Journal of Pharmaceutical & Biomedical Analysis
Vol. 1, No. 3, pp. 281–292, 1983
Printed in Great Britain

0731-7085/83 $3.00 + 0.00

The identification and stereochemical study of tetracycline antibiotics by ^{1}H nuclear magnetic resonance spectroscopy

A. F. CASY* and A. YASIN

School of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK

Abstract: The ^{1}H NMR spectra of seven tetracycline antibiotics of clinical importance are reported and assigned as solutes in DMSO-d_6 and other solvents. The data are analysed in terms of analytical utility and the provision of evidence of solute stereochemistry.

Keywords: *^{1}H NMR; tetracycline antibiotics; stereochemistry; conformation.*

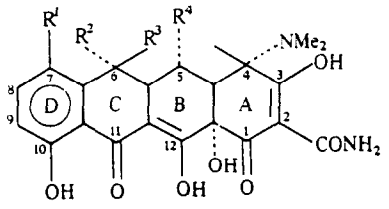
Introduction

The potential value of nuclear magnetic resonance (NMR) spectroscopy in differentiating groups of closely related compounds is generally accepted and it is surprising that the technique has received relatively little attention in regard to the analysis of the tetracycline antibiotics. About ten derivatives are now in frequent clinical use, all of which are based on the molecular framework and functionality array **1**, which dominates the physical properties, appearance and electronic and vibrational spectroscopic features of the group. In consequence, the more common analytical approaches are of little value for the characterization of individual members [1]. Similar problems of identification and detection apply to biotransformation products of therapeutic tetracyclines. Likewise, little systematic application of NMR data to problems of the relative configuration and conformational preference of tetracycline (TC) derivatives has been made, although much ^{1}H and ^{13}C spectral information on this group is scattered throughout the literature [2]. A comprehensive study of the NMR parameters of tetracyclines in clinical use and their common degradative contaminants has therefore been undertaken. ^{1}H NMR spectra of tetracyclines form the topic of the present report, while a ^{13}C study will be given later, both with emphasis at this stage upon differentiation of the bulk antibiotic substances and the provision of evidence of stereochemistry.

The most extensive ^{1}H NMR study of tetracyclines to date was reported in a paper in 1966 by Wittenau and Blackwood [3], who employed pyridine and trifluoroacetic acid (TFA) as solvents in all but one case. Use of these solvents may be criticized; pyridine

* To whom correspondence should be addressed.

Bridgehead carbons designated by 4a, 5a etc.



1

	R^1	R^2	R^3	R^4	Generic name	Abbreviation
1a	H	Me	OH	H	tetracycline	TC
1b	Cl	Me	OH	H	chlortetracycline	CTC
1c	Cl	H	OH	H	6-demethylchlor-tetracycline	6-demethyl CTC
1d	NMe_2	H	H	H	minocycline	—
1e	H	Me	OH	OH	oxytetracycline	OTC
1f	H		6=CH_2	OH	methacycline	—
1g	H	Me	H	OH	doxycycline	—

complicates spectral interpretations by its influence on ionization equilibria and by complexation effects, as discussed below, while use of the strong acid TFA seems inadvisable in view of the known acid-lability of most TC derivatives. In the present work, the ^1H NMR spectra of many commonly used TC derivatives have been recorded and critically evaluated in the polar and neutral solvents DMSO-d_6 and/or D_2O.

Materials and Methods

^1H NMR spectra

Spectra were obtained on a JEOL PS 100 NMR spectrometer operating at normal temperatures and were thus more highly resolved than those of an earlier 60 MHz study [3]. Samples were prepared in 5 mm o.d. tubes as approximately 10% m/v solutions in DMSO-d_6 and in other solvents with tetramethylsilane (TMS) as reference, and in D_2O with 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) as reference. The 220 MHz spectra were provided by the Physico-chemical Measurements Unit, Harwell, UK.

Materials

All materials used were commercial samples kindly supplied by Lederle Laboratories, Gosport (TC, CTC, 6-demethyl CTC, minocycline, all as hydrochlorides) and by Pfizer Ltd (Sandwich Kent, UK) (TC, OTC, methacycline, all as hydrochlorides, doxycycline hyclate, and TC base). The sample of 4-epi-TC HCl was kindly donated by Lederle Laboratories (Pearl River, NY, USA). Anhydro-TC HCl was prepared by the method of Asleson [4] and the 2-cyano analogues of TC and OTC were obtained by treating the antibiotics with dicyclohexylcarbodiimide [5].

Results and Discussion

^{1}H NMR chemical shift data are given in Table 1. The conventional numbering system of tetracyclines is shown in **1** while formulae **1a** to **1g** give details of structure, generic name and abbreviation (where appropriate) of the compounds examined in this study. Assignments of the aromatic protons of ring D, the 6-methyl and C_4 and C_7 dimethylamino proton resonances were straightforward, being based on known ^{1}H chemical shifts [6]. Other assignments are discussed below in terms of the various types of proton resonance.

Aromatic protons of ring D

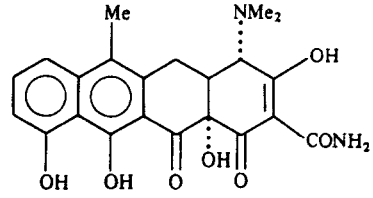
Examination of the 6.8–7.8 ppm region of the spectra readily permits the identification of those TC derivatives with two ring D substituents, since insertion of a substituent at C_7 changes the aromatic multiplicity pattern and integrals characterizing TC and OTC (i.e. three 1-proton resonances observed as a triplet and two doublets; items 1 and 8)* to an AB (or AX) two-proton doublet pair. This is exemplified by CTC and minocycline (items 5–7).

C_6-Methyl protons

The broad 3-proton resonance at 1.5 ppm of TC HCl (item 1) is a diagnostic feature of TC derivatives with methyl substituents at C_6; the broad nature of this and related resonances of other derivatives probably results from the operation of small, long-range coupling interactions. Electronegative atoms close to C_6, such as oxygen in OTC and chlorine in CTC, shift the resonance significantly downfield (items 8 and 5); absence of this signal narrows the range of possibilities for TC identification.

The 6-methyl resonance of doxycycline has an identical chemical shift to that of TC HCl, indicating that the shielding influences of 6-hydroxyl removal and 5-hydroxyl insertion are compensatory. The fact that the 6-methyl resonance appears as a notably broad singlet ($W_{1/2} \sim 12$ Hz) rather than as a doublet probably results from virtual long-range coupling [7, 8]† and is evidence that the C_6 and C_{5a} protons are strongly coupled. This is also evidence for a pseudo-equatorial conformation of the 6-methyl group of ring C, in accord with chemical evidence of configuration [9].

In the spectrum of anhydro-TC HCl (**2**), the 6-methyl proton resonance forms a sharp singlet at 2.4 ppm. The downfield shift of this signal ($\Delta\ \delta \sim 0.9$ ppm), as a result of the change of the C_6 carbon from sp^3 to sp^2 hybridization, yields a significant difference between the TC and anhydro-TC spectra (cf. items 1 and 13). This provides a useful means to monitor dehydration of the parent antibiotic.



2

* All item numbers refer to column 1 in Table 1.

† Direct long-range couplings may contribute to signal width, but cannot be the principal factor, taking into account the 6-methyl signal profiles of other derivatives.

Table 1
^{1}H chemical shifts of some tetracycline derivatives[a]

Item	Compound	Ring D aromatics[b]	C_6 methyl	C_4, C_7 NMe_2	Acidic protons $CONH_2$[c]	Acidic protons others	Methine protons C_4	Methine protons C_5	Methine protons C_{4a}	Methine protons C_{5a}	C_5 methylene	Miscellaneous
1	**1a** HCl	7.5 t 7.1, 6.9 d	1.5 bs	2.9 s	9.5 9.1	11.7 15.0	4.3 bs	—	[d]	[d]	[d]	—
2	**1a** base	7.5 t 7.1, 6.9 d	1.5 bs	2.5 s	9.1 8.6	11.9	3.3[e]	—	[d]	[d]	[d]	—
3	**1a** base in pyridine-d_5	[f]	1.8 s	2.6 s	10.1 9.9		3.7[e]	—	[d]	[d]	[d]	—
4	**1a** HCl in pyridine-d_5	[f]	1.8 s	2.6 s	10.1 9.9		3.7[e]	—	[d]	[d]	[d]	—
5	**1b** HCl	7.5 d 6.9 d	1.9 s	2.9 s	9.5 9.0	12.0	4.4 bs	—	[d]	[d]	[d]	—
6	**1c** HCl	7.6 d 6.9 d	—	2.9 s	9.6 9.1	11.6	4.4 bs 4.7 bs	— (C_6)	[d] [d]	[d] [d]	[d] [d]	— —
7	**1d** HCl	7.4 d 6.8 d	—	2.9 s 2.6 s	9.4 9.0	11.2	4.3 bs	—	[d]	[d]	[d]	—
8	**1e** HCl[g]	7.5 t 7.1, 6.9 d	1.7 bs	2.9 s	9.6 9.1	11.5 14.9	4.7 bs	3.8[h]	[d]	[d]	—	—
9	**1e** HCl in D_2O	7.5 t 7.1, 6.9 d	1.8 bs	3.1 s[i]	—	—	4.4 bs	4.0[j]	[k]	[k]	—	—
10	**1e** HCl in pyridine-d_5	[f]	2.3 bs	3.1 s	broad band 8–10.4 ppm		4.8 d	5.0 t		(3.5 m)	—	—
	D_2O added	[f]	2.3 bs	3.3 s	—	—	4.7 d	4.75 t		(3.4 m)	—	—
11	**1f** HCl	7.6 t 7.2, 7.0d	—	2.9 s	9.6 9.1	6.4 11.5 15.0	4.7 bs	[l]	3.1d[m]	[l]	—	5.6, 5.5[n]

Table 1 continued

Item	Compound	Ring D aromatics[b]	C_6 methyl	C_4, C_7 NMe_2	Acidic protons $CONH_2$[c]	Acidic protons others	Methine protons C_4	Methine protons C_5	Methine protons C_{4a}	Methine protons C_{5a}	C_5 methylene	Miscellaneous
12	**1g** HCl	7.5 t 6.9, 6.85 d	1.5[o]	2.9 s	9.6 9.1	11.5 15.1	4.8 bs	[p]	[d]	[d]	—	1.1 t[q] 3.45 q
13	**2** HCl[r]	7.6 m 6.9 d	2.4 s	2.9 s	9.7 9.3		4.4[e]	—	[d]	[d]	[d]	—
14	4-Epi-**1a** HCl	7.5 t 7.1, 6.9 d	1.5 bs	2.9 s 3.0 bs	9.5 9.3	11.7 15.1	4.75[e] 4.3 bs	—	[d]	[d]	[d]	—

[a] Hydrochloride salts in DMSO-d_6 unless otherwise stated; chemical shifts in ppm (to nearest 0.05 ppm, δ scale) relative to TMS or DSS in solvent D_2O. Resonance descriptions: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet; $W_{1/2}$, width at half maximum height. Residual proton signal of solvent near 2.5 pm present in all DMSO-d_6 spectra.

[b] Multiplet separations all between 7 and 8 Hz.

[c] Pair of broad absorption bands, $W_{1/2}$ = 12–15 Hz.

[d] Within 1.6–3.2 ppm envelope, unresolved.

[e] Narrow doublet, separation 3–4 Hz just resolved.

[f] Obscured by residual pyridine protons band.

[g] Comparable shift data with those of a 60 MHz study [3] except that the C_4- and C_5-proton assignments are reversed.

[h] Broad triplet, separations ~ 8 Hz.

[i] $W_{1/2}$ = 3 Hz before, and 10 Hz after, addition of DCl (~ 4% v/v).

[j] Doublet of doublets, separations 8.8 and 11 Hz (from 220 MHz spectrum).

[k] Overlapping doublets near 2.9 ppm.

[l] 2-proton multiplet near 3.7 ppm.

[m] Provisional assignments, separation ~ 10 Hz.

[n] Vinylic protons resonance of 6-methylene.

[o] Broad absorption, $W_{1/2}$ = 12 Hz.

[p] Obscured by methylene quartet of ethanol resonance.

[q] Separations ~ 7 Hz; ethanol of crystallization resonances.

[r] Comparable data with that of a 60 MHz study [22].

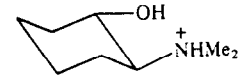
Dimethylamino protons

All spectra of hydrochlorides display intense resonances near 2.9 ppm attributable to the common C_4-quaternary substituent. The additional and similar resonance at 2.6 ppm in the spectrum of minocycline HCl reveals the C_7-quaternary amino feature of this derivative. Of the two *N*-dimethyl substituents, that at C_4 is the more basic and would be expected to be the preferred site of protonation of the mono-hydrochloride. This fact allows assignment of the two resonances, because *N*-protonation leads to a pronounced downfield shift of *N*-methyl resonances [10a].

The broad nature of the *N*-dimethyl resonance in the 220 MHz spectrum of OTC HCl (Fig. 1) requires comment, especially as Wittenau and Blackwood [3] describe the *N*-dimethyl signal of TC derivatives as appearing as two doublets in acidic solvents. The 220 MHz spectrum was run in D_2O plus DCl, i.e. under comparable low pH conditions. The broadening of the *N*-dimethyl resonance of OTC HCl in D_2O by addition of DCl (~ 4% v/v) was then confirmed at 100 MHz, when the signal half-width ($W_{1/2}$) increased from 3 to 10 Hz. Signal broadening as a result of C_4-quaternary ammonium coupling may be discounted, since a large excess of D_2O is present; it must therefore be due to non-equivalence of the two *N*-methyl environments at low pH. This situation will arise under conditions of slow proton exchange, because the two *N*-methyl groups are adjacent to an asymmetric centre at C_4; any restricted rotation about the C_4-N bond should enhance the magnitude of the *N*-methyl chemical shift difference.

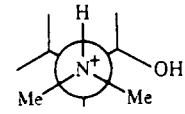
An analogous case is provided by the spectrum of the trans-2-dimethylaminocyclohexanol hydrochloride **3**, where distinct *N*-methyl signals are observed. This phenomenon has also been seen in the spectrum of the corresponding *cis*-isomer, but with less pronounced separation [11]. A similar situation may be readily envisaged for protonated TC derivatives. The phenomenon requires a slow rate of proton (or deuteron) exchange at the basic centre and hence is best observed when the pH is lowered. Separate lines should appear if the exchange rate is sufficiently slow, otherwise the two resonances overlap to form a broad band, as observed in Fig. 1.

NMe_2 signal: two singlets near 2.8 ppm; separation 7 Hz at 60 MHz in D_2O.



3

View down N–C bond of preferred conformation; Me subject to influence of OH substituent.

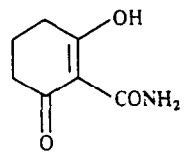


Acidic protons

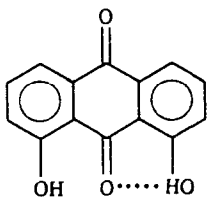
Tetracycline derivatives are remarkable for their high content of exchangeable protons. Most hydrochlorides possess the eight protons of TC HCl itself (hydroxyl at C_3, C_6, C_{10}, C_{12}, C_{12a}; the amino group of the C_2-amide; the protonated C_4-quaternary ammonium function). In principle all protons are detectable by comparing spectra in DMSO-d_6 before and after addition of D_2O. In practice, however, only four resonances attributable to acidic protons can be assigned with certainty and then only if sufficient field-offset is employed when recording the spectrum (Table 1).

Two groups have reported on the acidic proton resonances (chiefly those of TC [4, 12]) and a summary of assignments is made here. A pair of broad one-proton resonances near 9 and 9.6 ppm invariably appears in spectra run in DMSO-d_6. This is attributed to the amide protons on the basis of their absence in spectra of the corresponding 2-cyano analogues, and by reference to the spectra of model compounds such as **4** [4]. The appearance of these bands typifies protons exchanging between two different environments, such as could arise as a result of restricted rotation about the N–C amide bond [13].

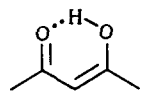
Broad resonances at 8.7 and 9.5 ppm in DMSO-d_6.



4



δOH 12.0ppm



δOH 15.1ppm

5 **6**

The narrower resonances at lower field near 12 and 15 ppm are assigned to the C_{10}- and C_{12}-hydroxyl protons, as there is evidence that these are intramolecularly hydrogen-bonded to nearby carbonyl oxygen at C_{11} and C_1 respectively [4]. The deshielding consequence of hydrogen bond formation upon acidic protons is well known, especially when intramolecular [10b]. Data on the models **5** and **6** support these assignments [12].

The C_6- and C_{12a}-hydroxyl groups probably form intermolecular hydrogen bonds with the solvent and absorb upfield of 8 ppm to form less well-defined bands due to overlap with other resonances. It is likely that the C_3-hydroxyl and quaternary ammonium proton absorptions are too broad for detection because of rapid exchange rates.

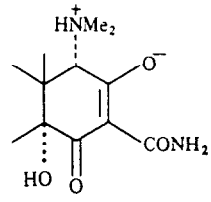
Acidic proton NMR data on TC derivatives are thus of little value for characterization purposes. For example, variations in acidic proton content, as in OTC with one extra and doxycycline with one less, cannot be exploited since diagnostic signals fall above 8 ppm and cannot be resolved. When solubilities permit, D_2O is preferred to DMSO-d_6 as an NMR solvent for TC derivatives, as it eliminates all separate acidic proton absorptions and makes for spectral simplicity, provided that the residual HDO band does not overlap any non-acidic proton signals.

Methine (C_4, C_{4a}, C_5 and C_{5a}) and C_5-methylene protons

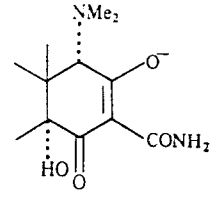
Only one resonance additional to the *N*-dimethyl and C_6-methyl signals could be resolved at a field higher than 6 ppm in the spectrum of TC HCl in DMSO-d_6. This was a broad one-proton singlet at 4.3 ppm and assigned to the C_4 proton, since this should be the most deshielded of the three methine protons; it is flanked by charged nitrogen and an enolic carbon atom. The spectra of all TC samples displayed a similar band in the

range 4.3–4.8 ppm (Table 1). The C_{4a}- and C_{5a}-methine and the C_5-methylene proton resonances must therefore constitute part of the unresolved envelope between 1.5 and 3 ppm. Wittenau and Blackwood [3] reported the PMR features of TC base in pyridine and gave 3.6 ppm as the chemical shift of the C_4-proton (see below), with a range of 2.2–3.2 ppm for the C_{4a}-, C_5- and C_{5a}-protons. They also quoted a range of 3.6–4 ppm for the C_4-proton resonance of a variety of derivatives, mostly bases, in pyridine or trifluoroacetic acid.

If assignment of the C_4-proton resonance of TC HCl is correct (as supported also by spin–spin decoupling experiments on TC base) [12], its chemical shift should be sensitive to ionization changes at the C_4-nitrogen and C_3-hydroxyl. Spectra of TC base in pyridine and DMSO-d_6 provide supportive data. In these spectra, the 4.3 ppm resonance of TC HCl in DMSO-d_6 shifts upfield to 3.7 ppm in pyridine, both signals forming narrow doublets (cf. items 2 and 3). Interpretation of these shifts is complicated by the fact that deprotonation of both the C_4-nitrogen and the C_3-hydroxyl will have a shielding influence on the C_4-proton [6, 10a]. The large upfield shift seen for TC base in DMSO-d_6 in which the zwitterion **7a** predominates [14] (A.F. Casy and A. Yasin, unpublished results) suggests that shielding due to 4-hydroxyl ionization dominates over deshielding arising from the quaternary ammonium group. The same factor may also account for the fact that the chemical shift of the quaternary ammonium group is at higher field that that of the HCl salt. An estimate of the magnitude of shielding consequent upon hydroxyl ionization was gained from data on *p*-nitrophenol (**8**).



7a

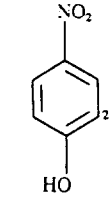


7b

δ_{2-H}

in DMSO-d_6 6.98 ppm

in DMSO-d_6/NaOH-D_2O 6.30 ppm



8

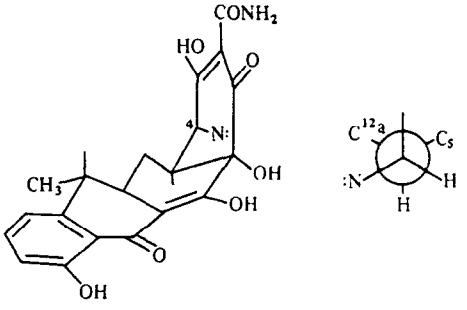
In pyridine the C_4-proton upfield shift from 4.3 to 3.7 ppm was less pronounced relative to that of its position in the spectrum of TC HCl in DMSO-d_6. In this case the feebly basic solvent would be expected to compete with the nitrogen of TC for the acidic proton. For this reason a large C_4-proton shift would be anticipated because of a rise in the population of the species **7b**, where both ionizable functions are in a favourable state for shielding. However, when pyridine complexes with polar molecules, protons in the vicinity of association sites are often markedly deshielded, because they fall within the aryl deshielding region of the solvent molecule as a result of the geometry of the

solute–solvent association mode [15, 16]. It is probable that the same deshielding mechanism operates in the case of TC-pyridine, where association may be through ion-pair formation of the type $O^- \ldots HN^+$, to shift the C_4-proton to lower field than its position in DMSO-d_6. Low field shifts of the C_4-*N*-dimethyl, C_6-methyl and C_3-amide proton resonances relative to shifts in DMSO-d_6 were also apparent (cf. items 2 and 3).

The narrow profile of the C_4-proton signals supports a tetracycline structure in which ring A is bent approximately at 90° to the plane of rings B, C and D (**9**). This would be the preferred solute conformation of all the TC derivatives examined, as established for CTC and some of its analogues in the solid state by X-ray diffraction [17–19]. In **9** the dihedral (torsion) angle relating the C_4- and C_{4a}-protons is close to 60°, while in the conformation in which all four rings are approximately planar the angle is close to 180°; such magnitudes are associated with small and large vicinal couplings respectively [10c, 20].

Conformation of hydrochloride of tetracycline derivatives; Newman diagram depicts view down C_4-C_{4a} bond.

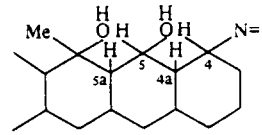
9



The spectra of most TC HCl samples displayed a low intensity broad band or narrow doublet to low field of the C_4-proton resonance, which is indicative of the presence of 4-epi-TC HCl as an impurity: i.e. a broad band near 4.8 ppm in DMSO-d_6 and a doublet with line separation ~ 3 Hz at 4.6 ppm in pyridine-d_5. A commercial sample of the 4-epi-TC HCl also displayed duplicate C_4-proton resonances in its spectrum (cf. item 14) and was judged to be an isomeric mixture with the 4-epi form as the major component. These signals provide the best means of assessing isomeric purity by ^{1}H NMR, since signal duplication in the *N*-dimethyl absorption region is complicated by possible non-equivalence effects as discussed above.

In the cases of OTC, methacycline and 6-demethyl CTC hydrochlorides, two methine resonances could be resolved in spectra in DMSO-d_6. In the OTC spectrum, the resonance at lower field (4.7 pm) was assigned to the C_4-proton, since it was a broad singlet comparable with the C_4-proton resonances of all other TC spectra. Moreover, its downfield position relative to the same resonance of TC HCl (4.3 ppm) accords with the additional deshielding influence of the 5-hydroxyl substituent of OTC. The higher field signal (3.8 ppm) formed a broad triplet in DMSO-d_6 and appeared as a well-resolved doublet of doublets in D_2O. Its multiplicity establishes that it is coupled to two protons (with apparent *J* values of 8.8 and 11 Hz). For this reason it must be attributable to the C_5- or C_{4a}-proton if long-range coupling can be discounted (cf. **10**). The C_5-proton is the most probable assignation because it is adjacent to electronegative oxygen, thus accounting for its low field shift, while the $^3J_{4a,4}$ coupling is small, as evidenced by the C_4-proton resonance, seen as a broad singlet; this coupling is too small to correspond with either of the two *J* values observed for the multiplet.

Partial structure of OTC showing C_{5a}, C_5, C_{4a}, C_4 coupled proton unit.



10

With the aim of resolving additional methine signals, a spectrum of OTC HCl in D_2O was run at 220 MHz. The spectrum over the region 1–6 ppm (Fig. 1) reveals the C_4-proton signal near 4.4 ppm and the doublet of doublets near 4 ppm; other resonances, however, remain within the broad *N*-dimethyl band centred at 3 ppm. The four sharp lines apparent on the right-hand shoulder of the *N*-dimethyl band can be reasonably attributed to the C_{5a}- and C_{4a}-protons, since separations a–c and b–d are 8.8 and 11 Hz respectively, corresponding with the separations of the 4-line signal near 4 ppm. Lines b and d are broader than a and c, and thus form the broad doublet expected for the C_{4a}-resonance. The signal centred at 3 ppm integrated as eight protons relative to the 3-proton intensity of the 6-methyl signal near 1.7 ppm.

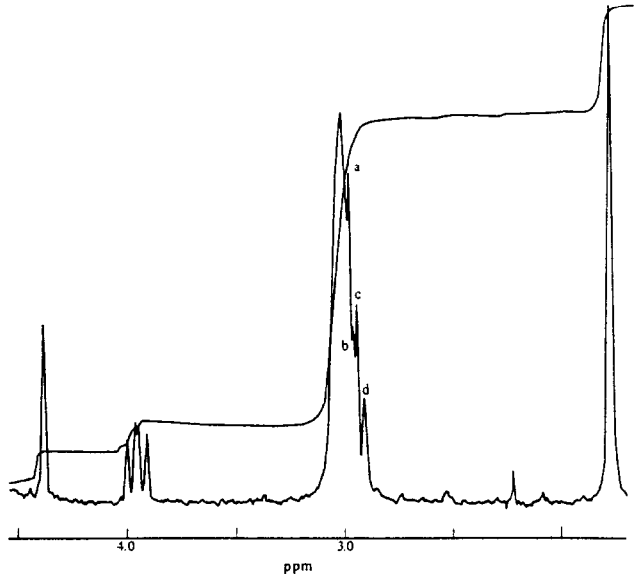


Figure 1
220 MHz ^1H NMR spectrum of oxytetracycline HCl in D_2O (plus ca. 4% v/v DCl); sweep width 1100 Hz.

A double resonance experiment supported these assignments; when the region allocated to the C_{4a}- and C_{5a}-multiplet near 2.9 ppm was irradiated, the doublet of doublets near 4 ppm collapsed to a singlet, while the broad absorption near 4.4 ppm became narrower. The order of magnitude of coupling constants within the C_{5a}, C_5, C_{4a}, C_4-unit of the OTC molecule as deduced from this analysis supports a structure similar to **9** as the preferred solute conformation of the hydrochloride in D_2O and also gives direct NMR evidence of the configuration at C_5 [21]. These arguments corroborate those of a study at 100 MHz [12] and reverse the original C_4- and C_5-proton resonance assignments

of OTC HCl in DMSO-d_6 [3]. These were unsupported by clear evidence of signal multiplicities. Different conclusions should also be made on conformational preference.

The spectrum of OTC HCl in pyridine-d_5 was equivalent to that of the corresponding anion **7b**, as shown by the identical spectra of TC base and TC HCl in the same solvent (cf. items 3 and 4). In pyridine-d_5, this spectrum showed pronounced downfield shifts of most resonances, as compared with shifts in DMSO-d_6 (cf. items 8 and 10), attributable to the deshielding influence of pyridine in the solute–solvent complexes, as already discussed. Of special note is the reversal of the relative field positions of the C_4- and C_5-proton signals, presumably due to the close association of solvent with the C_5-hydroxyl of OTC. Small changes in the separations of resonance bands also occurred, as can be clearly seen in those of the C_4- and C_5-protons; this is indicative of changes in conformation consequent upon complexation. The upfield shifts of resonances due to protons close to C_5- and the downfield shift of the *N*-dimethyl signal following addition of D_2O are interpreted as being due to a reduction in the extent of complexation of pyridine with OTC, as a result of its alternative association with D_2O. Similar spectral changes occurred when D_2O was added to TC HCl in pyridine-d_5.

Methine features of the spectrum of methacycline HCl in DMSO-d_6 (cf. item 11) were like those of OTC as regards the C_4- (broad singlet at 4.7 ppm) and C_5-proton signals (poorly resolved triplet near 3.7 ppm). The latter integrated as two protons and probably includes the C_{5a}-resonance, shifted downfield by the influence of the C_6-alkenic carbon. A doublet assigned to C_{4a} could be resolved to low field of the *N*-dimethyl singlet. All these features were confirmed in a 220 MHz spectrum, where the absorption band near 3.7 ppm was resolved as a single-proton broad triplet (3.75 ppm) and doublet (3.6 ppm), both resonances showing evidence of long-range coupling, in addition to vicinal coupling. Well-resolved line separations of the doublet at 3.0 ppm near the *N*-dimethyl signal and of the doublet to lower field were 11 and 9 Hz respectively. These magnitudes are comparable with those of corresponding resonances of OTC HCl.

6-Demethyl CTC HCl displayed two single-proton singlets near 4.5 ppm in its spectrum (cf. item 6). These are characteristic of structure, since all other TC spectra showed only one resonance of this type in the same region. The higher field resonance at 4.4 ppm is assigned to C_4 as before, while the signal at 4.7 ppm must arise from the C_6-proton, which is deshielded by its oxygen and chlorine neighbours. The low order of coupling ($^3J_{5a,6}$) evident from the profile of the C_6-proton signal indicates that the 6-hydroxyl of 6-demethyl CTC is pseudo-axial in the half-chair conformation of the molecule in DMSO-d_6, supplementing the rather sparse evidence of stereochemistry of this derivative [1].

Miscellaneous signals

The 6-methylene (vinyl) proton signal of methacycline, with two broad bands near 5.5 ppm, separated by 1–2 Hz and just resolved at 220 MHz, and the solvent resonances of doxycycline spectra (EtOH: 1.1 ppm triplet, 3.5 ppm quartet) are characteristic of structure in each case (cf. items 11 and 12). Moreover, comparative integrals of the ethanol triplet and 6-methyl resonances of the doxycycline spectrum confirm that half a mole of ethanol is involved in the crystallization of the hydrochloride salt.

Acknowledgements: We thank Mr D. Wood for skilled technical assistance, Lederle Laboratories and Pfizer Ltd for samples of tetracycline derivatives, and the Government of Dubai for financial support (to A.Y.).

References

[1] L. A. Mitscher, *The Chemistry of the Tetracycline Antibiotics*. Dekker, New York, 1978.
[2] E. P. Mazzola, J. A. Melin and L. G. Wayland, *J. Pharm. Sci.* **69**, 229–230 (1980) and references there cited.
[3] M. S. von Wittenau and R. K. Blackwood, *J. Org. Chem.* **31**, 613–615 (1966).
[4] G. L. Asleson, L. J. Stoel, E. C. Newman and C. W. Frank, *J. Pharm. Sci.* **63**, 1144–1146 (1974).
[5] A. Soder and W. Siedel, German Patent 1,091,564, 27 Oct. 1960; *Chem. Abstr.* **55**, 19882g (1961).
[6] L. M. Jackman and S. Sternhell, *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*, 2nd Edn. Pergamon Press, Oxford, 1969.
[7] J. I. Musher and E. J. Corey, *Tetrahedron* **18**, 791 (1962).
[8] E. D. Becker, *J. Chem. Educ.* **42**, 591–596 (1965).
[9] C. R. Stevens, J. J. Beereboom, H. H. Rennhard, P. N. Gordon, K. Murai, R. K. Blackwood and M. S. von Wittenau, *J. Am. Chem. Soc.* **85**, 2643–2652 (1963).
[10] A. F. Casy, *PMR Spectroscopy in Medicinal and Biological Chemistry*. Academic Press, London, 1971 (a) p. 58; (b) p. 398; (c) p. 87.
[11] S.-C. Wu, M.Sc. Thesis, University of Alberta, 1970.
[12] D. E. Williamson and G. W. Everett, Jr., *J. Am. Chem. Soc.* **97**, 2397–2405 (1975).
[13] R. J. Abraham and P. Loftus, *Proton and Carbon-13 NMR Spectroscopy*. Heyden, London, 1980.
[14] J. L. Colaizzi and P. R. Klink, *J. Pharm. Sci.* **58**, 1184–1189 (1969).
[15] P. V. Demarco, E. Farkas, D. Doddrell, B. L. Mylari and E. Wenkert, *J. Am. Chem. Soc.* **90**, 5480–5486 (1968).
[16] E. Wenkert and B. L. Mylari, *J. Am. Chem. Soc.* **89**, 174–176 (1967).
[17] J. Donohue, J. D. Dunitz, K. N. Trueblood and M. S. Webster, *J. Am. Chem. Soc.* **85**, 851–856 (1963).
[18] H. Cid-Dresdner, *Z. Kristallogr. Kristallogeom. Krystallphys. Krystallchem.* **121**, 170–189 (1968).
[19] K. Kamiya, M. Asai, Y. Wada and M. Nishikawa, *Experientia* **27**, 363–365 (1971).
[20] S. Sternhell, *Q. Rev.* **23**, 236–270 (1969).
[21] M. S. von Wittenau, R. K. Blackwood, L. H. Conover, R. H. Glauert and R. B. Woodward, *J. Am. Chem. Soc.* **87**, 134–135 (1965).
[22] F. Barbatschi, M. Dann, J. H. Martin, P. A. Miller, L. Mitscher and N. Bohonos, *Experientia* **21**, 162–163 (1965).

[*Received for review 8 June 1982; revised manuscript received 20 August 1982*]