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^{1}H Nuclear Magnetic Resonance Study of Restricted Internal Rotation of N^6,N^6-Dimethyladenine in Aqueous Solution†

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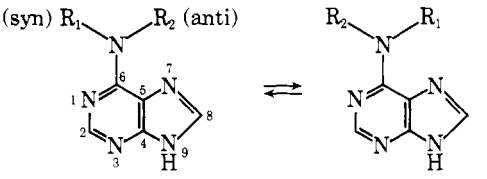
ABSTRACT: Kinetics of internal rotation about the C(6)–N(6) bond of N^6,N^6-dimethyladenine (M_2^6A) was investigated by ^{1}H nuclear magnetic resonance line-shape analysis of the methyl resonances (220 MHz). Rates of rotation were determined for M_2^6A deuterated at N(1) and for neutral M_2^6A. Activation parameters for monodeuterated M_2^6A at 22° are E_a = 13.8 kcal/mol, log A = 12.6, $\Delta G^\ddagger$ = 14.9 kcal/mol, $\Delta H^\ddagger$ = 13.2 kcal/mol, $\Delta S^\ddagger$ = −5.8 eu; for neutral M_2^6A: E_a = 15.5 kcal/mol, log A = 14.9, $\Delta G^\ddagger$ = 12.6 kcal/mol, $\Delta H^\ddagger$ = 14.9 kcal/mol, $\Delta S^\ddagger$ = 7.8 eu. Vertical stacking of bases interferes with internal rotation of the dimethylamino group.

Methylated nucleic acid bases play important roles in determining the activity and conformational properties of biomolecules of which they are components. The defense mechanism of certain strains of *Escherichia coli* against bacteriophage infection involves N(6) methylation of strategically located adenines of the bacterial DNA by modification methylases; by means of homospecific restriction endonucleases which do not hydrolyze the methylated DNA, bacteria preferentially degrade the phage DNA (Brockes et al., 1974; Marinus and Morris, 1974; for reviews see Arber, 1974 and Meselson et al., 1969). Modified nucleosides are located adjacent to the anticodon in certain types of tRNA: e.g., N^6-methyladenosine in *E. coli* valine tRNA (Saneyoshi et al., 1969). The exact reason for the presence of these minor components at this position is not certain, but it has been suggested that they may contribute to precise codon–anticodon pairing by altering the conformation of the anticodon loop (see review by Nishimura, 1972). N^6-substituted adenines display a wide range of biological and pharmacological activity: for example, various adenine derivatives, including N^6-methyladenosine and N^6,N^6-dimethyladenosine, inhibit tRNA methylation (Wainfan and Landsberg, 1973); N^6-phenyladenosine potentiates the effects of 6-mercaptopurine against leukemia L1210 (Grindley et al., 1973); and adenine derivatives inhibit accumulation of adenosine cyclic 3′,5′-monophosphate in fat cells (Fain, 1973). To understand the role of N^6-substituted adenines at the molecular level, we have initiated a program directed toward investigating the solution properties of the monomeric units.

As a consequence of the partial double bond character of the exocyclic C–N bond, the amino substituents of N(6)-methylated adenines are coplanar with the purine ring. One



of the resulting rotational isomers has R_1 syn to N(1); the other has R_2 syn to N(1). For monosubstituted adenines (R_2 = H) X-ray (McMullan and Sundaralingam, 1971; Bugg and Thewalt, 1972; Thewalt and Bugg, 1972; Sternglanz and Bugg, 1973a,b; Parthasarathy et al., 1974) and ^{1}H nuclear magnetic resonance (NMR) studies in nonaqueous solvents (Engel and von Hippel, 1974) indicate the preferred orientation for the alkyl substituent is syn to N(1). This orientation would block normal Watson–Crick base pairing if retained under physiological conditions. Engel and von Hippel (1974) demonstrated that Watson–Crick pairing was, in fact, blocked in a mixture of N^6-methyl-N^9-ethyladenine and 1-cyclohexyluracil; Hoogsteen pairing was favored. Ikeda et al. (1970) demonstrated that the 1:1 complex of poly(N^6-methyladenylic acid) with poly(U) is markedly destabilized by the presence of the methyl groups, even though, in this case, Watson–Crick base pairing occurs.

The kinetics and thermodynamics of syn–anti rotational isomerism of cytosine derivatives (Becker et al., 1965; Martin and Reese, 1967; Shoup et al., 1967, 1971, 1972; Engel

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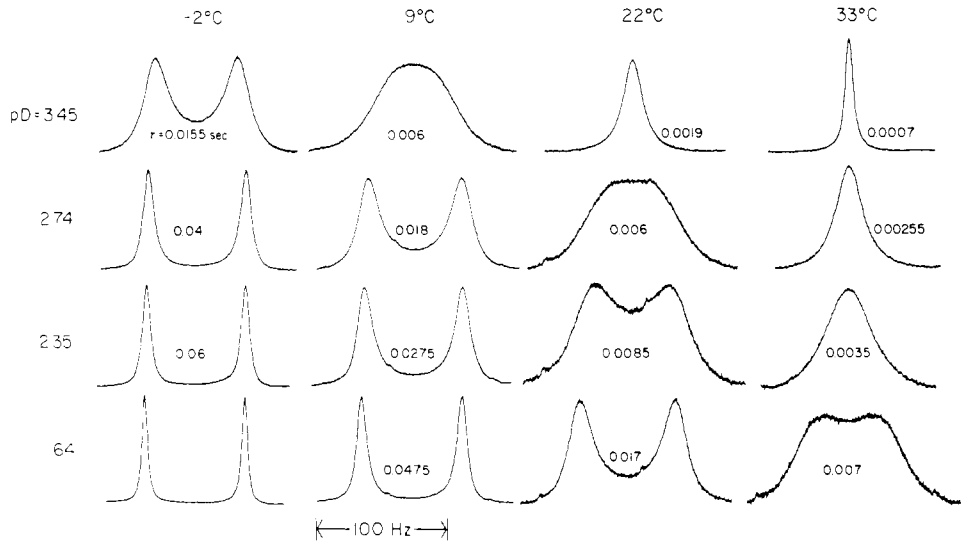


FIGURE 1: Temperature and pD dependence of the methyl resonances of N^6,N^6-dimethyladenine (0.15 *M*, 220 MHz).

and von Hippel, 1974) and adenine analogs (Martin and Reese, 1967; Neiman and Bergmann, 1968; Engel and von Hippel, 1974) in nonaqueous media have been studied by NMR. In neutral aqueous solution near room temperature the rate of internal rotation of the dimethylamino group of M_2^6A[1] is too rapid to measure by the NMR line-shape technique. Slower rates are encountered at low temperatures (Raszka, 1974) and in highly acidic media (protonation of the base diminishes the rate of internal rotation) (Pitner et al., 1975). We recently analyzed the kinetics of syn–anti isomerism of M_2^6A in highly acidic D_2O solution and determined the energy barrier of $M_2^6AD^+$ (M_2^6A deuterated on N(1); Pitner et al., 1975). Internal rotation of the biologically more interesting neutral base was too rapid to measure at the highest field strength attainable with the conventional magnet of our spectrometer (21.14 kG; 90 MHz). In the present study we have extended our measurements to neutral M_2^6A by using a spectrometer equipped with a superconducting magnet operating at 52 kG (220 MHz). Internal rotation rates of M_2^6A, as well as of $M_2^6AD^+$, were obtained from the pD dependence of the observed rate of rotation of mildly acidic solution (pD 1.6–3.5). In the less acidic solutions the effect of association of M_2^6A had to be taken into account.

Results

Methyl resonances of M_2^6A are shown as a function of pD and temperature in Figure 1. At low pD and temperature rotation about the exocyclic C–N bond is slow on the NMR time scale resulting in separate peaks for methyl protons syn and anti to N(1). The broadening and collapse of these peaks as pD or temperature is increased result from progressively more rapid internal rotation of the dimethylamino group. For each spectrum shown in Figure 1 and for corresponding spectra at pD 3.15, 3.76, and 3.98 the average lifetime (τ) of a methyl group in the syn or anti orientation was determined by comparing the spectrum with computer curves generated from equations derived by Gutowsky and Holm (1956).

It is well known that purine derivatives associate extensively in aqueous solution at neutral pD by vertical stacking of bases (Chan et al., 1964; Schweizer et al., 1965, 1968; Broom et al., 1967; Ts'o et al., 1969; Chan and Nelson, 1969); however, it has been demonstrated that this type of interaction does not occur between protonated bases found in more acidic solutions (Chan et al., 1964). This behavior is demonstrated by the concentration dependence of the H_2 and H_8 chemical shifts of M_2^6A at various pD's (Figure 2). For conciseness the average chemical shifts of these protons is plotted. Table I summarizes the chemical shifts of H_2, H_8, and the methyl protons at the concentration extremes. A distinct high field shift of these resonances indicates stacking of purine rings at pD 3.5–5.5 but not at pD 2.7. This shift reflects interactions of M_2^6A protons with anisotropic magnetic fields generated by circulating π electrons of adjacent bases.

The H_2 and H_8 chemical shifts are dependent both on the degree of protonation of N(1) and, as shown above, the extent of stacking; therefore, a pK_a determined by following the pD dependence of the H_2 and H_8 chemical shifts would be in error. For this reason the pK_a (4.10) for protonation of N(1) was determined by potentiometric titration. The fraction of neutral M_2^6A ($f_0 = [M_2^6A]/([M_2^6A] + [M_2^6AD^+] + [M_2^6AD_2^{2+}])$) was calculated from

$$pK_a = pD + \log [(1 - f_0)/f_0] \quad (1)$$

The concentration of the dideuterated species $M_2^6AD_2^{2+}$ is negligible in this pD range, since the pK_a for protonation of N(7) is −1.2 (Pitner et al., 1975). Consequently, the dication is not included in analysis of data in this study. Below pD 3.5 there is essentially no change in the chemical shifts of H_2 and H_8 as temperature is increased. This indicates that the degree of protonation is not changing. Consequently, the values of f_0 calculated at room temperature apply at all temperatures used in this study.

Discussion

At neutral pD the methyl resonances of M_2^6A appear as a single peak; rotation about the exocyclic C–N bond is too rapid for kinetic analysis by line-shape techniques. Raszka (1974) observed rapid rotation for N^6,N^6-dimethyladenosine at pH 7.5; he estimated a coalescence temperature of about 0°, although a detailed kinetic analysis was not presented. In more acidic solutions, however, rotation is slower and broadened spectra which can be analyzed are obtained

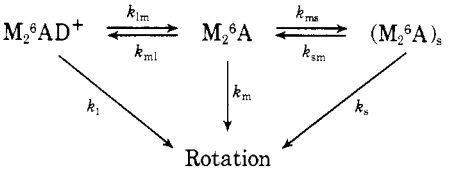
[1] Abbreviations used are: M_2^6A, N^6,N^6-dimethyladenine; M^6A, N^6-methyladenine.

Table I: Concentration Dependence of the Chemical Shifts of N^6,N^6-Dimethyladenine.

pD (±0.05)	Concn (M)	Chemical Shift[a] H-2	Δ	H-8	Δ	CH_3[b]	Δ
5.5	0.30	399	41	406	32	−26	36
	0.025	440		438		10	
4.0	0.30	432	41	436	31	0	34
	0.025	473		467		34	
3.5	0.30	451	27	451	21	15	23
	0.025	478		472		38	
2.7	0.30	470	8	465	8	30	7
	0.025	478		473		37	

[a] Hz to low field from internal tetramethylammonium chloride (90 MHz). [b] Center of broadened CH_3 resonance.

(Figure 1). Highly acidic solutions were used in our previous study of the rotation mechanism of $M_2{}^6A$ (Pitner et al., 1975); in those studies it was only necessary to consider two species, $M_2{}^6AD^{2+}$ and $M_2{}^6AD^+$, in the kinetic analysis. At the higher pD values in this study, however, the concentration of the neutral species is significant, and it must be included in the kinetic analysis; however, $M_2{}^6AD_2{}^{2+}$ can now be neglected. The following scheme accounts for the observed kinetics of internal rotation:



(2)

where the subscripts m and s refer to monomeric and stacked species of the neutral base. Single time-averaged peaks are observed for H_2 and H_8 rather than separate peaks for the neutral and monodeuterated species. This indicates that the protonation and stacking steps, represented by rate constants k_{1m}, k_{m1}, k_{ms}, and k_{sm}, are rapid on the NMR time scale. Therefore, the lifetime (τ) measured by NMR line-shape technique is given by

$$1/\tau = k_1 f_1 + k_m k_m + k_s f_s \quad (3)$$

where f_1 is the fraction of $M_2{}^6A$ in the deuterated form and f_m and f_s are similarly defined ($f_0 \equiv f_m + f_s$). Equation 3 can be rearranged to yield

$$1/\tau = k_1 + (k_m - k_1)(f_m + f_s) + (k_s - k_m)f_s \quad (4)$$

At fractions of neutral base less than 0.2, $1/\tau$ varies linearly with $(k_m + f_s)$ (Figure 3). This behavior is consistent with eq 4, since there is very little stacking at the lower pD's corresponding to these concentrations of neutral $M_2{}^6A$; f_s is very small and the last term in eq 4 is negligible. However, as the pD increases above 3.5, the fraction of stacked molecules becomes significant; $1/\tau$ no longer varies linearly with $(f_m + f_s)$, but assumes a value less than would be expected in the absence of stacking. Consequently, it follows from eq 4 that k_s is less than k_m, i.e., stacking interferes with rotation. A further indication of interaction between methyl groups and adjacent stacked molecules is the large upfield ring current shift of the methyl proton resonances with increasing $M_2{}^6A$ concentration (Table I). A downfield shift of these resonances would have been expected if the methyl groups were situated well beyond the edges of adjacent purine rings.

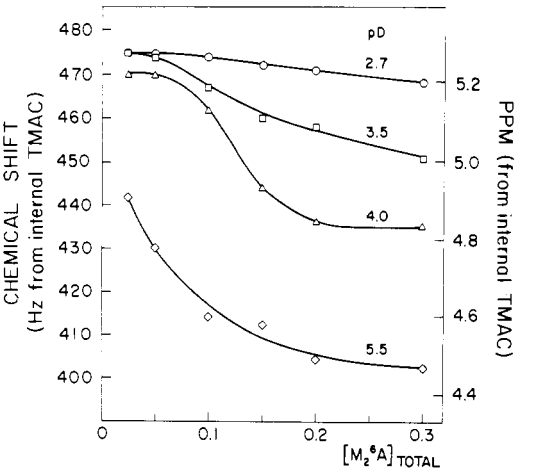


FIGURE 2: Concentration dependence of the average H_2 and H_8 chemical shifts of N^6,N^6-dimethyladenine at several pD (±0.05) values (28°, 90 MHz). The total concentration of neutral $M_2{}^6A$, both monomeric and stacked, is indicated on the abscissa.

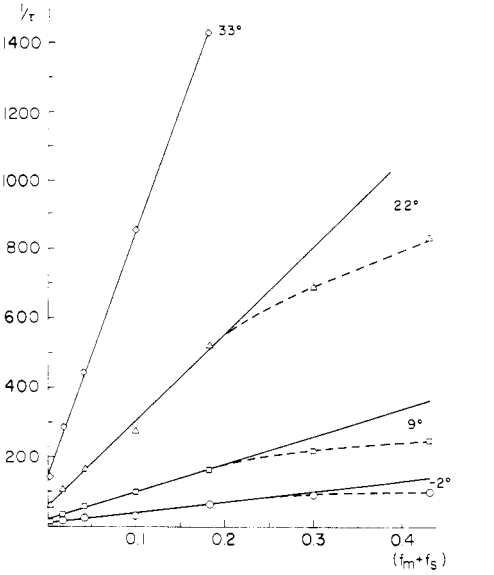


FIGURE 3: Reciprocal of the lifetime in the syn or anti orientation of the methyl groups of N^6,N^6-dimethyladenine vs. the fraction of neutral $M_2{}^6A$. Straight lines shown are linear least-squares fits for $f_m + f_s < 0.2$.

At each temperature k_1 can be determined from the intercept and k_m from the slope obtained by linear least-squares analysis of the initial portion of each curve in Figure 3 ($f_m + f_s < 0.2$). Arrhenius plots ($k = A \exp(-E_a/RT)$) for k_1 and k_m are presented in Figure 4 (the straight lines shown are weighted linear least-squares fits). The activation energy obtained from the k_1 plot for the monodeuterated species, 13.8 ± 0.7 kcal/mol, agrees within experimental error with our previous determination, 13.2 ± 0.6 kcal/mol, obtained at much higher acidity (Pitner et al., 1975). Similarly, the value of log A = 12.0 ± 0.6 agrees very well with the previous value of 11.4 ± 0.8. The energy of activation for rotation about the exocyclic C–N bond of the neutral monomeric species is 15.5 ± 0.2 kcal/mol, as determined from the k_m plot, and log A = 14.9 ± 0.15. These activation energies are comparable to barriers obtained in nonaqueous solution for methylated cytosines, 15–18 kcal/mol (Shoup et al., 1972), and methylated adenines, 11–15 kcal/mol (Martin and Reese, 1967; Engel and

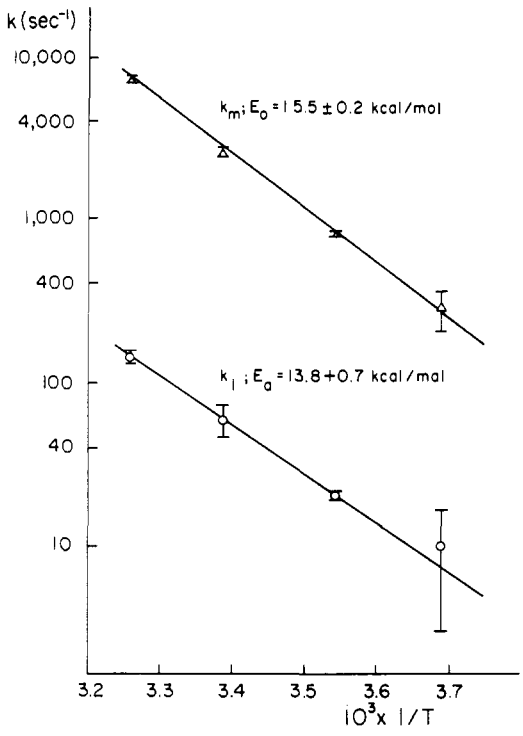
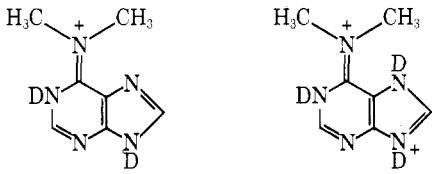


FIGURE 4: Arrhenius plots for rotation of the dimethylamino group of $M_2^6AD^+$ (k_1) and M_2^6A (k_m). Straight lines shown are weighted linear least-squares fits. Bars indicate errors in k_1 and k_m determined from the least-squares analysis indicated in Figure 3.

von Hippel, 1974). Molecular orbital calculations by Berthod and Pullman (1973) yield a barrier of 16 kcal/mol for M^6A. It might have been expected that the activation energy for rotation of the monodeuterated species would be larger than that of neutral M_2^6A as a result of contribution of resonance structures with a double bond between C(6) and N(6). However, Engel and von Hippel (1974) found



virtually no difference in activation parameters for M^6A and M^6AD^+ in a nonaqueous solvent. The difference obtained in the present case is probably due in part to differences in solvation in the aqueous medium of the neutral hydrophobic molecules and the more hydrophilic monodeuterated molecules. The Eyring equation ($k_1 = (kT/h)\exp(-\Delta G^\ddagger/RT)$) yields the following activation parameters at 22° for $M_2^6AD^+$: $\Delta G^\ddagger = 14.9 \pm 0.7$ kcal/mol, $\Delta H^\ddagger = 13.2 + 0.7$ kcal/mol, $\Delta S^\ddagger = -5.8$ eu; and for M_2^6A: $\Delta G^\ddagger = 12.6 \pm 0.3$ kcal/mol, $\Delta H^\ddagger = 14.9 \pm 0.3$ kcal/mol, $\Delta S^\ddagger = 7.8$ eu. The error limits are obtained from least-squares analysis and are only included for completeness; Shoup et al. (1972) have indicated that systematic errors in line-shape analysis are often more important than random errors. Entropies of activation are most sensitive to systematic errors; therefore, no error limits are included for $\Delta S^\ddagger$ values and no attempt is made to interpret the sign change in $\Delta S^\ddagger$ on going from $M_2^6AD^+$ to M_2^6A.

In the solid state stacking pattern of N^6-methyladenine determined by X-ray diffraction (Sternglanz and Bugg, 1973b), the N(6) methyl group of each molecule is directly above the center of the imadazole ring of the nearest neighboring molecule. Contribution from such a species to the average solution conformation of stacked aggregates would be consistent with the high field shift of methyl resonances with increasing concentration of M_2^6A (Table I). However, additional conformations must be present in order to explain the upfield shifts of H_2 and H_8.

This study indicates that rotation of M_2^6A is rapid at all pD values even though hindered in stacked aggregates. These results suggest that in aqueous solution rapid internal rotation may occur about the exocyclic C–N bond of M^6A residues in nucleic acids, unless rotation is impeded by formation of hydrogen bonds. The ability of such adenine derivatives to form Watson–Crick base pairs would therefore depend on the relative stability of their syn and anti isomers and on the energy of the hydrogen bonds. Extension of this study to adenine derivatives monoalkylated on N(6) and to polynucleotides containing such modified bases is required before definitive conclusions can be reached about the effect of N(6) alkylation of adenine on the conformation of biomolecules containing these derivatives.

Experimental Section

M_2^6A (Aldrich), D_2O, DCl, and NaOD (Stohler Isotopes) were used as commercially supplied. The purity and dryness of M_2^6A were confirmed by elemental analysis (Galbraith Laboratories Knoxville, Tenn.; Calcd for $C_7H_9O_5$: C, 51.50; H, 5.56; N, 42.94. Found: C, 51.53 ± 0.1; H, 5.55 ± 0.1; N, 42.88 ± 0.1; average of two determinations).

^1H NMR spectra were measured at 220 MHz on a Varian HR220 spectrometer and at 90 MHz on a Bruker HX-90-18 spectrometer. Tetramethylammonium chloride was chosen as the internal chemical shift reference because of its suitability for studies of nucleic acid interactions (Live and Chan, 1973). The formulas of Van Geet (1970) were used to calculate temperature from the chemical shifts of methanol (low temperature) and ethylene glycol (high temperature). The H_2 and H_8 resonances of M_2^6A were assigned by deuterium replacement of H_8 according to the method of Chan et al. (1964).

The pK_a of monodeuterated M_2^6A (4.10) was determined from the pD (pH meter reading plus 0.40) at half-titration of 3 ml of 0.15 *M* M_2^6A with 0.519 *M* DCl.

The energies of activation and corresponding standard deviations shown in Figure 4 were determined from a weighted linear least-squares annalysis of the data obtained in Figure 3. In this type of analysis each point is weighted by a factor inversely proportional to the square of its standard deviation. For example, the points at −2° (0.00369/°K) are weighted least in each analysis (standard deviations of k_1 and k_m are indicated by the bars). A nonlinear weighted fit to an equation of the form $k = A\exp(-E_a/RT)$ gave essentially identical results.

As pointed out by Shoup et al. (1972) systematic errors in line-shape analysis can arise from improper temperature calibration and incomplete knowledge of the temperature dependence of both line width and chemical shifts between protons at the two exchanging sites. The first of these errors was effectively eliminated in this study by using the equations of Van Geet (1970) to calculate temperatures from the calibrated spectra of methanol and ethylene glycol standard samples. Errors arising from any variation in line width were minimized by determining τ values for spectra in which the observed line widths were much greater than

either the line width in the absence of exchange or the line width for very rapid exchange (Shoup et al., 1972).

At all pD values at least one spectrum was measured below the coalescence temperature. At each pD for which at least two spectra were measured below the coalescence temperature, the chemical shift required to fit these spectra was constant. Therefore, the chemical shift determined for the low temperature spectrum of each set was assumed constant and was used to determine τ values for the other members of that set. The assumption of chemical shift constancy is probably good for all pD's for which stacking is unimportant; therefore, the rate constants determined at these low pD values were used to calculate activation parameters. However, the temperature dependence of stacking (Chan and Nelson, 1969) at higher pD's might be expected to alter the CH_3 chemical shifts. Nevertheless, the negative deviations from the straight lines shown in Figure 3 at fractions of neutral base greater than 0.2 are still at least semiquantitatively correct, because experimental spectra cannot be simulated assuming $1/\tau$ values which would lie on or above the straight lines (Figure 3). Shoup et al. (1972) have shown that the range of τ values which can be determined accurately by line-shape analysis is extended by increasing the chemical shift between exchanging sites. Therefore, even when stacking is important, the large chemical shifts (approx 75 Hz) allow τ values to be easily calculated for spectra beyond coalescence.

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