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METAL BINDING CHARACTERISTICS OF TETRACYCLINE DERIVATIVES IN DMSO SOLUTION

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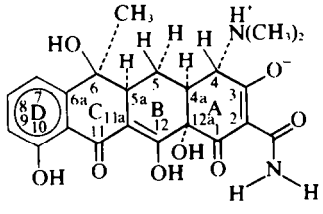
Abstract—A previous study of the site of metal binding in tetracycline has been extended to several derivatives of tetracycline in an effort to determine which specific functional groups are involved in binding to metal ions in DMSO solution and to explore relationships between antibacterial activity and metal binding characteristics. Proton NMR experiments using paramagnetic and diamagnetic lanthanide series ions as binding site probes indicate that the ring A tricarbonylmethane group is the binding site for tetracycline, 5-hydroxytetracycline, 4-epitetracycline and tetracyclinemethiodide in DMSO. No NMR evidence for metal binding is found for 4-dedimethylaminotetracycline. Binding to Mg^{2+} is also investigated by NMR for several of these compounds, and a discussion of conformational preferences of tetracycline derivatives in DMSO is presented.

The ability of antibiotics of the tetracycline series to bind metal ions has attracted interest for two decades. Work in this area has been stimulated by the possibility, yet unproven, that metal ions affect the biological action of tetracyclines by some means such as aiding transport or enhancing binding to bacterial ribosomes.[1-11] Tetracycline (**1**), hereafter abbreviated TC, offers a number of potential metal binding sites, and considerable effort has been made to determine which site or sites are primarily involved in metal binding.[12]



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A recent paper[13] from this laboratory describes a series of proton NMR experiments carried out on TC free base in DMSO-d_6 solution to which a series of paramagnetic and diamagnetic metal salts are added. Analysis of selective perturbations (shifting and broadening) of the TC NMR signals led to the conclusion that metal binding occurs through ring A functional groups. This has been subsequently corroborated by ^{13}C NMR studies.[14]

The NMR investigation has now been extended to several derivatives of TC in an effort to determine the specific functional groups involved and also to explore relationships between metal binding characteristics and antibiotic activity. Results of these studies in addition to a discussion of conformations adopted by TC derivatives in DMSO and use of praseodymium(III) as a metal ion probe are presented in this paper.

RESULTS AND DISCUSSION

Conformations of tetracycline derivatives in DMSO solution. Dreiding models show two limiting conformations for TC which may be interconverted by rotation about the C_{4a}–C_{12a} bond. These are shown schematically in Fig 1. In conformation A the 4-NMe_2 function is below the approximate plane described by rings B, C and D,

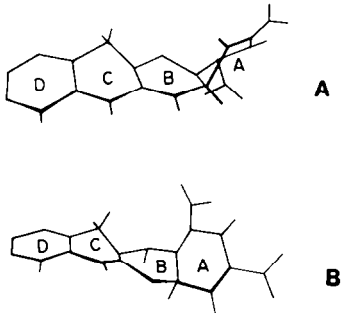

Fig. 1. Schematic representation of the limiting conformations of tetracyclines resulting from rotation about the C_{4a}–C_{12a} bond.

whereas in conformation B it is above this plane. Both these conformations have been found in X-ray crystallographic studies of tetracycline derivatives. The hydrochloride salts of 5-hydroxytetracycline, OxTC, and 7-chlorotetracycline, Cl-TC, are found in conformation A,[15,16] but the free base of 5,12a-diacetyl-5-hydroxytetracycline has conformation B.[17]

The C_4–H–C_{4a}–H NMR coupling constant has been used as a means of determining solution conformations of tetracycline derivatives.[18] In conformation A the dihedral angle[17] between these C–H bonds is around 74°, and the coupling constant is expected to be small relative to that in conformation B where the angle is close to 170°. Applying this relationship to tetracycline derivatives in DMSO solution, it appears that the hydrochloride salts of TC, Cl-TC and OxTC all have a conformation close to that shown in Fig. 1A whereas the corresponding free bases have a conformation more nearly like B. A possible explanation for this is that conformation B is stabilized in the zwitterion form by H-bonding between 4-$NHMe_2^+$ and C_3–O^-. These groups are further apart in conformation A. Principal differences in proton NMR spectra in DMSO-d_6 on going from the free bases to the hydrochloride salts are a downfield shift of at least 1 ppm for the C_4–H signal accompanied by loss of observable spin coupling,[19] and smaller downfield shifts of signals from the 4-NMe_2 protons and amide protons. Chemical shifts and signal assignments were reported earlier.[13] The NMR evidence for a conformational difference between these tetracyclines and their HCl salts is supported by the X-ray work

cited above and by CD spectra in DMSO in which TC and its hydrochloride salt have opposite Cotton effects at 260 nm.[20] Other derivatives such as tetracyclinonitrile and tetracycline methiodide appear, from lack of spin splitting of the C_4–H proton resonance signal, to exist in conformation A in DMSO.

Binding of tetracycline to praseodymium(III). In the previous NMR investigation of metal ion binding by TC, several anhydrous, first row transition metal salts and anhydrous nitrates of the rare earth ions La^{3+}, Nd^{3+} and Tb^{3+} were used as probes of the binding site.[13] Nd^{3+} proved to be the most useful of these ions. $Pr(NO_3)_3$ is the remaining rare earth nitrate which is easily prepared in anhydrous form,[21] and in hopes of finding a paramagnetic NMR probe more sensitive than Nd^{3+}, spectra of TC in DMSO-d_6 in the presence of various mole fractions of $Pr(NO_3)_3$ were recorded.

The effects of Pr^{3+} on the proton NMR signals of TC are essentially the same as those of Nd^{3+}, described in detail previously.[13] Notably, there is severe selective broadening of the amide and C_4–H signals accompanied by shifts of these and other signals. The rate of signal broadening with increasing $[Pr^{3+}]$ is qualitatively the same as with Nd^{3+}. In order to ascertain the diamagnetic contribution to the signal shifts, a series of spectra were recorded in the presence of La^{3+}. Here La^{3+} is presumed to bind to the same site as Pr^{3+}. By taking the difference in shifts observed at the same mole fractions of La^{3+} and Pr^{3+}, the *paramagnetic* contributions to the signal shifts are obtained. A plot of these shift differences is shown in Fig. 2. The plot closely resembles that obtained previously for Nd^{3+} except for the larger shift of the C_5–H signal with Pr^{3+} and the small shift observed for the C_{5a}–H signal. It appears that $Pr(NO_3)_3$ offers no advantage over $Nd(NO_3)_3$ for these studies.

5-Hydroxytetracycline. An NMR study of metal binding in OxTC was undertaken to determine whether its site of metal binding differs from that of TC. Proton NMR signal assignments and chemical shifts in DMSO were reported earlier.[13] Spectra of OxTC in DMSO-d_6 to which small amounts of a DMSO-d_6 solution of $Nd(NO_3)_3$ are added show essentially the same perturbations as found for TC in the presence of Nd^{3+}. The most obvious effects are pronounced selective broadening of the NMR signals assigned to C_4–H, C_5–H, and the amide protons. Some broadening is apparent also for the C_5–OH and C_6–OH signals. Shifts of several NMR signals in the presence of Nd^{3+} are observed. A plot of these shifts, corrected for diamagnetic contributions using $La(NO_3)_3$,[22] is shown in Fig. 3. The plot is qualitatively similar to that reported earlier for TC in the presence of Nd^{3+} except that shifts for OxTC are generally smaller. A paramagnetic shift for C_5–OH occurs with OxTC.

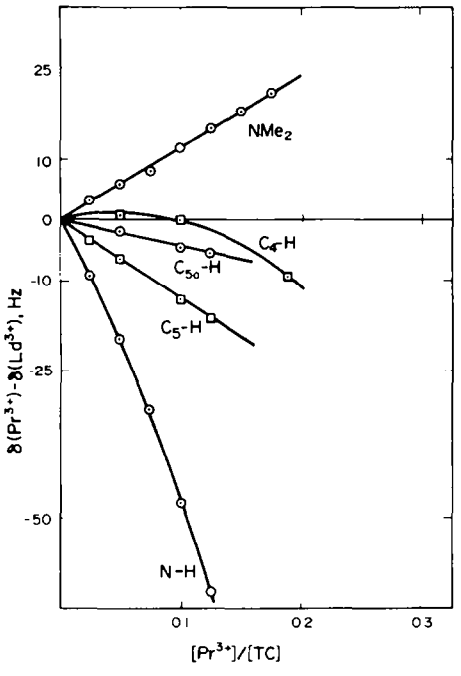

Fig. 2. Plot of proton NMR signal shifts for TC vs $[Pr^{3+}]/[TC]$ corrected for shifts observed at the same $[La^{3+}]/[TC]$.

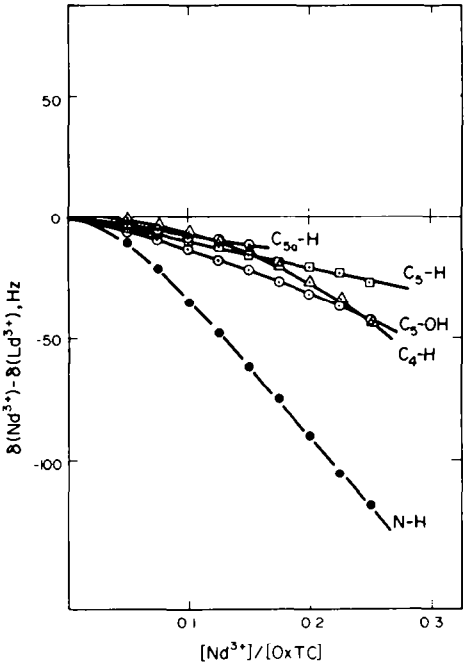

Fig. 3. Plot of proton NMR signal shifts for OxTC vs $[Nd^{3+}]/[OxTC]$ corrected for shifts observed at the same $[La^{3+}]/[OxTC]$.

The definite similarities in paramagnetic effects on the proton NMR spectra of TC and OxTC lead to the conclusion that binding sites, at least for lanthanide series ions,[23] must be the same for both. All the above paramagnetic effects involve protons on or adjacent to ring A, indicating that only ring A functional groups are involved in metal binding under these conditions. The absence of paramagnetic effects on signals at 11·6 and ~15 ppm, assigned[24] to C_{10}–OH and C_{12}–OH, respectively, rule out binding at these sites.

The effects of Mg^{2+} on the proton NMR spectrum of OxTC in DMSO-d_6 were also examined. Magnesium is the metal ion deemed most likely to be involved in the biological action of tetracyclines.[1,3,5–11] Again the results parallel those reported[13] in detail for TC in the presence of Mg^{2+} with regard to changes in signals assigned to the amide, C_4–H, and 4-NMe_2 and the appearance of new signals possibly arising from Mg^{2+}-induced conformation A. In addition, for OxTC, downfield and (small) upfield shifts were observed for signals assigned to C_5–OH and C_5–H, respectively. The latter shifts are also found for

OxTC in the presence of La^{3+}. The close parallel between effects observed for TC and OxTC lead to the conclusion that the site of Mg^{2+} binding is the same for both.

Tetracycline methiodide. Earlier work with tetracyclinonitrile suggested that the 4-NMe_2 group of tetracyclines is not significantly involved in metal binding, since conversion of the 2-carboxamide group to a nitrile drastically alters the metal binding characteristics of the drug.[13] However a more stringent test of the possible role of the NMe_2 group appeared necessary, thus the metal binding characteristics of tetracycline methiodide, TCMI, were examined by NMR. Binding by this group is not possible in TCMI due to the absence of an available electron pair.

In DMSO-d_6 solution the proton NMR spectrum of TCMI generally resembles that of TC·HCl. A signal at 7·7 ppm, not seen in the spectra of TC or TC·HCl, occurs for TCMI and is readily exchanged with D_2O. This signal is tentatively assigned to C_{12a}–OH.[25] Chemical shift values of readily apparent signals are given in Table 1. The absence of spin splitting for the C_4–H signal is evidence that TCMI assumes conformation A in DMSO as do the hydrochloride salts of other tetracycline derivatives. Indeed, TCMI may be regarded as a close analog of TC·HCl in which the 4-$NHMe_2^+$ group becomes 4-NMe_3^+.

In order to compare the metal binding characteristics of TC and TCMI directly, it is necessary to remove the 3-OH proton from TCMI, forming a species analogous to the zwitterion **1**. Since most strong bases are good ligands and would compete with the drug for available metal ions, the base chosen for this study was 1,8-bis(dimethylamino)naphthalene or "proton sponge." This base is strong,[26] soluble in DMSO, and is sterically prohibited from metal binding. Thus DMSO-d_6 solutions of TCMI containing slightly less than a 1:1 mole ration of base:drug were used for NMR studies of metal binding. The NMR spectrum of this solution shows a number of changes from that of TCMI. To a small extent these changes parallel those observed upon converting TC·HCl to TC (see above) except that no spin splitting of the C_4–H signal is evident. Signals arising from the proton sponge obscure the upfield member of the pair of amide signals in TCMI.

Spectra were recorded in the presence of various amounts of Nd^{3+} and compared with spectra obtained at the same mole fractions of La^{3+} up to metal/drug ratios of at least 0·4. Notable *differences* in spectra recorded in the presence of Nd^{3+} and La^{3+} occur for the C_4–H and amide signals. In the presence of La^{3+} these signals show only slight changes in linewidth over the entire range of La^{3+} concentrations. In contrast, in the presence of Nd^{3+} severe broadening of both these signals is evident even at a Nd^{3+}/TCMI mole ratio of 0·12. Selective broadening of the amide and C_4–H resonances is also observed for TC at somewhat larger Nd^{3+}/drug ratios.[27]

It must be concluded that the binding site (for lanthanide ions at least) is the same for TC and TCMI, and that the 4-NMe_2 function in TC is *not* involved significantly in metal binding. Other observations of significance here are: (1) the C_{10}–OH signal shows no selective broadening with Nd^{3+}, and (2) the signal assigned to C_{12}–OH becomes *sharper* with increasing Nd^{3+} or La^{3+} concentration. These observations rule out binding by the C_{10}–C_{11}–C_{12} oxygen atoms, leaving the ring A tricarbonylmethane group as the only plausible binding site under these conditions as concluded previously.[13] It is perhaps of interest to note that addition of La^{3+} to the TCMI-base mixture has a similar effect on the NMR spectrum as adding H^+ with regard to signal shifts and changes in linewidth of some signals, i.e. it reverses the effect of the base. The implications are that metal ions and protons seek the same binding site and both appear to have a preference for conformation A. The absence of spin coupling for C_4–H in deprotonated TCMI indicates that it does not convert to the limiting conformation B.

De-dimethylaminotetracycline. Removal of the 4-NMe_2 group from TC significantly reduces its *in vivo* and *in vitro* activity[28] and raises pKa_1, attributed primarily to deprotonation of the ring A tricarbonylmethane moiety, from 3·3 to 6·0.[29] It was of interest therefore to make a direct comparison of the nature of metal binding in 4-dedimethylaminotetracycline, dd-TC, and TC using the same NMR techniques. The proton NMR spectrum of dd-TC in DMSO-d_6 is similar to that of TC except for disappearance of the strong 4-NMe_2 signal and appearance of reasonably sharp signals attributed to the exchangeable protons C_1–OH, C_{12}–OH and C_{12a}–OH which are very broad or not apparent for TC. These signals were assigned by Asleson *et al.*,[24] and our chemical shift values (Table 1) are in good agreement with those reported previously.

As is the case with TCMI, one proton must be removed from dd-TC before it may be compared with TC free base. Thus proton sponge was used as a non-complexing base in a slightly less than 1/1 mole ratio relative to dd-TC. Addition of $Nd(NO_3)_3$ solution to the DMSO solution of dd-TC and base results in *none* of the selective effects observed for the proton NMR spectra of other TC derivatives described in this paper. No significant shifts or

Table 1. Proton NMR signal assignments for tetracycline derivatives in DMSO.[a]

	Position								
	2	3	4	5	6	7-9	10	12	12a
TCMI	9.4 9.5	b	3.4(CH_3) 4.5(H)[c]	2.5	1.5(CH_3) 5.0(OH)[c]	6.9-7.6	11.7	15.2	7.7[c]
dd-TC	8.7 8.9	18.3		1.9	1.5(CH_3) 4.8(OH)[c]	6.8-7.5	11.8	15.3	6.6[c]
ETC	8.5 9.1	b	2.5(CH_3) 4.0(H)[c]	2.0	1.5(CH_3) 4.8(OH)[c]	6.7-7.5	11.8	~15[d]	6.6[c]

[a]Chemical shifts in parts per million relative to TMS. [b]Signal not seen due to rapid exchange. [c]Assignments for C_6 and C_{12a} OH signals are tentative and may be in reverse order. [d]Very broad signal.

selective broadening is apparent for any NMR signal; the amide signals are fairly well resolved even at a Nd^{3+}/dd-TC ratio 1/3 at which point they are lost in the baseline for TC. Other series of spectra in DMSO were recorded using $Nd(NO_3)_3$ and $Mg(NO_3)_2$ with triethylamine as the base. Again, the effects observed in the case of TC are not seen.

It is clear that dd-TC shows relatively little tendency to bind Nd^{3+} or Mg^{2+} under these conditions. The NMR results obtained for TCMI indicate the 4-NMe_2 group is not involved in metal binding; thus the marked difference observed here between TC and its dedimethylamino derivative must result from the change in basicity of the tricarbonylmethane group.

4-Epitetracycline. The nature of metal binding in the C_4-epimer of tetracycline, ETC, was of interest because its antibacterial activity is considerably lower than that of TC or OxTC,[28] yet in contrast to the situation in dd-TC where the 4-NMe_2 group is removed, epimerization at C_4 has no drastic effect on the macroscopic pK_a values.[30] Although in our hands ETC was not obtained free of TC, separate NMR signals for the two stereoisomers in DMSO were observed for the C_4–H, C_6–OH, and amide protons. Also a signal occurring at 6·6 ppm is tentatively assigned to C_{12a}–OH of ETC.[25]

Addition of $Nd(NO_3)_3$ solution to the TC-ETC mixture results in strong selective broadening of the C_4–H and amide signals arising from *both* TC and ETC. Rates of broadening with increasing $[Nd^{3+}]$ appear identical for the two stereoisomers, but the downfield shifts of C_4–H and the 8·5 ppm amide signals for ETC are slightly more pronounced than for TC. In the presence of $La(NO_3)_3$ broadening and shifting of ETC signals parallels that observed for TC. The effects resulting from addition of $Mg(NO_3)_2$ to the mixture of TC and ETC in DMSO are essentially the same for C_4–H and amide NMR signals of both diastereomers and have been described previously in detail for TC.[13] We interpret these results as evidence that TC and ETC bind metal ions via the same functional groups with approximately the same avidity in DMSO.

SUMMARY AND CONCLUSIONS

Results of NMR experiments described in a previous paper indicate that TC free base in DMSO solution binds metal ions through ring A functional groups. It was concluded that O atoms of the tricarbonylmethane group are the most likely donors; however binding through the 4-NMe_2 group could not be ruled out.[13] The experiments described in this paper for TCMI show the 4-NMe_2 group is not significantly involved in metal binding. In the presence of selected metal ion probes, perturbations of proton NMR signals of TC, OxTC, ETC and TCMI are very similar and involve signals of protons associated with or adjacent to ring A. It must be concluded that the binding site is the tricarbonylmethane group in all these derivatives. In contrast, under these same conditions there is no NMR evidence for metal binding by dd-TC. The *in vitro* antibiotic activity of dd-TC is similar to that of ETC.[28] Both of these derivatives have much lower activity than TC or OxTC, and methiodide derivatives have little or no antibacterial activity.[28] Thus for these tetracyclines there is no obvious correlation between the nature of metal binding in DMSO and antibiotic activity.

EXPERIMENTAL

Compounds. TCMI, dd-TC, and ETC were prepared from TC or its hydrochloride salt using procedures described by McCormick *et al.*[31] In our hands, ETC was not obtained free of TC despite several attempts at purification. Elemental analyses or m.ps were satisfactory for the dedimethylamino and methiodide derivatives. Prior to the NMR experiments, tetracyclines were dried for several h under the following conditions: TC, OxTC, and ETC *in vacuo* at 60°; dd-TC *in vacuo* at 76°; TCMI *in vacuo* at 25°.

Anhydrous nitrate salts of Mg(II), Nd(III), and La(III) were prepared as described previously.[13] $Pr(NO_3)_3$ was prepared by treating the oxide with conc. HNO_3 followed by heating at 175° for 2 days. DMSO solutions of this material were filtered to remove traces of the oxide prior to the NMR experiments.

NMR experiments. NMR spectra were run on a Varian Model HA-100 instrument using dry DMSO-d_6 as the solvent and TMS as a reference. Stock solutions of anhydrous metal nitrates in DMSO-d_6 were prepared, and aliquots of these were transferred to NMR tubes containing tetracyclines using techniques described previously.[13]

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amide NMR signals occur at mole ratios of 0·25 for TC and 0·12 for TCMI. The rapid signal broadening for TCMI prevented accurate chemical shift measurements necessary for constructing a plot such as shown in Fig. 2 and 3.

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