized values are based on the mole fraction of free base present at pH 7.6; see text for discussion.

Spin-Labeling Studies. Levy et al.[24] have demonstrated the ability of organic-soluble Gd(III) chelates to enhance the relaxation rates of basic nitrogens selectively. Changes in ^{15}N spin–lattice relaxation rates on addition of the paramagnetic relaxation reagent (PARR) were found to be strongly dependent on the availability of lone-pair electrons on individual nitrogens to coordinate with the gadolinium ion of the paramagnetic complex and hence could be related to nitrogen basicities and steric factors. Spin-labeling effects for competing sites within a substrate molecule or in different molecules were monitored by determining the electron–nuclear relaxation times or, under *favorable* conditions,[24] by preferential quenching of the NOE. We decided to test the feasibility of this approach to the nitrogen assignments of such functionally more complex systems as the aminoglycosides, which are complicated by the presence of numerous oxygen-containing groups that may provide additional sites for Lewis acid–base interactions with the PARR. For the method to be successful, two basic criteria must be satisfied: (a) a major portion of the ^{15}N relaxation enhancement must involve complexing of the spin label directly to the nitrogen and (b) the nuclear order of complexes for different nitrogens with the PARR must be the same or if the nuclear orders differ, then their stability constants for complexation should be similar. Several investigations of paramagnetic, cupric complexes with aminoglycosides using ^{13}C NMR as a probe have clearly indicated that the amino groups are the primary binding sites with cupric ion,[25] although both simple ammine complexes[25a] and chelated complexes[25b,c] with vicinal hydroxylamino group pairs have been observed, depending on whether the vicinal oxygen function is –OR or –OH, respectively. Octahedral lanthanide metal complexes are also able to coordinate with basic sites in a substrate molecule, because of the ability of the lanthanide ion to expand its coordination sphere.

(22) For example, the magnitude of the protonation shifts can be rationalized in the following manner: Because of reduced steric accessibility, hydrogen bonding (HO–H···$\ddot{N}H_2$–R) of solvent molecules with an axial amino group should be less favorable than that with an equatorial amino group. Since the direction of shifts on hydrogen bonding should be the same as for protonation, the ^{15}N chemical shifts of equatorial amino groups would tend to be further downfield, and hence the protonation shifts would appear to be smaller. Similar effects have been observed for ^{15}N solvent shifts of basic nitrogen groups in other compounds; see: Botto, R. E.; Westerman, P. W.; Roberts, J. D. *Org. Magn. Reson.* **1978**, *11*, 510–515.

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However, X-ray data[26] on stable $M(dpm)_3 \cdot 2(ligand)$ complexes (where M = Eu or Ho) suggest that chelated complexes with aminoglycosides would be energetically unfavorable, owing to severe steric restriction and the pseudoapical positions adopted by ligands in these complexes.

Earlier studies[27] have shown that (1,10-diaza-4,7,13,16,21-pentaoxabicyclo[8.8.5]tricosane)gadolinium(III) chloride ([Gd(2.2.1)]$^{3+}$ cryptate) is a useful "shiftless" relaxation reagent for the ^{15}N NMR spectroscopy of solutions of nitrogenous materials in water and polar organic solvents. As an extension of the initial studies, the use of this lanthanide complex as a spin label for aqueous solutions of neomycin B has been examined, with the hope that observed selectivities of the relaxation effects based on steric factors that might govern the coordination ability with the PARR would lead to the unambiguous assignment of all ^{15}N signals of the antibiotic.

Natural-abundance, ^{15}N spin–lattice relaxation (T_1) data obtained for diamagnetic and paramagnetic solutions of neomycin B at pH 7.6 are presented in Table V, together with the ^{15}N chemical shifts and diamagnetic NOE data. The fullness of the NOEs indicates that all nitrogens are relaxed largely by the dipolar mechanism (T_1^{dd}). The smaller NOE observed for the N-3 amino group is consistent with its being essentially unprotonated at the pH (7.6) used for the experiment.

Although it would have been desirable to perform the spin-labeling experiments directly on a solution of **1b** (free base), the pH of the solution of **1b** was adjusted to 7.6 for several reasons: (a) the [Gd(2.2.1)]$^{3+}$ complex is extremely labile in highly basic, aqueous solutions,[27] (b) the nitrogen resonances are well dispersed at this pH, and (c) there remain measurable concentrations of the unprotonated form present, at equilibrium, for each amino group (see Figure 1), so as to permit coordination with the PARR. It is also readily apparent from the ^{15}N chemical shift titration data that, at pH 7.6, the relative concentrations of the free base forms of the individual amino groups in the antibiotic are very different and must be taken into account.

Electron–nuclear relaxation arises from the interaction of the nuclear spin with the spin of an unpaired electron and may result from several different mechanisms.[28] The observed electron–nuclear relaxation rate has been described by[29]

$$1/T_1^e(\text{obsd}) = qp/[\tau_M + T_1^e(c)] + 1/T_1^e(t) \qquad (1)$$

where q is the number of substrate ligands in the coordination sphere of the metal complex, p is the ratio of the concentrations of the paramagnetic ion and ligand, and τ_M is the mean residence time of the ligand in the first coordination sphere of the metal. The electron–nuclear relaxation time, $T_1^e(c)$, in the first term of eq 1 is due to both the spin–dipolar and scalar interactions modulated by rotational reorientation of the PARR–substrate complex[28,30] and applies when a substrate is bound directly to the paramagnetic metal ion. The "outer-sphere" relaxation time[31] [$T_1^e(t)$] depends only on the number of paramagnetic ions per unit volume of solution[32] and, therefore, is independent of the ligand concentration.

Equation 1 thus requires a linear dependence of $T_1^e(\text{obsd})$ on the [PARR]/[ligand] ratio. Indeed, with the substrate in large excess and at a constant concentration of $Gd(dpm)_3$, ^{15}N $1/T_1^e(\text{obsd})$ values for pyridine have been shown to increase as a linear function of inverse pyridine concentration.[24] Moreover, from ^{15}N relaxation data, the outer-sphere contribution to ^{15}N relaxation in pyridine under these conditions was shown to be negligibly small, a result that was supported by the estimated ^{15}N parameter, $T_1^e(t) = 60$ s, obtained by correcting ^{13}C relaxation data for the smaller magnetogyric ratio of ^{15}N.[24] For the interaction of neomycin B with the [Gd(2.2.1)]$^{3+}$ complex, the outer-sphere contributions to ^{15}N relaxation would be expected to be even smaller than that found for $Gd(dpm)_3(py)_2$, because the greater bulk of the cryptate ligand would increase the effective ^{15}N–Gd distance. In the subsequent discussion, therefore, the outer-sphere contribution is regarded as being unimportant.

For the case of several, competing nitrogen sites within a single substrate molecule, the ratio of ^{15}N $T_1^e(\text{obsd})$ parameters can be used to determine the spin-labeling efficiency. However, for **1b**, the extent to which each nitrogen site coordinates with the PARR, at a given pH, is undoubtedly limited by the relative concentration of each free amino form. The equilibria leading to complexation with the PARR are

$$R\text{–}NH_3^+ \underset{+H^+}{\overset{K_a}{\rightleftharpoons}} R\text{–}NH_2 + PARR \overset{K_s}{\rightleftharpoons} R\text{–}NH_2{:}PARR \qquad (2)$$

where the relative concentrations of free amine for individual amino groups can be expressed by

$$[R\text{–}NH_2]^{rel} = K_a/([H^+] + K_a) \qquad (3)$$

In order to simplify treatment of these multicomponent equilibria, it is assumed that the protonation equilibria of the six amino sites would not be significantly perturbed by the presence of the PARR at a concentration only 1% that of the neomycin, especially since at any instant of time, this concentration of PARR is divided between complexing with the six competing sites. In this event, the pK_a values in Table IV may be used in eq 3 to calculate $[RNH_2]^{rel}$ for each amino group. If the amine is present in large excess, and if it is assumed that the values of K_s are reasonably large,[24] then the observed electron–nuclear relaxation rates should be directly proportional to the ratio of the concentration of the amine–PARR complex to the total concentration of both the protonated and unprotonated forms. Since the relative proportions of individual nitrogen sites in the molecule must be equal and can be taken as unity, the ^{15}N relaxation rates become simply proportional to $[R\text{–}NH_2]^{rel}$. This makes it possible to normalize the $T_1^e(\text{obsd})$ values with respect to relative concentrations of free amine at pH 7.6, and, thus, the $T_1^e(\text{norm})$ values presented in Table V are the products of the T_1^e values measured and $[R\text{–}NH_2]^{rel}$.

Whereas the experimental T_1^e values in Table V are comparable and do not show obvious trends for different nitrogens, the $T_1^e(\text{norm})$ values fall into two distinct groups. For those nitrogens that have been assigned unambiguously from the chemical shift evidence, the $T_1^e(\text{norm})$ data clearly demonstrate a specific, spin-labeling effect for N-6′ and N-6‴. Clearly, these should be the most accessible nitrogen sites in the antibiotic. Nitrogens N-2′ and N-2‴, which are adjacent to *O*-glycosidic linkages, should be considerably less accessible to the PARR. Thus, the observed selectivities in relaxation enhancement are presumably due to steric effects.

Specific assignments of the N-1 and N-3 signals[33] are now possible, on the basis of the data in Table V. Since the PARR is expected to have more ready access to coordination with N-1 than N-3, the resonance at −340.3 ppm can be assigned to N-1 [cf. $T_1^e(\text{norm})$ values of 1.1 and 4.0 for N-1 and N-3, respectively]. The signals for N-1 and N-3 in neomycin B derivatives **1a–c** reported in Table I are subsequently assigned with the aid of Figure 1. Furthermore, these assignments are consistent with the relative basicities that are expected for N-1 and N-3 on the basis of the inductive effects of their neighboring 6-hydroxy and 4-alkoxy substituents, respectively. Previous measurements of pK_a values

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(33) Definitive assignment of N-1 and N-3 was a particular problem in previous ^{15}N studies of the nebramycins; see ref 8 and 9.

Table VI. ^{15}N Chemical Shifts, T_1, and NOE Data for Neomycin B Free Base in Dimethyl Sulfoxide in the Presence or Absence of 2.9×10^{-3} M $[Gd(2.2.1)]^{3+}$ Cryptate

assignment[a]	δ_{N_i} ppm[b]	T_1(dia), s[c]	NOE(dia)[c]	NOE(para)[c]	% reduction NOE[d]
N-1	−346.6	0.6	−2.8	−1.4	50
N-3	−344.3	0.5	−3.1	−2.0	35
N-2'	−355.6	0.3	−2.6	−2.0	23
N-6'	−363.0	0.8	−3.4	−1.7	50
N-2'''	−360.7	0.4	−3.7	−2.8	24
N-6'''	−361.7	0.7	−3.6	−1.8	50

[a] Based on NOE data and confirmed by data shown in Figure 3. [b] Negative values denote upfield shifts from an external capillary of 1.0 M $NH_4{}^{15}NO_3$ for diamagnetic solutions; measured from an external capillary of saturated $NH_4{}^{15}NO_3$ in H_2O and calculated as $[\delta^{satd}\ NH_4{}^{15}NO_3 - 1.2]$. [c] Estimated accuracy ±5–10%. [d] Calculated as ([NOE(dia) − NOE(para)]100)/NOE(dia).

of amino sugar derivatives indicate that 3-amino-3-deoxyglycopyranosides are slightly more basic than their 2-*O*-methyl derivatives.[34] On this basis, the pyranosyloxy group at C-4 of the deoxystreptamine moiety of **1b** may be expected to have a larger inductive effect (−*I*) than the hydroxyl group at C-6. Consequently, N-1 is expected to be more basic than N-3, as indeed was observed. The pK_a values for N-1 and N-3 also agree with those that have been measured and assigned to N-1 and N-3 in apramycins.[9]

Because the gadolinium cryptate complex is extremely stable in solutions of neomycin B free base in dimethyl sulfoxide, it is possible to compare data from spin-labeling experiments on the free base with that obtained from aqueous solutions at pH 7.6. The relevant data for diamagnetic and paramagnetic solutions of **1b** in dimethyl sulfoxide are shown in Table VI and may be compared with the data in Table V. The ^{15}N T_1 values for the diamagnetic solution in dimethyl sulfoxide are considerably shorter than those for aqueous solutions of the same concentration, and, in fact, the reductions in T_1 values are seen to be proportionate to the increased solution viscosity: experimental values, η_{H_2O} = 1.51 and η_{Me_2SO} = 4.70 cP. However, the NOE values observed (Table VI) for the dimethyl sulfoxide solution are significantly smaller than those expected if relaxation were governed entirely by the dipolar mechanism. The smaller NOE values may possibly result from residual traces of paramagnetic impurities, although an appreciable effort was made to minimize their concentration in the solution (see Experimental Section). Furthermore, the ^{15}N T_1 values obtained for dimethyl sulfoxide solutions containing 2.4×10^{-3} M $[Gd(2.2.1)]^{3+}$ cryptate were only slightly smaller than (or in some cases equal to) those obtained for diamagnetic solutions. Consequently, in order to observe measurable spin-labeling effects in this case, it was necessary to measure the NOE for both diamagnetic and paramagnetic solutions. These NOE data may be compared in Table VI, and the percentage reduction in NOE in the presence of the PARR can clearly be used as a measure of the spin-labeling efficiency at each nitrogen site. Assuming (initially) a similar order of ^{15}N chemical shifts, the observed selectivities in spin-labeling effects found for **1b** in dimethyl sulfoxide solution parallel those shown for aqueous solutions in Table V, with the exception of N-2''' and N-6'''. For these nitrogens, the spin-labeling results predict that the relative order of their ^{15}N line positions in dimethyl sulfoxide solution is the reverse of that found for aqueous solution (see Tables I and VI). This fact has been confirmed by separate studies of the variation in the ^{15}N chemical shifts of **1b** with increasing molar ratios of water in dimethyl sulfoxide. The results illustrated in Figure 3 demonstrate that crossing of the N-2''' and N-6''' signals occurs at a water mole fraction of ~0.5.

Spin-labeling experiments of the type reported here offer a semiquantitative method for the determination of steric accessibility factors at nitrogen sites in complex antibiotic structures. Information provided by the relaxation measurements, in conjunction with a knowledge of the relative base strengths of the nitrogen groups, should serve as an excellent guide for future chemical modifications of the nitrogen sites in antibiotics.

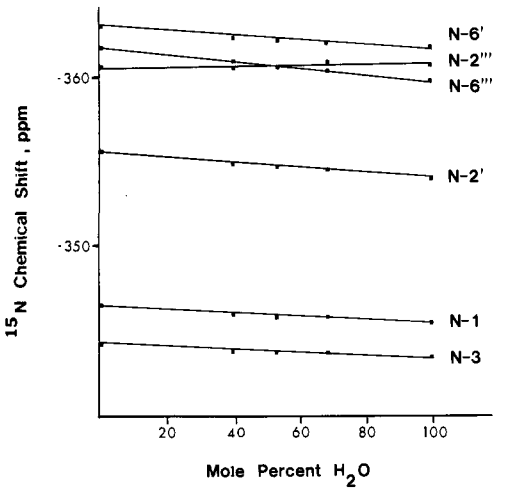

Figure 3. Variation of ^{15}N chemical shifts of neomycin B (**1b**) in dimethyl sulfoxide solution with proportion of water added.

Experimental Section

Neomycin sulfate[11] [Anal. $(C_{23}H_{46}N_6O_{13}\cdot 2.5H_2SO_4\cdot 5H_2O)$ C, H, N, S] and 2-amino-2-deoxy-α-D-glucopyranose were purchased from Sigma Chemical Co. 2-Acetamido-2-deoxy-α-D-glucopyranose, 6-amino-6-deoxy-α-D-glucopyranose hydrochloride, and 2,6-diamino-2,6-dideoxy-α-D-glucopyranose dihydrochloride were obtained from United States Biochemical Corp. All D-glucopyranose derivatives were used without further purification.

Methanolysis of Neomycin Sulfate. The experimental procedure employed was similar to that of Ford and co-workers[35] but was revised to include the additional purification steps described below:

Neamine tetrahydrochloride isolated in the manner described previously[35] was dissolved in methanol (200 mg/10 mL). Anhydrous ether was added dropwise to the solution until it became turbid, and the insoluble matter was removed by filtration through a medium-porosity sintered-glass funnel. The purity of the product remaining in the mother liquor was monitored by TLC on silica gel (plastic sheets, Kodak) by employing 2:1 (v/v) methanol:acetone as solvent and detection with 1% ninhydrin in 1-butanol. This precipitation procedure was repeated until the major impurity, unreacted neomycin hexahydrochloride (R_f 0.05), had been removed. By use of this procedure, neamine tetrahydrochloride (R_f 0.38) could be isolated with greater than 99% purity.

Methyl neobiosaminide B dihydrochloride (R_f 0.61) was isolated in >98% purity as a mixture of α and β anomers by dissolution of the crude methanolysate in a minimum amount of methanol followed by titration with anhydrous ether. In this case, the major impurity was the less soluble neamine tetrahydrochloride. The identity and purity of both products were verified by their ^1H NMR spectra.

Separation of Neomycin B from Neomycin C and General Procedure for Generation of Aminoglycoside Free Bases. In a typical preparation, a solution of 2.2 g of neomycin sulfate (9:1 mixture of neomycin B and C) in a minimum volume of water was passed and washed through 40 mL of Amberlite IRA-900C (OH form) until the eluate tested negatively with 1% ninhydrin in pyridine. For most purposes, a 50% excess of the ion-exchange resin was used. The initial 40-mL volume of eluate was found to contain mostly neomycin C (violet to ninhydrin); subsequent fractions, 50–175 mL, were combined and lyophilized to give pure neomycin B (blue to ninhydrin) in yields ranging from 85–95%.

Preparation of *N*-Acetylaminoglycosides. In general, 120 mL of anhydrous methanol and 40 mL of acetic anhydride were used for acetylation of each 15 mequiv (based on amine content) of free base, dissolution of which usually occurred within 0.5 h. The reaction mixture was stirred at room temperature overnight or until an aliquot from the solution produced a negative reaction toward ninhydrin. The product was precipitated from solution by the addition of 300 mL of anhydrous ether and was filtered, washed with several small aliquots of anhydrous ether,

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and dried under vacuum at 35 °C. Typical yields were better than 95%.

NMR Experiments. Proton-decoupled, ^{15}N NMR spectra were recorded at 9.4 T (40.53 MHz for ^{15}N and 400.134 MHz for ^{1}H) on a Bruker Instruments spectrometer, Model WM-400, in the pulse Fourier transform mode, with quadrature phase detection.

^{15}N measurements were made by using 15-mm sample tubes that contained 50–100 mM solutions of the aminosaccharides in 85:15 (v/v) H_2O:D_2O. The operating parameters included a spectral width of 20 kHz, a pulse width of 40 μs (75° flip angle), an acquisition time of 0.4 s, and a pulse repetition time of 3.0 s. Two-level, broad-band proton decoupling performed at high power (5 W) during acquisition of data and at low power (1 W) during the relaxation delay provided decoupled spectra *with* the NOE and avoided excessive heating, so that the samples remained at 26 ± 2 °C. In a typical experiment, 16K of memory was allocated for data acquisition and was then increased to 32K (16K real data) by zero filling. Before Fourier transformation of the data, 2 Hz of exponential line broadening was applied. Transformed spectra had a digital resolution of 1.2 Hz (or 0.03 ppm) per data point. The ^{15}N chemical shifts are estimated to be accurate to ±0.1 ppm. Many samples gave no useful ^{15}N NMR spectra until paramagnetic impurities had been removed. This was accomplished by passing the NMR solution through a small column of Chelex-100 ion-exchange resin[36] into a sample tube that had been pretreated with a solution of ethylenediaminetetraacetic acid, disodium salt. The pH of solutions for NMR was adjusted with ~1 N and ~4 N NaOH or HCl.

The pK_a values, ^{15}N protonation shifts ($\Delta\delta_N$), and ^{15}N chemical shifts (δ_N) of the protonated amino groups of neomycin B (see Table IV) were computed[23] by fitting the ^{15}N chemical shift titration data (Figure 1) to the function $\delta_N = \Delta\delta_N 10^{-pK}10^{pH}/(1 + 10^{-pK}10^{pH}) + \delta_{NH_3^+}$ by use of the on-line, mathematical modeling laboratory component (MLAB) of the NIH/EPA Chemical Information System. Experimental ^{15}N chemical shift titration curves were plotted by using MLAB and compared with the appropriate theoretical curves calculated from the parameters obtained from the best fit. These parameters were confirmed by separate computations in which the curve fitting and plotting were programmed on a Univac 1108 system.

(36) A chelating, cationic exchange resin sold by Biorad Laboratories.

^{15}N spin–lattice relaxation times (T_1) were measured by using the fast inversion–recovery (FIRFT) sequence[37] modified to include two-level, broad-band proton decoupling (vide supra). A fixed delay of 2.0 s was used between pulse sequences and ten variable delays (τ values) ranging from 0.03 to 15.0 s. Sample temperatures were maintained at 25 ± <1 °C throughout an entire T_1 experiment by using low decoupling power during variable and fixed delay times. The free induction decay for each τ value was acquired in 4K of computer memory by using a spectral width of 4 kHz and was then expanded to 16K (8K real data) by zero filling; finally, exponential line broadening (1 Hz) was applied [digital resolution = 0.5 Hz (or 0.01 ppm) per data point]. The 90° pulse (44.5 μs) was determined for 80% formamide in D_2O. Degassed solutions of neomycin B free base were treated with Chelex-100 resin prior to T_1 measurements. Paramagnetic solutions were prepared by the direct addition of solid (1,10-diaza-4,7,13,16,21-pentaoxabicyclo[8.8.5]tricosane)gadolinium(III) chloride, $[Gd(2.2.1)]Cl_3$, which was synthesized according to the procedure of Gansow et al.[38]

Pulse sequence recycling times used in nuclear Overhauser experiments were $>8T_1$. Spectrometer parameters identical with those for ^{15}N relaxation measurements were employed.

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Affinities of Phosphoric Acids, Esters, and Amides for Solvent Water

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Abstract: The equilibrium constant for transfer of trimethyl phosphate to the vapor phase from dilute aqueous solution was found to be 3×10^{-7}, which is lower than that of methyl acetate by a factor of approximately 10^4. As each alkyl group was removed from trialkyl phosphates, affinity for solvent water was enhanced by an additional factor of more than 10^4, as indicated by distribution coefficients between chloroform and water. Differences in free energy of solvation between reactants and products were found to be more than sufficient to account for the favorable free energy of hydrolysis of phosphate esters in water, so that these reactions would be endergonic in the vapor phase. Distribution properties of phosphoric amides, methyl ethylene phosphate, and triethylphosphine oxide were also investigated.

Derivatives of phosphoric acid are widespread constituents of lipids, nucleic acids, proteins, and carbohydrates and serve as reactive intermediates in the action of several enzymes. These compounds are distinguished from other classes of biological molecules by their extreme polarity. In considering possible influences of solvation effects on equilibria of their chemical transformations in water and on their binding affinities for biological receptors, it would be helpful to have quantitative information about the relative affinities of various phosphorus derivatives for watery surroundings. Substituted phosphonic acids, for example, have sometimes been prepared as analogues of unstable phosphoric acid derivatives in the hope that they might serve as stable enzyme inhibitors: are they more or less hydrophilic than the parent compounds? Vapor pressure measurements have shown that the large negative free energy of carboxylic ester aminolysis, a central reaction in protein biosynthesis, is more than completely matched by the difference in free energy of solvation between reactants and products.[1] Can the negative free energy of hydrolysis of phosphoric acid esters[2-4] be analyzed in similar terms?

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