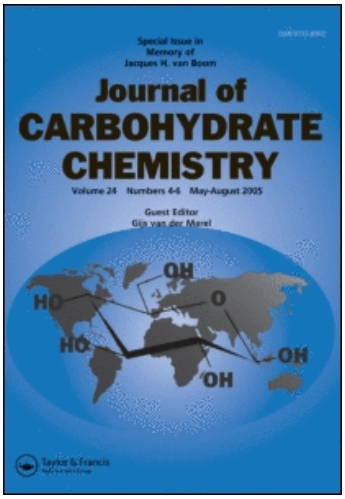

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TWO-DIMENSIONAL PROTON J-RESOLVED NMR SPECTROSCOPY OF NEOMYCIN B

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ABSTRACT

The ^1H NMR spectrum of a solution of neomycin B free base (Structure 1) in D_2O has been assigned completely by two-dimensional, homonuclear J-resolved NMR spectroscopy and spin decoupling at 400 MHz. Proton chemical shifts and proton-proton couplings are reported for all glycoside residues in neomycin B along with their computer simulated spectra. The $^4C_1$ chair conformation has been assigned to the 2,6-diamino-2,6-dideoxy-β-L-idopyranosyl (ring D) portion of the antibiotic (1b) by analysis of the proton coupling constants and chemical shifts. The β-furanose form of the ribosyl portion (ring C) has been assigned. Vicinal proton couplings for the 2-deoxystreptaminyl group (ring B) are consistent with a chair conformation in which all ring substituents are equatorial, and proton chemical shift assignments are based on protonation studies. A computer simulated composite of the individual calculated spectra is presented for comparison with the experimental spectrum of neomycin B.

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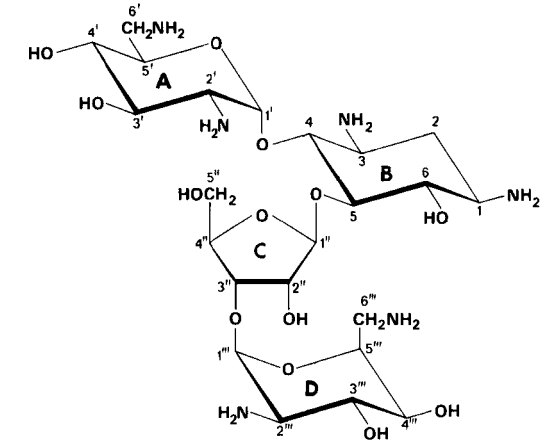

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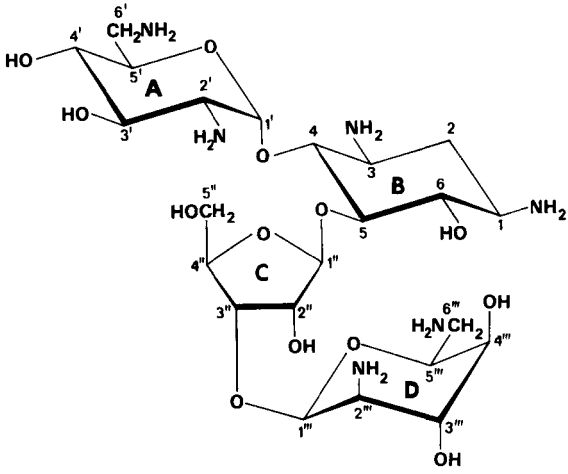

b

Structure 1. Conformation of neomycin B found (a) in the literature[4] and (b) by ^{1}H NMR at 400 MHz.

INTRODUCTION

Despite more than thirty years of considerable research effort devoted to the isolation and characterization of the neomycin antibiotics,[1] the complete solution conformation of neomycin B (1) has not been defined with certainty. The focus of controversy concerns the ground state conformation of the 2,6-diamino-2,6-dideoxy-β-L-idopyranosyl portion (ring D) in neomycin B. In fact, the conformation of ring D has been a subject for debate since the very early work of Rinehart and coworkers[2] who had observed that N-acylated derivatives of this aminoglycoside were not readily oxidized by periodate and had invoked conformational arguments to explain this extraordinary behavior.

NMR spectroscopy has enjoyed an increasingly important role in the structural elucidation of aminoglycosides in recent years. Unfortunately, ^{1}H spectra of the neomycins have not been amenable to complete analysis of ring protons apart from the anomeric hydrogen atoms because of severe overlap of resonance multiplets. Indeed, early ^{1}H NMR studies[3,4] at low field did provide a direct method for assignment of the configuration of anomeric linkages between specific sugar residues. Later, results from ^{13}C NMR studies[5] demonstrated that conformations of idopyranosides can vary considerably between the extreme $^{4}\underline{C}_{1}$ and $\underline{^{1}C}_{4}$ forms depending on several factors. However, ^{13}C NMR analysis of neomycin B derivatives led to inconclusive results concerning the discrete ground state conformation of the 2,6-diamino-2,6-dideoxy-L-idopyranosyl portion (ring D) in the antibiotic.[6] More recently, ^{15}N NMR analysis[7] of several derivatives of neomycin B and its hydrolytic products implied that the N-2''' (ring D) shifts were consistent with the $^{4}\underline{C}_{1}$ conformation (axial orientation of the 2-amino group), although, as in previous work, the possibility of a "twist" conformation could not be entirely excluded.

The development of two-dimensional (2D) NMR methods[8] in recent years has facilitated the routine analysis of complex

spectra for a wide variety of organic substances. In particular, homonuclear J-resolved 2D spectroscopy[8-12] is especially useful for the simplification of complex proton NMR spectra since it has the particular advantage that the chemical shift and coupling information can be separated and displayed in different dimensions.

This paper describes a ^1H NMR investigation of neomycin B free base at 400 MHz. Even at this reasonably high spectrometer frequency, the proton spectrum of neomycin B is not completely dispersed. Consequently, homonuclear 2D J-resolved NMR has been applied to facilitate the separation of all proton resonances in the NMR spectrum of the antibiotic, thereby allowing the solution conformations of its various rings to be assigned unambiguously.

RESULTS AND DISCUSSION

The complete 400 MHz proton NMR spectrum of a solution of neomycin B free base in D_2O is shown in Figure 1. The intense resonance of HOD from the exchangeable hydroxyl and amino protons has been eliminated from the spectrum for simplification. Although proton resonances in the high-field and low-field regions of the spectrum appear well dispersed, there is severe overlap of

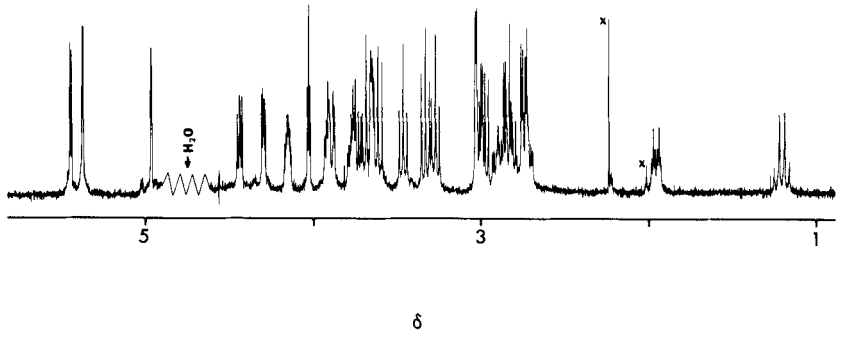

FIG. 1. ^1H NMR spectrum of a solution of neomycin B free base in D_2O at 400 MHz. X denotes impurities.

the spin multiplets in the central region. Rings A-D of neomycin B can be considered as four isolated 6-7 spin systems of protons, thus complete analysis of the entire proton spectrum by conventional spin decoupling methods requires a large number of separate experiments. Nevertheless, the minimum number of decoupling experiments required for total analysis was performed, leading to the assignment of all multiplet patterns and spin coupling constants in the proton spectrum. It should be pointed out, however, that assignments made exclusively by spin decoupling techniques in complex spectra containing overlapping multiplets can suffer from some ambiguities, owing to the presence of non-selective off-resonance effects. Therefore, confirmation of the spin-spin couplings for neomycin B was obtained from a single homonuclear 2D J-resolved NMR experiment.

Proton 2D J-resolved spectroscopy is based on spin-spin coupling modulation[13] of resonance intensities in a 1D spin-echo experiment.[14,15] Thus, the pulse sequence utilized in the 2D experiment is simply $(\pi/2) - \underline{t_1}/2 - (\pi) - \underline{t_1}/2 - \underline{t_2}(AQ)$. The initial $\pi/2$ pulse nutates the equilibrium magnetization vector aligned along the $\underline{z}$-axis into the $\underline{x}$-$\underline{y}$ plane, and this transverse magnetization is then allowed to evolve during the total incremental delay time $\underline{t_1}$. Individual lines of multiplets are phase modulated as $\underline{t_1}$ is varied, at frequencies determined by the spin coupling constants and the nature of the multiplet patterns. It is important to realize that this phase modulation is maintained throughout the entire $\underline{t_1}$ period due to the fact that proton spin states coupled to the protons giving rise to multiplets are inverted during the second $\underline{t_1}/2$ delay period as a result of the π pulse. Because it is a broadband pulse, it simultaneously reverses the orientation of all protons in the system. Thus, the slower and faster rotating spin coupling components of a given multiplet do not refocus at time $\underline{t_1}$ as occurs with components that have fanned out due to magnetic field inhomogeneity.

A set of free induction decay signals is acquired during time $\underline{t_2}$ for a series of closely spaced values of $\underline{t_1}$, and the resulting

data matrix is Fourier transformed first with respect to $\underline{t}_2$ to give a new data matrix whose columns are amplitude modulated by both the chemical shift and coupling constant frequencies. After a second Fourier transformation (of the columns in the $\underline{t}_1$ dimension), tilting of the doubly transformed matrix then gives a data matrix which is purely either $\underline{f}_1$ or $\underline{f}_2$ in each dimension. In the tilted 2D matrix, the spin multiplet patterns are orthogonal to the shift axis and hence can be separated along this axis as long as there is some difference in chemical shift.

The complex regions of the 2D J-resolved proton NMR spectrum of neomycin B are shown in Figure 2. The J dimension and chemical shift dimension are $\underline{f}_1$ and $\underline{f}_2$, respectively. Resolution of the data is facilitated by the proton-decoupled proton spectrum (bottom traces) obtained by projection of the 2D spectrum onto its chemical shift axis.[16] However, it should be pointed out that the software available at the time this work was performed gave nonadditive line intensities in the projection spectra. Consequently, peak areas are not a quantitative index of actual proton content for each resonance. Proton chemical shifts presented in Table 1 have been rigorously assigned by 2D analysis of the spin coupling patterns, and by 1D homonuclear decoupling experiments to confirm spin-spin coupling connectivities. Cross sections[16] of the 2D spectra (Figure 2) taken perpendicular to the chemical shift axis yield individual J spectra devoid of chemical shift information and whose spacings represent spin-spin coupling only. A sequential plot of these J spectra is presented in Figure 3. The 2D method yields complete resolution of all 27 inequivalent proton spin multiplets, despite their incomplete resolution in the 1D spectrum of the antibiotic. Optimum resolution enhancement was applied to the J spectra by Gaussian, exponential filtering of the interferograms. A complete set of CH coupling constants for individual glycoside residues is derived from analysis of the J spectra and these values are also presented in Table 1.

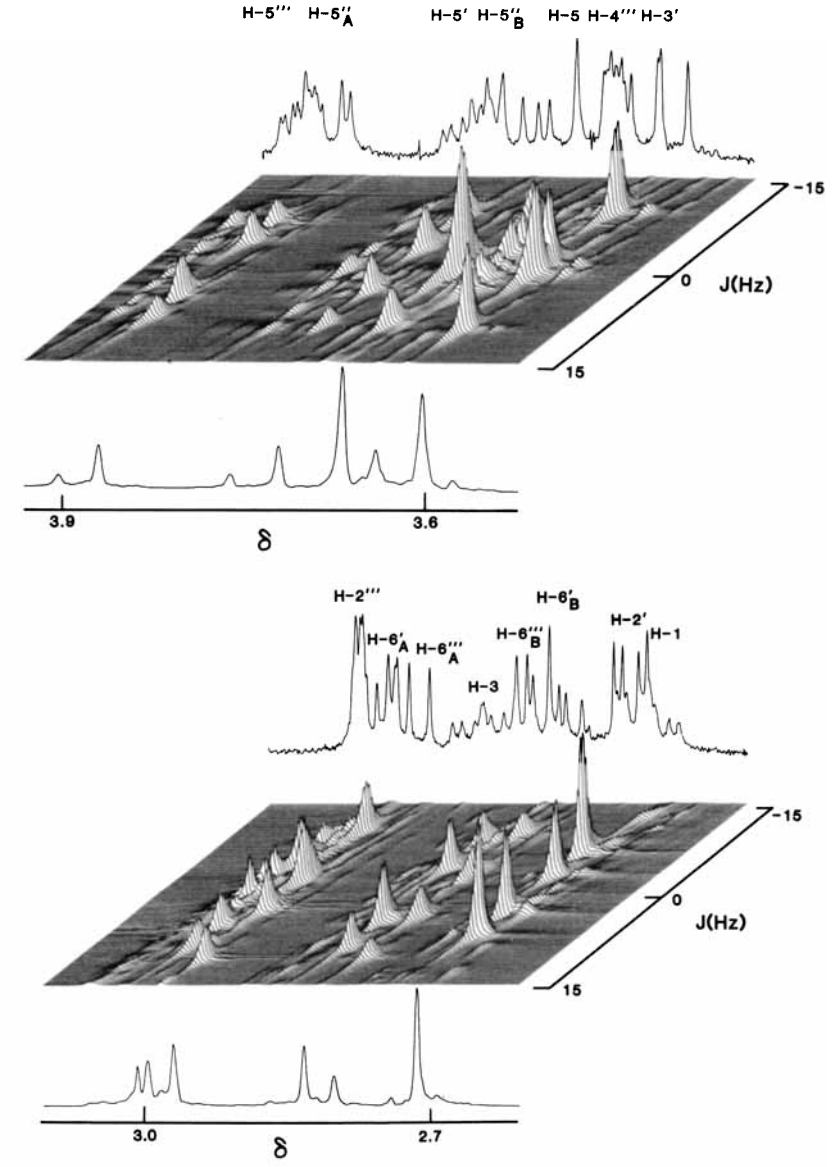

FIG. 2. Selected regions of the 2D J-resolved ^{1}H NMR spectrum of neomycin B. Proton assignments for rings A-D are those depicted in structure 1.

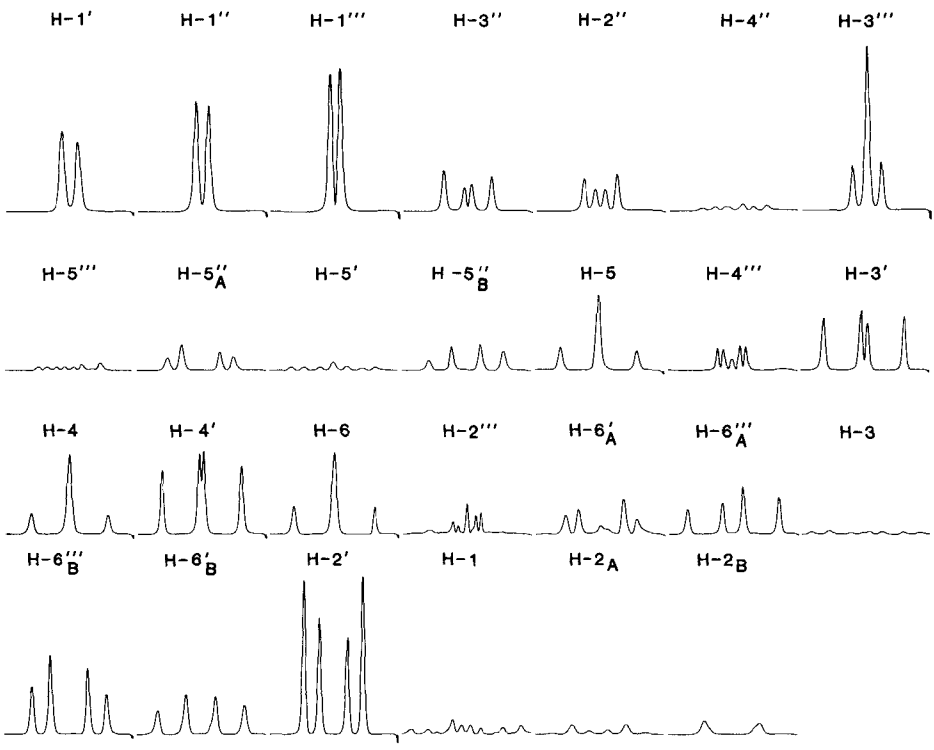

FIG. 3. Resolution enhanced, 2D ^{1}H NMR J spectra of neomycin B. Proton assignments as in FIG. 2.

Confirmation of the analysis of the 2D J-resolved data was obtained by simulation of the six or seven spin systems comprised by the CH protons of rings A-D of neomycin B. Individual simulated spectra for rings A-D are shown in Figure 4. The refined chemical shifts and coupling constants presented in Table 1 were determined by repetitive analysis of the spin systems for the individual rings until good agreement between the experimental and calculated composite spectra was obtained (see Figure 5). The composite simulated spectrum for the entire neomycin B molecule was obtained by addition of the four simulated spectra in Figure 4

TABLE 1. Proton NMR Parameters for Individual Glycoside Residues of Neomycin B in D_2O at 400 MHz.[a]

Sugar Moiety[b]	H-1	H-2 (H-2')	H-3	H-4	H-5 (H-5')	H-6 (H-6')
RIB	5.37	4.29	4.43	4.14	3.88 (3.73)	--
DOS	2.72	1.95 (1.20)	2.89	3.46	3.68	3.26
2,6-DAG	5.45	2.74	3.61	3.33	3.77	3.00 (2.82)
2,6-DAI	4.96	3.02	4.02	3.64	3.91	2.97 (2.83)

	$J_{1,2}$ ($J_{1,2'}$)	$J_{1,6}$	$J_{2,3}$ ($J_{2',3}$)	$J_{2,4}$	$J_{3,4}$	$J_{4,5}$ ($J_{4,5'}$)	$J_{5,6}$ ($J_{5,6'}$)	$J_{X,X'}$[c]
RIB	2.7		5.1		6.2	3.1 (5.3)		-12.4
DOS	4.1 (12.4)	9.7	4.1 (12.4)		9.5	9.1	9.7	-12.4
2,6-DAG	3.9		10.3		9.1	9.7	3.4 (6.9)	-13.6
2,6-DAI	1.8		3.3	1.1	3.3	1.5	8.6 (4.4)	-13.5

[a] Chemical shifts in ppm downfield from internal TSP; coupling constants in Hertz.
[b] RIB = ribofuranosyl; DOS = 2-deoxystreptaminyl; 2,6-DAG = 2,6-dideoxy-2,6-diaminoglucopyranosyl; 2,6-DAI = 2,6-diamino-2,6-dideoxyidopyranosyl.
[c] $J_{X,X'} = J_{5,5'}$ for RIB, $J_{2,2'}$ for DOS, and $J_{6,6'}$ for 2,6-DAG and -DAI.

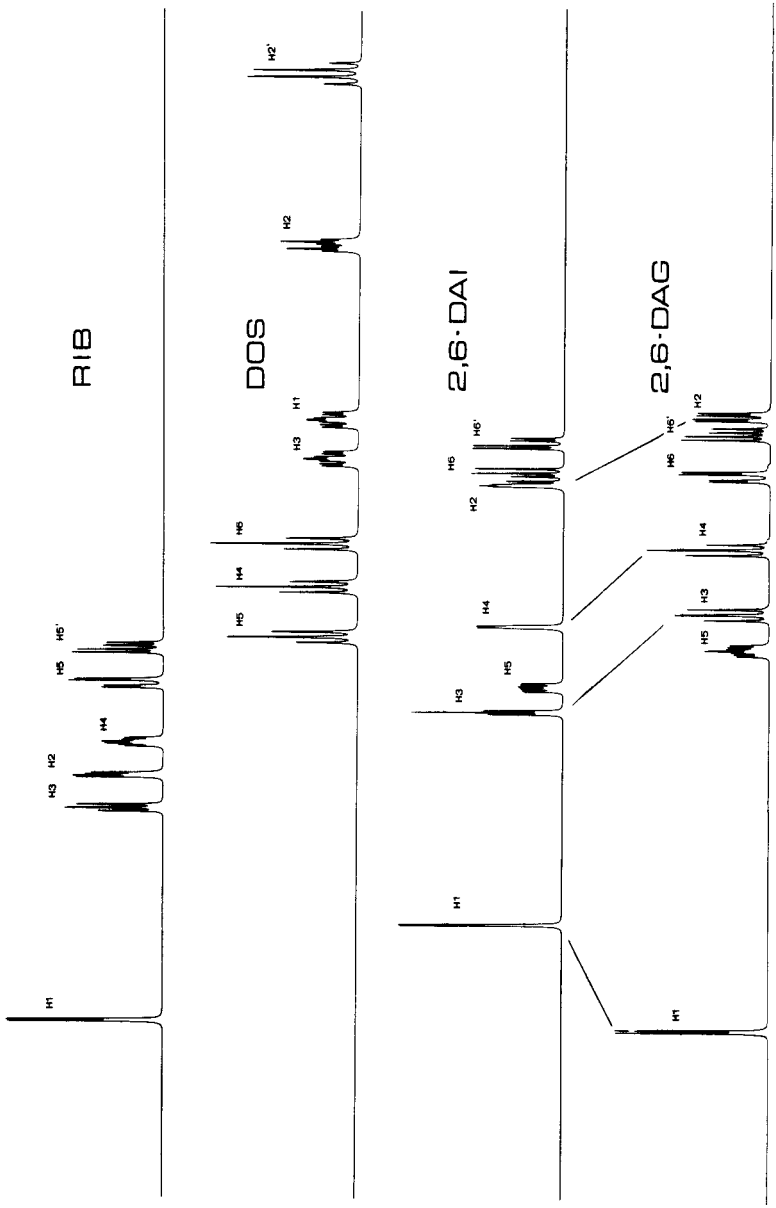

FIG 4. Simulated spectra of individual rings A-D in neomycin B. Proton assignments as in TABLE 1.

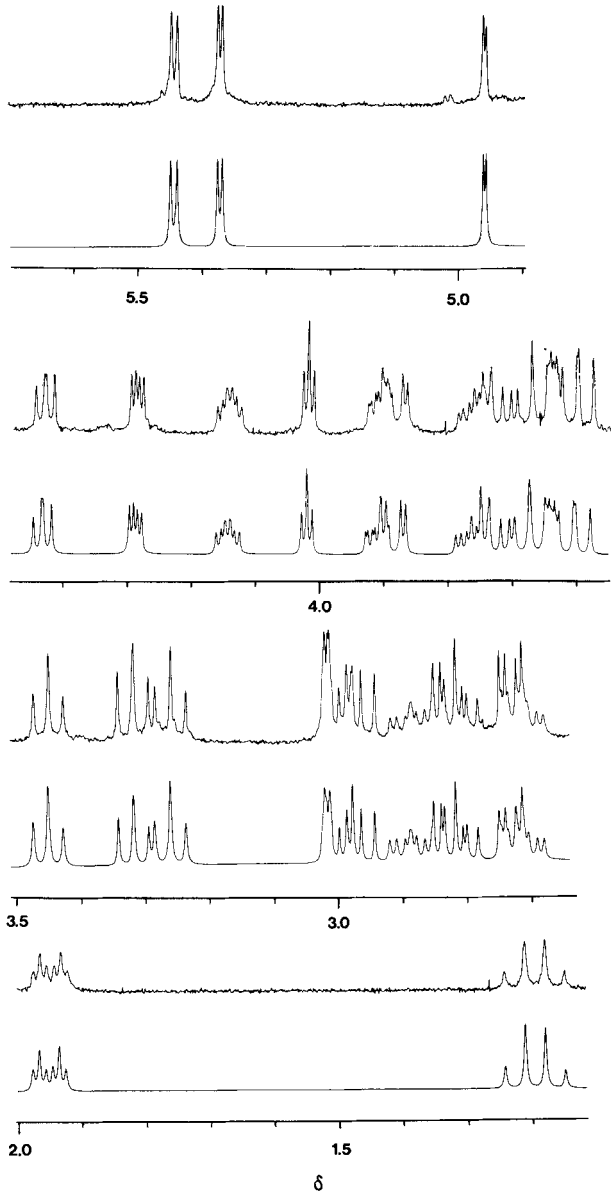

FIG. 5. Experimental (top) and simulated composite (bottom) ^{1}H NMR spectra of neomycin B in D_2O at 400 MHz.

(see Experimental section). The chemical shift and coupling constant values obtained in this manner were very similar to those measured from the 2D J-resolved NMR experiment.

The values of the vicinal CH proton-proton coupling constants found for the 2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl (2,6-DAG) portion are consistent with those expected for the $^4C_1$ conformation (ring A in 1). The large values for $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ of 10.3, 9.1, and 9.7 Hz, respectively, indicate that the H-2/H-3, H-3/H-4 and H-4/H-5 pairs have a trans-diaxial orientation. The small value $J_{1,2}$ 3.9 Hz is consistent with the gauche orientation (dihedral angle of ~60°) for H-1 and H-2 as would be expected for the α-anomer.[17-19] Similar couplings have been observed previously for 2-amino-2-deoxy-α-D-glucopyranose derivatives examined under similar conditions.[20]

Analysis of the complete set of CH coupling constants for the 2,6-diamino-2,6-dideoxy-β-L-idopyranosyl (2,6-DAI) portion corroborates the striking difference in the ground state conformations associated with rings A and D in neomycin B (see structure 1b). For this residue, the small values for $J_{1,2}$, $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ (1.8, 3.3, 3.3, and 1.5 Hz, respectively) are clearly consistent with gauche orientations for all H-1 -- H-4 vicinal hydrogen pairs. Thus, the magnitudes of the couplings are those predicted for the $^4C_1$ chair conformation (1b) of the 2,6-DAI residue. Furthermore, the observation of a long range coupling $J_{2,4}$ 1.1 Hz over four bonds is consistent only with conformation 1b, in which H-2, C-2, C-3, C-4, and H-4 are constrained to a planar "W" arrangement. Similar proton-proton couplings (1-2 Hz) over four bonds have been observed previously in saturated systems when atoms are aligned in this particular steric arrangement.[21] In addition, spin decoupling experiments were carried out to confirm the presence of this coupling.

Further supporting evidence for this structure may be deduced from a comparison of the proton chemical shifts (Table 1) of the 2,6-DAG and 2,6-DAI residues in the antibiotic. The chemical shifts of H-2, H-3, and H-4 for the 2,6-DAI group are consistently

to lower field (0.28-0.41 ppm) of those in 2,6-DAG (ring A), which is indicative of equatorially oriented protons in the former residue.[22,23] In contrast, H-1 appears to higher field (0.06 ppm) of the anomeric proton of the second 2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl residue (ring D) in neomycin C,[24] which is characteristic of the axial orientation (β-configuration). The small magnitude of the axial-equatorial shift difference observed here is readily explained by the deshielding effects of the axial 2-amino and 3-hydroxyl groups on the shift of the axial anomeric proton in 2,6-DAI. Deshielding of proton chemical shifts (~0.3 ppm) of several pento- and hexo-pyranoses has been observed previously when ring protons and polar substituents (-OH, $-NH_2$) assume either a 1,2 trans-diaxial or 1,3 syn-diaxial relationship.[25-29] Apparently, the $^4C_1$ conformation placing the bulky aglycone at C-1''' and aminomethyl group at C-5''' in equatorial orientations is the one preferred. However, in view of the fact that H-2 and H-4 must assume a planar "W" arrangement in this conformation, it is interesting that the values for $J_{1,2}$ and $J_{4,5}$ are rather small. Although the small value of $J_{4,5}$ in ring D certainly resembles the situation in galactose derivatives with HO-4 axial and C-6 equatorial,[27] it is possible that the $^4C_1$ conformation for the 2,6-DAI moiety does not have ideal geometry because of distortion induced by the syn-axial interaction between the amino and hydroxyl groups at C-2''' and C-4'''.

Proton coupling constants in Table 1 for the β-ribofuranosyl (RIB) portion can be compared with those measured for methyl α- and β-D-ribofuranosides under similar conditions (Table 2). Although the value of $J_{1,2}$ is intermediate between those determined for the methyl ribofuranosides, the remaining couplings ($J_{2,3}$ = 5.1, $J_{3,4}$ = 6.2, and $J_{4,5}$ = 3.1 Hz) are more characteristic of those for the β-anomer. Dihedral angles calculated from a modified Karplus relationship[18] derived for saccharides imply that the furanose ring of the RIB portion is distorted toward a planar conformation, presumably a consequence of steric interactions from the bulky aglycones at C-1" and C-3".

TABLE 2. Proton Coupling Constants in Methyl α- and β-D-Ribofuranosides[a]

Anomer	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{4,5'}$	$J_{5,5'}$
α	4.2	6.7	3.7	3.7	4.9	-12.2
β	1.1	4.6	6.8	3.3	6.5	-12.4

[a]In Hertz.

The magnitudes of the couplings for the 2-deoxystreptaminyl (DOS) portion support a chair conformation in which H-1, H-2', and H-3, H-4, H-5, and H-6 vicinal hydrogen pairs have a trans-diaxial relationship to each other. However, based on the proton chemical shift and coupling information presented in Table 1, proton resonances apart from H-2 and H-2' could not be rigorously assigned. Specific assignments were made by comparing values of the shifts obtained upon protonation of the amino group at C-1 (Table 3). In our earlier ^{15}N NMR studies of neomycin B,[7] N-1 and N-3 were assigned unambiguously on the basis of spin-labeling experiments using a paramagnetic relaxation reagent. Moreover, data obtained from ^{15}N chemical shift titration curves of the individual nitrogen nuclei confirmed that N-3 is essentially unprotonated in solutions of neomycin B sulfate at the native pH. Thus, the protonation shifts for the DOS moiety presented in Table 3 are the chemical shift differences observed between spectra of neomycin B sulfate and neomycin B free base recorded under identical conditions. Proton assignments for neomycin B sulfate were made by means of spin decoupling experiments.

Table 4 shows the effect of protonation of both amino groups on the proton chemical shifts of a solution of 2-deoxystreptamine in D_2O. These values were then used to predict those found for the DOS residue of the antibiotic. By analogy, H-1 and H-6 could be assigned readily on the basis of the large values (+0.51 and

TABLE 3. Effect of First Protonation on Proton Chemical Shifts of 2-Deoxystreptamine Moiety in Neomycin B

$\Delta\delta^a$						
H-1	H-2	H-2'	H-3	H-4	H-5	H-6
+0.51	+0.31	+0.51	+0.34	+0.17	+0.15	+0.58

[a]Positive values denote downfield shifts in ppm; for proton chemical shift assignments see Table 1.

TABLE 4. Effect of Protonation on Proton Chemical Shifts of 2-Deoxystreptamine in D_2O

	δ^a				
Form (pD)	H-1,3	H-2	H-2'	H-4,6	H-5
Dihydrochloride (5.3)	3.35	2.47	1.83	3.55	3.43
Free Base (11.0)	2.74	2.00	1.21	3.16	3.29
	$\Delta\delta^b$				
	+0.61	+0.47	+0.62	+0.39	+0.14

[a]In ppm downfield from internal TSP.

[b]Positive values denote downfield shifts in ppm, $\Delta\delta = \delta^{2HCl} - \delta^{Base}$.

+0.58 ppm, respectively) of their protonation shifts. The remaining protons were subsequently assigned by performing spin decoupling experiments to confirm spin-spin coupling connectivities.

EXPERIMENTAL

Preparation of Solutions. Samples for 1D ^1H NMR were prepared as 15-20 mmol/L solutions in deuterium oxide (99 atom %D). For 2D, J-resolved ^1H NMR, neomycin B free base (7.1 mg) was lyophilized twice with deuterium oxide (100 atom %D), and was then dissolved in deuterium oxide (0.3 mL, 100 atom %D).

NMR Spectroscopy. ^1H NMR spectra were recorded at 400 HMz and at 24°C, using a Bruker Instruments[24] Model WM-400 spectrometer in the pulse-Fourier transform mode, with quadrature detection. The 180° pulse width (26 μs) was determined directly for the solutions of neomycin. 1D NMR spectra were acquired by means of the Bruker FTQNMR program, using a spectral width of 3.6 kHz, a 90° pulse (13 μs), a 32,768 point data set, an acquisition time of 4.5 s, and a total relaxation delay of 9.0 s. Optimum resolution enhancement was achieved by Gaussian, exponential filtering of the free induction decay signal using a line broadening of -0.4 Hz and a Gaussian broadening fraction of 0.3. The frequency domain spectrum (16,384 points) had a digital resolution of 0.2 Hz (0.0006 ppm) per point. The HOD signal was suppressed by selective irradiation at its resonance frequency during the fixed delay prior to data acquisition. Homonuclear spin decoupling experiments with solvent suppression were accomplished by switching the irradiation frequency from the HOD resonance to the desired decoupling frequency, just before data acquisition. Adequate power for solvent suppression and decoupling was 0.1-0.3 W. Proton chemical shifts were measured with reference to the methyl resonance of internal sodium 4,4-dimethyl-4-silapentanoate-2,2,3,3-d_4 (TSP). 2D, J-resolved ^1H NMR spectra were acquired by means of the Bruker FTNMR2D program, using an 8192 x 128 data

matrix, and spectral widths of 2 kHz and 31.25 Hz in the chemical shift and coupling constant dimensions, respectively. The use of a spectral width of 31.25 Hz for the J-spectra allowed an optimal digital resolution for all of the spin multiplets of neomycin that were overlapped in its 1D ^{1}H NMR spectrum. However, this spectral width was not large enough to permit correct representation of the well separated, but wide quartet (H-2' = H-2_B) at highest field.

Spectrum simulation. The six or seven spin systems comprised by the CH protons of rings A-D of neomycin B were each simulated separately by using the Bruker PANIC program. A software patch was written for co-adding four simulated spectra to give a composite simulated spectrum for the entire neomycin molecule. Some of the chemical shifts were then adjusted manually to improve the fit of the composite simulated spectrum to the experimental spectrum.

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24. Only by comparing H-1 shifts of the two sugars having identical C-1 substituents can a valid estimate of the axial-equatorial shift difference be assessed. The H-1 chemical shift of the 2,6-DAG residue (ring D) of neomycin C free base was determined to be δ5.02; unpublished results.

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30. Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation by the National Bureau of Standards, nor does it imply that the materials or equipment are necessarily the best available for the purpose.