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Carbohydrate Research 271 (1995) 55–63

CARBOHYDRATE RESEARCH

The complete ^{1}H NMR assignments of aminoglycoside antibiotics and conformational studies of butirosin A through the use of 2D NMR spectroscopy

James R. Cox, Engin H. Serpersu *

Department of Biochemistry, University of Tennessee, Knoxville, TN 37996-0840, USA

Received 10 August 1994; accepted 22 November 1994

Abstract

The complete proton assignments of the aminoglycoside antibiotics, butirosin A, kanamycin A and kanamycin B, at pH 6.5 have been made through the use of various homonuclear and heteronuclear 2D NMR methods. Butirosin A NOESY experiments suggest a stacking arrangement between the xylose and 2,6-diamino-2,6-dideoxyglucose rings, while the 2-deoxystreptamine ring and its substituent, the (*S*)-4-amino-2-hydroxybutyryl group, extend away from the stacked rings. Informative long-range NOEs were observed for butirosin A but not with kanamycin A or kanamycin B. Many intra-ring NOEs were observed with all three aminoglycosides that confirm the proton assignments made in this study.

Keywords: Butirosin A; Kanamycins A and B; Aminoglycoside antibiotics

1. Introduction

Aminoglycosides are antibiotics that represent a broad spectrum of molecules that are active against staphylococci, a wide range of gram-negative bacteria, and gram-positive bacilli [1]. However, the antibacterial spectrum of many aminoglycosides, including kanamycin A, kanamycin B, and butirosin A, has been limited by resistance through several types of enzymatic covalent modifications [2]. The elucidation of the mechanisms of these enzymatic aminoglycoside modifications will be an important step in confronting this public health crisis.

* Corresponding author.

SSDI 0008-6215(95)00048-8

The proton assignments of these aminoglycosides are necessary in studies to determine the conformation and arrangement of bound substrates [3,4]. Such structure and function studies are essential in understanding the molecular interactions between modifying enzymes and antibiotics that will be useful in the design of more effective drugs and/or inhibitors.

Proton assignments of other aminoglycosides, tobramycin [5,6], amikacin [7], neomycin B [8], and apramycin [9] have also been made through the use of 2D NMR methods. This paper describes the proton assignments and presents a suggested solution structure of butirosin A as determined by various homonuclear and heteronuclear NMR studies. Also reported are the proton assignments of kanamycin A and kanamycin B at pH 6.5 which are suitable for studies of enzyme–substrate interactions.

2. Experimental

Materials.—Kanamycin A, kanamycin B, and butirosin A were obtained as sulfate salts from Sigma Chemical Co. (St. Louis, MO). D_2O (99.9%) was obtained from Wilmad Glass Co. (Buena, NJ).

Sample preparation for NMR spectroscopy.—Solutions for NMR spectroscopy were prepared by dissolving the sulfate salts directly in 99.9% D_2O. Sample concentrations were 100 mM in all experiments except for NOESY (^1H–^1H nuclear Overhauser and exchange spectroscopy) experiments in which the sample concentrations were 10 mM at pH 6.5.

NMR spectroscopy.—All NMR spectra were obtained on a wide-bore Bruker AMX 400 MHz spectrometer at 27° C. 1D data sets were collected over a spectral width of 6024 Hz with an acquisition time of 2.72 s. A total of 16k–32K data points of 8–16 transients were collected. All ^1H chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

Two-dimensional ^1H–^1H COSY, NOESY, and HOHAHA [10–12] (homonuclear Hartmann-Hahn spectroscopy) data sets were collected in the phase-sensitive mode using the time-proportional phase increment method [13]. The transmitter offset was placed on the HDO resonance, which was irradiated during the relaxation delay of 2.0 s to suppress the water signal. A total of 201–423 FIDs of 2K were collected. The spectral width was 2809 Hz, and 32–112 scans per FID were acquired. The HOHAHA pulse program [10,11] contained a MLEV 17 spin lock pulse [12] that was preceded and followed by two 2.5 ms trim pulses. Mixing times of 14.7 to 85.8 ms (including trim pulses) were employed. Mixing times of 300, 400, and 500 ms were used in NOESY experiments with butirosin A. The data were zero filled to 1K points (0.5K in NOESY experiments) in t_1 and were multiplied by sin (COSY) and $\sin^2$ (HOHAHA, NOESY) window functions in both dimensions before Fourier transformation. A 2K × (202–248) data matrix was acquired for ^{13}C–^1H COSY experiments [14–16]. Proton decoupling was achieved with the WALTZ 16 sequence [17], and 80–96 transients were obtained for each value of t_1. The data were zero filled to 512 points in t_1, and Gaussian multiplication was used in both dimensions.

3. Results and discussion

The structures of kanamycin A, kanamycin B, and butirosin A are shown in Fig. 1. The primed ring is 6-amino-6-deoxyglucose in kanamycin A and 2,6-diamino-2,6-dideoxyglucose in kanamycin B and butirosin A. The double-primed ring is 3-amino-3-deoxyglucose in kanamycin A and kanamycin B and xylose in butirosin A. The unprimed ring in kanamycin A and kanamycin B is 2-deoxystreptamine. The unprimed ring in butirosin A is 2-deoxystreptamine modified at N-1 with an (*S*)-4-amino-2-hydroxybutyryl group (AHB), which is denoted with a triple prime.

Kanamycin A and kanamycin B.—The complete proton assignments of the sulfate salts kanamycin A and kanamycin B at pH 6.5 are presented in Table 1. Due to the similarities of the two antibiotics, only the assignment strategy for kanamycin A will be

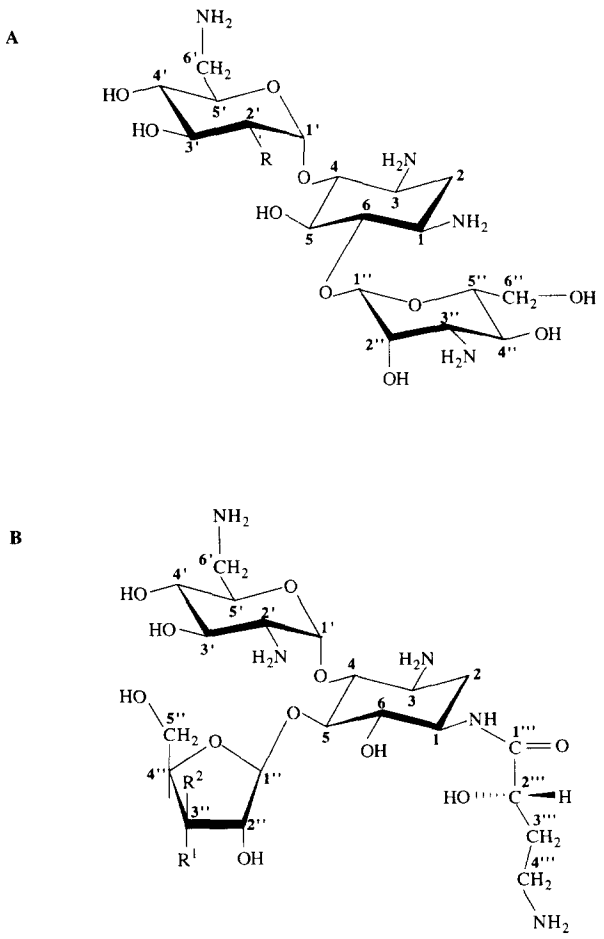

Fig. 1. The structures of (A) kanamycin A (R = OH) and kanamycin B (R = NH_2), and (B) butirosin A (R^1 = H, R^2 = OH) and butirosin B (R^1 = OH, R^2 = H).

Table 1
^{1}H NMR chemical shifts (ppm) and coupling constants [a] (Hz, in parentheses) for kanamycin A, kanamycin B, and butirosin A

Proton(s)	Kanamycin A	Kanamycin B	Butirosin A
1	3.155	3.449	3.904(4.4)
2a	1.429(12.6)	1.773(12.8)	1.829(13.0)
2e	2.117(12.6, 4.1)	2.336(12.8, 4.4)	2.197
3	3.057(10.1, 3.6)	3.276	3.453
4	3.488(9.75, 3.6)	3.784	4.036
5	3.690(4.3)	3.800(4.7)	3.864(9.1)
6	3.416(9.4)	3.712(9.2)	3.599(10.5, 9.1)
1′	5.493(3.8)	5.800(4.2)	6.154(3.7)
2′	3.588(3.8)	3.276(4.2)	3.405
3′	3.724(9.0, 3.6)	3.885	4.060
4′	3.319(9.7)	3.376(9.3)	3.427
5′	3.963(9.7)	3.984(9.3)	3.904
6′	3.358(8.1, 3.2)	3.422	3.473(13.5)
	3.100(8.1, 5.2)	3.174(8.6)	3.206(13.5, 8.0)
1″	5.040(3.7)	5.085(3.8)	5.334
2″	3.669(10.5, 3.7)	3.859(3.8)	4.272
3″	3.180(10.5)	3.401	4.173
4″	3.462(10.0)	3.633(10.3)	4.330
5″	3.895(10.2)	3.909	3.761(12.0, 8.2)
6″	3.755(2.2)	3.768(4.9)	—
2‴		—	4.305
3‴	—	—	1.986(6.7)
	—	—	2.127
4‴	—	—	3.122

[a] Coupling constants were calculated from chemical shift values read from the computer screen. If coupling constants were not calculated, it was due to spectral overlap.

briefly presented. The anomeric protons and the axial and equatorial protons on C-2 were the first protons of kanamycin A to be assigned [18], and these assignments are confirmed in this study. ^{1}H–^{13}C COSY and ^{1}H–^{1}H COSY experiments were employed to assign the remaining protons. The ^{1}H–^{1}H COSY (not shown) spectrum allowed assignment of H-2″, H-3, and H-1, which are confirmed in the ^{1}H–^{13}C COSY spectrum (not shown). The ^{1}H–^{1}H COSY spectrum also allowed assignment of H-2′ which was ambiguous in the ^{1}H–^{13}C COSY spectrum.

The ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY spectra were quite crowded between 3 and 4 ppm. Also, C-3′ and C-5″ share a common ^{13}C resonance at 50 ppm and C-2′ and C-4′ share a common ^{13}C resonance at 48 ppm in the ^{1}H–^{13}C COSY spectrum. Therefore, HOHAHA spectra of kanamycin A were obtained to avoid these ambiguities. HOHAHA experiments allow magnetization diffusion through isotropic mixing [19,20].

The HOHAHA experiments were performed with five different mixing times ranging from 14.7 to 77.7 ms. Fig. 2 shows an expanded view of the ^{1}H–^{1}H COSY and two HOHAHA spectra that resolved the ambiguities and permitted the complete proton assignments to be made. As an example, panels C and D of Fig. 2 show expanded views of a HOHAHA experiment with a mixing time of 45.4 ms. In panel C it is evident that a

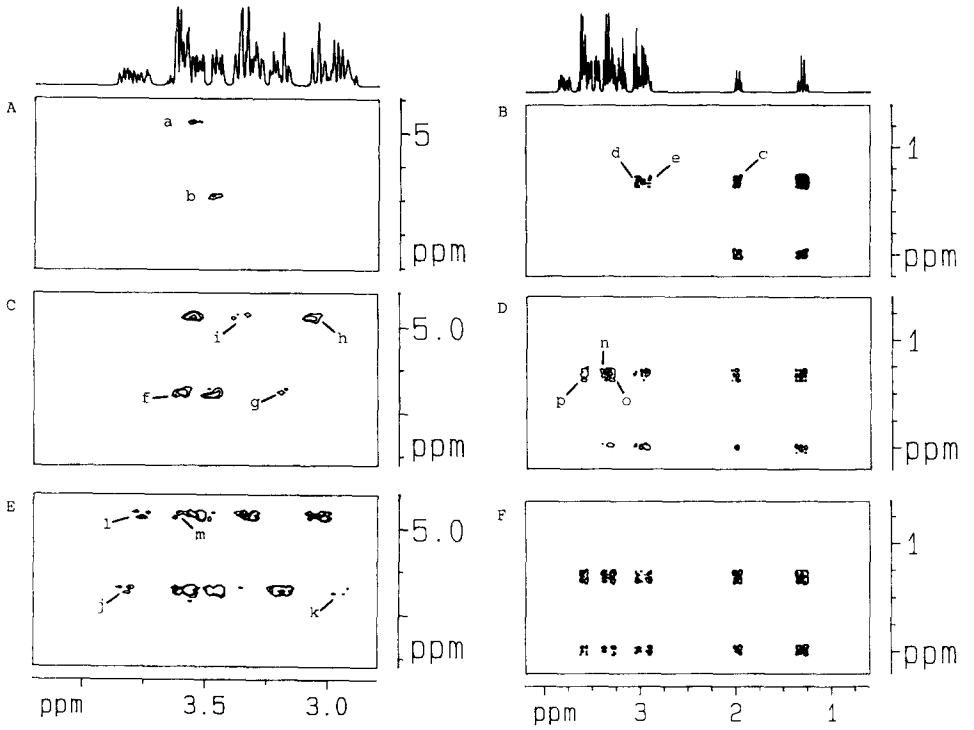

Fig. 2. Expanded regions of homonuclear NMR spectra of kanamycin A used to follow the appearance of cross peaks. Panels A and B are from the $^{1}H-^{1}H$ COSY spectrum, panels C and D are from a HOHAHA spectrum with a mixing time of 45.4 ms, and panels E and F are from a HOHAHA spectrum with a mixing time of 61.6 ms. Labeled cross-peaks in panel A: H-1″–H-2″ (a) and H-1′–H-2′ (b). Panel B: H-2a–H-2e (c), H-1–H-2 (d), and H-2–H-3 (e). Panel C: H-1′–H-3′ (f), H-1′–H-4′ (g), H-1″–H-3″ (h), and H-1″–H-4″ (i). Panel D: H-2–H-4 (n), H-2–H-6 (o), and H-2–H-5 (p). Panel E: H-1′–H-5′ (j), H-1′–H-6′ (k), H-1″–H-5″ (l), and H-1″–H-6″ (m). See text for further details.

strong cross-peak for H-1′–H-3′ (bottom row, f) has already formed, while a weaker cross-peak for H-1′–H-4′ (bottom row, g) is barely visible with the 45.4 ms mixing time. Assignment of H-3′ in this experiment clears up the ambiguity between H-3′ and H-5″ in the $^{13}C-^{1}H$ COSY experiment. The rest of the assignments for kanamycin A and kanamycin B were completed in a similar fashion through the use of COSY and HOHAHA spectra. The proton assignments of kanamycin A and kanamycin B have been made previously as free bases at high pH [21], and the assignments of kanamycin A [1] were also made previously as the free base and the sulfate salt [22]. Since the aminoglycoside chemical shifts are pH sensitive, direct comparison with the previous studies is not feasible.

NOESY spectra ($\tau_m = 500$ ms) of kanamycin A and kanamycin B (not shown) showed no long-range NOEs. However, many intra-ring NOEs and NOEs between

[1] The pH at which the assignments were made was not given in the manuscript (Ref [22]).

protons across the α-glycosidic linkages were observed that confirmed the proton assignments made in this study.

Butirosin A.—The complete proton assignments of butirosin A are also presented in Table 1. Partial proton assignments of butirosin A and related analogs have been made previously [23]; however, this work provides the complete proton assignments for this antibiotic. The strategies outlined above for kanamycin A were also employed to make the butirosin A proton assignments. The $^{1}H-^{13}C$ COSY spectrum of butirosin A was even more crowded than the heteronuclear spectra of the kanamycins.

Thus, HOHAHA experiments with mixing times between 21.2 and 85.8 ms were performed (total of four experiments) to aid in the assignment process. As with kanamycin A and kanamycin B, the cross-peaks in the HOHAHA spectra were assigned based on their appearance as the isotropic mixing time was increased.

NOESY spectra of butirosin A showed short- and long-range NOEs that give an insight into its conformation as well as the arrangement of the sugar rings. A NOESY spectrum ($\tau_m = 500$ ms) is presented in Fig. 3 (with some important cross-peaks marked), and a list of observed NOEs appear in Table 2. The same ^{1}H NOE cross-peaks were also observed in NOESY spectra obtained with shorter mixing times ($\tau_m = 300$ and 400 ms). Thus, the observed NOEs are unlikely to be due to spin diffusion and should represent proximity between the protons of butirosin A.

NOE cross-peaks were detected between H-1′ and H-4 across the α-glycosidic linkage and between H-1″ and H-5 across the β-glycosidic linkage. The most interesting observation was the detection of a strong NOE cross-peak between the H-5′ and H-5″ protons, which is marked in Fig. 3. This observation suggests that, in solution, the

Table 2
Various NOEs detected in $^{1}H-^{1}H$ NOESY spectrum ($\tau_m = 500$ ms) of butirosin A

Deoxystreptamine ring	2,6-Diamino-2,6-dideoxyglucose ring	Xylose ring	AHB [a] chain
Intra-ring/chain ^{1}H NOEs			
1–3	1′–2′	1″–2″	2‴–3‴
1–5	2′–3′	2″–3″	2‴–4‴
1–6	3′–5′	3″–4″	3‴–3‴
2a–2e	4′–5′	4″–5″	3‴–4‴
2a–3	5′–6′	—	—
2a–4	6′–6′	—	—
2a–6	—	—	—
2e–3	—	—	—
2e–1	—	—	—
3–5	—	—	—
4–5	—	—	—
4–6	—	—	—
5–6	—	—	—
Inter-ring/chain ^{1}H NOEs			
	1′–4 (across α-glycosidic linkage)		
	1″–5 (across β-glycosidic linkage)		
	1–2‴ (long-range)		
	5′–5″ (long-range)		

[a] AHB is the triple-primed chain: (*S*)-4-amino-2-hydroxybutyryl group.

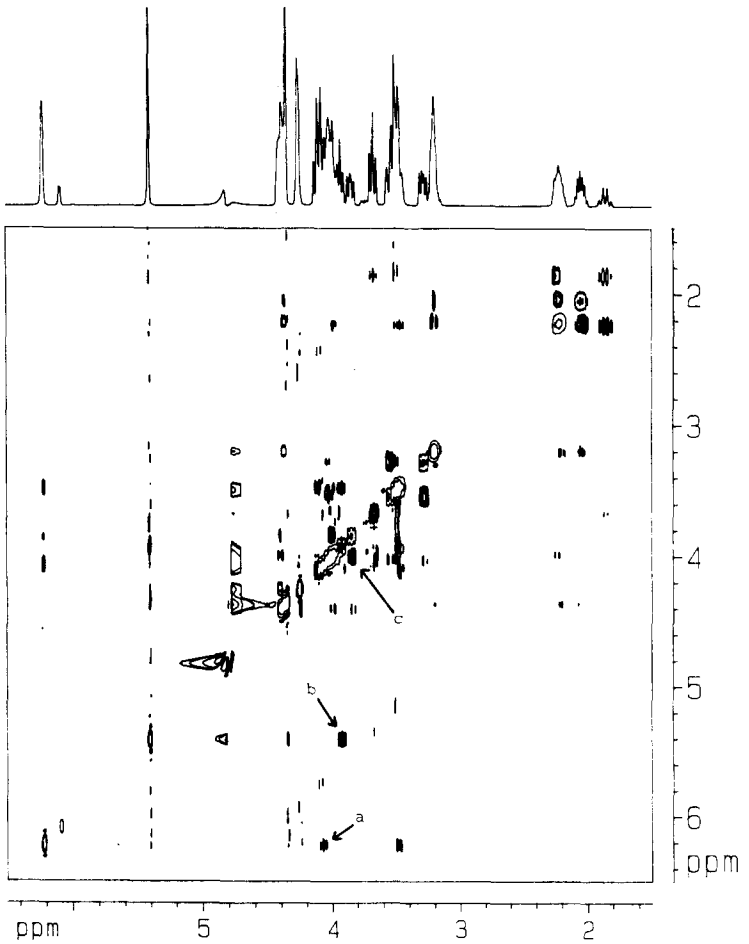

Fig. 3. 400 MHz 2D NOESY spectrum of butirosin A with a mixing time of 500 ms. A total of 2K data points with a spectral width of 3205 Hz were collected, and 256 increments with 48 scans per increment were acquired. The data were zero filled to 0.5K in t_1 and multiplied with a shifted $\sin^2$ function before Fourier transformation. Labeled cross-peaks: H-1′–H-4 (a) across the α-glycosidic linkage, H-1″–H-5 (b) across the β-glycosidic linkage, and H-5′–H-5″ (c) a long-range NOE.

xylose ring faces the 2,6-diamino-2,6-dideoxyglucose ring to bring these protons near each other. A solution conformation of butirosin A consistent with the observed NOEs is shown in Fig. 4. The predicted stacking arrangement of the xylose and 2,6-diamino-2,6-dideoxyglucose rings was made based on the observed H-5′ and H-5″ NOE cross-peak and the absence of NOE cross-peaks between H-5′ and H-2″ and between H-3′ and H-5″. The H-2‴ proton of the (*S*)-4-amino-2-hydroxybutyryl group was positioned near H-1 based on the observed NOE between these protons. The ring conformations shown in Fig. 4 are based on the observed intra-ring NOEs, which are consistent with the proton assignments made in this study. The conformation of butirosin A presented in

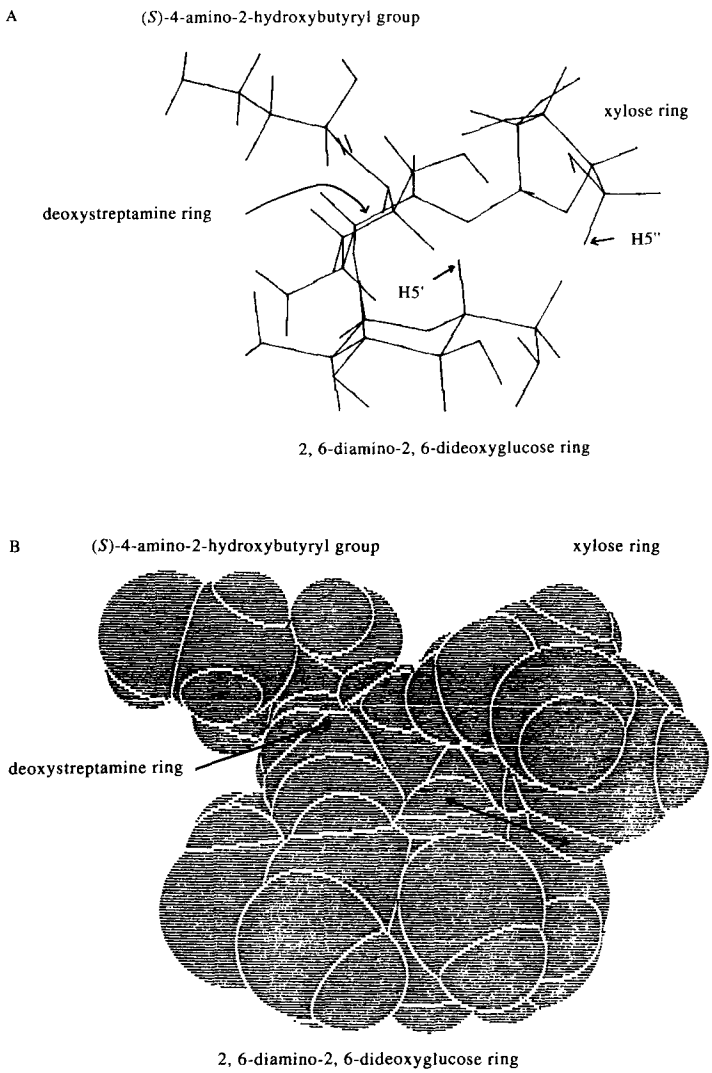

Fig. 4. The proposed solution conformation of butirosin A presented as a line (A) and space filling (B) model. The conformation was derived from observed short- and long-range NOEs. A long range NOE was observed between H-5′ and H-5″, which is marked by a double-headed arrow in the space-filling model.

Fig. 4 was constructed using Desktop Molecular Modelling software (Oxford University Press) and does not show any van der Waals overlap between atoms.

In summary, it appears that, in solution, the xylose and 2,6-diamino-2,6-dideoxyglucose rings of butirosin A are in a stacked arrangement, while the (*S*)-4-amino-2-hydroxybutyryl group extends away from the deoxystreptamine ring.

Acknowledgements

We thank Dr. Gerry Wright of McMaster University for getting us interested in this project and John Lamerdin for his help with the NMR spectroscopy. This work was supported by a grant from NIH (GM-42661).

References

[1] D.A. Cox, K. Richardson, and B.C. Ross, in P.G. Sammes (Ed.), *Topics in Antibiotic Chemistry*, Vol. 1, Part A, Halsted Press, New York, 1977, pp 5–11.
[2] H. Umezawa and S. Kondo, in H. Umezawa and I.R. Hooper (Eds.), *Aminoglycoside Antibiotics*, Springer, New York, 1982, Chapter 6.
[3] W.F. Wolkers, J.D. Gregory, J.E. Churchich, and E.H. Serpersu, *J. Biol. Chem.*, 266 (1991) 20761–20766.
[4] J.D. Gregory and E.H. Serpersu, *J. Biol. Chem.*, 268 (1993) 3880–3888.
[5] J.E. Van Pelt, E.S. Mooberry, and P.A. Frey, *Arch. Biochem. Biophys.*, 280 (1990) 284–291.
[6] L. Szilagyi, *Carbohydr. Res.*, 170 (1987) 1–17.
[7] N.H. Anderson, H.L. Eaton, K.T. Nguyen, C. Hartzell, R.J. Nelson, and J.H. Priest, *Biochemistry*, 27 (1988) 2782–2790.
[8] D.G. Reid and K. Gajjar, *J. Biol. Chem.*, 262 (1987) 7967–7972.
[9] L. Szilágyi and Z.S. Pusztahelyi, *Magn. Reson. Chem.*, 30 (1992) 107–117.
[10] D.G. Davis and A. Bax, *J. Am. Chem. Soc.*, 107 (1985) 2820–2821.
[11] L. Braunschweiler and R.R. Ernst, *J. Magn. Reson.*, 53 (1883) 521–528.
[12] A. Bax and D.G. Davis, *J. Magn. Reson.*, 65 (1985) 355–360.
[13] D. Marion and K. Wütrich, *Biochem. Biophys. Res. Commun.*, 113 (1983) 967–974.
[14] A. Bax, *J. Magn. Reson.*, 53 (1983) 517–520.
[15] V. Rutar, *J. Magn. Reson.*, 58 (1984) 306–310.
[16] J.A. Wilde and P.H. Bolton, *J. Magn. Reson.*, 59 (1984) 343–346.
[17] A.J. Shaka, J. Keeler, and R. Freeman, *J. Magn. Reson.*, 53 (1983) 313–340.
[18] R.U. Lemieux, T.L. Nagabhushan, K.J. Clemetson, and C.N. Tucker, *Can. J. Chem.*, 51 (1973) 53–66.
[19] D.G. Davis and A. Bax, *J. Am. Chem. Soc.*, 107 (1985) 2820–2821.
[20] R.P. Hicks, J.K. Young, and D. Moskau, *Concepts Magn. Reson.*, 6 (1994) 115–130.
[21] G.I. Eneva and S.L. Spassov, *Spectrochim. Acta*, 47A (1991) 875–880.
[22] R.U. Lemieux and K. Bock, *Jn J. Antibiot.*, 32 (Suppl.) (1979) 163–177.
[23] P.W.K. Woo and T.H. Haskell, *J. Antibiot.*, 35 (1982) 692–702.