Communications to the Editor

Carbon-13 Nuclear Magnetic Resonance Spectral Analysis of Tetracycline Hydrochloride and Related Antibiotics

Sir:

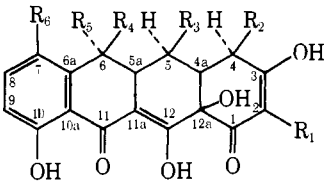
The application of NMR spectroscopy to the study of tetracyclines has not been extensive, due primarily to the limited solubilities of tetracyclines in the solvents of choice. However, ^{1}H NMR has been applied in the study of tetracyclines to elucidate stereochemical and structural features,[1-11] to determine the microscopic dissociation constants,[12,13] to monitor the kinetics of epimerization at the 4-position,[14] and to study the binding sites of a number of metal ions.[15] The only prior application of ^{13}C NMR to tetracyclines has been the spectrum of tetracycline hydrochloride published in a catalog of ^{13}C NMR spectra[16] which reported a partial assignment. This paper will verify the assignments that have been made and assign the remainder of the signals.

Carbon-13 NMR offers several advantages over ^{1}H NMR for the study of tetracyclines. First, the number of signals observed in the ^{13}C NMR spectrum is twice that observed in the ^{1}H NMR spectrum giving a greater number of probes. Second, the ^{1}H NMR spectrum contains a number of signals that are overlapping or poorly resolved while the proton broad band decoupled ^{13}C NMR spectrum contains 21 well-resolved signals.

The ^{13}C NMR spectra were obtained on a Bruker HX-90-E spectrometer operating at 22.63 MHz. The accumulated interferograms were Fourier transformed by a Nicolet B-NC 12 computer. The samples were 0.1-0.2 *M* solutions in either DMSO-d_6 with tetramethylsilane as an internal standard or D_2O with dioxane as an internal standard. Samples were spun in 10-mm tubes at approximately 25°.

Tetracycline hydrochloride (**1**), oxytetracycline hydrochloride (**2**), 6-deoxyoxytetracycline hydrochloride (**3**), and tetracycline base (**4**) were obtained from Pfizer, Inc.[17] The chlortetracycline hydrochloride (**5**) and demethylchlortetracycline hydrochloride (**6**) were purchased from American Cyanamid Co. The 6-methyleneoxytetracycline hydrochloride (**7**) was obtained from Wallace Laboratories.

Tetracycline methiodide (**8**),[18] 2-cyanotetracycline (**9**),[19] and 2-carbamoylcyclohexane-1,3-dione (**10**)[20] were synthesized according to the literature.



	R_1	R_2	R_3	R_4	R_5	R_6
1	$CONH_2$	$N(CH_3)_2 \cdot HCl$	H	CH_3	OH	H
2	$CONH_2$	$N(CH_3)_2 \cdot HCl$	OH	CH_3	OH	H
3	$CONH_2$	$N(CH_3)_2 \cdot HCl$	OH	CH_3	H	H
4	$CONH_2$	$N(CH_3)_2$	H	CH_3	OH	H
5	$CONH_2$	$N(CH_3)_2 \cdot HCl$	H	CH_3	OH	Cl
6	$CONH_2$	$N(CH_3)_2 \cdot HCl$	H	H	OH	Cl
7	$CONH_2$	$N(CH_3)_2 \cdot HCl$	OH	$=CH_2$		H
8	$CONH_2$	$N(CH_3)_3 \cdot I$	H	CH_3	OH	H
9	CN	$N(CH_3)_2$	H	CH_3	OH	H

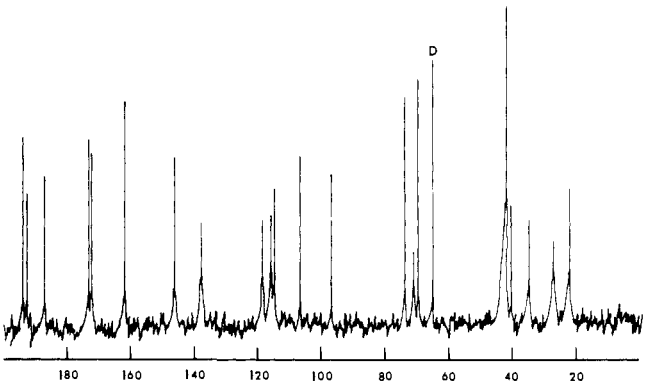


Figure 1. Tetracycline hydrochloride 0.2 *M* in D_2O.

Table I. Results of Gated Decoupling Experiment for Tetracycline–HCl

		J_{C-H} in Hz		
δ, ppm	Splitting	D_2O	DMSO-d_6	Assignment
22.1	Quartet	128	127	C-6 Methyl
27.3	Triplet	129	128	C-5
35.0	Doublet	134	*a*	C-4a
42.3	Doublet	≈135	*a*	C-5a
43.1	Quartet	≈145	*a*	$N(CH_3)_2$
70.7	Doublet	146	144	C-4
116.8	Doublet	162	162	C-9
118.6	Doublet	162	162	C-7
138.4	Doublet	162	162	C-8

[a] Obscured by DMSO-d_6 solvent.

The proton broad band decoupled spectrum of tetracycline hydrochloride (**1**), shown in Figure 1, contains 21 signals (for $C_{22}H_{24}N_2O_8 \cdot HCl$) in addition to the signal from the dioxane standard. The two dimethylamine group carbons were observed as one signal as might be expected assuming free rotation about the C-4 carbon–nitrogen bond.

Since ten of the carbons in tetracycline are directly bonded to a proton, a gated decoupling experiment was used to identify these carbons. The results are presented in Table I. The one triplet that was observed can directly be assigned to the C-5 carbon while the two quartets can be assigned to the methyl group at C-6 and the dimethylamine group. The downfield signal at 43.1 ppm was assigned to the dimethylamine carbons because of the substituent effect of the nitrogen. The two doublets at 35.0 and 42.3 ppm were assigned to the C-4a and C-5a carbons, respectively, by comparison with the C-4a and C-5a signals in **6** listed in Table II. The removal of the C-6 methyl affects the C-5a carbon much more than it affects the C-4a carbon. The C-4 carbon was assigned due to its coupling to the proton, the chemical shift characteristic of a tertiary carbon bonded to a nitrogen, and the comparison with the respective signal in **8**. The C-7 and C-9 carbon signals were assigned by comparison with **5, 6, 11,** and **12** (Chart I). The C-8 carbon was assigned by comparison with the model compounds **11** and **12.** It is interesting to note that the order of the signals for the C-7, C-8, and C-9 carbons is the same as for the analogous proton signals in the ^{1}H spectrum.[14]

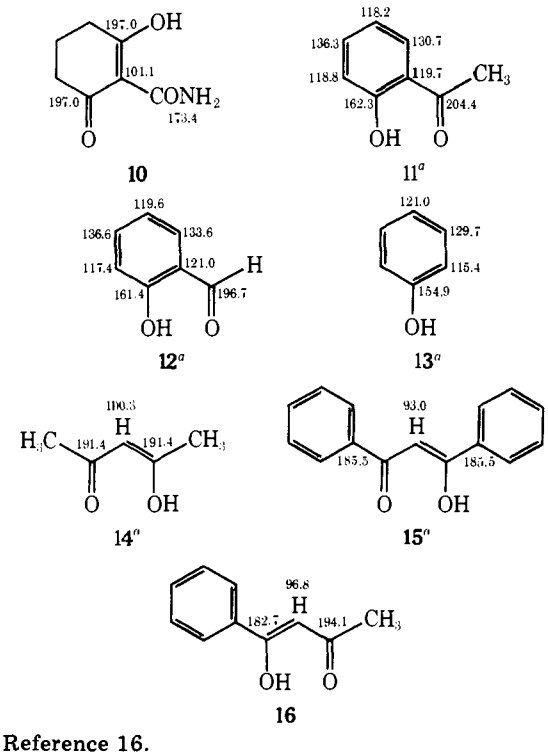
The signals for the C-6 and C-12a carbons have chemical

Table II. Chemical Shift Values and Assignments for Some Tetracycline Antibiotics

	CH_3	C-5	C-4a	C-5a	$N(CH_3)_2$	C-6	C-4	C-12a	C-2	C-11a	C-10a	C-9	C-7	C-8	C-6a	C-10	$CONH_2$	C-12	C-3	C-1	C-11
											in D_2O										
1	22.1	27.3	35.0	42.3	43.1	70.4	70.7	74.4	97.1	107.3	115.1	116.8	118.6	138.4	146.7	161.9	173.0	173.2	187.3	193.9	194.3
2	24.1	65.7	42.4	50.8	43.1	71.2	67.2	73.8	97.1	105.5	114.9	116.6	118.8	138.4	146.9	161.8	171.6	173.0	187.3	193.5	194.8
3	15.9	66.2	42.9	46.3	42.1	39.4	69.1	73.9	96.3	108.2	115.8	116.9	116.9	137.7	147.8	161.3	170.3	172.5	187.2	194.1	194.1
6	*b*	28.6	35.1	37.7	42.6	65.1	70.5	74.8	97.2	105.9	116.5	120.3	124.4	138.6	139.7	160.8	173.1	173.6	187.4	193.4	193.9
7	114.3[c]	66.0	41.8	45.4	42.1	140.7	66.5	74.5	97.0	106.7	115.4	117.7	118.2	138.2	143.4	160.8	169.5	172.7	187.3	193.8	194.2
											in DMSO-d_6										
1	22.5	27.0	34.8	41[a]	41[a]	67.8	67.9	73.1	95.5	106.9	114.4	115.2	116.9	136.5	148.0	161.4	172.1	175.1	187.3	193.0	193.5
2	24.7	64.4	41[a]	49.8	42[a]	68.9	64.8	72.6	95.3	105.4	114.5	114.8	117.1	136.5	148.9	161.2	172.0	173.6	187.2	193.1	193.8
3	15.8	64.5	38[a]	45.1	41[a]	41[a]	67.9	73.0	95.0	107.2	115.4	115.4	115.9	136.6	147.8	161.1	171.7	173.8	187.4	192.6	193.7
4	22.9	22.4	37[a]	40[a]	42[a]	68.1	69.4	74.3	98.3	105.8	114.4	115.4	116.8	136.4	148.0	161.4	172.6	176.8	192.4	192.4	192.9
5	25.0	27.1	34.9	42[a]	41[a]	70.4	68.1	73.2	95.6	106.1	117.0	118.9	121.2	139.7	143.6	160.7	172.1	175.7	187.3	192.0	193.4
6	*b*	28.5	34.9	37[a]	41[a]	63.9	67.8	73.7	95.6	105.2	115.9	119.0	122.1	137.0	140.7	160.1	171.9	176.2	187.3	191.7	193.5
7	113.7[c]	63.9	41[a]	44.0	41[a]	140.6	65.1	73.5	94.8	105.1	114.4	116.4	117.2	137.0	142.6	160.7	171.6	173.6	187.3	192.1	193.5
8	22.4	26.8	36.0	42[a]	53.8	67.9	75.4	72.1	97.3	106.5	114.4	115.3	117.1	136.6	147.8	161.4	172.8	174.8	186.2	192.2	192.9
9	22.5	26.4	34.1	41[a]	41[a]	67.8	69.8	73.5	83.5	105.9	114.6	115.4	117.0	132.8	148.2	161.6	119.2[d]	178.7	181.9	188.1	192.9

[a] Partially or totally obscured by the DMSO-d_6 solvent signal. [b] This carbon not present in compound. [c] $=CH_2$. [d] $C\equiv N$.

Chart I. Chemical Shifts of Selected Carbons in Model Compounds



[a] Reference 16.

shifts in the range expected for quaternary carbons bonded to an oxygen. The upfield signal at 67.8 ppm was assigned to the C-6 and the downfield signal at 73.1 ppm to the C-12a on the basis of comparison with **3, 6,** and **7.** The C-6 carbon is shifted upfield in **3** and **6** and is shifted downfield in **7** while the C-12a carbon signal changes less than 1 ppm.

The chemical shift range for the C-2 and C-11a carbon signals was determined by comparing spectra of a number of β-diketones and **10.** The final assignment was made by comparison with the signals in **9.** The C-2 carbon is shifted upfield 12 ppm when the amide group is dehydrated to the nitrile. The C-10a signal assignment was made by comparison with **11** and **12.** The C-6a carbon signal was assigned because of changes noted in this signal for **5, 6,** and **7.** The C-10 assignment was made by comparing the signals observed for **11, 12,** and **13.** The chemical shift range for the amide carbon and the C-12 carbon was determined by comparison with other amide compounds and the model compound **16.** The change in the upfield signal in **9** allowed the final assignment.

The final three signals belong to the C-1, C-3, and C-11 carbons. While model compounds **10, 15,** and **16** helped determine the chemical shift range, the final assignments of the signals at 187.3, 193.0, and 193.5 ppm to the C-3, C-1, and C-11 carbons, respectively, were made by monitoring the signals as a function of pH. The signals at 187.3 and 193.0 started to shift at pH 3, however, the signal at 193.5 did not change until pH 7. This was consistent with previous assignments of the first pK_a of the A ring system.[12,21]

Carbon-13 NMR spectroscopy can be applied in several ways to study tetracycline chemistry. The spectrum of tetracycline can be monitored as a function of pH. In this manner the deprotonation of tetracycline with increasing pH can be followed allowing for many more probes than in the ^1H study.[12] The shifts observed with ^{13}C NMR are also much larger than ^1H NMR with shifts of up to 16 ppm observed.[22] A full paper will discuss this topic more completely. ^{13}C NMR can also be used to monitor metal ion com-

plexation and preliminary results indicate that certain carbon signals are preferentially broadened in the presence of paramagnetic metal ions.[22,23]

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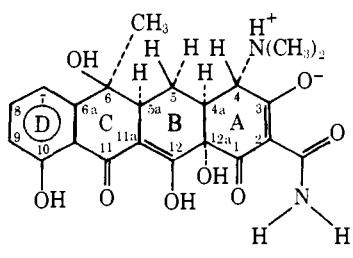
A ^{13}C Nuclear Magnetic Resonance Analysis of the Metal Binding Site in Tetracycline

Sir:

Recently a paper from this laboratory reported results of a proton NMR investigation directed toward establishing the site or sites of metal binding in tetracycline(I), abbreviated TC.[1] The study involved adding small amounts of



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paramagnetic and diamagnetic metal salts to DMSO-d_6 solutions of TC free base and observing selective effects of these salts on the proton NMR signals of TC. The results

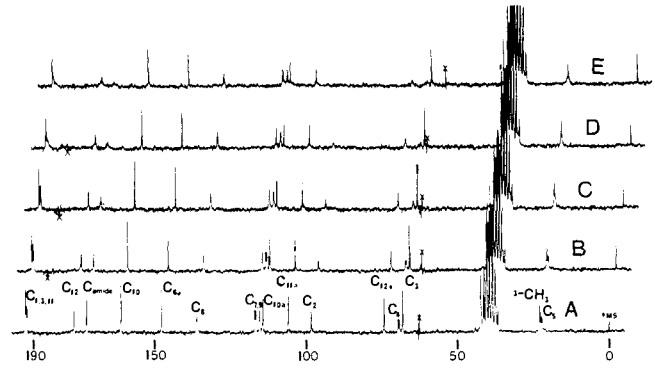

Figure 1. Natural abundance ^{13}C NMR spectra of tetracycline free base in DMSO-d_6 at Nd^{3+}/TC mole ratios of 0 (A), 0.017 (B), 0.034 (C), 0.050 (D), and 0.067 (E).

strongly indicate that binding occurs through ring A functional groups for each metal ion investigated. These conclusions have now been corroborated by a carbon-13 NMR study using lanthanide series ions as binding site probes. Results of the carbon-13 work are presented here.

The carbon-13 NMR spectrum[2] of TC (0.25 *M*) in DMSO-d_6 shows 18 of the possible 21 signals (Figure 1A). Asleson and Frank have made complete carbon-13 NMR signal assignments for TC in DMSO utilizing a series of TC derivatives.[3] Their assignments are used here. The solvent resonances (37–42 ppm) obscure signals assigned to carbons at positions 4a and 5a and in the dimethylamine group.

After recording the spectrum of TC free base, small amounts of a 0.21 *M* solution of anhydrous $Nd(NO_3)_3$ in DMSO-d_6 were added to the TC solution such that Nd^{3+}/TC mole ratios were 0.017, 0.034, 0.050, and 0.067. A spectrum was recorded after each addition of $Nd(NO_3)_3$. Similarly, another series of spectra containing increasing amounts of anhydrous $La(NO_3)_3$ was recorded. The paramagnetic Nd^{3+} ion was found previously to be particularly effective in causing selective broadening and shifting of proton resonance signals of TC.[1] As the mole fraction of Nd^{3+} is increased, a selective broadening occurs for signals assigned to C_{12}, the amide-C, C_2, C_{12a}, C_4 and two carbonyl signals in the group[4] containing C_1, C_3, and C_{11} as shown in Figure 1. Signal shifts in the presence of Nd^{3+} are no more than a few hertz. The largest shift occurs for C_5 which is about 10 Hz downfield of the free TC resonance at a Nd^{3+}/TC mole ratio of 0.067. These effects do not arise entirely from interaction of unparied electrons on Nd^{3+} with ^{13}C nuclei, however, since broadening and shifting of some of the same ^{13}C NMR signals of TC are also observed in the presence of the diamagnetic La^{3+} ion. With increasing La^{3+}/TC ratio, signals attributed to C_{12}, the amide-C, C_2, and *one* of the C_1, C_3, C_{11} carbonyls broaden, and the C_5 signal shifts downfield. Signal shifts in the presence of La^{3+} and Nd^{3+} are of the same magnitude and sign. Broadening is more pronounced for Nd^{3+} than for La^{3+} in most cases. Signal broadening in the presence of La^{3+} must arise from environmental averaging between bound and free TC.

In principle the location of the bound paramagnetic ion can be determined by analyzing the relative relaxation times and isotropic shifts of the perturbed NMR signals. However, one must first correct for signal perturbations caused by a diamagnetic ion binding at the same site. In the previous proton NMR study, this was done by examining *differences* in the spectra of TC in the presence of Nd^{3+} and in the presence of the same mole fraction of La^{3+}. When this procedure is employed for the carbon-13 spectra, the following conclusions emerge. (1) The effects of Nd^{3+}