carried out at the Ohio State University Computer Center using the IBM 370-105 computer and displayed on a Calcomp plotter. The results in Tables I-VI represent the best fits of calculated to experimental spectra obtained by iterating the NMR parameters and rate constants. The error in the fit was 5% of the rate constant in the coalescence region, where line shape is most sensitive to changes in rate constant, to 10-12% approaching the slow and fast exchange limits.

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References and Notes

(1) T. L. Brown, *J. Pure Appl. Chem.*, **23**, 447 (1970).
(2) B. J. Wakefield, "The Chemistry of Organolithium Compounds", Pergamon Press, Oxford, England, 1974.
(3) D. Margerison and T. P. Newpont, *Trans. Faraday Soc.*, **59**, 2058 (1963); M. Weiner, C. Vogel, and R. West, *Inorg. Chem.*, **1**, 654 (1962); M. L. Lewis and T. L. Brown, *J. Am. Chem. Soc.*, **92**, 4664 (1970).
(4) T. L. Brown, *Adv. Organomet. Chem.*, **3**, 365 (1965).
(5) R. Zerger, W. Rhine, and G. Stucky, *J. Am. Chem. Soc.*, **96**, 6048 (1974).
(6) T. F. Schaaf, W. Butler, M. D. Glick, and J. P. Oliver, *J. Am. Chem. Soc.*, **96**, 7593 (1974).
(7) K. C. Williams and T. L. Brown, *J. Am. Chem. Soc.*, **88**, 4134 (1966); L. M. Seitz and T. L. Brown, *ibid.*, **88**, 4140 (1966); D. P. Novak and T. L. Brown, *ibid.*, **94**, 3793 (1972); R. L. Kieff and T. L. Brown, *J. Organomet. Chem.*, **77**, 289 (1974); R. L. Kieft, D. P. Novak, and T. L. Brown, *ibid.*, **77**, 299 (1974).
(8) M. Witanowski and J. D. Roberts, *J. Am. Chem. Soc.*, **88**, 736 (1966).
(9) G. Fraenkel, D. T. Dix, and M. J. Carlson, *Tetrahedron Lett.*, 579 (1968).
(10) L. M. Seitz and T. L. Brown, *J. Am. Chem. Soc.*, **88**, 2174 (1966).
(11) G. Fraenkel, C. E. Cottrell, and D. T. Dix, *J. Am. Chem. Soc.*, **93**, 1704 (1971).
(12) J. I. Kaplan and G. Fraenkel, *J. Am. Chem. Soc.*, **94**, 2709 (1972).
(13) A. A. Bothner-By, Quantum Mechanics Program Exchange, Department of Chemistry, Indiana University, Bloomington, Ind. Modified for use in the Nicholet 1080 Computer by Dr. J. W. Cooper, Tufts University.
(14) Note that not all diastereomers need be magnetically nonequivalent.
(15) D. Margierson and J. D. Pont, *Trans. Faraday Soc.*, **67**, 353 (1971).

^{15}N Nuclear Magnetic Resonance Spectroscopy. The Nebramycin Aminoglycosides

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Abstract: The ^{15}N NMR spectra of some of the components of the nebramycin complex are reported. Resonances are assigned through comparisons of spectra of the individual factors. The observed chemical shifts are broadly consistent with the current knowledge of substituent effects in ^{15}N NMR spectroscopy. Titration curves can be used to obtain pK_a values for each of the nitrogens of a compound. The results of this study were used to confirm the identification of a monoacetyl derivative of tobramycin.

Recent studies of ^{13}C nuclear magnetic resonance (NMR) of the aminoglycoside antibiotics have vastly simplified the problem of structure elucidations of these compounds.[1] Furthermore, the effect of protonation of the basic amines on ^{13}C chemical shifts[1f] has been used to guide attempts to modify chemically the structure of an aminoglycoside.[2] The recent report of the measurement of ^{15}N NMR spectra of amino sugars[3] indicates yet another potentially useful method of studying the structures and reactivities of the aminoglycoside antibiotics. In this paper, we report our own initial explorations of the ^{15}N NMR spectra of these compounds.

The subjects of the present investigation were some of the various biologically active factors of the nebramycin complex of aminoglycoside antibiotics isolated from *Streptomyces tenebrarius*. The structures of the compounds studied are detailed in Table I. Most of these structures have been established by previous workers;[1f,4] the structures of factors 3 and 7 were established in these laboratories and will be reported elsewhere.[1g]

Experimental Section

^{15}N NMR spectra were measured on a JEOL PFT-100 spectrometer operating at 10.09 MHz and equipped with a ^{1}H decoupler, a deuterium field-frequency lock, and a JEOL EC-100 data system. The free induction decay was collected into 8K of computer memory, using a spectral width of 5 kHz. Samples were pulsed with a repetition rate of 2 s, using a 45° pulse angle. The conditions of data collection and transformation would be anticipated to contribute ca. 1.7 Hz to the natural line width.

Samples of concentration 0.5-1.0 M were prepared in H_2O-D_2O (ca. 9:1). No special precautions were taken to exclude paramagnetic impurities, and the solutions were not degassed. pH was adjusted, using approximately 6.6 N KOH and HCl solutions.

In some cases, spectra taken in acidic solutions required much longer scanning. At pH 11, a spectrum of apramycin was taken utilizing a decoupling technique designed to retain nuclear Overhauser enhancement without collapsing scalar coupling;[5] this spectrum did not differ in any material way from that obtained from normal noise-modulated proton decoupling.

^{15}N chemical shifts were measured relative to external $^{15}NH_4Cl$ (2.9 M) in 1 M HCl contained in a coaxial 2-mm capillary. In all cases, the resonances of the sample had the same phase as the standard sample.

Results

^{15}N chemical shifts measured in this study are presented in Table II. In acidic or basic solutions, the various nitrogen resonances are well resolved; at some intermediate pH values, peak overlaps occur, but they could easily be detected through their relative peak heights. In the case of apramycin in alkaline solution, the spectrum was remeasured in the absence of the external standard to detect the underlying resonance. Chemical shifts in alkaline solution are probably accurate to ±0.1 ppm. In acidic media, greater peak width reduces the accuracy to 0.2 ppm. In some cases, the pH drifted significantly during

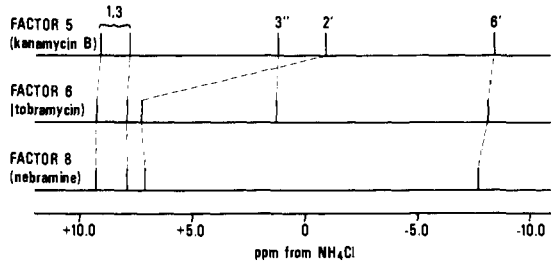


Figure 1. Comparison of the ^{15}N chemical shifts of factors 5, 6, and 8 in basic solution (pH ca. 10).

collection of the spectra, and these are so indicated in Table II.

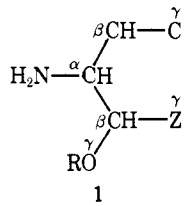
Discussion

Assignment of Resonances. Resonances of individual nitrogen nuclei were assigned through comparisons of the spectra of these structurally similar molecules. Figure 1, which compares the spectra of factors 5, 6, and 8 in alkaline solution, exemplifies the logic used in these assignments. The peak near −8 ppm occurs only in those factors which include an aminomethyl (CH_2NH_2) group and is therefore assigned to N(6′); further arguments supporting this assignment are presented below. The positions of the four resonances of factor 8 are nearly unchanged in the spectrum of factor 6, suggesting that the chemical shifts of nitrogens 1, 3, 2′, and 6′ are little influenced by the additional 3-deoxy-3-aminoglucose unit of the latter. The fifth resonance of factor 6 is therefore assigned to N(3″). Comparison of the spectra of factors 5 and 6 shows that the chemical shifts of four of the nitrogen nuclei are not dependent on the oxidation of carbon 3′. The nitrogen resonance that does change is inferred to be the adjacent N(2′). The two resonances near 8–9 ppm are therefore assigned to nitrogens 1 and 3 by elimination. The latter two have been tentatively assigned specifically on the basis of protonation shifts (vide infra).

Similar arguments lead to the assignments presented in Table II. In factors 2 and 7, we cannot presently assign the resonances of nitrogens 7′ and 4″ specifically.

Substituent Effects. From surveys of the ^{15}N NMR spectra of simple molecules, a series of substituent effects have been derived.[6] For both the amines and the hydrazines, the chemical shift change associated with α substitution is generally small and downfield. The β effect is also deshielding but substantially larger, averaging about 20 ppm. While the γ effect is generally shielding, the magnitude appears to be dependent upon the identity of the atom at the γ position. Thus, the effect of a γ carbon is about −2.5 ppm, while an oxygen in this position is more strongly shielding (−6 to −9 ppm).[6b]

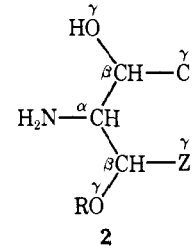
The direct use of these parameters in calculating nitrogen chemical shifts in molecules as complex as the aminoglycosides is probably not justified. It is interesting to note, however, that observed *differences* in chemical shift can be explained in terms of these substituent parameters. Nitrogens in environments such as **1**, for example, fall into a relatively narrow range of



1

chemical shifts (ca. 9 to 7 ppm). Exemplifying this type are nitrogens 1 and 3 of the 2-deoxystreptamine ring. It is interesting that the chemical shifts of the nitrogens of this type seem broadly independent of whether Z is carbon or oxygen (e.g., N(2′) of factors 2, 6, and 8).

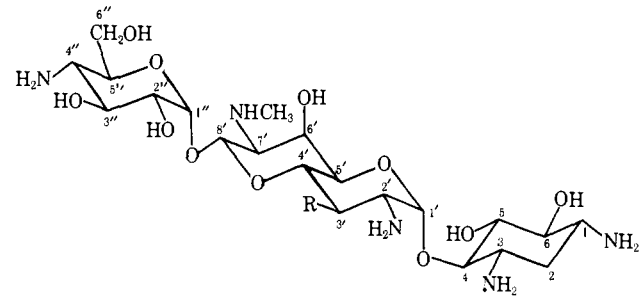
Also included in Table II are several examples of nitrogens in local environments such as **2.** This type of nitrogen differs



2

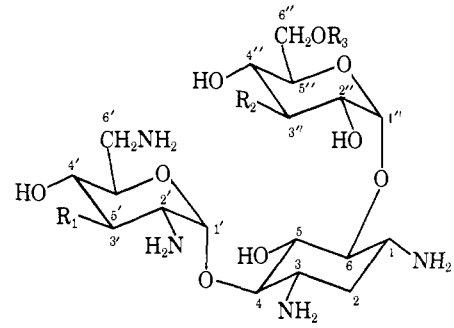
from that of **1** only in having one more γ oxygen. In terms of the above substituent parameters, one might expect the chemical shifts of nitrogens of **2** to be higher field than those

Table I. Structures and Names of Aminoglycoside Antibiotics Studied in This Work



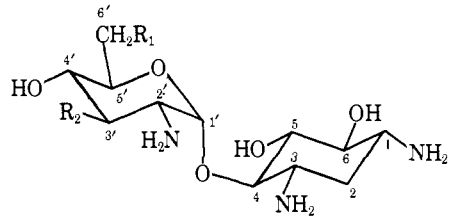
1a

Factor	R	Common name
2	H	Apramycin
7	OH	Oxyapramycin



1b

Factor	R_1	R_2	R_3	Common name
3	OH	OH	H	
4	OH	NH_2	$CONH_2$	
5	OH	NH_2	H	Kanamycin B
5′	H	NH_2	$CONH_2$	
6	H	NH_2	H	Tobramycin



1c

Factor	R_1	R_2	Common name
8	NH_2	H	Nebramine
9	OH	H	Lividamine
10	NH_2	OH	Neamine

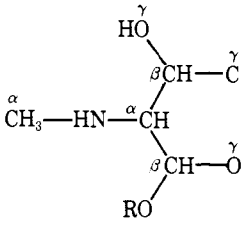
Table II. ^{15}N NMR Data[a] on Nebramycin Factors

Factor No.	pH	N(1)[b]	N(3)[b]	N(2')	N(6')	N(7')[c]	N(3'')	N(4'')[c]	Other
2	3	17.2	15.7	16.5		11.4		ca. 10.0	
	4	17.2	15.7	16.6		11.4		10.2	
	5	16.9	15.7	16.6		11.0		10.0	
	6	15.1	15.4	16.1		8.6		9.2	
	7	11.9	14.6	14.6		3.9		6.5	
	8	10.5	12.4	10.0		1.2		0.9	
	9	9.3	9.3	7.9		ca. 0		−0.8	
	10.3	9.1	8.2	7.5		ca. 0		−1.1	
	11[d]	8.8	7.9	7.0		0		−1.5	
3	4	16.7	14.6	11.1	3.9				
	10.4	9.1	7.7	−1.0	−8.5				
4	4	16.7	14.8	11.3	3.9		9.9		$OCONH_2$, 52.9
	10.5	9.1	7.6	−0.9	−8.5		1.1		$OCONH_2$, 52.0
5	4[d]	16.1	14.9	11.0	3.9		9.9		
	9.5	9.3	7.9	−0.8	−7.9		1.2		
	11	9.1	7.8	−0.9	−8.4		1.2		
5'	11	9.2	7.6	7.5					$OCONH_2$, 51.9
6	3	17.2	15.2	16.3	4.5		10.4		
	4	17.1	15.2	16.2	4.5		10.4		
	5	16.6	15.1	16.2	4.4		10.3		
	6	13.8	14.4	16.0	4.1		9.9		
	7	11.7	12.8	14.4	3.4		7.5		
	8	10.6	10.0	10.6	1.0		3.5		
	9	9.9	ca. 8.1	ca. 8.1	−3.8		1.8		
	10	9.4	8.0	7.4	−6.8		1.3		
	11	9.3	7.9	7.3	−8.1		1.2		
N(6')-Acetyl-6	10	8.6	7.7	7.1	94.1		1.1		
7	4	16.9	15.5	11.0		11.4		9.9	
	9.5	8.4	8.0	−1.0		0		−1.2	
8	10.7	9.3	7.9	7.1	−7.7				
9	9.1	9.2	8.1	7.4					

[a] Chemical shifts are in ppm from external NH_4Cl. [b] These assignments are tentative. [c] These resonances cannot be distinguished in assignment. [d] pH drifted significantly during measurement of spectra.

of **1.** This is indeed observed, nitrogens of type **2** falling in the range of chemical shifts 1 to −1 ppm. Again, the chemical shift is not strongly dependent on whether Z is carbon or oxygen. Perhaps more surprising is the fact that replacement of a γ-hydroxyl group with a hydroxymethyl group, as in the local environment of N(4'') of factors 2 and 7, also shows little effect on the nitrogen chemical shift.

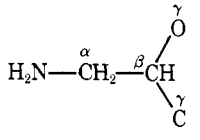
The α effect observed with amines and hydrazines[6] might lead one to expect that N-methylation of the nitrogen would lead to a detectable downfield shift. In fact, the N(7') nuclei of factors 2 and 7, which conform to structural type **3,** have not



3

yet been specifically assigned. By elimination, however, this nitrogen must come into resonance at either 0 or −1.5 ppm, either of which is compatible with the range of chemical shifts noted above for **2.** Apparently, the α effect will be less important in these complex molecules, possibly being outweighed by the opposing effects of γ substitution.

Finally, we may consider structural type **4,** the aminomethyl



4

group. The obvious difference here is the reduction in the number of β substituents, which should result in nitrogen nuclei of type **4** having substantially higher field chemical shifts. This supports our assignment of the peak near −8.5 ppm to this nitrogen (vide supra). Comparison of the shifts of types **1** and **4** suggests that the β effect in these systems is similar to the 20-ppm shifts observed in simpler molecules.[6]

At present, it is not possible to identify any chemical shifts associated with δ or ε substitution. The close correspondence of the chemical shifts of the 2-deoxystreptamine nitrogens (1 and 3) of factors 2, 7, and 8 to those of factors 3, 4, 5, and 6 indicates that these effects must be rather small.

The Effects of pH. One would anticipate that pH would have a substantial effect on the chemical shifts of these basic nitrogens.[6c] As shown in Table II, protonation of these amines leads to downfield shifts ranging about 7 to 12 ppm. Closer inspection of the available data leads to more specific correlations between the observed protonation shifts and the structural type as defined above.

For amines of type **1,** the protonation shifts are 6.9 to 7.9 ppm when R is a glycosidic unit and Z is carbon. When R = **H,** as in nitrogen 1 of factors 2 and 7, the protonation shift is slightly larger (ca. 8.5 ppm); such a small difference may not be significant, however, in view of possible differences in solvation in acidic and basic solutions. For type **1** nitrogen nuclei in which R is an aminocyclitol and Z is oxygen rather than carbon, the protonation shifts are again larger (9.0–9.5 ppm).

In the case of nitrogen nuclei of environment **2,** the protonation shifts range from 8.7 to 12.2 ppm. In cases in which Z is carbon and R is H, the protonation shift is ca. 9 ppm. As before, if the nitrogen is at position 2 of a pyranose structure, such that Z is oxygen and R is an aminocyclitol, the protonation shift is larger (ca. 11 to 12 ppm).

As was mentioned above, the resonances of nitrogens 7' and 4'' of factors 2 and 7 cannot presently be differentiated in as-

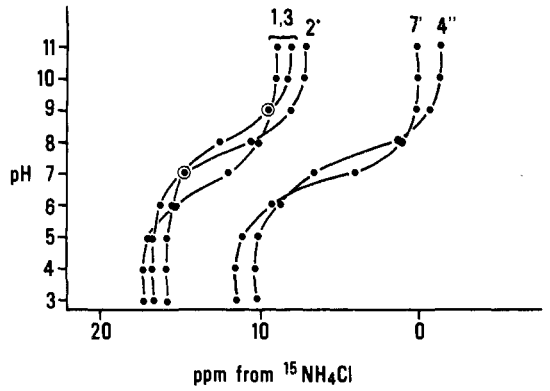


Figure 2. Titration curves for the ^{15}N resonances of factor 2 (apramycin).

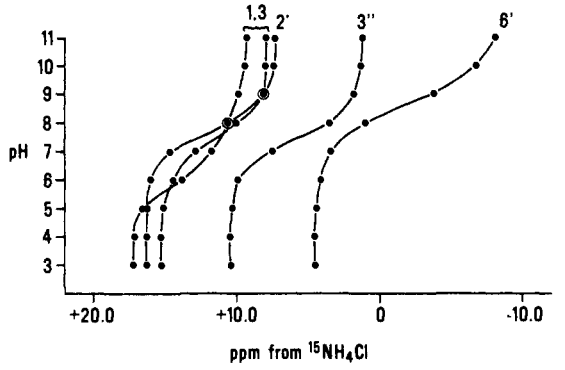


Figure 3. Titration curves for the ^{15}N resonances of factor 6 (tobramycin).

signment. The environment of N(4″) can be described as type **2** with Z = oxygen and $R^{\gamma}O = CH_2OH$, while N(7′) is a methylamine of type **3**. It is apparent from Table II that the protonation shifts of these two nitrogens are very similar (ca. 11.5 ppm). The aminomethyl type of nitrogen (**4**) shows a consistently large protonation effect (12.3–12.6 ppm). Nonbasic nitrogens, such as in a carbamoyl group, show only a very small pH dependence (cf. factor 4).

More complete titration data are available for factors 2 and 6 (Figures 2 and 3, respectively). The resonance assignments for each have been confirmed at the basic and acidic extremes of these curves but not at intermediate pH values. It is seen, however, that the points can be joined by a series of smooth sigmoid curves. Practically speaking, it is apparent that it is preferable to measure the spectra of these compounds at pH values greater than 9 or less than 5; between these limits, the problem of peak overlapping is more acute. The curves also show that all the nitrogens are essentially fully protonated at pH 4, and most are fully deprotonated at pH 11. pK_a values for the individual nitrogens can be extracted from Figures 2 and 3 and appear in Table III. The value shown for N(6′) of factor 6 must be taken as a lower limit, inasmuch as this nitrogen does not appear to have been fully deprotonated at pH 11 (cf. Figure 3). This nitrogen is obviously the most basic of the molecule. The pK_a values of nitrogens 1 and 3 are quite different in the two factors, suggesting that the basicities of these two nitrogens are dependent upon whether the deoxystreptamine ring is mono- or disubstituted.

Applications. It is obvious from the above that ^{15}N NMR spectroscopy is useful in confirming the complementing information derived from ^1H, ^{13}C NMR, and mass spectroscopies.[1] In some cases, ^{15}N NMR spectroscopy can provide particularly simple solutions to otherwise vexing problems. An example is the case of the monoacetate of tobramycin (factor 6).[1g]

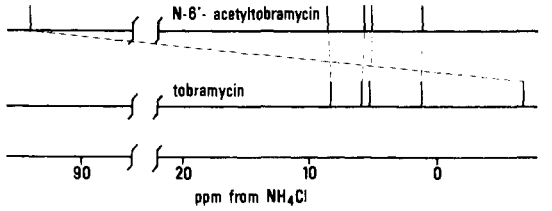


Figure 4. Comparison of the ^{15}N chemical shifts of factor 6 (tobramycin) and its *N*-6′-acetyl derivative (pH ca. 10).

Table III. pK_a Values of Individual Nitrogens of Nebramycin Factors 2 and 6

Factor	N(1)[a]	N(3)[a]	N(2')	N(6')	N(7')[b]	N(3'')	N(4'')[b]
2	6.6	8.2	7.7		6.7		7.5
6	6.2	7.4	7.6	8.6		7.4	

[a] These assignments are tentative. [b] These two nitrogens could not be distinguished in assignment.

When tobramycin is allowed to react with limited amounts of acetic anhydride in aqueous tetrahydrofuran, one major acetylated product is isolated.[1g] From comparisons of the ^{13}C NMR spectrum of this monoacetate with that of the free antibiotic,[1f] the structure of this product was deduced to be *N*-6′-acetyltobramycin. The same conclusion is readily reached by simply comparing the ^{15}N NMR spectrum of this acetate to that of tobramycin (Figure 4). It is immediately obvious that the substituted nitrogen must be N(6′), which is deshielded by acetylation by more than 100 ppm. The remaining nitrogens stay relatively unchanged.

Finally, because of the large protonation shifts shown by the nitrogen resonances, ^{15}N NMR spectroscopy may provide more sensitive and direct information regarding the basicities of the individual amine nitrogens. It should be no surprise, for example, that nitrogen 6′ of tobramycin is the most easily acetylated site in the molecule. Not only is this nitrogen the most basic, but it is also the most sterically accessible. ^{15}N NMR spectra of the aminoglycosides may therefore be expected to yield important information to chemists working with these molecules.

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References and Notes

(1) (a) N. Neuss et al., *Helv. Chim., Acta*, **53**, 2314 (1970); (b) P. W. K. Woo and R. D. Westland, *Carbohydr. Res.*, **31**, 27 (1973); (c) J. B. Morton, R. C. Long, P. J. L. Daniels, R. W. Tkach, and J. H. Goldstein, *J. Am. Chem. Soc.*, **95**, 7464 (1973); (d) S. Omoto, S. Inouge, M. Kojima, and T. Niida, *J. Antibiot.*, **26**, 717 (1973); (e) K. Boch, C. Pedersen, and H. Heding, *ibid.*, **27**, 139 (1974); (f) K. F. Koch, J. A. Rhoades, E. W. Hagaman, and E. Wenkert, *J. Am. Chem. Soc.*, **96**, 3300 (1974); (g) K. F. Koch, K. E. Merkel, S. C. O'Connor, J. W. Paschal, and D. E. Dorman, to be published; (h) T. L. Nagabhushan, W. N. Turner, P. J. L. Daniels, and J. B. Morton, *J. Org. Chem.*, **40**, 2830 (1975); (i) T. L. Nagabhushan, P. J. L. Daniels, R. S. Jaret, and J. B. Morton, *ibid.*, **40**, 2835 (1975).
(2) J. J. Wright, Abstracts of the 15th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, D.C., Sept. 24–26, 1975, p 91.
(3) B. Coxon, *Carbohydr. Res.*, **35**, C1 (1974).
(4) (a) K. F. Koch and J. A. Rhoades, *Antimicrob. Agents Chemother.*, 309 (1970); (b) K. F. Koch, F. A. Davis, and J. A. Rhoades, *J. Antibiot.*, **26**, 745 (1973); (c) T. Oda, T. Mori, and Y. Kyotani, *ibid.*, **24**, 503 (1971); (d) S. O'Connor et al., *J. Org. Chem.*, in press.
(5) R. Freeman, H. D. W. Hill, and R. Kaptein, *J. Magn. Reson.*, **7**, 327 (1972).
(6) (a) R. L. Lichter and J. D. Roberts, *J. Am. Chem. Soc.*, **94**, 2495 (1972); (b) J. P. Warren and J. D. Roberts, *J. Phys. Chem.*, **78**, 2507 (1974); (c) R. L. Lichter, "Determination of Organic Structures by Physical Methods", Vol. 4, F. C. Nachod and J. J. Zuckerman, Ed., Academic Press, New York, N.Y., 1971.