Tetrahedron Vol. 43, No. 12, pp. 2789 to 2803, 1987
Printed in Great Britain.

0040-4020/87 $3.00 + .00
Pergamon Journals Ltd.

AN NMR SPECTROSCOPIC STUDY OF AZADIRACHTIN AND ITS TRIMETHYL ETHER

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(*Received in UK 23 February* 1987)

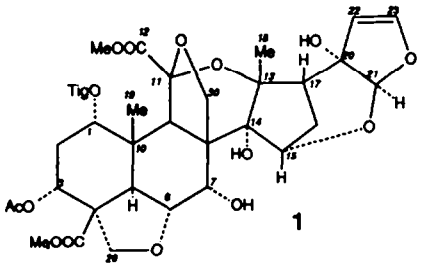
Abstract - The neem tree Azadirachta indica A. Juss and the related Chinaberry tree Melia azedarach L. are attracting considerable interest, particularly because of their insect-repelling properties.[1,2] The most potent constituent is azadirachtin, a limonoid[3] which exerts strong physiological[4] and phagorepellent activities.[5] Incorporating the earlier chemical and spectroscopic studies carried out by Morgan and coworkers[6] and applying the technique of continuous wave proton decoupling in partially relaxed Fourier transform ^{13}C NMR, we proposed structure 1 for azadirachtin in 1975.[7] Since evidence for this complex structure was not convincing, we have been using this molecule as a test sample for the application of modern NMR methods, however, without arriving at a conclusive structure.[8] After proposals of revised structures by Kraus and coworkers, Ley and coworkers, and ourselves starting in mid-1985,[9] its structure was finally established as 2 by Kraus, et al.[10] and by Ley, et al.[11] In the following we summarize the results of our NMR studies and some of the unique difficulties encountered during the investigation of this compound and its 7,11,20-trimethyl ether.

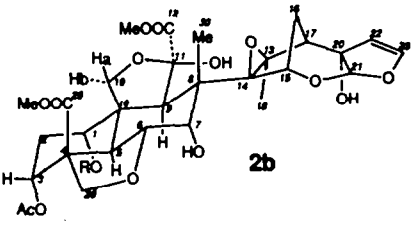
Problems with Structural Analysis of Azadirachtin using NMR Spectroscopy.

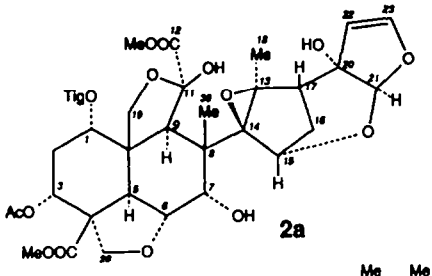
It is a suprisingly difficult task to determine the structure of azadirachtin, $C_{35}H_{44}O_{16}$ by NMR spectroscopy for the following reasons. Since azadirachtin contains so many oxygen atoms the connectivity of the molecular framework is hard to establish, by either carbon or proton NMR because spin-spin coupling through oxygen reduces the value of the coupling constants to such a low value as to make experiments involving coupling through oxygen either difficult and/or inconclusive.

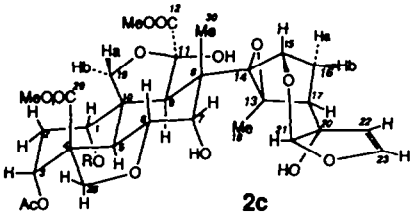
The oxygens also serve to physically separate the spin 1/2 nuclei thereby reducing the amount of information available from proton nOe measurements. In principle, ^{17}O NMR might seem to offer an attractive solution to these problems. However, in practice, the quadrupolar nature of this nucleus produces broad lines in the spectra of molecules of the size of azadirachtin and moreover, reduces the possibility of distance measurements via the nOe, since nOe is a consequence of dipolar relaxation. These factors coupled with the low receptivity of ^{17}O to NMR measurements serve to make ^{17}O NMR studies

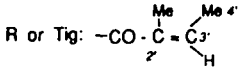
of little use for structural determination. In essence, the oxygens act as spectroscopically silent centers.











While proton nOe experiments can provide relative distance information between protons within the molecule, they cannot, in general, be used to locate hydroxyl groups because of spin-diffusion amongst the exchangeable protons. In addition, the NMR spectra of azadirachtin are temperature-dependent, indicating conformational flexibility of the molecule, which further complicates the interpretation of the nOe results. These temperature dependent effects are consistent with the idea of restricted rotation about a single bond.[12] Thus, we have chosen azadirachtin as a test case to demonstrate the problems associated with structural assignment by NMR spectroscopy, and use the data to assemble the structure of azadirachtin by treating it as if it were an unknown.

In order to facilitate structural determination by NMR spectroscopy, a derivative of azadirachtin was prepared in which all three hydroxyl groups were methylated. The rationale behind the derivatization was to provide a related compound in which the sites of methylation might be more readily assigned than the sites of the hydroxyl groups in the parent compound. The trimethyl ether was chosen, since in the past, attempts at locating the hydroxyls by the use of acetylation shifts in carbon NMR spectra failed because the acetylation of azadirachtin yields only a mono-acetate.[7a]

Experimental Section. The spectrometers used in this work were a Bruker WM-300, WM-250 and a Nicolet NT-300. ^{1}H NMR spectral data are reported in ppm relative to the residual proton absorptions in the deuterated solvent $CDCl_3$ (7.24), DMSO-d_6 (2.50); ^{13}C NMR spectra are also reported in ppm relative to the signal of the solvent $CDCl_3$ (77.0) and DMSO (39.5). High resolution mass spectra were recorded on a VG 70-70 EQ instrument.

Isolation of Azadirachtin.[13] Azadirachtin has always been difficult to isolate in sufficient quantities due to the large volume of fats and oils contained within the seeds of *Azadirachta indica*. A rapid and greatly simplified method has now been devised to obtain azadirachtin in high yield.[13]

Ground neem seeds (2 kg) were defatted with hexane and extracted with 95% ethanol. The ethanolic extract was subjected to two quick efficient partitionings: the first, between petroleum ether and 95% methanol/water to remove any remaining oils and other nonpolar materials; followed by a second, between water and ethyl acetate to remove water-soluble proteins and sugars.

A large-scale preliminary purification of azadirachtin from the remaining matrix was accomplished by flash chromatography (EtOAc/hexane 3:1) in a 600 ml sintered glass funnel packed with lc-grade silica gel. The residue from the azadirachtin containing fractions was found to dissolve in hot carbon tetrachloride, which upon cooling yielded azadirachtin as a microcrystalline material. This key crystallization step is not an exacting process and is reproducible. Even material containing only 30 - 40 % azadirachtin can be purified in this manner to remove the remaining impurities of like polarity, which are not otherwise easily separable on the preparative scale.

Final purification was accomplished by flash chromatography ($CHCl_3$/ CH_3CN 3:1) to remove small amounts of more polar compounds that co-crystallized from carbon tetrachloride. The procedure yielded essentially pure azadirachtin (> 5 g) as a clear colorless glass.

Preparation of azadirachtin-7,11,20- trimethyl ether. This was prepared by a modified procedure of Johnstone.[14] To a 25 ml round bottomed flask containing 3.5 ml of DMSO (Aldrich, HPLC grade, stored over molecular sieves) was added 235 mg (4.2 mmole) of finely powdered KOH. After stirring for five minutes, 0.5 ml (8.0 mmole) of methyl iodide was added followed by 200 mg (0.28 mmole) of solid azadirachtin. After stirring for five minutes no starting material remained; stirring for longer periods only complicated the isolation procedure and decreased the overall yield due to the formation of a tetramethyl ether derivative resulting from hydrolysis of the 3-acetyl group. The reaction mixture was diluted with 20 ml of water and extracted three times each with 30 ml CH_2Cl_2. The organic layers were combined and concentrated. The residue was purified by flash chromatography (hexane-ethyl acetate 1:1) to yield 85 mg of the trimethyl ether (40% yield, purified). The mass spectrum showed the presence of a molecular ion with elemental composition of $C_{35}H_{50}O_{16}$ (m/e calc. 762.3055, obs. 762.3077).

NMR Measurements. The proton spectra of azadirachtin and its trimethyl ether were assigned by a combination of decoupling and COSY two-dimensional experiments. It is interesting to note that, in our hands, it was easier to demonstrate the coupling between 7-OH to 7-H in $CDCl_3$ solution by decoupling rather than by COSY two-dimensional experiments. Steady-state proton nOe difference spectra were measured at 333 K using self-compensating 90∞ observe pulses of the form 90∞(y), 270∞(-x), 360∞(x) in order to eliminate selective population transfer effects.[15] A presaturation time of 5 secs was used in all experiments since inversion-recovery T_1 relaxation time measurements[16] indicated that the proton T_1 values lay between 0.2 secs (for 8-methyl) and 1.8 secs (for 23-H). The solutions in $CDCl_3$ were 0.02-0.05 M and were degassed ultrasonically. The samples were sealed under argon. They were not spun during the nOe experiments and a 2 Hz exponential line-broadening factor was used to minimize the artifacts caused by incomplete subtraction. The spectra had a digital resolution of 0.5 Hz. All nOe experiments were repeated at least three times.

Kinetic nOe measurements[17] were also carried out as differences between on- and off- resonance experiments. Self-compensating 90∞ observe pulses were again used with a recycle delay of 10 secs with periods of 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 sec of selective frequency irradiation to allow the truncated nOe to build up. The data were analysed with a least squares fitting procedure to the best fit single exponential; no evidence of bi-exponential decay was obseved.

The proton bearing carbons of azadirachtin and its trimethyl ether were assigned by a combination of DEPT experiments, two-dimensional carbon-proton

J-spectra and two-dimensional carbon-proton chemical shift correlation experiments with magnetization transfer through one-bond couplings (for an introduction to these modern NMR methods see ref 18). However, since twelve of the 35 carbons are quaternary, these were assigned by the use of long-range carbon proton coupling in the carbon spectrum. This was achieved by the use of INAPT experiments[19] and two-dimensional COLOC experiments.[20] The INAPT (Insensitive Nuclei Assigned by Polarization Transfer) experiment is basically a modified version of the well-known refocused INEPT experiment.[18] This modification entails the use of frequency selective proton pulses with delays set to optimize transfer through long-range proton carbon spin coupling. The INAPT experiments were performed with a proton decoupler field strength of $\gamma h_2 /2 \pi = 25$ Hz to generate a selective 90° pulse width of 10 millisecs. The polarization transfer delays were optimized for $J_{CH} = 6$ Hz. The COLOC (COrrelation spectroscopy via LOng-range Couplings) experiment is a modified two-dimensional heteronuclear chemical shift correlation experiment which has been optimized for the transfer of magnetization via long-range spin coupling, with the added advantages of refocusing magnetic inhomogeneity during the evolution time and providing complete homonuclear proton decoupling in the final two-dimensional spectrum. The delays in the COLOC experiment were set to optimize carbon-proton coupling constants of 10 Hz.

All two-dimensional experiments were performed using quadrature detection in both dimensions, appropriate phase cycling and the absolute-value mode of display. In general, 256 x 1K data sets were acquired and processed with sine bell resolution enhancement in both dimensions. The F_1 dimension was zero filled once to yield 512 x 512 data matrices.

Results and Discussion

General Considerations. Structure **3** represents the conformation which best accounts for observed nOe's and other NMR data. The tiglate is shown hydrogen-bonded to the 7-OH in order to account for nOe observations indicating that the tiglate is tucked under the molecule (see below). The carbon and proton assignments in $CDCl_3$ at 333 K are set out in Table 1. The NMR data are temperature dependent. For the sake of consistency the data are reported at 333 K since the table compares the values for azadirachtin with those of the trimethyl ether, and some of the carbons of the trimethyl ether cannot be observed at tempertures lower than 333 K. The assignment of the five isolated proton spin systems has been discussed before.[6-8] In agreement with work by others,[10, 21] the only discrepancy with our previous studies[7a] is that the assignments of 7-H and 15-H are now reversed. A method of linking these five spin systems is needed, as the remaining signals are singlets and

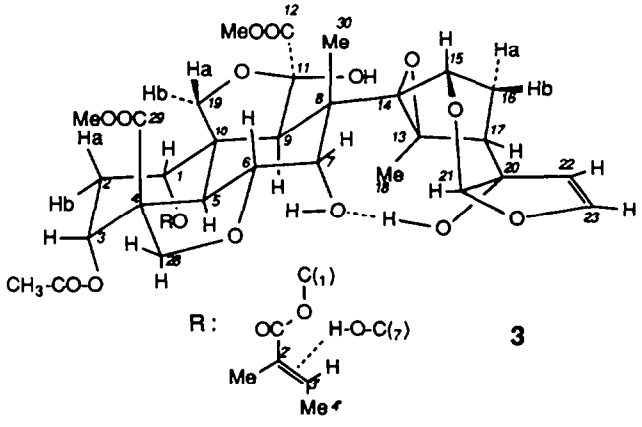


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Table 1. Comparison of the Proton and Carbon NMR Data of Azadirachtin and its 7,11,20 - Trimethyl Ether, 333 K in $CDCl_3$

	Azadirachtin			Trimethyl ether	
Position	H	C	Position	H	C
1	4.78 (dd, 3.4, 2.7)	71.1 (d,155)	1	4.72 (dd, 3.0, 2.7)	71 9
2a	2.36 (ddd, 16 8, 2.7, 2.4)	30.0 (t, 130)	2a	2 49 (ddd, 16.8, 3 0, 2.7)	29.7
2b	2 20 (ddd, 16 8, 3 4, 3.0)		2b	2 19 (ddd, 16 8, 3 4, 2.7)	
3	5.49 (dd, 3.0, 2.4)	67.4 (d, 155)	3	5.45 (dd, 3.4, 2.7)	67 5
4		53.0 (s)	4	52 9	
5	3.34 (d, 12.4)	37.4 (d, 125)	5	3 52 (d, 12 4)	38 3
6	4.57 (dd, 12.4, 3 0)	74.1 (d, 150)	6	4 53 (dd, 12 4, 2 4)	75 2
7	4.68 (d, 3.0)	74 8 (d, 154)	7	3.78 (d, 2.4)	85.3
8		45 9 (s)	8	47 3	
9	3 34 (s)	45.3 (d, 137)	9	3 89 (br s)	47 1
10		50.8 (s)	10	50 4	
11		104.5 (s)	11	107 8	
12		171.9 (s)	12	170 1	
13		70 5 (s)	13	72.3	
14		68 7 (s)	14	70 7	
15	4 64 (d, 3.7)	76.8 (d, 160)	15	4.89 (d, 3)	77 2
16a	1.69 (ddd, 13 4, 5.4, 3 7)	25 4 (t, 137)	16a	1.82 (ddd, 12 4, 6 0, 3 0)	26 9
16b	1.28 (d, 13 4)		16b	1.15 (d, 12 4)	
17	2 32 (d, 5 4)	48 9 (d, 140)	17	2 31 (d, 6 0)	46 5
18	1.97 (s)	18 5 (q, 128)	18	1 75 (s)	18 9
19a	3.74 (d, 9.7)	69.4 (t, 150)	19a	3.65 (d, 9 4)	69 2
19b	4 14 (d, 9 7)		19b	4 02 (d, 9 4)	
20		83.8 (s)	20	88.6	
21	5 62 (s)	109 0 (d, 183)	21	5 55 (s)	104 4
22	5 00 (d, 3 0)	108 3 (d, 178)	22	4 85 (d, 2.7)	103 5
23	6 41 (d, 3.0)	147 3 (d, 193)	23	6 45 (d, 2 7)	148 1
28a	3.76 (d, 9.1)	73.2 (t, 152)	28a	3 63 (d, 8 4)	72.4
28b	4.03 (d, 9.1)		28b	3 94 (d, 8.4)	
29		173.5 (s)	29		173 9
30	1 73 (s)	21.6 (q, 128)	30	1 28 (s)	20 0
12-OMe	3 65 (s)	53 2 (q, 135)	12-OMe	3.63 (s)	52.6
29-OMe	3.77 (s)	52 6 (q, 136)	29-OMe	3.75 (s)	52 4
3-OAc	1 91 (s)	20.8 (q, 125)	3-OAc	1 89 (s)	20 6
1'		166.4 (s)	1'	166 9	
2'		129 2 (s)	2'	129 2	
3'	6 91 (qq, 7 0, 1.3)	137.4 (d, 157)	3'	6 98 (qq, 7 0, 1 7)	137.6
4'-Me	1.75 (dq, 7.0, 1.0)	14 2 (q, 127)	4'-Me	1 78 (dq, 7 0, 1.3)	14 2
5'-Me	1 83 (dq, 1 3, 1.0)	11 9 (q, 128)	5'-Me	1 75 (dq, 1 7, 1 3)	11 7
7-OH	2.73 (br. s)		7-OH	7-OMe 3.48 (s)	59.9
11-OH	4 94 (s)		11-OH	11-OMe 3 32 (s)	51 9
20-OH	2.75 (br. s)		20-OH	20-OMe 3.13 (s)	50 1
C=O (OAc)		169.6 (s)	C=O (OAc)		169 6

offer little information about connectivity.

Since the NMR spectra of azadirachtin have been found to be temperature dependent, the most immediate problem is to locate the site in the molecule that gives rise to these effects, since our earlier structure would be unlikely to produce such large temperature dependent features.

Temperature Dependent Effects on Proton Spectra of Azadirachtin. The proton assignments of azadirachtin in DMSO-d_6 at room temperature are set out in Table 2. The proton NMR spectrum of azadirachtin in DMSO-d_6 (Figure 1) shows two broad signals at 4.22 δand 4.58 δassigned, respectively, to 7-H and 15-H; they are actually broader than two of the three hydroxyl groups (which disappear upon deuterium exchange). Similar broadening occurs at the signal for 9-H (not shown). Figure 2 demonstrates the effect of temperature at the low frequency end of the spectrum in DMSO. At room temperature, the resonance of the 8-Me group (1.48 δ) is significantly broadened with respect to that of the 3-OAc group (1.86 δ), while the resonance of 13-Me (1.83 δ) is not clearly discernible from that of the tiglate 5'-methyl. The linewidths of the signals from 2-H's, 7-H, 9-H, 15-H, 4'-Me and 8-Me are all reduced by heating the sample to 333 K. At room temperature in DMSO the 2-H protons appear as a broad signal (2.2 δ)

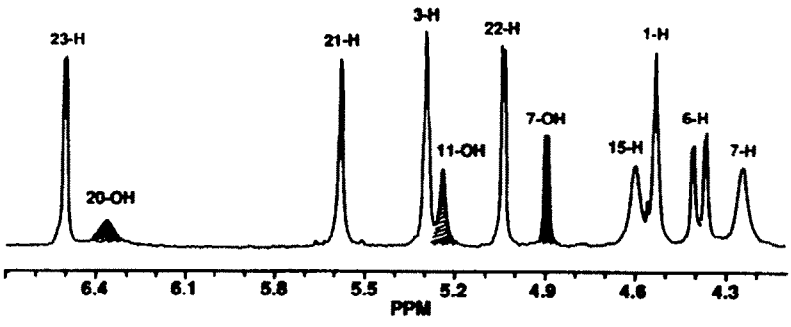


Figure 1. Proton NMR spectrum of azadirachtin in DMSO d_6 at 297K. The hatched resonances are exchangeable in DMSO/D_2O.

Table 2. Proton NMR Data for Azadirachtin, 297K in DMSO-d_6

Position	δ (multiplicity, J)	Position	δ (multiplicity, J)
1	4.52 (t, 3)	17	2.22 (d, 5)
2a	2.2 (broad)	20-OH	6.35 (broad)
2b	2.2 (broad)	21	5.57 (s)
3	5.30 (t, 3)	22	5.02 (d, 3)
5	3.48 (d, 12)	23	6.48 (d, 3)
6	4.36 (dd, 12, 3)	19a	3.84 (d, 9)
7-H	4.22 (broad)	19b	3.66 (d, 9)
7-OH	4.88 (d, 3)	28a	3.92 (d, 10)
8-Me	1.48 (broad)	28b	3.48 (d, 10)
9	3.4 (broad)	3-OAc	1.86 (s)
11-OH	5.22 (s)	3'-H	6.93 (dq, 2, 7)
13-Me	1.83 (s)	4'-Me	1.74 (dq, 2, 7)
15	4.58 (broad)	5'-Me	1.83 (broad)
16a	1.52 (broad m)	29-OMe	3.67 (s)
16b	0.96 (d, 13)	12-OMe	3.78 (s)

superimposed upon that of 17-H (2.22 δ), whereas at room temperature in $CDCl_3$ the 2-H protons in chloroform appear as a complex multiplet (2a-H, 2.33 δ, ddd, 16.8, 2.6, 2.8 ; 2b-H, 2.24 δ, ddd, 16.8, 2.6, 2.9) as shown in the insert of Figure 2, which was obtained by an inversion recovery sequence (180°pulse - 0.5 sec delay - 90°pulse - observe) which nulls the magnetization from the interfering resonance of 17-H.

Cooling a solution of azadirachtin in $CDCl_3$ to 220 K, broadens the signals from 2a-H, 7-H, 9-H, 15-H and 8-Me. The temperature dependence of the 2a-H and tiglate 4'-methyl protons possibly indicates movement of the tiglate side-chain, while those of the other protons reflect restricted rotation within the skeletal framework.

Cooling a solution of azadirachtin in either THF-d_8 or DMF-d_7 to 180 K produces doubling of every proton signal. Integration of the spectra show that the two rotamers are present in a ratio of *ca.* 1:2. These temperature-dependent spectra suggest that the major difference between the two rotamers involves both the conformation of the tiglate group and the conformation around a single bond, the latter being somewhere in the region near 7-H, 9-H, 15-H and 8-methyl.

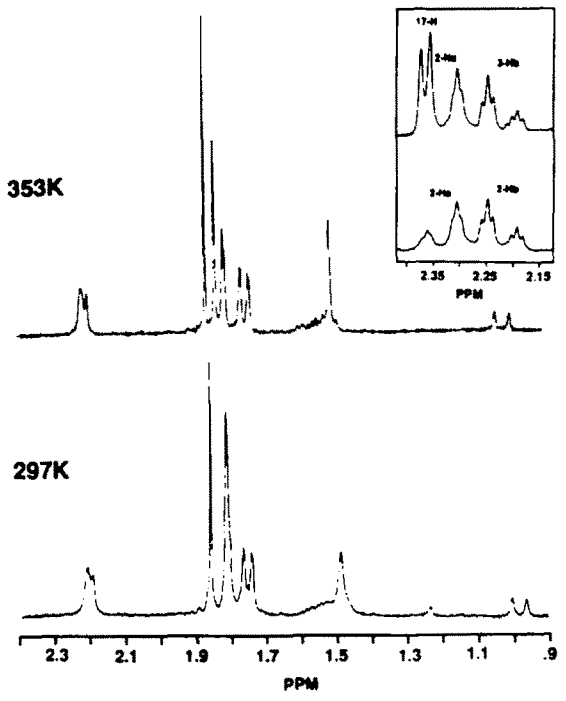


Figure 2. *Temperature effects in the proton spectrum of azadirachtin for the low frequency region in DMSO-d_5. Bottom spectrum 297K. Top 353K.*

Insert. *Top - azadirachtin, chloroform, room temperature. Bottom - after inversion recovery sequence.*

Proton nOe Studies of Azadirachtin. Preliminary nOe experiments at room temperature (297 K) involving presaturation of 7-OH (3.20 δ) produced small positive nOe effects at 7-H, 21-H and 13-Me but also much more intense negative effects (saturation transfer) to both other hydroxyls and also to a very broad resonance (80 Hz wide at half height, centered at 1.8 δ) which we assume to be due to water bound to azadirachtin. Irradiation of the hydroxyl at 3.07 δ (20-OH) produces the same result. The chemical shifts of the hydroxyl proton at 5.04 δ (now assigned to 11-OH) is so close to that of 22-H at 5.03 δ that it is not possible to irradiate them separately. Thus, irradiation at 5.04 δ hydroxy proton results in intensification of signals due to 17-H, 23-H and 8-methyl, with only minor saturation transfer to the other two hydroxyls (both negative) and no measurable transfer to water.

The observation that saturation transfer between the hydroxyls is not equal in both directions is consistent with the idea that two of the hydroxyls (7-OH and 20-OH) are strongly hydrogen bonded, but the remaining hydroxyl (11-OH) is not (structure 3). However, this information could probably have been deduced from the normal proton spectrum since one of the hydroxyls is much sharper than the other two. Thus nOe measurements of the hydroxyl protons have revealed little (if anything) about their exact position of attachment to the backbone of the molecule. In fact these experiments are of less use than the decoupling experiments which at least positively assigned 7-OH.

NOe measurements of the nonexchangeable protons are equally confusing. Preliminary nOe experiments at room temperature involving irradiation of 8-methyl showed saturation transfer of the order of 10% to all of the hydroxyl groups; in retrospect, this was presumably caused by water in the chloroform solution which had the same chemical shift as that of the 8-methyl. In order to remove these complications from saturation transfer the $CDCl_3$ solution was shaken with D_2O.

Another problem with nOe studies of azadirachtin is that since the normal proton spectra are temperature dependent one might predict that the nOe results should also be temperature dependent. Therefore, in order to avoid complications arising from slow conformational interconversions, we chose to measure the nOe difference spectra at as high a temperature as is convenient in $CDCl_3$, *i.e.*, 333 K, since the structure of azadirachtin was our major concern, not the conformation of the rotamers. The high temperature nOe data thus obtained are summarized in Table 3. There is some indication from the data (enhancement of 23-H signal upon irradiation of 21-H, see below) that deuterium exchange does not actually prevent magnetization transfer via the hydroxyls, but merely reduces it.

Irradiation of 19b-H produces enhancements at 1-H and 19a-H, while irradiation of 19a-H produces enhancements at 19b-H, 8-methyl and 6-H. This

Table 3. Steady-State Proton Nuclear Overhauser Enhancements in Azadirachtin in $CDCl_3/D_2O$ at 333 K

Irradiate	Enhanced proton signals
1-H	$-COOCH_3$(3%), 2a(2%), 2b(2%), 19b(1%)
6-H	19a(3%)
7-H	21(9%), 8-Me(5%), 6(2%)
15-H	8-Me & 16a(9% total for both), 16b(4%), 21(3%)
19b-H	19a(5%), 1(3%)
19a-H	19b(4%), 8-Me(2%), 6(1%)
21-H	7(5%), 23(3%)
22-H	23(13%)
13-Me	9(6%), 17(3%), 3'(2%)
8-Me	15(6%), 6(6%), 7(4%), 19a(3%)
3-OAc	5(2%), 3'(7%)
3'-H	4'(11%), 3-OAc(5%), 13-Me(4%), 7(4%), 5(3%)

serves to identify which of the 19-H protons is closer to the 8-methyl group, and suggests that this methyl group was assigned to the wrong position, *i. e.*, 10-methyl in our earlier structure **1**. Irradiation of 8-methyl produces a 6% enhancement at 15-H, 6% at 6-H, 4% at 7-H and 4% at 19a-H. Since the enhancement of 6-H is larger than that of 7-H, this might suggest that 8-methyl is closer to 6-H than to 7-H, which is not the case. This is an example of a general problem of structural assignment from nOe data, which is that steady-state NOE data do not necessarily reveal distance information when there are multiple pathways for relaxation. Fortunately, this issue can be

clarified by the use of kinetic nOe measurements.[17] The kinetic nOe results obtained by irradiation of 8-methyl show the best fit single exponentials corresponding to rate constants of 1.4 (±0.05) sec^{-1} for 15-H, 1.6 (± 0.04) sec^{-1} for 7-H, 1.8 (±0.1) sec^{-1} for 6-H, and 3.1(±0.2) sec^{-1} for 19a-H. Distance ratios can be obtained from the ratios of the time constants, and show that 8-methyl is indeed closest to 15-H then 7-H, 6-H and 19a-H, respectively.

Irradiation of 13-methyl produces a 6% enhancement at 9-H, 3% at 17-H and 2% at 3'-H (tiglate). Irradiation of 7-H leads to enhancement at 21-H (9 %), 8-methyl (5 %) and 6-H (2 %), demonstrating the close approach of 7-H and 21-H. However, the reverse experiment, *i.e.*, the irradiation of 21-H, while producing an enhancement of 7-H (5%) as expected, also produces a rather unexpected result of a nOe between 21-H and 23 -H (3%). Since 21-H and 23-H are on opposite sides of a dihydrofuran ring, and thus have a separation of at least 4 Å, we assume that this effect is caused by dipolar relaxation of both 21-H and 23-H by 20-OH, despite the fact that the hydroxyl protons have been deuterium exchanged. Irradiation of the 3'-H (tiglate) produces enhancements of 11% at 4'-protons, 5% at the 3-acetate, 4% at 13-methyl, 4% at 9-H and 3% at 5-H, presumably demonstrating that the tiglate sidechain points in towards the bond C5-C6 as shown in **3**. This leads us to suppose that the 20-OH is hydrogen bonded to the 7-OH, which in turn is π-hydrogen bonded to the double-bond of the tiglate sidechain. The 11-OH would be free from hydrogen bonding in our scheme.

In summary then, the nOe results provide information about the close proximity of 7-H and 21-H, suggest that a methyl is attached to C-8, that the 19-H methylene group is close to 1-H and that the tiglate sidechain is tucked under the molecule; they also locate the 13-methyl on the opposite face of the molecule from 8-methyl. Thus, we were able to disprove various earlier structures; [7,21] however, these nOe results do not enable us to predict the correct structure and do not show that azadirachtin exists in different conformations. From the temperature dependent effects we can assume that azadirachtin has a single bond linking two "halves" of the structure. The location of this single bond must be in the vicinity of 8-Me, 7, 9 and 15-H. A suitable candidate for such a link is between C-8 and C-14.

Long Range Carbon Proton Coupling in Azadirachtin. In order to determine the connectivity of the backbone of the molecule, long range spin coupling in the carbon spectrum was studied by a variety of techniques. The most obvious of these was simple low power selective frequency proton decoupling.[8] Figure 3 shows the effect of low power irradiation of 3-H (at 5.49 δ in the proton spectrum at room temperature in $CDCl_3$) whilst observing the high frequency end of the carbon spectrum. The 169.4 δ (dq) carbonyl signal collapses to a quartet; similarly irradiation of the protons of the

acetate methyl group (at 1.94 δin the proton spectrum) collapses this carbonyl to a doublet, showing that the acetate is attached to 3-H.

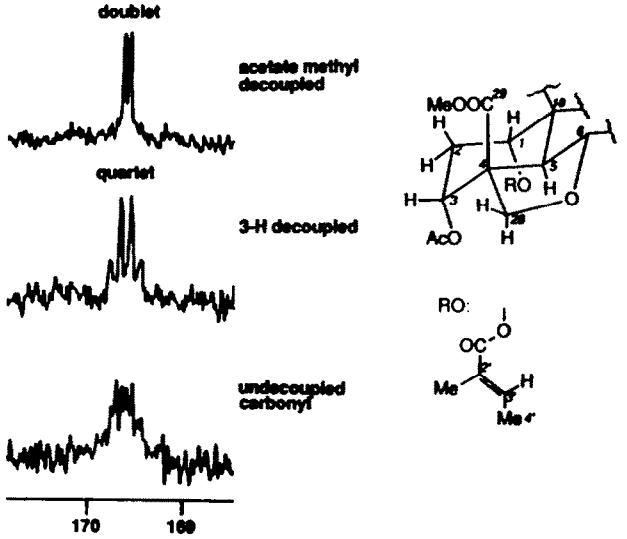


Figure 3. *The top two spectra of the 169.6 ppm carbonyl resonance are simplified due to low power selective frequency proton decoupling. LSPD experiment.*

Table 4. *Long-Range Proton-Carbon Spin Coupling in Azadirachtin*

COLOC data (J = 10 Hz)	INAPT data (J = 6 Hz)
1-H ⇒ C-3, C-5	1-H ⇒ C-1', C-5, C-10
2-H ⇒ C-4, C-10	2-H ⇒ C-3, C-4, C-10
3-H ⇒ C-1, C-4, C-5	
5-H ⇒ C-4, C-6	5-H ⇒ C-4, C-6, C-10, C-29
6-H ⇒ C-7	
7-H ⇒ C-6	
7-OH ⇒ C-8	
9-H ⇒ C-2, C-5, C-8, C-10	9-H ⇒ C-8, C-10, C-11, C-12, C-30
	12-H(OMe) ⇒ C-12
15-H ⇒ C-14	
16a-H ⇒ C-13, C-20	16a-H ⇒ C-13, C-15, C-20
16b-H ⇒ C-14	
17-H ⇒ C-13	17-H ⇒ C-13, C-14, C-15, C-20
18-H ⇒ C-13, C-14	
	19-H ⇒ C-1, C-5
	21-H ⇒ C-15, C-20, C-23
	22-H ⇒ C-20, C-21
23-H ⇒ C-20	
30-H ⇒ C-7, C-8	
	3'-H ⇒ C-1'

Further studies of the long range carbon proton spin coupling utilized the INAPT [19] and COLOC [20] experiments. These data are compared in Table 4. Both of these experiments enabled us to assign all the non-protonated carbons. However a considerable amount of chemical insight is necessary to interpret these results since the observation of long-range coupling between carbons and protons does not determine the number of bonds between the coupled nuclei. For example, the COLOC data demonstrate long-range coupling between a quaternary carbon at 53.0 ppm (C-4) and 2a-H, 3-H and 5-H, whereas the INAPT data (Figure 4) show that 2a-H and 5-H couple to two quaternary carbons at 53.0 (C-4) and 50.8 ppm (C-10). Since 3-H fails to couple to the 50.8 ppm carbon, and because the INAPT data indicate that 9-H is also coupled to this

carbon, the 50.8 and 53.0 ppm signals must belong to C-10 and C-4, respectively. The data also show that C-5 is coupled to both 1-H and 19-H and so provide a link between C-5 ,C-10 and C-19. In addition, it is seen that C-6 is coupled to 7-H and 5-H , and this demonstrates the link between C-5 and C-6. Other useful long range couplings are those of both 16a-H and 23-H to C-20, and those of the protons of 13-Me and 17-H to C-13 which led to the assignments of both these nonprotonated carbons.

An example of the scope and limitation of these experiments is demonstrated by the problems of locating the methyl groups. The COLOC data indicate that the protons on the 8-methyl group (1.73 ppm) are coupled to C-7 (74.8 ppm) and to a quaternary carbon at 45.9 ppm (C-8). Since 9-H is also coupled to the carbon at 45.9 ppm, this signal at 45.9 must be due to C-8. Similarly, with 13-methyl, the COLOC data show long range coupling between both C-13 and C-14 to the protons attached to C-18 (13-methyl), while the INAPT data does not offer any information at all in this case, because the proton spectrum is too crowded in this region to permit selective irradiation

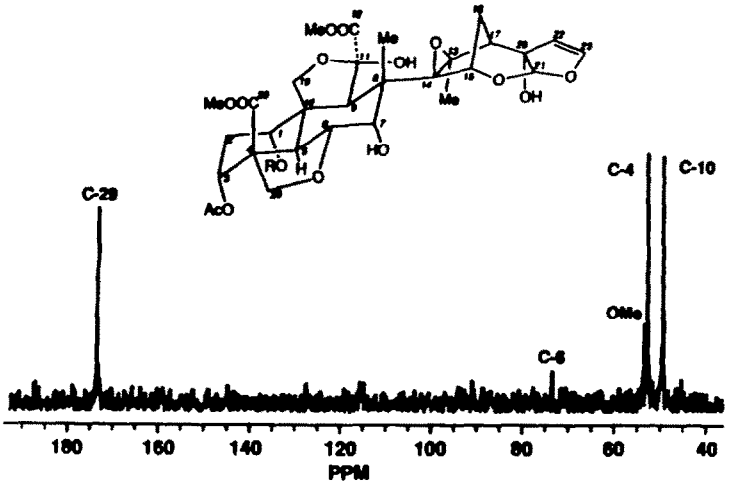


Figure 4. *75 MHz Carbon-13 NMR spectrum resulting from selective irradiation of 5-H showing long-range heteronuclear coupling to C-4, C-6, C-10 and C-29.*

of this methyl group. Most importantly, no coupling between the hydroxyl protons and their neighbouring carbons could be detected by any of these techniques.

We can still not prove that the single bond lies between C-8 to C-14, because it has not been possible to observe long range coupling between C-14 to the protons of 8-methyl. However, the variable temperature results indicate 8-methyl to be more temperature dependent than 13-methyl, which in turn suggests that 13-methyl is not adjacent to the site of the single bond, and thus a single bond between 8 and 13 is unlikely. We need two more hydoxyl groups and an ether-type linkage to fit the molecular weight/number of degrees of unstaturation. Since the temperature dependence of the spectra also indicate that the ether-type linkage cannot link the two halves of the

molecule in a rigid framework, we are forced to consider the possibility of an epoxide linking C-13 and C-14, with an OH at both positions 11 and 20. Since it is not generally possible to locate the position of an epoxide by NMR, we need to be able to locate the hydroxyl groups; however it has not been possible to locate all three unambigously from studies of free azadirachtin.

Comparisons between Proton Spectra of Azadirachtin and its Trimethyl Ether. The most interesting aspect of the shifts which occur upon methylation is that the protons which experience the major effects are also those which are most temperature dependent. For instance, 7-H is shielded by 0.90 ppm, 9-H and 15-H are deshielded by 0.55 and 0.25 ppm respectively. Large effects are also seen at the 8- and 13-methyl groups, which are shielded by 0.45 and 0.22 ppm respectively. On the other hand, despite the nOe data which suggest that 7-H and 21-H are close together, 21-H neither very temperature dependent nor shifted appreciably upon methylation.

Temperature Dependent Effects on Proton Spectra of Azadirachtin 7,11,20- trimethyl ether. The temperature dependent features of the proton spectra of azadirachtin are enhanced by the replacement of hydroxyls by methyl ethers. Figure 5 illustrates the effect of temperature on the proton spectrum of azadirachtin trimethyl ether in $CDCl_3$. The most dramatic effects are the appearance at 323 K of the signals from 7, 9, and 15-H, which are hardly visible at room temperature. The resonance of the 8-Me sharpens dramatically as does 2a-H.

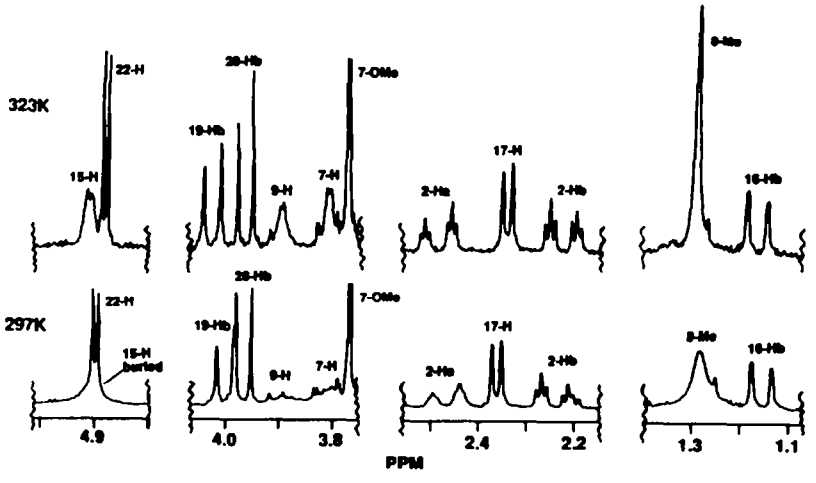


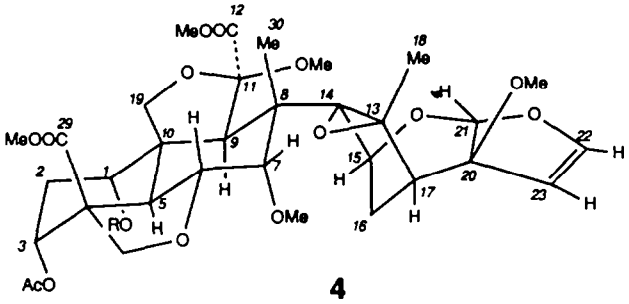
Figure 5. Temperature effects on the proton NMR spectra of azadirachtin-7,11,20-trimethyl ether in chloroform. Bottom spectra, 297K. Top spectra, 323K of the same region.

Proton nOe Studies of Azadirachtin-7,11,20-trimethyl ether. Here again the measurements were conducted at elevated temperatures. The results are summarized in Table 5. Of particular interest, is the signal from 11-OMe which is enhanced by irradiation of either 8-methyl or 13-methyl, while

irradiation of 11-OMe itself produces enhancements at 21-H (4%), 15-H (3%), 9-H (2%), 13-Me (1%) and 8-Me (1%). The data in Table 5 enable us to assign the proton signals from the new methoxyl groups. The conformation of the trimethyl ether which best accounts for the major nOe's is shown in structure **4**.

Table 5. Steady-State Proton Nuclear Overhauser Enhancements in Azadirachtin 7,11,20- Trimethyl Ether at 333K in $CDCl_3$

Irradiate	Enhanced proton signals
7-H	7-OMe(4%), 6(3%), 21(2%), 15(2%)
17-H	22(7%), 13-Me(3%), 20-OMe(2%)
21-H	20-OMe(8%), 11-OMe(4%), 23(2%)
22-H	23(5%), 17(3%), 20-OMe(2%), 7-OMe(1%)
8-Me	11-OMe(3%), 15(3%), 7(1%)
13-Me	17(3%), 11-OMe(2%), 20-OMe(2%)
7-OMe	7(1%), 21(1%)
11-OMe	21(4%), 15-H(3%), 9-H(2%), 13- Me(1%), 8-Me(1%)
20-OMe	22(3%), 21(2%), 11-OMe(1%), 17(1%)



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Carbon NMR Spectra of Azadirachtin Trimethyl Ether. Figure 6 demonstrates the effect of raising the sample temperature from 297 to 333 K on the carbon spectrum of this derivative. At 333 K all the linewidths are reduced and the signals assigned to C-13 and 14 become visible, whereas at room temperature they cannot be observed. The resonance assigned to 8-methyl also shows a dramatic sharpening upon increasing the sample temperature. This again points to a single bond in the region near 8-methyl, C-13 and 14. Methylation deshields C-7, 11, and 20 by 10.5, 3.3 and 4.8 ppm respectively. The only other major effects are the shielding of C-21 and 22 by 4.6 and 4.8 ppm respectively.

Long Range Carbon Proton Coupling in Azadirachtin-7,11,20 - trimethyl ether. The key points of the INAPT data for this derivative are the couplings between the protons of the new methoxy groups at 3.47 δ(7-OMe) with C-7, the protons at 3.31 δ (11-OMe) with C-11,and protons at 3.13 δ(20-OMe) with C-20. Thus all three hydoxyls in azadirachtin are finally located. The stereochemistry of the epoxide link can be deduced from the nOe results of 13-methyl.

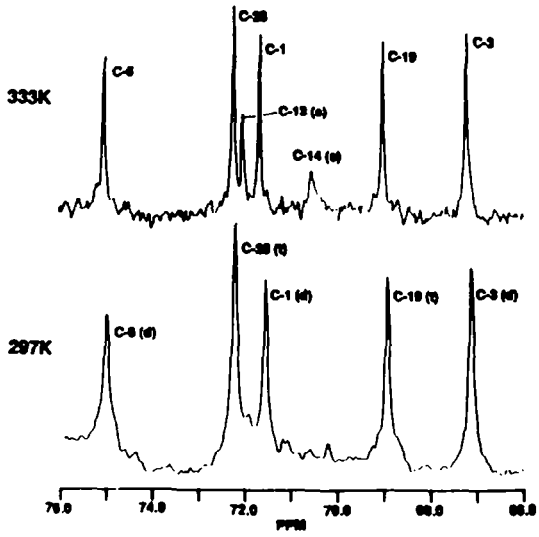

Figure 6. *Temperature effects in the carbon NMR spectrum of azadirachtin-7,11,20-trimethyl ether in chloroform. Bottom spectra, 297K. Top spectra, 333K of the same region.*

Conclusions

We have demonstrated that it is possible to deduce the structure of azadirachtin from the results of NMR spectroscopy; however, it has involved the assimilation of data from variable temperature results, proton nOe studies and long range proton carbon spin coupling. None of these techniques can produce the answer by themselves, and a considerable amount of chemical insight is necessary in the interpretation of the results.

The major problem with this work is concerned with the location of the oxygens. There is no NMR experiment which can conclusively locate an oxygen, because there is no suitable dipolar isotope of oxygen, the most abundant magnetic isotope (0.037%) being oxygen-17 which is quadrupolar (spin 5/2). This means we have to find links across the oxygen, and deduce its presence by the absence of anything we can detect, such as a quaternary carbon atom. For example, even if one were to tolerate the low sensitivity of the two-dimensional INADEQUATE experiment [22] which uses carbon-carbon coupling to provide carbon-carbon connectivity information, the results would still not locate the epoxide bridge. It is rather ironic to note that the best evidence we have for the epoxide comes, not from modern NMR experiments, but from the observation of the temperature dependence of the spectra.

Acknowledgment. We are grateful to Dr. John C. James for measuring the spectra shown in Figure 3,[8] and to Dr. Richard R. Izac (Philip Morris Research Center, Richmond, Virginia) for a supply of neem seeds. Koji Nakanishi is grateful to Professor David Taylor for discussions, but also for kindly agreeing to write a summary article on azadirachtin and the three full papers appearing in this issue, and to Professor W.D. Ollis, editor, for arranging them to appear collectively in *Tetrahedron*. We also thank NSF for partial support (PCM-8115599) of the NMR facility at the University of Missouri.

References and Footnotes.

(1) Two conferences have been held on the neem tree: First Internat. Neem Conf., Rottach-Egern, Germany, 1980; Second Intern. Neem Conf., Giessen, Germany, 1983. A quarterly newsletter devoted to neem research is published by the Indian Agricultural Research Institute, New Delhi-11012.
(2) *Chem. & Eng. News* **1985**, pp 46-51.
(3) For a recent review on the limonoids from Meliaceae, see: Taylor, D.A.H. In "Progress in the Chemistry of Organic Natural Products", Herz, W.; Grisebach, H.; Kirby, G.W. Ed.; Springer-Verlag, Wien, New York; Vol. 45,1984; pp 1-102.
(4) (a) Rembold, H.; Garcia, E. de Souza *J. Insect Physiol.* **1984**, *30*, 939; (b) Redfern, R. E.; Hayes, D. K.; Warthen, J. D.; DeMilo, A. B.; McGovern, T. P. *Ann. Rev. Chronopharmacol.* **1984**,*1*,239; (c) Koul, O. *Entomol. Exp. Appl.* **1984**, *36*, 85.
(5) (a) Butterworth, J. H.; Morgan, E. D. *J. Insect. Physiol.* **1979**,*17*, 969; (b) Gill, J. S.; Lewis, C. T. *Nature (London)* **1971**, *232*, 402; (c) Ruscoe, C. N. E. *Nature (London)* **1972**, *236*, 159.
(6) Butterworth, J.H.; Morgan, E.D.; Percy, G.R. *J. Chem. Soc. Perkin Trans.* **1972**, *1*, 2445.
(7) (a) Zanno, P.R.; Miura, I.; Nakanishi, K. *J. Am. Chem. Soc.* **1975**, *97*, 1975; (b) Nakanishi, K. In "Recent Advances in Phytochemistry", Runeckles, V.C., Ed.; Plenum Press: New York, 1975; Vol. 9, Chapter 11.
(8) James, J. C. Ph. D. Thesis, Columbia University, 1982.
(9) Mostly communication through mail and telephone and exchange of paper drafts; only one of the transitional structures were published. We thank the other two proups for this exchange of information.
(10) Kraus, W; Bokel, M.; Klenk, A.; Pohnl, H.*Tetrahedron lett.*, **1985**,*26*, 6435.
(11) Broughton, H. B.; Ley, S. V.; Slawin, A. M. Z.;D. J. Williams, D. J.; Morgan, E. D. *J. Chem. Soc. Chem. Commun..*, **1986**, 46.
(12) Gullo, V. P.; Muira, I.; Nakanishi, K.; Cameron, A. F.; Connolly, J. D.; Duncanson, F. D.; Harding, A. E.; McCrindle, R.; Taylor, D. A. H. *J. Chem. Soc. Chem. Commun.*, **1975**, 345.
(13) Schroeder, D.R.; Nakanishi, K. submitted for publication.
(14) Johnstone, R. A. W.; Ross, M. E.*Tetrahedron*, **1979**, *35*, 2169.
(15) Shaka, A. J.; Bauer, C.; Freeman, R. *J. Magn. Reson.*, **1984**,*60*, 479.
(16) L. D. Colebrook, L. D.; Hall, L. D. *Org. Magn. Reson.*, **1983**,*21*, 532.
(17) Sanders, J. K. M.; Mersh, J. D. *Prog. Nucl. Magn. Reson. Spectrosc.*, **1982**, *15*, 353.
(18) Turner, C. J. *Prog. Nucl. Magn. Reson. Spectrosc.*, **1984**,*16*, 311.
(19) Bax, A.; Ferretti, J. A.; Nashed, N.; Jerina, D. M. *J. Org. Chem.*, **1985**, *50*, 3029.
(20) Kessler, H.; Griesenger, C.; Zarbock, J.; Loosi, H. R. *J. Magn. Reson.*, **1984**, *57*, 331.
(21) Bilton, J. N.; Broughton, H. B.; Ley, S. V.; Lidert, Z.; Morgan, E. D.; Rzepa, H. S.; Shepard, R. N. *J. Chem. Soc., Chem. Commun.*, **1985**, 968.
(22) Bax, A.; Freeman, R.; Frenkiel, T. A.; Levitt, M. H. *J. Magn. Reson..*, **1981**, *43*, 478.