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Review

Application of ^{13}C nuclear magnetic resonance spectroscopy to the analysis and structural investigation of tetracycline antibiotics and their common impurities

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Abstract: The spectral assignations of all the main resonances in the ^{13}C nuclear magnetic resonance spectra in DMSO-d_6 or D_2O are presented and reviewed for ten of the principal tetracyclines in therapeutic use: tetracycline, chlortetracycline, 6-demethylchlortetracycline, minocycline, oxytetracycline, methacycline, meclocycline, doxycycline, rolitetracycline and lymecycline. NMR techniques employed include: proton noise-decoupled spectra, off-resonance decoupled spectra, nuclear Overhauser enhancement and the INEPT technique (insensitive nuclei enhanced by polarization transfer). ^{13}C NMR is also examined as a means of detecting and identifying the principal degradation and isomerization products of tetracycline antibiotics. The analytical potential of ^{13}C NMR is discussed with respect to quantitative analysis of impurities, studies on sites of protonation and metal-binding studies.

Keywords: *^{13}C NMR spectroscopy; tetracycline antibiotics; isomeric impurities; degradation products.*

Introduction

The value of ^{13}C nuclear magnetic resonance (NMR) spectroscopy in differentiating groups of closely related compounds is well known [1, 2]. Its specificity in this respect is particularly appropriate to the identification and analysis of tetracycline antibiotics. About ten derivatives are all based on the molecular framework and functionality array **1**. This so dominates their appearance and physical properties, that electronic and vibrational spectra within the group are similar [3] and therefore of little value for the unambiguous characterization of individual members. Similar considerations apply to impurities, degradation and isomerization products of tetracyclines, the presence of

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which may seriously impair therapeutic efficacy. Although some NMR studies of tetracycline antibiotics based on ^{1}H and ^{13}C data have been reported, these are relatively few in number, considering the medicinal importance of the compounds. The ^{1}H work has recently been reviewed and extended [4]. It is the intention of the present review, therefore, to make a comparative examination of ^{13}C NMR spectral information on the tetracyclines in order to draw attention to its analytical potential and to its capability of providing evidence about fine points of structure, such as those relating to ionic equilibria and stereochemistry.

^{13}C NMR investigations have only become generally possible since the advent of commercial Fourier transform (FT) spectrophotometers, capable of overcoming the problems of reduced sensitivity of ^{13}C signals compared with ^{1}H NMR. These are attributable to the low (1%) natural abundance of ^{13}C and to its smaller magnetic moment. In the present review, published data are supplemented by original data obtained in the authors' laboratory, recorded to provide spectra under standard instrumental and solute-state conditions.

Materials and Methods

All the previously unpublished spectral data reported in the present work were obtained using a Jeol FX90Q FT NMR spectrometer, operating at 22.5 MHz. Samples were presented in 5 or 10 mm (o.d.) tubes as approximately 10% solutions in deuterated dimethylsulphoxide (DMSO-d_6) with 2% tetramethylsilane (TMS) as reference, or as solutions in D_2O (sometimes water) with dioxane as internal reference. The deuterium of the solvent generates the lock signal. Solutions were not degassed because such treatment does not usually significantly affect the spectra in this series of compounds.

DMSO-d_6 is of value as a common solvent for all compounds (HCl salts and bases), but has the disadvantage of giving a ^{13}C resonance band (septet centred on 39.5 ppm), which obscures several tetracycline signals, notably those due to C_4-NMe_2 and C_{5a}. Resolution in this region may be improved by recording spectra in protonated DMSO (one signal near 40 ppm) or in water, since many compounds precipitate from D_2O during spectral accumulation. When the latter solvents are used, a capillary tube of D_2O is included to provide the lock signal.

Proton noise-decoupled ^{13}C NMR spectra, supplemented by off-resonance (OFR) decoupled spectra and a gated-decoupling programme that retains the nuclear Overhauser enhancement (NOE) to recover fully proton-coupled spectra, were commonly collected in the work reported here. The latter two techniques aid identification of methyl, methylene, methine and quaternary carbons. Differentiation of these carbon atoms has been facilitated most recently by a technique described as INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) and related pulse sequences that employ various fractions of the average coupling constants $^{1}J(^{1}H/^{13}C)$ [5].

In certain cases spectral assignments have been corroborated by consideration of spin-lattice relaxation times (T_1), as measured by the inversion recovery method employing the sequence (180° pulse — delay τ — 90° pulse), each cycle being interrupted by a pulse delay of about 10 s to allow full relaxation [6]. NOE factors, which provide evidence of the mechanism of relaxation, were determined by comparing the resonance intensities after an appropriate number of scans of spectra (typically 2000), recorded under proton noise-decoupling and gated-decoupling conditions, both with use of a 90° pulse and 50 s pulse delay [7]. Signals that have intensity ratios near three can be assumed to derive

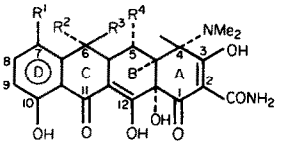
from carbons that relax predominantly by dipole–dipole (DD) mechanisms. In such cases, individual T_{1DD} values depend upon the sixth power of the distance of the carbon nucleus from nearby protons; hence the relative magnitudes of T_{1DD} values for quaternary carbons can be related to the counts of β- and γ-protons, thereby aiding spectral assignments [8].

The principal sources of additional data considered in this review were Asleson and Frank [9], who employed a 22.63 MHz Bruker HX90E spectrometer, and Mazzola *et al.* [10], who used a Varian CFT-20 instrument operating at 20 MHz.

Results and Discussion

^{13}C NMR chemical shifts and spin-lattice relaxation time data are given in Tables 1 and 2 respectively, which comprise or summarize the results of the authors and of several investigators [9, 10]. The molecular structures, numbering system, generic names and abbreviations used for the compounds are tabulated with **1**. For convenience, reference

Scheme 1



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Bridgehead carbons designated by 4a, 5a etc.

	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	$C_6{=}CH_2$		OH	Methacycline	—
1g	Cl	$C_6{=}CH_2$		OH	Meclocycline	—
1h	H	Me	H	OH	Doxycycline	—
1i	Replace C_2-$CONH_2$ in **1a** by $CONHCH_2NC_4H_8$				Rolitetracycline	—
1j	Complex of **1a** with CH_2O and lysine				Lymecycline	—

data (spectral parameters of model compounds, etc.) of importance to the ^{13}C spectral assignment of the tetracyclines are presented at the end of this review. Evidence of assignments and points of potential analytical utility will be outlined below.

Tetracycline hydrochloride

Because of the central role played by TC HCl (**1a** HCl) in the ^{13}C NMR studies, and as an insight into assignment methodology used in the field, the original arguments of Asleson and Franks [9] are presented here in some detail, together with some more recent evidence. Once assignment of the parent compound is established, the spectra of analogues may be assigned on the basis of similarities to, and differences from, the standard spectrum.

Resonances due to 19 of the 21 different types of carbon are apparent in the ^{13}C NMR

Table 1
^{13}C chemical shifts of some tetracycline derivatives[a]

Position	**1a**(TC)	**1a**[c]	**1a** base	**1b**(CTC)	**1c**[e]	**1d**[f]	**1e**(OTC)	**1e**[c]
1	192.9	193.8	192.3	192.0	191.6	192.9	192.9	193.6
2	95.7	97.5	98.5	95.8	95.7	95.6	95.4	97.7
3	187.2	187.2	191.8	187.3	187.2	187.4	187.1	187.2
4	67.9	70.4	69.8	68.9	67.9	68.0	64.9	67.4
4a	35.3	35.4	37.4	35.9	35.1	34.3	42[b]	43.3
5	26.9	26.9	22.5	27.6	28.2	29.6	63.2	65.9
5a	42[b]	42.3	40.8[b]	42[b]	36.6	31.5	49.8	51.2
6	67.9	70.7	68.1	70.5	64.8	33.7	68.9	71.2
6a	147.9	146.9	148.0	143.6	140.6	142.0	148.7	147.7
7	116.9	118.7	116.9	121.1	122.1	136.5	118.9	118.7
8	136.5	138.5	136.4	139.7	136.9	128.5	136.6	138.4
9	115.2	116.8	114.5	120.6	118.9	114.8	114.9	116.7
10	161.3	162.0	161.4	160.7	160.0	157.4	161.2	162.6
10a	114.4	114.9	114.4	117.0	115.8	116.0	114.4	115.2
11	193.4	193.8	192.9	193.3	193.3	193.6	193.7	194.6
11a	106.8	107.1	105.9	106.1	105.1	108.2	105.3	105.6
12	175.0	174.1	176.8	175.8	176.1	174.3	173.5	173.5
12a	73.1	74.4	74.3	73.3	73.7	73.9	72.5	74.0
C_2-$CONH_2$	172.1	173.5	172.4	172.1	171.9	171.8	171.9	172.5
C_4-NMe_2	42[b]	43.3[d]	42.4[b]	42.8[b]	42[b]	41.2[b,g]	41.8[b]	43.6
C_6-Me	22.5	22.3	22.8	25.1	—	—	24.6	24.5

Position	**1f**	**1g**[k] base	**1h**[j]	**1h**[j]	C_6-epi-**1h**	**1i**[k,m]
1	192.1	190.3	192.6	192.4	192.5	191.8
2	95.0	98.8	95.2	96.2	95.0	101.2
3	187.3	187.1	187.4	187.5	187.2	189.6
4	65.3	64.9	68.1	69.5	64.7	72.5
4a	41[b]	40	41[b]	39.4	32.9	38.6
5	64.1	65.5	64.6	66.4	63.8	22.5
5a	44.2	45.4	45.2	46.9	43.3	41.6
6	140.6[h]	136.9[h]	41	42.3	41.2[l]	68.0
6a	142.6	138.6	147.7	148.4	150.0	148.0
7	117.3	119.6	115.5	116.7	118.7	116.7
8	137.0	137.8	136.7	137.3	137.1	135.9
9	116.4	118.3	115.5	116.3	115.6	115.1
10	160.7	159.8	161.1	162.7	161.3	161.4
10a	114.4	116.6	115.4	116.3	114.3	114.5
11	193.5	191.3	193.6	194.8	193.7	192.0
11a	105.2	105.4	107.2	107.8	103.5	105.3
12	173.6	178.2	173.5	173.6	174.8	180.1
12a	73.5	75.4	73.0	74.0	73.8	74.8
C_2-$CONH_2$	171.6	170.5	171.7	172.4	171.6	168.3
C_4-NMe_2	41[b]	41.7	41[b]	42.9	41.2[l]	42.3
C_6-Me	113.7[h]	117.7[h]	15.8	15.8	16.6	22.9

Table 1 (continued)
^{13}C chemical shifts of some tetracycline derivatives[a]

Position	C_4-epi-**1a**	**7**(anhydro-TC)	**8**(β-apo-OTC) base	**8**(α-apo-OTC) base
1	191.3	192.8	195.6	195.1
2	95.5	97.8	98.3	98.9
3	187.8	187.4	197.6	191.2
4	66.0	66.9	63.5	63.0
4a	43.3	42[b]	45.0	34.4
5	19.8	28.7	70.9	69.4
5a	42[b]	121.6	143.4	141.6
6	68.0	130.5	114.9	113.4
6a	148.0	138.8	138.4	138.9
7	117.5	114.9	115.3	115.9
8	136.5	132.4	129.6	130.0
9	115.9	111.3	113.9	111.8
10	161.4	157.9	156.3	162.4
10a	114.5	112.1	105.2	102.5
11	193.0	163.6	154.7	159.8
11a	106.6	108.6	108.9	108.9
12	175.1	199.3	168.3	168.2
12a	73.7	76.2	76.0	75.5
C_2-$CONH_2$	172.9	172.1	173.1	172.6
C_4-NMe_2	42[b]	42[b]	42[b]	42[b]
C_6-Me	22.4	14.1	13.8	14.0

[a] Values are reported in ppm from tetramethylsilane and in solvent DMSO-d_6, unless otherwise stated; most data are from the authors' collection of spectra, accumulated using 1500 to 2000 pulses and correspond closely with those of previous reports [9, 10]. Some assignments of signals of the same multiplicity and similar chemical shift may be interchanged. Samples were examined as HCl salts unless otherwise stated.

[b] Signal within solvent multiplet.

[c] In H_2O(D_2O capillary) with dioxane (67.4 ppm) or methanol (50.0 ppm) as internal reference.

[d] Intensity > twice that of resonance at 42.3 ppm.

[e] The four highest field signals were clearly resolved in D_2O (MeOH as reference): 43.3 (highest intensity), 38.5, 36.8 and 28.8 ppm.

[f] The six highest field signals were clearly resolved in MeOH-D_2O (dioxane as reference); the lowest field pair of the set (45.0, 42.4 ppm) had equal intensities that were several times greater than those of the remaining peaks.

[g] C_7-NMe_2 signal at 44.4 ppm.

[h] Alkenic carbon.

[i] Ethanol resonances: Me (18.4 ppm), CH_2 (56.0 ppm).

[j] In methanol (D_2O capillary).

[k] cf. [10].

[l] C_6 and NMe_2 signals overlap.

[m] Additional signals: 23.6(β) and 50.1 ppm (α) pyrrolidine carbons, 56.6 ppm CH_2N.

Table 2
Spin-lattice relaxation times (T_1) of some tetracycline derivatives

Carbon[a]	TC HCl T_1(s)(DMSO-d_6)[b]	TC HCl T_1(s)(H_2O)	OTC HCl T_1(s)(H_2O)	Doxycycline HCl[d] T_1(s)(DMSO-d_6)
11	2.27	3.03	2.40	1.60
1	2.20	3.03	1.97	1.53
3	1.48	2.63	1.32	1.06
12	1.30	2.35	1.55	1.02
$CONH_2$	0.87	1.85	1.01	0.58
10	1.27	2.17	1.29	0.93
6a	2.17	3.20	2.32	1.00
8	0.09	0.14	0.09	0.05
7	0.09	0.17	0.08	0.02[e]
9	0.09	0.15	0.09	0.02[e]
10a	3.90	4.58	3.07	2.22
11a	2.09	2.97	1.82	1.29
2	2.83	3.77	2.54	1.37
12a	1.54	2.39	1.44	1.07
4	0.18	0.21	0.09	0.04
6	1.06	2.22	1.02	0.05[c]
5a	0.87	2.53	0.09	0.07
NMe_2	c	0.16	0.33	0.06[c]
4a	c	0.13	0.10	0.07[c]
5	c	0.16	0.07	0.05
C_6-Me	0.12	0.20	0.06	0.07

[a] Arranged (with some exceptions for OTC and doxycycline) in order of decreasing chemical shift.
[b] Values reported by Asleson [22] are shorter, e.g. 1.9, 1.8, 1.36 s for C_{11}, C_2, and C_3 respectively, but show comparable variations.
[c] Signals overlap solvent band and some were not recorded.
[d] Ethanol of crystallization resonances (56.0 and 18.4 ppm) were both characterized by T_1 = 3.5 s.
[e] Calculated from point of zero intensity.

spectrum of TC HCl run in DMSO-d_6, and of these, only two overlap (C_4 and C_6). The solvent band obscures the missing pair (NMe_2 and C_{5a}) as is apparent from a spectrum run in water. The spectra in Fig. 1 well demonstrate the superior resolving power of ^{13}C relative to ^1H NMR spectroscopy [11], which results from the approximately ten-fold wider spread of chemical shift values of ^{13}C nuclei. Off-resonance (OFR) multiplets provide evidence of C_6-Me and C_4-NMe_2 (quartet), C_5 (triplet) and C_4, C_{4a}, C_{5a}, C_7, C_8 and C_9 (doublet) assignments.

All resonances due to protonated carbons are broad and of low intensity compared with most quaternary carbon signals, a fact attributed to the very fast relaxation rates of protonated carbons (Table 2), which operate in the viscous solutions necessary for spectral measurements. These resonances are narrower and more intense in spectra of aqueous solutions of reduced viscosity. This observation is common to all tetracycline spectra, and may be put to advantage in that it allows the ready identification of the C_7, C_8 and C_9 resonances of ring D (typified in Fig. 1). Within this trio, phenolic models (ref. [9] and Table 3) clearly allow assignment of C_8 (136.5 ppm), but C_7 and C_9 shifts are close and may be interchanged.

Of the three resonances assigned to C_4, C_{4a} and C_{5a} (OFR doublet), that at lowest field (67.9 ppm) is assigned to C_4 since this carbon is subject to α-deshielding by nitrogen and to several α/β affects due to surrounding carbon atoms. C_{4a} and C_{5a} shift assignations

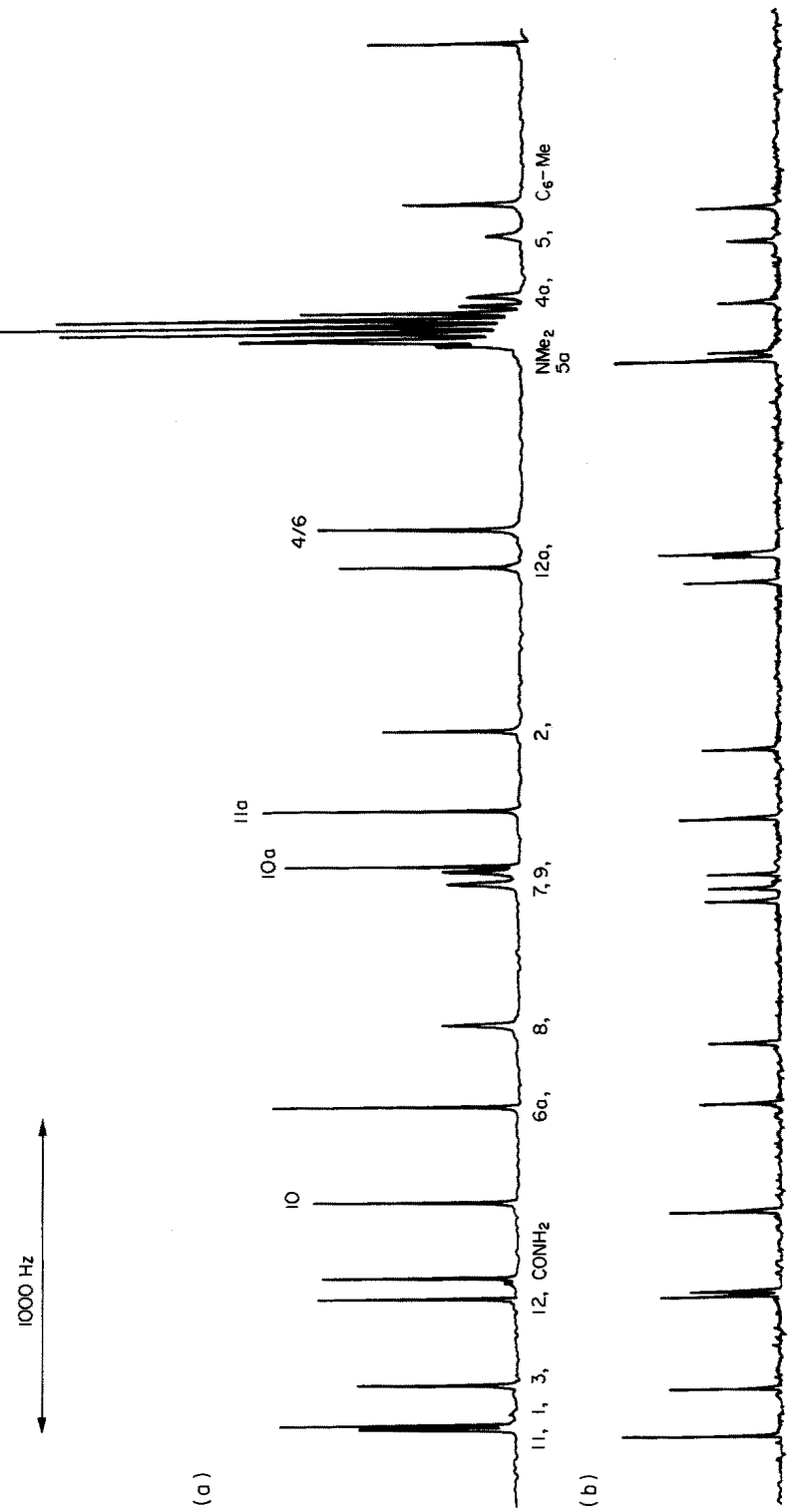


Figure 1
Proton noise-decoupled ^{13}C NMR spectrum of tetracycline hydrochloride in (a) DMSO-d_6 and (b) H_2O. The assignments given apply to both spectra. The broad nature and relatively low intensities of resonances C_8, C_7, C_9, C_{4a} and C_5 (due to protonated carbons) in spectrum (a) should be noted, as should the fact that disparities in signal intensities of resonances C_{6a} through C_2 are much smaller in spectrum (b). The NMe_2 and C_{5a} resonances, obscured by the solvent multiplet in spectrum (a), are clearly resolved in spectrum (b).

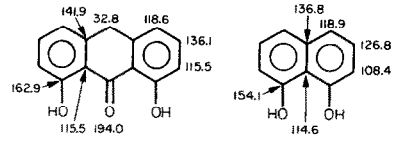
follow from comparisons with corresponding shifts of 6-demethyl CTC, the C_{5a} signal being the more sensitive to removal of C_6-methyl.

The major assignment problem concerns the 12 quaternary carbon signals. Resonances at 67.9 and 73.1 are assigned to C_6 (overlaps C_4 signal) and C_{12a} (saturated carbons linked to oxygen) respectively, the lower field shift being little altered in 6-deoxy OTC, 6-demethyl CTC and methacycline. Measurements of T_1 are supportive, the C_6 signal showing the lower value in accord with its richer proton environment (Table 2). Assignment of the lowest field pair of resonances (C_{11}, 193.4 ppm and C_1, 192.9 ppm) is made by reference to model data on α,β -unsaturated ketones. When monitoring signals as a function of pH, the C_1 signal and that at 187.2 ppm shift at pH 3, while the C_{11} signal does not alter until pH 7 is reached [12].* The first pK_a of TC is associated with the A ring system, as discussed below [13]. The same pH study allows assignment of the next higher field resonance (187.2 ppm) to C_3, as confirmed by comparative T_1 values: C_{11} 2.27 s, C_1 2.2 s, C_3 1.48 s in DMSO-d_6 (Table 2).

By reference to models the amide carbonyl resonance is located at either 172.1 or 175 ppm; the higher field value is chosen on the basis of T_1 measurements. The resonance at 161.3 ppm, typical of the C–O bond in phenols with ortho-carbon substituents, is attributed to C_{10}, while C_{12} (another unsaturated carbon linked to oxygen) must give rise to the 175 ppm signal.

The last four unassigned resonances are attributable to unsaturated carbons unattached to oxygen (C_2, C_{6a}, C_{10a} and C_{11a}). The highest field signal of this group (95.7 ppm) is due to C_2, according to chemical shift data on models including the 2-cyano analogue of TC, since the C_2 signal shifts upfield by 12 ppm when the amide group is dehydrated. The C_{6a} assignment also rests on comparisons with models, such as dithranol† (**2**), and on the sensitivity of the C_{6a} resonance to structural changes at C_6. The same model (**2**), together with 1,8-dihydroxynaphthalene (**3**), provides evidence for the C_{10a} assignment at 114.4 ppm.

By elimination, the 106.8 ppm resonance is attributable to C_{11a}. Relaxation time measurements of this quartet support these assignments, in that lower T_1 values are found for the C_{11a} and C_{6a} carbons (each with one β-hydrogen) than for the C_2 and C_{10a} carbons, which lack β-hydrogens (Table 2).



2 (in $CDCl_3$) **3**

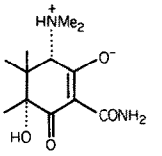
Tetracycline base

Most chemical shift values for TC base (**1a**) carbons are very close to those of the hydrochloride salt. The fact that the NMe_2 carbon resonance undergoes no significant downfield shift when the salt is converted to the free base [14, 15], coupled with the downfield shift (+4.6 ppm) of the C_3 signal, since α-deshielding by a phenolic function is

* Data in [12] for C_1 and C_{12} of Fig. 2, and for C_8 and C_{10} of Fig. 7 have been erroneously interchanged.

† Chemical shifts shown on formulae are expressed in ppm relative to TMS in DMSO-d_6, unless otherwise stated.

enhanced after ionization [1], is evidence that the zwitterion **4** is a significantly populated species of the base in DMSO-d_6. This is in agreement with potentiomeric titration data [16] and ^{1}H NMR data [4].



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Chlortetracycline hydrochloride

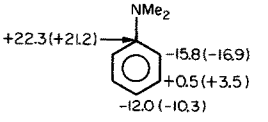
The chief difference between TC and CTC HCl (**1b** HCl) spectra is the replacement of the C_7 OFR doublet at 116.9 ppm of TC by a singlet at 121.1 ppm, in accord with the presence of a chloro-substituent at C_7. Resonances of carbons in the vicinity of C_7 are also affected by chlorination in approximate agreement with the shielding parameters of chlorine in aromatic systems ([1] and Table 3). One result of this is the resolution of the C_4 and C_6 signals which overlap in the spectrum of TC HCl.

6-Demethylchlortetracycline hydrochloride

This derivative (**1c** HCl) differs from CTC solely in the absence of a methyl substituent at C_6, so that only two resonances appear upfield of the solvent band (the C_6-Me OFR quartet is absent). Shifts of the C_6, C_{6a}, C_{5a} (upfield) and C_5, C_7 (downfield) signals relative to corresponding CTC resonances may be explained by removal of the α/β- and γ-shielding influences of the methyl group respectively.

Minocycline hydrochloride

Apart from its additional dimethylamino substituent (at C_7), this derivative (**1d** HCl) represents a further simplification of TC HCl in that it lacks both methyl and hydroxyl substituents at C_6. The spectrum shows four broad signals to high field, and 16 signals to low field of the solvent multiplet, plus one within this band. The six highest field signals are well resolved in the spectrum run in methanol-D_2O as solvent. In contrast to a previous report [10], resonances at 44.4 and 41.2 ppm of the group are assigned to the C_7- and C_4-NMe_2 carbons, respectively, on the basis of the protonated state of the more basic 4-amino group, since the α-N-protonation shift is negative [14, 15]. The C_6 resonance of TC HCl suffers a pronounced upfield shift (about 36 ppm) as a result of the absence of C_6 substituents, which also accounts for the shifts of C_{5a} (upfield) and C_5 (downfield). Reordering of some of the lower-field resonances of TC following insertion of a C_7-NMe_2 group must occur as a result of the major shielding influences of this substituent (cf. **5**).



Shielding parameters in ppm relative to benzene and, in parentheses, benzaldehyde

5

Assignments of carbons of ring D have been made that accord best with the combination of C_7-NMe_2 and C_{10}-OH shielding effects. These indicate that shielding magnitudes at carbons *o*- and *p*- to C-NMe_2 are attenuated in the tetracycline as compared with values found in the monocyclic aromatic models.

Analysis of the four highest field resonances gives difficulty because their close placing leads to overlap of OFR multiplicities. The INEPT programme, however, is admirably suited to a problem such as this, since it yields a spectrum (Fig. 2) which clearly shows resonances at 34.3 and 31.5 ppm to be due to methine, and those at 33.7 and 29.6 ppm to be methylene carbons. Moreover, the solvent band is much reduced in intensity, revealing the two NMe_2 resonances.

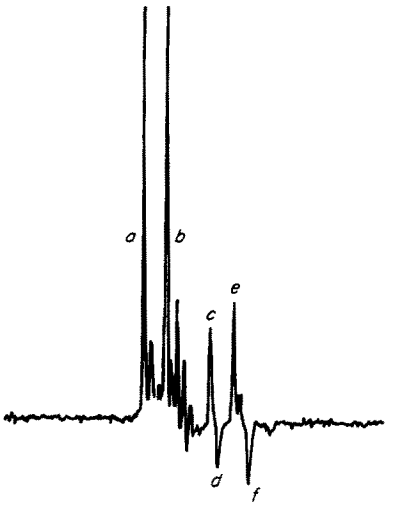


Figure 2
INEPT ^{13}C NMR spectrum of minocycline HCl in DMSO-d_6 (0–50 ppm region, CH, CH_3 signals positive, CH_2 signals negative). Assignments: *a* 44.4 ppm, C_7-NMe_2; *b* 41.2 ppm, C_4-NMe_2; *c* 34.3 ppm, C_{4a} (CH); *d* 33.7 ppm, $C_6(CH_2)$; *e* 31.5 ppm, C_{5a}(CH); *f* 29.6 ppm, $C_5(CH_2)$.

Oxytetracycline hydrochloride

The spectrum of this derivative (**1e** HCl), together with that of its relatives **1f–1h**, lacks the C_5 methylene resonance near 27 ppm (OFR triplet), present in all the spectra discussed so far. Instead, these spectra display a lower field resonance near 63 ppm (OFR doublet), a change due to the common C_5-OH substituent of the group. In this series the C_{4a} and C_{5a} signals also show downfield shifts relative to the corresponding TC values, although these are smaller in magnitude, as would be anticipated from a β-parameter. Spin-lattice relaxation times (Table 2) of OTC carbons support assignments as in the case of TC; cf. for example comparative T_1 values of C_6 and C_{12a}, and C_1, C_3 and C_{11}.

Methacycline and meclocycline hydrochlorides

The hydrochloride of methacycline (**1f** HCl) and meclocycline (**1g**) base differ from OTC in having a methylene function attached to C_6. Their spectra are notable amongst the rest of the group in having no signals upfield of the solvent band. The C_7, C_9 and C_{10a} trio of signals near 115 ppm of the OTC spectrum are duplicated and joined by a fourth resonance in the same region (OFR triplet) assigned to the methylene carbon. The C_6 signal of OTC moves downfield by about 70 ppm following the change from sp^3 to sp^2 carbon, while C_{6a} (no longer subject to β-deshielding by oxygen) moves upfield. The

other resonances have similar chemical shifts to those of OTC. Chemical shift differences between C_2, C_6 and the C_6-methylene carbons serve to distinguish the two derivatives (cf. Table 3).

Doxycycline hydrochloride and its 6-β-methyl epimer

The spectrum of doxycycline (**1h** HCl) displays 23 signals as compared with 21 for OTC. The extra two are associated with the ethanol of crystallization (18.4 ppm quartet; 56.0 ppm triplet); three overlap near 115 ppm and three are masked by the solvent band. The absence of the C_6-OH substituent is reflected in the upfield shifts of C_6 and neighbouring carbon resonances, most pronounced for C_6 (−28 ppm), moderate for C_6-methyl (−8.8 ppm) and C_{5a} (−4.6 ppm) by comparison with corresponding shifts of OTC. The near coincidence of the C_7, C_9 and C_{10a} resonances is a notable feature of the spectrum.

Spin-lattice relaxation data (Table 2) are useful in confirming the ethanol assignments; these carbons have the longest T_1 values of the set (3.5 s). The remaining results accord with the assignments in the usual way. The C_{10} and C_{6a} carbons have comparable relaxation times, unlike the same carbons of TC and OTC, because replacement of C_6-OH by hydrogen makes the proton environment of the two carbons similar.

The C_6 epimer of doxycycline (C_6-epi-**1h**) is a probable impurity since catalytic reduction of its precursor (methacycline) is not stereospecific [17]. The spectrum of the C_6 epimer of doxycycline hydrochloride differs notably from that of the more active antibiotic in: (1) clear resolution of C_7, C_9 and C_{10a} resonance bands; (2) reduced separation of C_4 and C_5 signals ($\Delta\delta$ 0.6 ppm, 3.5 ppm for doxycycline); (3) resolution of the C_{4a} resonance to high field of the solvent band; and (4) absence of ethanol resonances. Thus the presence of the C_6-epimer in a doxycycline sample may be readily detected from its ^{13}C NMR spectrum. Furthermore, the absence of such diagnostic signals is evidence of isomeric purity, as was the case for the sample reported here. Most chemical shift differences between corresponding carbons of doxycycline and its epimer are consistent with those anticipated from replacement of an α- by a β-C_6-methyl. For example, upfield shifts of C_{10a} and C_{11a} resonances due to γ-shielding (steric polarization), are observed. Upfield shifts at C_4 (3–4 ppm) and C_{4a} (8.1 ppm), however, cannot be so explained and may be due to conformational changes.

Rolitetracycline and lymecycline

Rolitetracycline (**1i**) and lymecycline (**1j**) are both Mannich bases formed from tetracycline, formaldehyde and pyrrolidine or lysine. The spectrum of rolitetracycline is very similar to that of TC base, but has the distinguishing feature of additional signals at 56.6, 50.1 and 23.6 ppm due to the pyrrolidino-methylene unit. An attempt to record the spectrum of lymecycline in D_2O (it is insoluble in DMSO-d_6) at the usual concentration (50–60 mg in 0.5 ml solvent) failed because of the rapid formation of a precipitate from the initially clear solution. The spectrum of the supernatant liquid displayed six resonances characteristic of lysine (176, 55.9, 40.5, 31.3, 27.7 and 22.7 ppm) [18], plus one extra resonance (35.9 ppm) probably due to hydrated formaldehyde. However, the spectrum of the precipitate in DMSO-d_6 showed the material to be TC base. It proved possible to record a spectrum of a more dilute solution (4 mg in 0.5 ml D_2O stored for 24 h) at higher frequency (100.6 MHz) which displayed all the lysine and many of the TC signals. It is evident that lymecycline is a highly unstable complex that rapidly reverts to its component parts on dissolution.

Common impurities of TC and OTC

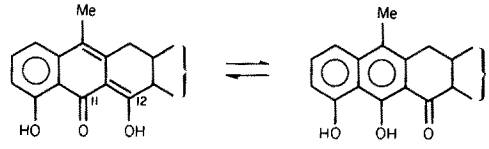
4-Epitetracycline hydrochloride. In acidic media TC epimerizes to give the inactive 4-epimer (C_4-epi-**1a**) which differs from the parent in having a *trans* rather than a *cis* C_4-NMe_2/C_{12a}-OH configuration [3]. Chemical shift differences in the spectra of TC and C_4-epi-TC HCl are thus only expected for carbons in the region of the epimerization centre. In fact, all signals to low field of 65 ppm are similar in the spectra of the two isomers, except that the C_4 and C_6 resonances are resolved in the C_4-epi-TC spectrum. The C_5 resonance becomes the highest field signal after epimerization, while that of C_{4a} moves to low field of the solvent multiplet. The upfield shift of C_5 may be explained as due to the γ-shielding influence of NMe_2, since the position of this group now makes it γ-gauche to C_5 (see **6**). Steric factors may also account for the shift of the C_{4a} signal. The presence of C_4-epi-TC in a TC spectrum is therefore best detected by the appearance of a resonance upfield of the 22.5 ppm C_6-methyl signal.

View along C_4–C_{4a} bond of half-chair conformation or ring A

6

Anhydrotetracycline hydrochloride. This dehydration product of TC HCl (**7** HCl), a common impurity of the antibiotic formed together with C_4-epi-TC in media of acidic pH [3], may be formulated as the tautomeric mixture **7**; ^{13}C NMR data indicate structure **7b** with both rings C and D aromatic to be the principal component as discussed below.

Partial structures: ring A not shown



7

The proton noise-decoupled spectrum of anhydro-TC HCl displays the required 21 signals (2 to high, 17 to low field and 2 within the solvent multiplet). Many carbons have similar environments to corresponding carbons of TC (e.g. C_1, C_3, C_{10} and $CONH_2$) and may be assigned by direct chemical shift comparisons. In **7b** C_{11} is phenolic (whereas in TC it is ketonic and assigned to the lowest field resonance) and is assigned to the 163.6 ppm signal (see below). Conversely, C_{12} of TC (enolic) becomes ketonic and is accordingly assigned to the lowest field resonance of the anhydro derivative at 199.3 ppm.

The two signals immediately upfield of the $CONH_2$ signal have shifts close to the C_{10} resonance of TC HCl and are thus linked to the C_{10} and C_{11} carbons, both of which are phenolic in **7b**. Since C_{11} is closer to the C_{12} carbonyl and does not possess β-hydrogens attached to carbon, the lower field signal (NOE spectrum, sharp singlet) is assigned to C_{11}, while the C_{10} chemical shift must be 157.9 ppm (NOE spectrum, narrow doublet due to one β C–H).

An upfield shift of the C_{6a} resonance is expected by comparison with that of TC HCl (147.9 ppm) as a result of the absence of β-deshielding by oxygen; data for 1,8-

dihydroxynaphthalene (**3**) supports a 138.8 ppm assignment. Carbon resonances C_7–C_9 in ring D may be identified by their broad, low intensity nature. Of the two resonances between the C_8 and C_7 signals, that at 130.5 ppm is assigned to C_6 (see **3** and *p*-cresol of Table 3) and that at 121.6 ppm to C_{5a} (both carbons are aliphatic in TC HCl). The broad nature of the lower field signal in the NOE spectrum is consistent with the resonance of a carbon with three β-hydrogens.

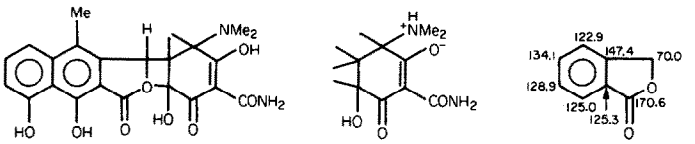
The remaining resonances may be all assigned from model data (e.g. C_{10a}, 112.1 ppm from **3**) and by direct comparison with the spectrum of TC HCl, since little difference in the environment of corresponding carbons is involved. Of the higher field resonances of TC HCl, that of C_{4a} moves downfield into the solvent multiplet, while the C_6-methyl signal moves upfield (absence of a α-deshielding by oxygen) after dehydration.

α- *and* β-*apo-oxytetracycline bases*. Under acidic conditions OTC degrades to α- and β-apo-oxytetracycline, products formulated as lactones **8** of unknown stereochemistry [19]. Samples were prepared in our laboratories. The spectrum of the major β-isomer displayed the required number of signals (19 to low, 1 to high and 1 within the solvent band), confirming that no loss of carbon had occurred during the transformation.

Spectral analysis is carried out in relation to the zwitterion structure **9** (the samples were isolated as free bases) containing a C_1, C_{12a}-ketol unit. Only one of the two lowest field resonances (197.6 and 195.6 ppm) can be due to a carbonyl carbon (C_1), because the chemical shift values are too great for the C_{12} (lactone) or amide carbonyl (see below). Assignment of one of these resonances to C_3 is appropriate, however, because an enolic carbon has a chemical shift greater than 190 ppm when ionized, as indicated by data on TC base and HCl discussed above.

The next two resonances to high field are assigned to $CONH_2$ (173.1 ppm) and the lactone carbonyl (168.3 ppm), a decision on the latter shift being aided by data for phthalide (**10**). Resonances at 156.3 and 154.7 ppm are attributed to the phenolic carbons C_{10} and C_{11} (cf shifts of analogous carbons of anhydrous-TC), while the C_7, C_8 and C_9 assignments follow in the usual way from the characteristic signal appearance (proton noise-decoupled spectrum, low intensity and broad; NOE doublet). Comparisons with model data (anhydro-TC and **10**) also lead to the assignment of the C_{5a} (143.4 ppm) and C_{6a} (138.4 ppm) resonances.

Assignment of the remaining quaternary carbon signals, except that of C_2 (98.2 ppm), presents some difficulty. Chemical shifts of the three signals still to be allocated (105.2 ppm and two near 115 ppm) are appropriate to C_{10a} and C_{11a} but seem too low for C_6 (130.6 ppm in anhydro-TC), unless the configuration about C_5 is such that C_6 is subject to steric polarization by the bulky ring A moiety (wherein the C_6 shift provides evidence of stereochemistry).



8 **9** (ring A only) **10**

The presence of four tertiary carbon (CH) signals (all doublets in the NOE spectrum) between the C_2 resonance and the solvent multiplet supports the ketol structure **8**. The three to lower field are assigned to C_4, C_5 and C_{12a} (all carbons linked to oxygen or

nitrogen), while the highest field signal must arise from C_{4a}. The 63.5 ppm resonance is probably due to the C_4 carbon by comparison with C_4 shifts of TC derivatives. The C_6 methyl signal (13.8 ppm) is close to that of anhydro-TC.

The spectrum of the α-isomer of **8** may be assigned by analogy. Differences in chemical shifts are mostly small and are most notable at C_3 (α, shifts to higher field), C_{10} and C_{11} (α, shifts to lower field) and C_{4a} (α, 34.4 ppm; β 45.0 ppm). An interpretation of ^1H and ^{13}C NMR spectral differences between the isomers **8** in terms of stereochemistry will be given elsewhere. Detection of apo-OTC impurities in the spectrum of an OTC sample is best made by observation of signals to high and low field respectively of the resonance extremes of the parent compound.

Analytical Potential of ^{13}C NMR Data for Tetracyclines

Identification and purity analysis

This presentation and analysis of chemical shift data demonstrates the specific nature of individual tetracycline ^{13}C NMR spectra. This fact allows the ready identification of a compound within the group by spectral examination as, for example, in Table 3, which is based primarily on the higher field resonances. ^{13}C NMR spectra complement related ^1H

Table 3
Procedure for the rapid identification of a tetracycline antibiotic[a] from its ^{13}C NMR spectrum (HCl salt or base in DMSO-d_6)[b]

Number of signals resolved by 1 ppm or more to high field of solvent multiplet (δ<36.5 ppm)	TC derivative	Additional diagnostic features
Nil	Methacycline	140.6 sharp C_6 113.7 broad C_6-CH_2 95.0 sharp C_2
	Meclocycline (base)	136.9 sharp C_6 117.7 broad C_6-CH_2 98.8 sharp C_2
One	OTC[c](24.6)	49.8(C_{5a})
	TC base (2 signals close to 22.7 ppm)	No signal near 50
Two	6-Demethyl CTC	Low field pair (35.1, 28.2), both broad
	Doxycycline[d]	High field pair, one sharp (18.4), one broad (15.8)
	Rolitetracycline (base)	Closely located pair of signals near 23 ppm, one broad due to two near-overlapping signals (22.5, 22.9) and one at 23.6.
Three	TC[e](35.3, 26.9, 22.5) CTC(35.9, 27.6, 25.1)	C_4/C_6 coincide at 67.9 C_4 68.9, C_6 70.5
Four	Minocycline	34.3, 33.7, 31.5, 29.9 all broad

[a] Within the group TC, CTC, 6-demethyl CTC, minocycline, OTC, methacycline, meclocycline, doxycycline and rolitetracycline.
[b] Proton noise-decoupled spectra; chemical shifts in ppm from tetramethylsilane.
[c] Low intensity signal near 13.8 is indicative of α/β-apo-OTC impurities.
[d] Low intensity signals at 16.6 (between the upfield doxycycline signals) and 32.9 are indicative of C_6-β-epi-doxycycline impurity.
[e] Low intensity signals at 19.8 and 14.1 are indicative of C_4-epi-TC and anhydro-TC impurities respectively.

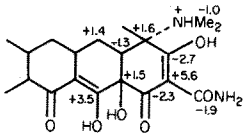
data [4] in the qualitative analysis of tetracycline antibiotics in the bulk state and provide superior information in certain cases, notably in the detection of products of degradation and isomerization.

The potential of ^{13}C NMR in relation to the quantitative analysis of pharmaceuticals and especially mixtures of TC antibiotics is far greater than for ^{1}H NMR [11], due to the occurrence of resonance signals unique to each derivative and free from overlap. Thus, in the spectrum of a 3:1 mixture of TC and OTC in DMSO-d_6 (Fig. 3), although many resonances coincide, several isolated lines characteristic of one or other of the pair of derivatives are observed which could readily serve as analytical signals. The intensity ratios are indicative of constituent concentrations and can, with due allowance for full relaxation of spin states between pulses and with the control and optimization of instrumental parameters [20], provide accurate quantitative information.

Studies on sites of protonation

Since chemical shifts of ^{1}H and ^{13}C nuclei close to acidic and basic centres of a molecule are sensitive to the degrees of ionization of such functions, NMR spectroscopy provides a valuable means of investigating sites of protonation. In the case of tetracycline hydrochloride the technique has been applied both to proton [21] and ^{13}C spectra [12, 22]. In each case chemical shift changes consequent upon raising the pH of a solution of the salt in a step-wise manner were recorded, when ^{13}C spectra provide the greater number of centres of observation. The data also allowed calculation of microscopic dissociation constants. The ^{13}C NMR study confirms the view that first-proton loss occurs within the tricarbonyl system of ring A (pK_a 4.4 in 1:1 D_2O–DMSO), in that major shifts observed over the pH range 1–6 were due to ring A and neighbouring carbons (cf. **11**). Over the pH range 6–13 the C_{10} resonance showed only a minor shift (+0.4 ppm) while the C_{12} signal moved 13 ppm downfield, evidence that proton loss associated with pK_a 9.7 took place chiefly at the C_{12} rather than C_{10}-hydroxyl, a reversal of conclusions of the proton study.

Chemical shift changes (ppm, + downfield, − upfield) of TC HCl carbons after a pH rise from 1 to 6 (partial formula)



II

Shift patterns (several resonances, notably those of amide CO, C_1 and C_3, gave C-shaped plots of pH *vs* shift) have yet to be satisfactorily explained in terms of shielding mechanisms. The C_8 resonance, well-removed from either the A or B (NMe_2) sites, was employed to measure the degree of protonation of the C site comprising rings C and D, and to aid calculation of the microscopic dissociation constants.

Metal-binding studies

Asleson and others [22, 23] obtained evidence for sites at which tetracycline binds metals by observing the differential broadening of ^{13}C resonance signals that occurred when a spectrum of TC HCl (at pH 8.9–9.4 in 1:1 D_2O–DMSO) was recorded in the presence of various metal ions (paramagnetic Cu^{2+} and Nd^{3+}; diamagnetic Ca^{2+} and Mg^{2+}). In general for all of the metal ions studied, an increase in the concentration of

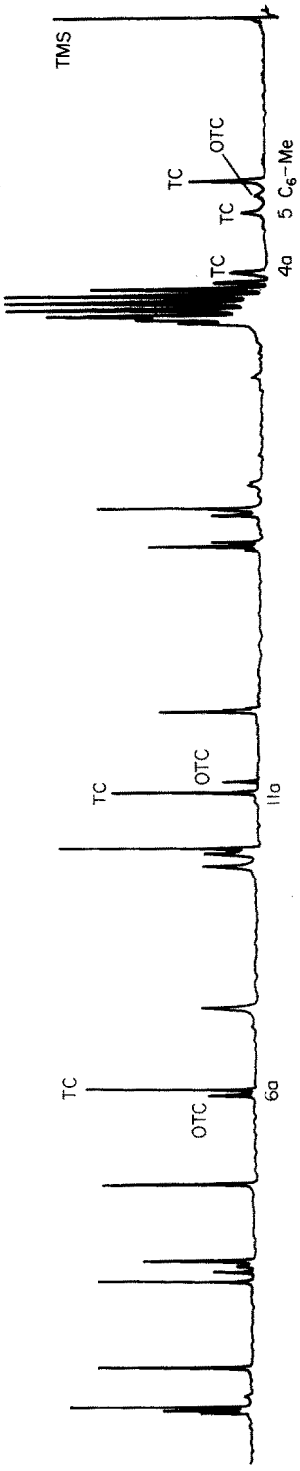


Figure 3
Proton noise-decoupled ^{13}C NMR spectrum of a 3:1 mixture of TC and OTC hydrochlorides in DMSO-d_6 (frequency scale as in Fig. 1). Resonances of potential analytical value are marked. In the corresponding 100 MHz ^1H NMR spectrum of the mixture, extensive overlap of TC and OTC resonances occurred and only the C_4-H signals were well resolved.

metal ion had the effect of decreasing the intensity of all the carbon signals of TC. All of the metal ions appeared to bind at the C_{11}, C_{12} site at lower pH, while secondary binding was seen in the A ring at higher pH values. No evidence of preferential binding was observed either from the C_{10} carbon or carbons near nitrogen, which suggests that binding at the C_{10}, C_{11} site or that involving basic nitrogen does not occur under the experimental conditions employed. Somewhat different results were reported by Gulbis and Everett [23, 24], who studied TC base in DMSO and found evidence that binding involved the functional groups of ring A.

Reference Data

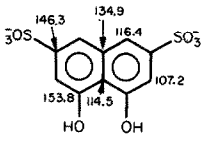
Many of the spectral assignments discussed in this review are based upon the ^{13}C chemical shifts of model compounds reported by Asleson and Frank [9], together with additional data summarized in this section on the basis that much of it is novel. ^{13}C chemical shifts of a series of simple benzene derivatives as solutes in DMSO-d_6 are given in Table 4. This work was considered necessary, as most literature values for the compounds refer to pure liquids or solutions in non-polar solvents. The results establish the shielding parameters of the 1,4-substituent combinations of OH–Cl, OH–Me and OH–NMe_2 required to aid assignments of ring D of the TC derivatives and ring C of anhydro-TC. Shifts are close to reported values [1] and in some cases differences may be related to hydrogen-bonding effects [25].

Table 4
^{13}C chemical shifts of some mono- and di-substituted benzene derivatives in DMSO-d_6[a]

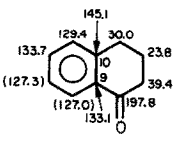
Substituent	C_1	C_2	C_3	C_4	C_5	C_6	Other
OH	156.8	115.9	129.9	120.3	129.9	115.9	
1-OH,4-Cl	156.4	117.1	129.2	123.1	129.2	117.1	
1-OH,4-Me	155.3	115.3	129.9	127.5	129.9	115.3	Me 20.2
NH_2	148.2	114.7	129.1	116.8	129.1	114.7	
NMe_2	150.7	112.6	128.9	116.4	128.9	112.6	Me_2 40.1
CHO	135.3	(128.2)	(127.8)	133.1	(127.8)	(128.2)	CO 191.3
1-NMe_2,4-CHO	154.3	111.1	131.5	125.0	131.5	111.1	Me_2 39.5 CO 189.6
1-OH,4-NH_2	148.6	115.8	115.8	140.4	115.8	115.8	
after NaOH	161.2	(119.5)	(119.0)	134.0	(119.0)	(119.5)	
SO_3^- calcium salt	147.1	(125.5)	(127.7)	128.9	(127.7)	(125.5)	
1-SO_3^-, 4-Me Na salt	144.7	(126.0)	(128.5)	138.7	(128.5)	(126.0)	Me 21.0
1-SO_3H, 4-Me	144.2	(128.4)	(131.8)	143.5	(131.8)	(128.4)	Me 23.5

[a] In ppm from tetramethylsilane (benzene 128.4 ppm); shifts in parentheses may be interchanged.

Shift data for dithranol, 1,8-dihydroxynaphthalene and other compounds are shown in formulae **2**, **3**, **12** and **13**, all as solutes in DMSO-d_6. Assignments for **3** and **12** (chromotropic acid) were made on the basis of the spectra of phenol and benzene sulphonic acid derivatives (Table 4). The ^{13}C NMR spectrum of dithranol (**2**) confirms its 1,8-dihydroxy-9-keto structure [26]. Assignments were based on the spectrum of 2-hydroxyacetophenone [1]. The chemical shifts of phthalide (**10**) serve as useful references for apo-derivatives of OTC and follow directly from those of α-tetralone (**13**) and the report of Hughes *et al.* [27]. The C_9 and C_{10} shifts of **13** were confirmed from T_1 relaxation time values because of conflicting assignments in the literature [28, 29].



12 (Na salt)



13 (in D_2O)

T_1 s
C_9 17.7 (1 β-H)
C_{10} 5.0 (3 β-H)

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