Determination of the N–H Bond Lengths for Simple Peptides in the Solid State by Natural Abundance ^{15}N Two-Dimensional Separated Local Dipolar Field (2D-SLDF) NMR Spectroscopy#

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(Received May 13, 1993)

The N–H bond lengths of simple petides were determined under the condition of high-resolution spectroscopy by analyzing the dipolar side-band patterns obtained from samples of natural abundance, as recorded by ^{15}N two-dimensional (2D) separated local dipolar field (SLDF) NMR spectroscopy combined with cross-polarization and magic-angle spinning (CP-MAS) techniques. It was found that the dipolar side-band pattern obtained from the one-dimensional (1D) cross section taken from the 2D spectrum can be conveniently used to distinguish the ^{15}N signal of the N–H group from that of the N–C group, whose peak positions are very close to each other. Further, spectral simulations of the ^{15}N dipolar patterns of the peptides were performed in order to determine the N–H bond lengths of the amino acid residues of natural abundance at the C-terminus. These values were determined to be 1.07, 1.12, and 1.09 Å for Ala–Gly, Gly–Pro–Ala, and Ala–Pro–Gly, respectively, with an accuracy of 0.01—0.02 Å. The obvious difference in the bond lengths between the last two compounds suggests that the degree of interchain packing, as estimated from the N–H bond lengths, significantly differs between Gly–Pro–Ala and Ala–Pro–Gly. This finding is consistent with the previous data regarding the presence or absence of ring-puckering motion at the Pro residue, as examined by the ^{13}C spin-lattice relaxation times in the laboratory frame.

The hydrogen bond has been well recognized as being the most important molecular interaction which leads to the determination of the three-dimensional structure, dynamics and molecular recognition for a number of molecular systems, especially for biological molecules.[1—4] Undoubtedly, a detailed determination of the molecular geometry is crucially important for identifying and interpreting the underlying principles of the hydrogen bond system, X–H···Y, in any molecular system, where X–H and Y are designated as being the proton donor and proton acceptor, respectively. Accurate hydrogen bond geometries are available only from single-crystal neutron-diffraction studies, since the X–H covalent bond lengths determined by X-ray diffraction are generally too short (by about 0.3 Å).[4] The neutron-diffraction studies of biological molecules, however, are in many instances hampered by a difficulty to grow large single crystals. It is thus essential to measure the X–H bond lengths by an alternative means other than the diffraction methods in order to gain insight into the manner of hydrogen bonding.

A two-dimensional (2D) separated local dipolar field (SLDF) spectroscopy[5] combined with cross-polarization magic-angle spinning (CP-MAS)[6] provides an excellent means for this purpose by allowing us to determine N–H or C–H bond lengths by analyzing the dipolar side-band pattern under the condition of high resolution.[6—8] In fact, this technique has been applied to measure the N–H bond lengths in DNA[9] and peptides.[10,11] As to the accuracy of the bond lengths studied by this technique, it was demonstrated[11] that the thus-obtained N–H bond lengths were consistently 0.035 Å longer than the corresponding neutron-diffraction results, presumably due to differences in averaging, resulting from the molecular motion for the two experiments. Accordingly, a systematic accumulation of data on the N–H bond lengths of a variety of biological molecules seems to be very important. Nevertheless, these previous experiments[9—11] were carried out using ^{15}N-enriched samples, because of the low sensitivity of ^{15}N NMR. It thus seems to be very useful to extend this approach to the system of natural abundance, since a variety of hydrogen bond systems can be studied in more detail by using recently developed instruments with much improved sensitivity.

We attempted to record the ^{15}N 2D SLDF/CP-MAS NMR spectra of some simple peptides in order to explore the possibility for a precise determination of the N–H bond lengths from samples of natural abundance, and to gain insight into the manner of interchain packing, as estimated from the evaluated N–H bond lengths.

Experimental

Peptide samples (alanylglycine (Ala–Gly), alanylprolylglycine (Ala–Pro–Gly) and glycylprolylalanine (Gly–Pro–Ala) were purchased from Sigma Chemical Co., USA, and were used without further purification. The samples were contained in a pencil-type Zirconia rotor and spun by compressed air. All NMR spectra were recorded in the solid state on a Chemagnetics CMX-400 NMR spectrometer at a resonance frequency of 40.6 MHz for ^{15}N nuclei. The pulse sequence employed in this 2D work (SLDF/CP-MAS NMR) is outlined in Fig. 1. A BLEW12[12] homonuclear decoupling pulse-sequence was used during the evolution period and a continuous-wave (cw) decoupling pulse was applied during the detection period in order to eliminate any heteronuclear

#This paper is dedicated to the memory of the late Professor Hiroshi Kato.

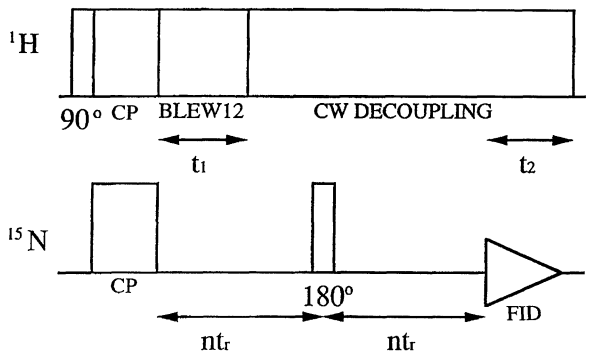

Fig. 1. Pulse sequence used to obtain the ^{15}N two-dimensional separated local dipolar field (2D-SLDF/CP-MAS) NMR spectra.

dipolar interactions. The 90° pulse length of the ^{1}H channel was 5 μs for the excitation and homonuclear decoupling. The contact and repetition times for this experiment were 2 ms and 3 s, respectively. For recording the 2D NMR spectra, 48 t_1 values were generally employed and 400 transients were accumulated for each t_1 value. The typical acquisition time for obtaining the ^{15}N 2D SLDF/CP-MAS NMR spectrum was 32 h. A 180° pulse was irradiated in the ^{15}N channel at the position of the nt_r time after the end of the contact time to refocus the isotropic components of the ^{15}N chemical-shift interaction. It was essential to choose the position of the 180° pulse so as to be synchronized with the rotor period, where t_r is the rotor cycle and n is integer number. We thus controlled the spinning rate of the sample rotation as accurately as ±1 Hz by the aid of a spinning-rate controller provided by Chemagnetics. The ^{15}N chemical shifts were first calibrated with respect to the ^{15}N chemical shift of the amino nitrogen of glycine, and were then converted to the value from NH_4^+ (11.59 ppm higher field from the ^{15}N chemical shift of the amino nitrogen of glycine in the solid state).[13] All of the experiments were performed at ambient temperature. The 2D NMR data were processed using a Silicon Graphics Personal IRIS workstation along with Hare Research FELIX software. A spectral simulation was performed using an NEC 9801 personal computer equipped with a transputer (INMOS, UK) using the SLFDIP program, which was written in FORTRAN 77 language based on the theory described in the text. The source program was then compiled using a Parallel Fortran compiler supplied by 3L Ltd, UK.

Results and Discussion

Determination of N–H Bond Lengths. Figure 2 shows the ^{15}N 1D CP-MAS NMR spectrum (left trace) and the cross sections taken from the 2D-SLDF/CP-MAS-NMR spectrum of Gly–Ala (right traces). The ^{15}N resonance lines can be readily assigned to the amino and peptide nitrogens from the higher to the lower field, respectively, on the basis of the chemical-shift data.[14,15] In addition, the cross section of the peptide ^{15}N NMR spectrum shows a pronounced dipolar side-band pattern, while the cross section of the amino nitrogen does not show a side-band pattern (Fig. 2, right trace). This observation is consistent with the fact that the amino group rotates rapidly about the C_3 axis,[16] thus reducing the dipolar coupling to a large extent. Figure 3 shows the ^{15}N 1D CP-MAS NMR spectrum and the cross sections of the ^{15}N 2D-SLDF/CP-MAS NMR spectrum of Gly–Pro–Ala. It is emphasized that the two ^{15}N peptide signals, which appear to be very close to each other at around 110 ppm (Table 1), can be easily distinguished by the dipolar patterns of the cross sections. Namely, since the cross section of the resonance in the higher field showed many side bands, it is assigned to the peptide nitrogen of the Gly residue, since the nitrogen in this case is bonded directly to the proton. In contrast, the cross section in the lower field resonance did not show any side band. Therefore, this peptide nitrogen was assigned to the Pro residue, because this nitrogen is bonded directly to the carbon atom. The cross section of the amino nitrogen does not show a side band because of rapid averaging in the dipolar interaction (as mentioned above). A similar behavior of the cross sections of the ^{15}N 2D-SLDF/CP-MAS NMR spectra was also obtained for Ala–Pro–Gly (spectra not shown).

These dipolar side-band patterns from the peptide nitrogen provide information concerning the N–H bond lengths. To evaluate the N–H bond lengths accurately, a spectral simulation was performed according to the following procedure. The transition frequencies of the two time-dependent resonance lines due to a N–H dipolar interaction under the condition of MAS at a frequency of ω_r, when the carrier frequency is set to the center of the two resonances, can be given by

$$\omega_D(t) = \pm\frac{D}{2}\left\{\sin^2\beta\cos 2(\alpha+\omega_r t) + \sqrt{2}\sin 2\beta\cos(\alpha+\omega_r t)\right\}, \quad (1)$$

with

$$D = \frac{\gamma_N\gamma_H h}{2\pi r^3}\cdot S.$$

Here, α is the azimuthal and β the polar angle defined by the internuclear vector with amplitude r in a coordinate system with the z-axis parallel to the rotor axis. S is the scaling factor of the heteronuclear dipolar interaction; the theoretical value of 0.475 for a BLEW12[12] was used in the calculation. The angle from the y-axis with which the initial magnetization aligns after time t

Table 1. ^{15}N Chemical Shift Values of Gly–Ala, Gly–Pro–Ala, and Ala–Pro–Gly in the Solid State Taken by CP-MAS Experiment

	^{15}N chemical shifts (ppm)[a]		
	$^{15}NH_3$	^{15}N–H	^{15}N–C
Gly–Ala	6.3	106.3	—
Gly–Pro–Ala	5.8	105.8	111.1
Ala–Pro–Gly	20.3	86.0	111.4

a) With respect to the ^{15}N chemical shift value of $^{15}NH_4NO_3$ (0 ppm).

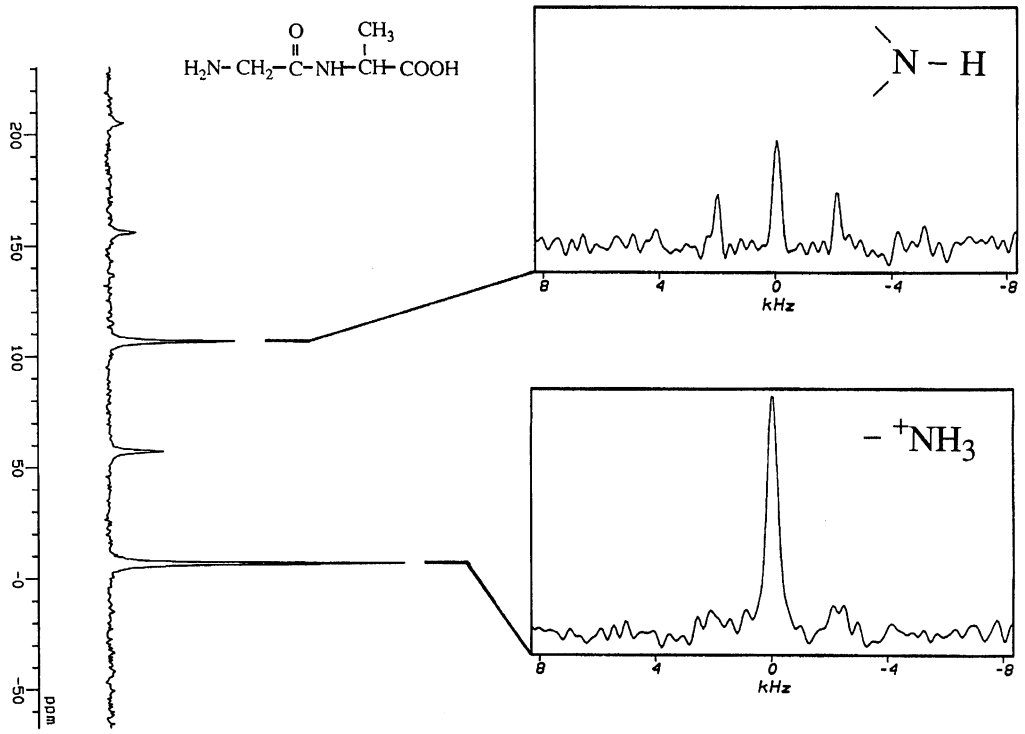

Fig. 2. ^{15}N 1D CP-MAS NMR spectrum (left) and the cross sections taken from the 2D-SLDF/CP-MAS spectrum of Gly–Ala (right).

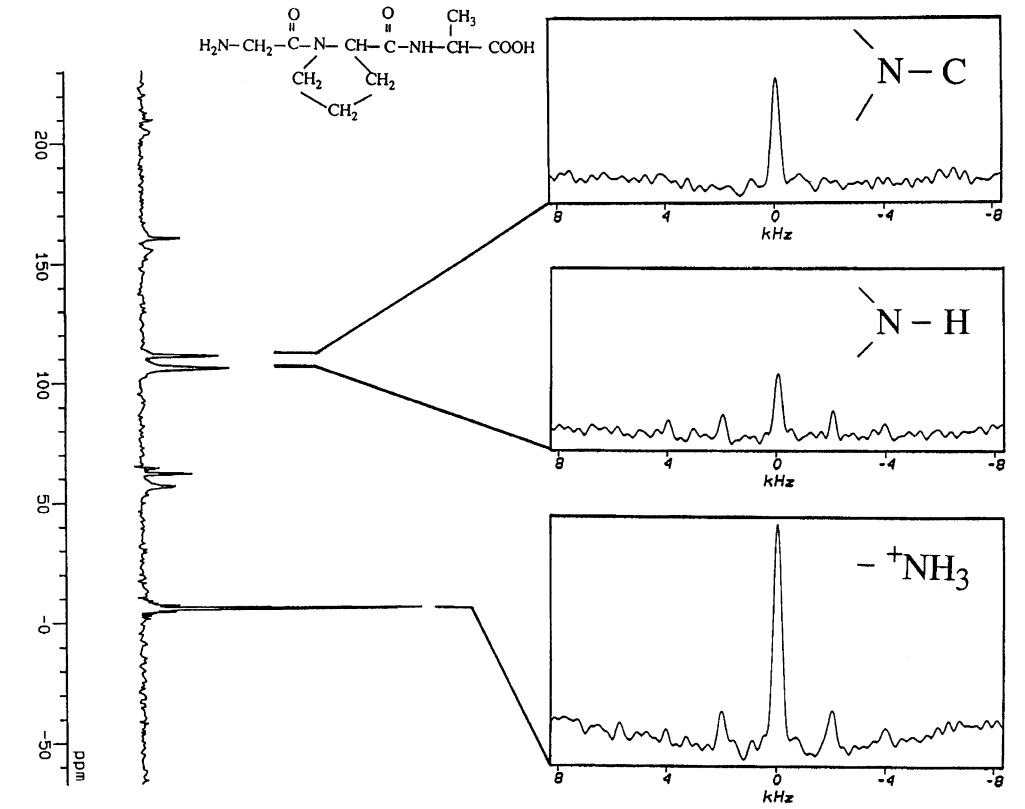

Fig. 3. ^{15}N 1D CP-MAS NMR spectrum (left) and the cross sections taken from the 2D-SLDF/CP-MAS NMR spectrum of Gly–Pro–Ala (right).

during the evolution period of the 2D-SLDF pulse sequence is given by

$$\Phi(\alpha,\beta,r,t) = \int_0^t \omega_D(t')dt'. \quad (2)$$

Using this angle, the free-induction decay signal for ^{15}N magnetization with N–H dipolar coupling is given by

$$g(t) = \exp[i\Phi(\alpha,\beta,r,t)]. \quad (3)$$

After averaging over all orientations in the powder sample, and Fourier transformation, dipolar side-band spectrum is obtained as

$$G(\omega) = \int_{-\infty}^{\infty} < g(t) > e^{-i\omega t}dt. \quad (4)$$

In the numerical computation, powder averaging was performed by incrementing the angle by 10° over the range of 0 to 360° and 0 to 90° for α and β, respectively. Equation 4 was computed by a fast Fourier-transform algorithm elaborated by Cooley and Tukey.[17] Figure 4 shows the simulated spectra for a series of different N–H bond lengths when the rotor frequency is 2 kHz. It is emphasized that the side-band pattern is sensitively varied by changing the N–H bond lengths. It is, therefore, possible to determine the bond length with an accuracy of 0.01—0.02 Å by carefully comparing the ratio of the center to the side-band intensities of the experimentally obtained dipolar spectrum with that of the simulated one.

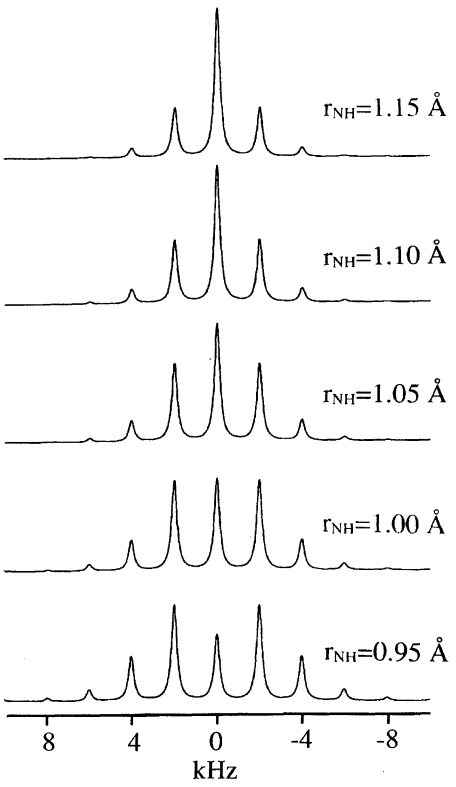

Fig. 4. Simulated spectra for the ^{15}N dipolar side band patterns with a series of different N–H bond-lengths (2kHz spinning frequency and linewidth 300 Hz).

We thus determined the N–H bond lengths of the peptide nitrogens for three petides at the *C*-terminus residue to be 1.07, 1.12, and 1.09 Å for Gly–Ala, Gly–Pro–Ala, and Ala–Pro–Gly, respectively (Fig. 5). It is now obvious that a rather precise determination of the N–H bond lengths is generally feasible from simple peptides, even if they are of natural abundance.

Significance of the N–H Bond Lengths. Empirically, it was demonstrated on the basis of the neutron-diffraction data that the X–H bond-length (X=O or N) is inversely proportional to the X···Y length of the X–H···Y hydrogen bond system.[18] This means that the N–H length would be prolonged to some extent when the proton donor X–H is closer to form a hydrogen bond with a proton acceptor, Y. In fact, Kuroki et al.[19] proved the existence of this trend between the

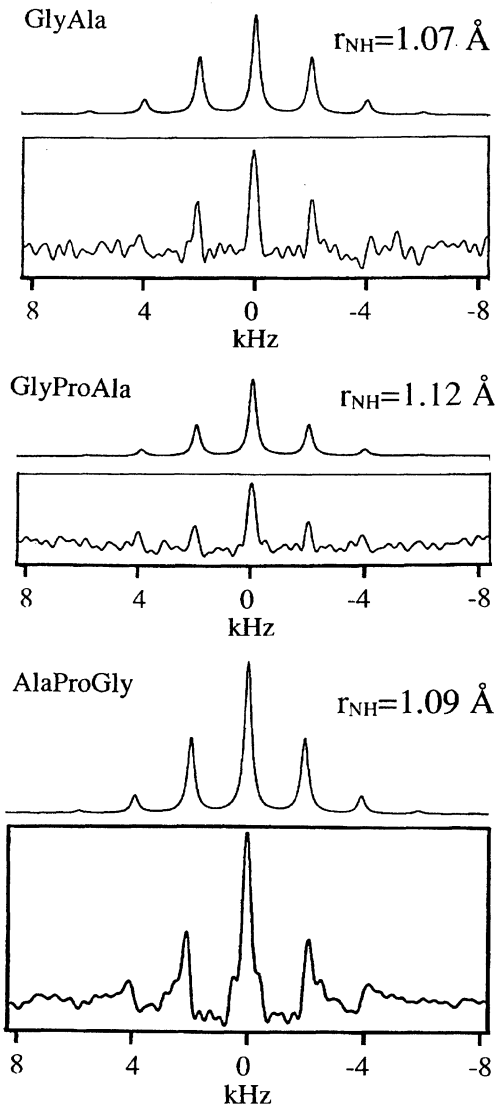

Fig. 5. Comparison of the experimental (in the box) and simulated spectra of the dipolar side band patterns for Gly–Ala, Gly–Pro–Ala, and Ala–Pro–Gly. The simulated spectra were obtained for a 2 kHz spinning frequency and with a linewidth of 300 Hz.

N–H lengths and the N···O lengths of two hydrogen-bonded N-methylacetamide on the basis of an *ab initio* molecular orbital calculation. It is therefore expected that the observed difference in the N–H bond lengths by 0.03 Å results in a more pronounced change in the N···O length if the above-mentioned empirical relation can be applied to the present situation. In this connection, we previously showed[20] that the pyrrolidine ring of the Pro residue in Ala–Pro–Gly undergoes a rapid puckering motion in the solid state with a time scale of 10^{-8} s, as detected by a measurement of the ^{13}C spin-lattice relaxation time in the laboratory frame, while that of Gly–Pro–Ala does not. Such a puckering motion in the solid state was noted for collagen fibril and some collagen-like polypetides, and is caused by the presence of free space due to weak molecular packing around the Pro residue. Therefore, the present observation strongly suggests that the absence of the ring-puckering motion in Gly–Pro–Ala can be explained in terms of the tightly packed environment. For this reason, it appears that a precise determination of the N–H bond length by this approach is very useful to examining the manner of interchain packing of the peptide molecules.

Another interesting feature of the N–H bond length is related to a correlation with the isotropic ^{15}N chemical shifts, as proposed by Kuroki et al.[19] They observed that the relation of the ^{15}N chemical shift (δ_{obs}) to the bond length (R_{NH}) can be empirically expressed as

$$\delta_{obs} = 39.32R_{NH} + 57.73. \quad (5)$$

It is thus possible to evaluate the bond length in terms of the ^{15}N chemical shifts if such a relationship is established for an individual hydrogen bond system. The existence of this sort of relation is conceivable, since the ^{15}N chemical shifts are very sensitively displaced by the formation of the hydrogen bonds,[21—23] even though the direction of the displacement of the ^{15}N shift is opposite, depending on whether the nitrogen atom is involved in either the proton donor or acceptor.

In conclusion, a systematic determination of the N–H bond lengths is worthwhile from the view point to gain insight into either the precise geometries or to make a rough estimate of the extent of molecular packing of the hydrogen bond system. Undoubtedly, the present ^{15}N 2D SLDF/CP-MAS approach can be a very promising means to this end, because such measurements can be performed with samples of polycrystalline or amorphous solids.

This work was supported, in part, by a Grant-in-Aid for Scientific Research No. 04554017 from the Ministry of Education, Science and Culture.

References

1) G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond," W. H. Freeman & Co., San Francisco and London (1960).
2) S. N. Vinogradov and R. H. Linnell, "Hydrogen Bonding," Van Nostrand Reinhold Company, New York (1970).
3) P. Schuster, G. Zundel, and C. Sandorfy, "The Hydrogen Bond, Recent Developments in Theory and Experiments," North Holland Publishing Company, Amsterdam (1976).
4) G. A. Jeffrey and W. Saenger, "Hydrogen Bonding in Biological Structure," Springer-Verlag, Berlin (1991).
5) R. K. Hester, J. L. Ackerman, V. R. Cross, and J. S. Waugh, *Phys. Rev. Lett.*, **34**, 993 (1975).
6) M. G. Munowitz, R. G. Griffin, G. Bodenhausen, and T. H. Huang, *J. Am. Chem. Soc.*, **103**, 2529 (1981).
7) J. Herzfeld, J. E. Roberts, and R. G. Griffin, *J. Chem. Phys.*, **86**, 597 (1987).
8) W. P. Power and R. E. Wasylishen, *Annu. Rep. NMR Specrosc.*, **23**, 1 (1991).
9) J. A. DiVerdi and S. J. Opella, *J. Am. Chem. Soc.*, **104**, 1761 (1982).
10) M. H. Frey, S. J. Opella, A. L. Rockwell, and L. M. Gierasch, *J. Am. Chem. Soc.*, **107**, 1946 (1985).
11) J. E. Robert, G. S. Harbison, M. G. Munowitz, J. Herzfeld, and R. G. Griffin, *J. Am. Chem. Soc.*, **109**, 4163 (1987).
12) D. P. Burum, M. Linder, and R. R. Ernst, *J. Magn. Reson.*, **44**, 173 (1981).
13) A. Shoji, T. Ozaki, T. Fujito, K. Deguchi, S. Ando, and I. Ando, *J. Am. Chem. Soc.*, **112**, 4693 (1990).
14) G. C. Levy and R. L. Lichter, "Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy," John Wiley & Sons, New York (1979).
15) M. Witanowski and G. A. Webb, "Nitrogen NMR," Plenum Press, London (1973).
16) E. R. Andrew, W. S. Hinshaw, M. G. Hutchins, and R. O. I. Sjöblom, *Mol. Phys.*, **32**, 795 (1976).
17) J. W. Cooley and J. W. Tukey, *Math. Comput.*, **19**, 297 (1965).
18) I. Olovsson and P.-G. Jönsson, in Ref. 3, Vol. 2, pp. 393—456.
19) S. Kuroki, S. Ando, I. Ando, A. Shoji, T. Ozaki, and G. A. Webb, *J. Mol. Struct.*, **240**, 19 (1990).
20) H. Saitô and M. Yokoi, *J. Biochem.*, **111**, 376 (1992).
21) H. Saitô, K. Nukada, H. Kato, T. Yonezawa, and K. Fukui, *Tetrahedron Lett.*, **1965**, 111.
22) H. Saitô and K. Nukada, *J. Am. Chem. Soc.*, **93**, 1072 (1971).
23) H. Saitô, Y. Tanaka, and K. Nukada, *J. Am. Chem. Soc.*, **93**, 1077 (1971).