ions may be considered not bonded or uncoordinated. At least, their bond order is much less than one. On this basis, Rb(3) in dehydrated $Rb_{11}Na_1$-A is termed zero-coordinate, zero being the sum of integers, all zero, describing its bond orders to its nearest neighbors. For this structure, the rounded-off value of 1.0 Å used in the criterion stated above may be raised to 1.5 Å.

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Supplementary Material Available: listings of the observed and calculated structure factors for both structures (Supplementary Tables A and B) (4 pages). Ordering information is given on any current masthead page.

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Chemical–Structural Properties of Tetracycline Derivatives. 3. The Integrity of the Conformation of the Nonionized Free Base[1]

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Abstract: A second crystal structure modification of anhydrous oxytetracycline free base, OXY, has been obtained by slow evaporation of a warm, water free toluene solution. A crystal structure analysis has been carried out with intensity data measured from a cooled crystal (*ca.* −150 °C). The crystal displays space group symmetry $P2_12_12_1$ with one molecule per asymmetric unit and lattice parameters: a = 8.836 (1), b = 12.416 (1), and c = 18.760 (2) Å. The crystal was found to consist of fully associated (nonzwitterionic) OXY molecules displaying the same conformation observed in the first modification, but differences in inter- and intramolecular hydrogen bonding are observed. A reinterpretation of reported proton NMR data[12,14] confirms the proposal[2] that the tetracycline derivatives are likely to be nonzwitterionic in nonaqueous solvents. Some consequences of the misconception that the free base derivatives are zwitterionic in Me_2SO, particularly with respect to metal bonding, are discussed.

Crystal structure analyses have been reported for two forms of oxytetracycline, OXY, free base,[2] a dihydrate and an anhydrous form. The structure analyses have demonstrated that the molecular moieties in the crystals differ both in chemical structure and in conformation. The dihydrate crystals consist of zwitterionic microspecies in which the dimethylamine group at C(4) is protonated, while the negative charge is distributed over the A ring chromophore in a manner correlatable with the orientation of the amide substituent at C(2). The anhydrous crystals were found to contain nonionized molecular moieties with a clearly enolic A ring chromophore. Conformationally, the zwitterion was found to be very similar to the several examples that have been reported for the fully protonated cationic derivatives,[3,4] while the nonionized molecule displayed the conformation initially proposed by Schach von Wittenau and Blackwood[5] from NMR studies and subsequently observed for crystalline 5,12a-diacetyloxytetracycline.[6] The report presenting these structures[2] raised the possibility that both modifications of the free base might be important to the biological activity of the broad spectrum tetracycline antibiotics. While the zwitterionic form is quite likely adopted in aqueous rich regions of the body, it was felt that the nonionized form presented a more suitable model for the more hydrophobic regions.

A second crystal modification of anhydrous oxytetracycline, OXY(II), had been crystallized under very similar conditions to those which produced the first modification, OXY(I).[2] Its crystal structure analysis was undertaken to determine whether or not the free base had adopted an alternative conformation and/or chemical structure, a distinct possibility in view of the possible A ring tautomerism.[3a,7]

Experimental Section

Anhydrous OXY free base was dissolved in warm (~70 °C) water-free toluene and maintained on a hot plate at approximately this temperature to allow slow evaporation, with refluxing, of the solvent. Crystals, displaying rectangular prismatic morphology rather

Table I. Refined Lattice Parameters for the Two Crystal Modifications of Anhydrous OXY

	OXY(II)	OXY(I)[2]
a	8.836 (1)	10.297 (1)
b	12.416 (1)	10.770 (2)
c	18.760 (2)	18.369 (3)

Table II. Fractional Coordinates for Carbon, Nitrogen, and Oxygen Atoms[a]

Atom	10^4x	10^4y	10^4z
C(1)	7851 (3)	3096 (2)	3676 (1)
O(1)	8732 (3)	2362 (2)	3822 (1)
C(2)	8102 (3)	3894 (2)	3117 (1)
C(2am)	9609 (3)	4056 (3)	2798 (1)
N(2am)	10776 (3)	3450 (3)	2994 (1)
O(2am)	9792 (2)	4797 (2)	2339 (1)
C(3)	6943 (3)	4565 (2)	2893 (1)
O(3)	7159 (3)	5310 (2)	2412 (1)
C(4)	5330 (3)	4508 (2)	3168 (1)
N(4)	4161 (3)	4466 (2)	2604 (1)
C(4m2)	4525 (5)	3725 (3)	2024 (2)
C(4m1)	3714 (4)	5516 (3)	2316 (2)
C(4a)	5048 (3)	3570 (2)	3687 (1)
C(5)	3679 (3)	3808 (2)	4164 (1)
O(5)	2988 (2)	2815 (2)	4386 (1)
C(5a)	3991 (3)	4552 (2)	4818 (1)
C(6)	3050 (3)	5599 (2)	4775 (1)
C(6m)	1365 (3)	5304 (3)	4807 (2)
O(6)	3446 (3)	6077 (2)	4103 (1)
C(6a)	3464 (3)	6401 (2)	5361 (1)
C(7)	2475 (4)	7221 (2)	5540 (2)
C(8)	2870 (4)	8000 (2)	6048 (2)
C(9)	4263 (4)	7975 (2)	6379 (2)
C(10)	5298 (4)	7179 (2)	6192 (1)
O(10)	6689 (3)	7218 (2)	6502 (1)
C(10a)	4909 (3)	6372 (2)	5687 (1)
C(11)	6056 (3)	5590 (2)	5464 (1)
O(11)	7400 (3)	5661 (2)	5698 (1)
C(11a)	5644 (3)	4773 (2)	4954 (1)
C(12)	6759 (3)	4142 (2)	4671 (1)
O(12)	8225 (2)	4225 (2)	4845 (1)
C(12a)	6431 (3)	3262 (2)	4134 (1)
O(12a)	6025 (2)	2300 (2)	4499 (1)

[a] The numbers in parentheses are the estimated standard deviations in the last significant digit.

Table V. Bond Distances for the Nonionized Oxytetracycline Free Base Derivatives

Atoms	OXY(II)	OXY(I)[a]
C(1)-O(1)	1.229 (4)	1.233 (3)
C(1)-C(2)	1.460 (4)	1.438 (3)
C(1)-C(12a)	1.535 (4)	1.527 (3)
C(2)-C(3)	1.386 (4)	1.391 (3)
C(2)-C(2am)	1.474 (4)	1.473 (3)
C(2am)-O(2am)	1.270 (4)	1.274 (3)
C(2am)-N(2am)	1.328 (4)	1.324 (4)
C(3)-O(3)	1.306 (4)	1.304 (3)
C(3)-C(4)	1.517 (4)	1.523 (3)
C(4)-C(4a)	1.539 (4)	1.546 (3)
C(4)-N(4)	1.479 (4)	1.471 (3)
N(4)-C(4m1)	1.466 (4)	1.468 (4)
N(4)-C(4m2)	1.460 (4)	1.462 (4)
C(4a)-C(5)	1.533 (4)	1.542 (3)
C(4a)-C(12a)	1.530 (4)	1.532 (3)
C(5)-C(5a)	1.561 (4)	1.563 (3)
C(5)-O(5)	1.438 (3)	1.435 (3)
C(5a)-C(6)	1.544 (4)	1.533 (3)
C(5a)-C(11a)	1.508 (4)	1.516 (3)
C(6)-C(6a)	1.528 (4)	1.538 (3)
C(6)-C(6m)	1.534 (4)	1.530 (4)
C(6)-O(6)	1.437 (3)	1.444 (3)
C(6a)-C(7)	1.383 (4)	1.383 (4)
C(6a)-C(10a)	1.416 (4)	1.421 (4)
C(7)-C(8)	1.403 (4)	1.400 (4)
C(8)-C(9)	1.379 (5)	1.381 (4)
C(9)-C(10)	1.392 (4)	1.397 (4)
C(10)-C(10a)	1.421 (4)	1.416 (4)
C(10)-O(10)	1.361 (4)	1.355 (4)
C(10a)-C(11)	1.464 (4)	1.453 (4)
C(11)-C(11a)	1.441 (4)	1.451 (4)
C(11)-O(11)	1.270 (3)	1.270 (3)
C(11a)-C(12)	1.366 (4)	1.365 (3)
C(12)-C(12a)	1.515 (4)	1.520 (3)
C(12)-O(12)	1.339 (3)	1.337 (3)
C(12a)-O(12a)	1.423 (3)	1.424 (3)

[a] Bond distances from crystal modification OXY(I) are those reported by Stezowski.[2]

than the hexagonal prismatic form displayed by the OXY(I) crystals, formed on the sides of the beaker.

A 0.10 × 0.25 × 0.35 mm crystal was selected for space-group determination and subsequent data collection. The Laue symmetry and systematic extinctions displayed by the OXY(II) crystal allowed the unambiguous assignment of the crystal symmetry to space group $P2_12_12_1$. All crystallographic data were measured with the above crystal enclosed in a thin-walled glass capillary and cooled to ~ −150 °C on a Syntex $P\bar{1}$ autodiffractometer equipped with a low-temperature device (Syntex LT-1); measurements were made with monochromatized Mo Kα radiation, λ = 0.71069 Å. The lattice parameters, Table I, were refined by least-squares techniques[8] with 47 automatically centered 2θ values in the angular range $27.4 < 2\theta < 41.5°$.

Integrated intensities were measured in an ω scan mode for which the scan range was 0.75°; the scan rate was allowed to vary from 2.0 to 24.0° min^{-1} as a function of maximum peak intensity. Background radiation was measured on each side of the reflection, 1.0° from the theoretical reflection center, for one-half the scan time. Three reference reflections, monitored after each 129 data were measured, displayed neither systematic nor significant deviations from their initial intensities. Of the 6932 unique reflections measured, $(\sin\theta)/\lambda_{max}$ = 0.904 Å^{-1}, 3100 were classified as objectively observed under the criterion $I \geq 3\sigma(I)$. The data were corrected for Lorentz and polarization effects.

Structure Determination and Refinement. The initial model for the crystal structure was determined by direct methods. All carbon, nitrogen, and oxygen atoms were located in the first E map and all hydrogen atoms were located in subsequent difference electron density maps. The structural model was refined by variable block-block diagonal least-squares techniques. The blocks were constructed so that the parameters, fractional atomic coordinates and anisotropic temperature factor coefficients, associated with one carbon, nitrogen, or oxygen atom and those of any hydrogen atom bonded to it were refined in one block; the hydrogen atoms were refined with isotropic temperature factors. The single scale factor was refined in a separate block. Examination of the data indicated the lack of an extinction problem. Those data classified as unobserved for which the calculated intensity was greater than the arbitrary cutoff value ($3\sigma(I)$) were also used in the refinement. The data were empirically weighted with the equation $\sigma_c^2(F) = \sigma_o^2(F) + 0.125F + 0.001F^2$. In this manner, 394 variables were refined with 4879 contributing data to give standard residuals: R = 0.054, R_w = 0.065, and σ = 1.29.[9]

Results and Discussion

The refined fractional atomic coordinates and anisotropic temperature factor coefficients are presented in Tables II and III (deposited[9]), respectively; the refined fractional atomic coordinates and isotropic temperature factor coefficients for the hydrogen atoms are presented in Table IV.[9]

The crystals of OXY(II) consist of nonionized OXY molecules in a conformation nearly identical with that displayed

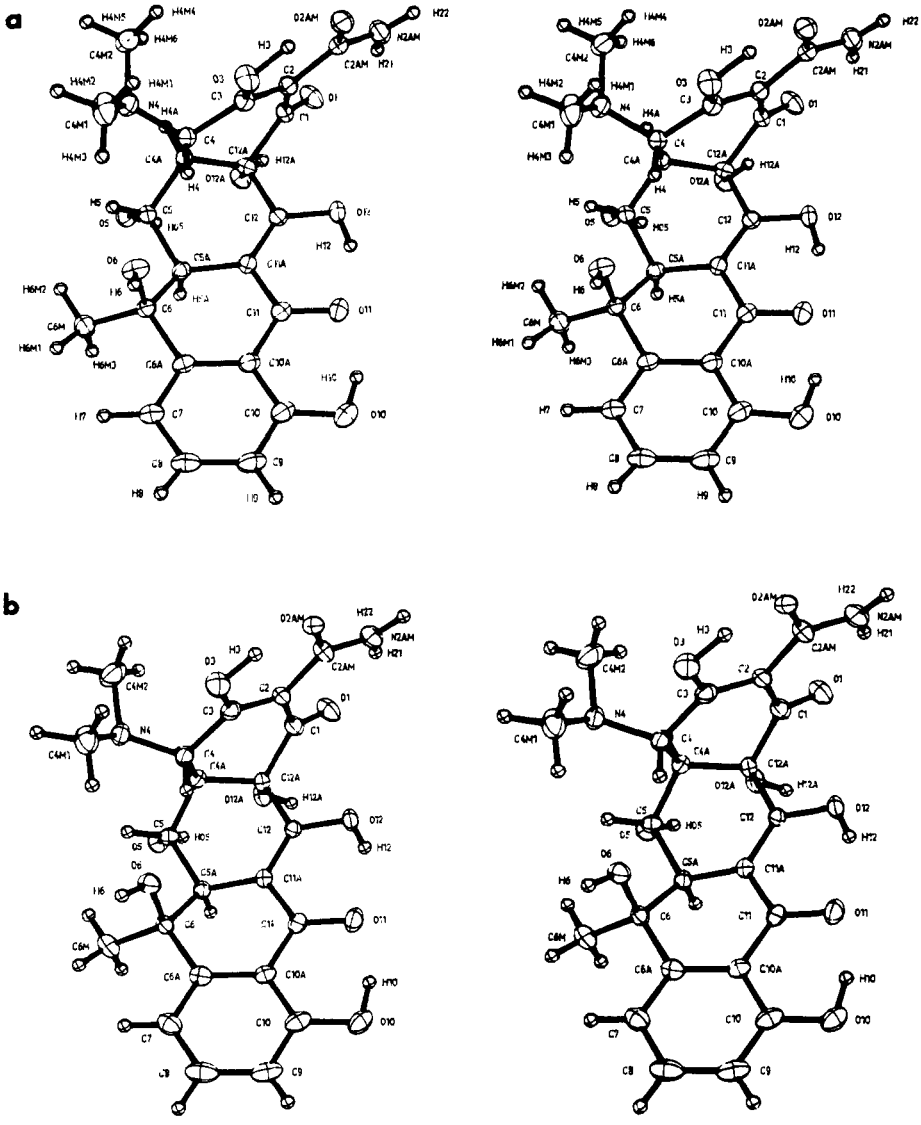

Figure 1. Stereoscopic projections[10] of nonionized OXY free base molecules. The thermal ellipsoids of the carbon, nitrogen, and oxygen atoms are depicted at the 65% probability level for the refined anisotropic thermal parameters. Hydrogen atoms are depicted with uniform isotropic thermal parameters (B = 0.5 Å^2). Figure 1a presents the results of the OXY(I) crystal structure analysis,[2] while Figure 1b presents the molecule as observed in this analysis, OXY(II).

in the OXY(I) crystals,[2] the molecular entities of both analyses are presented in the stereoscopic projection[10] in Figure 1 for ready comparison. Similarly, bond distances between carbon, nitrogen, and oxygen atoms are presented for both examples in Table V. Bond angles and a selected set of dihedral angles, Tables VI and VII, have been deposited;[9] the latter values in this crystal modification vary by no more than 7° from those reported for OXY(I).[2] In addition to the similarities in the conformations displayed by the molecule in the two crystalline modifications, and also in 5,12a-diacetyloxytetracycline,[6] the tabulated bond distances also indicate the structural integrity displayed by the nonionized free base. In the comparison between the OXY(I) and OXY(II) molecules, there is only one bond distance that differs by more than three estimated standard deviations (3σ = 0.012 Å), that for the C(1)-C(2) bond, 1.460 (4) and 1.438 (3) Å for OXY(II) and OXY(I), respectively. This difference may in part reflect the changes in electron density of the oxygen atom of the carbonyl group at C(1) in the OXY(II) molecule as the result of less intramolecular hydrogen bonding relative to that presented by OXY(I). In the latter modification, there is an intramolecular hydrogen bond between the hydroxyl group at C(12a) and this carbonyl group, whereas in OXY(II) this carbonyl group is hydrogen bonded only to atom H(21) of the amide group at C(2).

The differences in crystal packing[9] giving rise to crystal forms OXY(I) and OXY(II) are closely assignable to differences in intermolecular hydrogen bonding rather than to changes in conformation or chemical structure. OXY(I) displays intermolecular hydrogen bonds between the oxygen atom of the amide group and the 6-hydroxyl group and between the oxygen atom of the 5-hydroxyl group and the NH_2 moiety of the amide group. The intermolecular hydrogen bonding in OXY(II) is between the oxygen atom of the 5-hydroxyl group and the hydrogen atom of the 12a-hydroxyl group[11] and between the hydroxyl oxygen atom O(10) and the hydrogen atom of the 6-hydroxyl group. The hydrogen bonding in this and other crystal structure analyses of tetracycline derivatives is discussed in more detail in the report of the crystal structure analyses of α-6-deoxyoxytetracycline hydrohalides.[3a]

The likelihood that the tetracycline free base derivatives adopt a nonionized chemical structure in anhydrous or nearly anhydrous solutions was pointed out in the discussion of the conditions under which the zwitterionic and nonionized forms

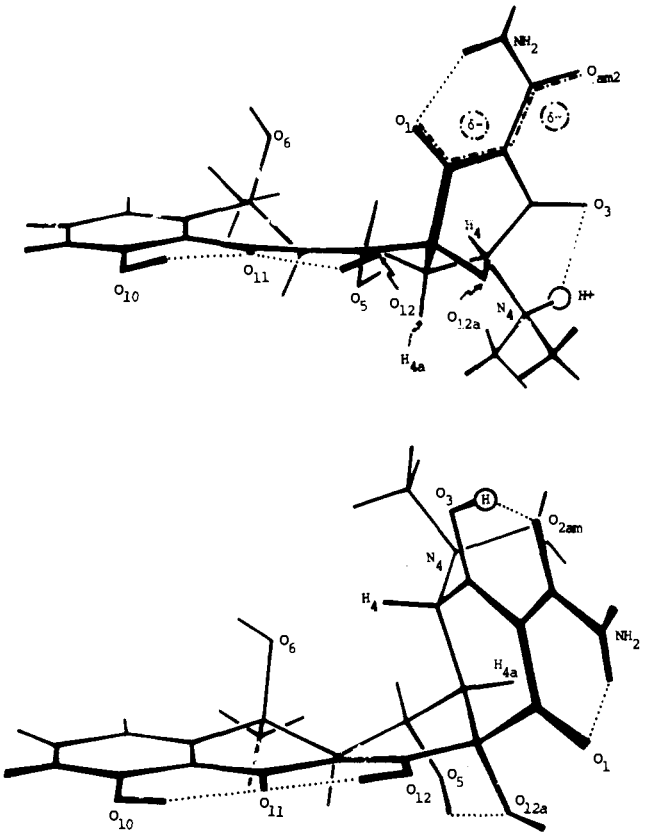

Figure 2. The crystallographically observed conformations for OXY free bases. The upper figure presents the conformation of the zwitterionic form,[2] which corresponds to conformation A of Gulbis and Everett.[12] This form displays a protonated dimethylamine group, designated by the presence of H^+ in the figure, and a negatively charged A ring chromophore.[2] The nonionized free base, depicted in the lower figure, provides an example of conformation B[12], in which the enolic A ring chromophore is stabilized by strong hydrogen bonding between the hydroxyl group and the carbonyl moiety of the amide substituent, see atoms O(2am) and O(3) above and in Figure 1. The observed intramolecular hydrogen bonding is indicated by dotted lines connecting the relevant hydrogen and oxygen atoms (detailed atom identification is provided in Figure 1). The drawings presented were constructed from ORTEP plots.[10]

of the free base have been obtained in the crystalline form.[2] A recent report describing NMR investigations of various tetracycline derivatives in Me_2SO[12] provides strong evidence to support this hypothesis, though it was not interpreted as such.

The C(4)H-C(4a)H coupling constants were utilized by Gulbis and Everett[12] to demonstrate that the hydrochloride salts of tetracycline (TC), chlorotetracycline (CITC), and OXY present the same conformation in Me_2SO as that reported from their crystal structure analyses[3b,4] and that their respective free bases adopt a conformation in this solvent similar to that reported for crystalline 5,12a-diacetyloxytetracycline free base.[6] Examples of these conformations, which they have designated conformation A and B, respectively, are presented in Figure 2. The authors present, as a possible explanation for the conformation of the above mentioned free base derivatives, their conformation B, a proposal that B is stabilized in the zwitterionic form as the result of hydrogen bonding between the protonated dimethylamino group and a negatively charged oxy substituent at C(3). This proposal is in direct conflict with the earlier crystallographic data[3,4,6] as well as with our hypothesis concerning the chemical structure of the free base when conformation B is adopted.[2]

The proposed hydrogen bonding occurs with regularity in conformation A rather than in conformation B, in which it has not yet been observed. The orientation of the dimethylamino groups revealed by the crystal structure analyses of OXY·HCl[3b] and CITC·HCl, both of which display conformation A, provided an early indication of this hydrogen bonding, though it should be noted that the appropriate hydrogen atoms were not located in either analysis and that the existence of the above hydrogen bond was somewhat obscured because the hydrogen atom of the dimethylamino group also interacts with the chloride anion in both of these structures. The report[6] for the crystal structure analysis cited for the example of conformation B presented 5,12a-diacetyloxytetracycline free base in a nonzwitterionic form and described the enolic character of the A ring chromophore in some detail. Crystal structure analyses for $TC \cdot 6H_2O$ and $OXY \cdot 2H_2O$ have demonstrated the suitability of conformation A for the zwitterionic free bases[2] and have confirmed the hydrogen bonding between the dimethylammonium group and the oxy substituent at C(3). Thus we feel that the crystallographic evidence is incompatible with the proposal that conformation B is adopted for the zwitterionic free base.[13]

In contrast to the points raised above, the observations reported by Gulbis and Everett for tetracycline methiodide (TCMI) and tetracycline nitrile (TCN) are consistent with the hypothesis from this laboratory that conformation B is stabilized by a strong hydrogen bond in the enolic A ring chromophore and is thus indicative of the nonionized form when observed for the free bases. The authors report that neither TCMI nor TCN were found to adopt conformation B. It can be readily demonstrated that the modifications in chemical structure giving rise to both of these derivatives preclude formation of the stabilizing hydrogen bond, Figure 2. The TCMI analogue of the usual tetracycline free bases lacks the hydrogen atom necessary for the formation of the enolic A ring chromophore, while TCN lacks the important amide carbonyl group, which serves as the proton acceptor in the formation of the hydrogen bond present in all crystal structure analyses in which conformation B has been observed.[2,6] The reported observation of conformation B in Me_2SO for all those examples in which the enolic form of the A ring chromophore was both structurally accessible and able to be stabilized by strong intramolecular hydrogen bonding provides substantial evidence in support of our proposal that this form may be adopted in the hydrophobic regions of biological systems and thus contribute to the antimicrobial activity of the tetracyclines.[2]

Based upon extensive NMR investigations for several tetracycline derivatives in the presence of various metal ions, Everett and co-workers[12,14,15] have concluded that the free bases in Me_2SO utilize A ring oxy substituents to form metal complexes. The authors have repeatedly referred to the zwitterionic character of the uncomplexed ligands in this solvent and have further indicated that they consider the complexed ligand to be zwitterionic as well. The reinterpretation of the chemical structure of the free ligand in Me_2SO (nonzwitterionic) that we have presented might be expected to effect the interpretation of changes in the NMR spectra of the tetracyclines resulting from the presence of metal ions. It seems appropriate for us to present some of our thoughts concerning that interpretation.

The proposed complex site utilizes the carbonyl group of the amide and a second oxy moiety, O(3) or in the alternative tautomeric form of the chromophore O(1), of the tricarbonyl methane system. It is precisely these groups which are involved in the strong intramolecular hydrogen bonding in the enolic structure of the chromophore displayed in conformation B. Clearly this hydrogen bonding must be disrupted if the complexed ligand is zwitterionic. In view of our interpretation of the stabilizing effect of his hydrogen bond on conformation B, one might then expect a conformational change to occur upon formation of the zwitterion. Indeed, a $HgCl_2$ complex with

zwitterionic OXY free base has been demonstrated to present the ligand in conformation A[16] and to display the proposed interactions with the A ring oxy substituents. The authors have not discussed any change in conformation as the result of complex formation, but we believe their reported data are generally consistent with such a change.

Gulbis and Everett[12] have indicated that, relative to the free bases, the acidic forms of the tetracyclines show a downfield shift in the signals of the dimethylamino protons, the amide protons, the C(4a)H, and the C(4)H signals, and loss of observable spin coupling. In view of the nonionized structure of the free bases in Me_2SO, the formation of the fully protonated derivatives may be considered to reflect the effects of protonation of the dimethylamino group and the change from conformation B to conformation A. Similar shifts, but of differing magnitudes, are observed for many of the same signals when metal ions are present with the free bases. Mechanistically, the observed shifts may arise in part from transfer of the proton of the enolic free base to the nitrogen atom of the dimethylamino group and the associated conformational change from B to A. The most sensitive indicator of a transition from one conformation to the other, changes in the C(4)H-C(4a)H coupling constant, is most likely obscured by the reported signal broadening.[12,14]

The observations of Gulbis and Everett[12] concerning dedimethylaminotetracycline (dd-TC) provide further substantiating evidence for the proposal that complex formation of the usual tetracycline derivatives is accompanied by a structural change in the ligand. The lack of the dedimethylamino group prevents the formation of the zwitterion and strongly favors the enolic structure of the A ring chromophore. None of the characteristic shifts displayed by the tetracycline derivatives possessing the dimethylamino substituents was observed in the NMR spectra of dd-TC in the presence of metal ions. We interpret these observations as an indication that dd-TC has retained conformation B and probably the enolic structure of the A ring as well. In view of the nonprotonated structure of the dimethylamine group of the usual tetracyclines in this solvent, it seems possible that the 1,8-bis(dimethylamino)-naphthalene or the triethylamine used as bases were inadaquate for the removal of the desired proton and thus did not activate the chromophore for metal complex formation.

In summary, while our interpretation of the chemical structure of the free bases of the broad spectrum tetracycline antibiotics in Me_2SO differs with that of Everett and co-workers,[12,14,15] it does not necessarily conflict with their interpretation of the structure of the complexed ligand (zwitterionic) nor with their proposed binding site (oxy substituents of the A ring chromophore) in this solvent system. The conditions utilized by Everett and co-workers differ greatly from those used by other investigators.[16-20] The tetracycline derivatives clearly display considerable structural flexibility, which manifests itself in environmentally induced changes in ionization state and conformation. The probability that their interaction with metal ions is subject to similar effects has been discussed.[21] It seems consistent with our interpretation of these effects that the observations of Everett and co-workers[12,14,15] are relevant to the interaction of the tetracycline free bases in the relatively hydrophobic regions of the body. In view of the possibility that Mg ions may be involved in active transport of these antibiotics,[22] further clarification of the anomalies the authors' report for this metal under the conditions they have investigated should be persued.

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Supplementary Material Available: refined anisotropic temperature factor coefficients for the carbon, nitrogen, and oxygen atoms (Tables III and IV); bond angles (Table VI); a selected set of dihedral angles involving these atoms (Table VII); stereoscopic packing diagrams of OXY(I) and OXY(II); and observed and calculated structure factor amplitudes (42 pages). Ordering information is given on any current masthead page.

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(13) As a point of interest, Gulbis and Everett[12] report that an examination of molecular models indicates that conformation B allows a closer approach between atoms O(3) and N(4) than does conformation A. In the crystalline examples,[2] the O(3)–N(4) distances in the crystalline zwitterionic examples, conformation A, are 2.647 and 2.617 Å for TC(±) and OXY(±), respectively, while for conformation B they are 2.845 and 2.871 Å in OXY(I) and OXY(II), respectively. Thus the closer approach between the nitrogen atom of the dimethylamine group and the oxy substituent at C(3) clearly occurs in conformation A and not in conformation B as was proposed.
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