Chemical-Structural Properties of Tetracycline Derivatives. 7. Evidence for the Coexistence of the Zwitterionic and Nonionized Forms of the Free Base in Solution[1a]

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Abstract: Circular dichroism spectra have been measured for oxytetracycline free base dissolved in an ethanol/water solvent system over a wide range of composition. The spectra demonstrate a solvent-dependent equilibrium between the zwitterionic form and the nonionized form. The zwitterion clearly predominates in water-rich solutions, whereas the nonionized form becomes the species of preference when the ethanol content exceeds ca. 85% (v/v). This dependence upon the thermodynamic environment supports the concept that the nonionized form of the free base is the important lipid-soluble tetracycline species and suggests, in the context of the known three-dimensional structure of biologically active and nonactive tetacycline derivatives, that the conformational-structural adaptability of tetracycline free bases is a significant factor in their broad spectrum antibacterial action.

Earlier crystallographic studies[2] have demonstrated that oxytetracycline (OTC) free base can adopt two well-defined chemical structures, one a zwitterion, OTC(+), and the other a nonionized free base form, OTC(0), each having a specific molecular conformation. The zwitterionic conformation is very similar to that observed for OTC·HCl[3] and OTC·HBr.[4] Solution studies[5] have established this to be the conformation adopted by the medicinally important tetracyclines in acidic or neutral aqueous solution. A second solution form of OTC derivatives was proposed[6] to explain ^{1}H NMR data in nonaqueous solvents. The solid-state structure of the second major conformational form was first established[7] for 5,12a-diacetyloxytetracycline (DAOTC), crystallized from 2-propanol in the nonionized DAOTC(0) form.

^{1}H NMR data reported[8] for other tetracycline free bases in dimethyl sulfoxide have been interpreted[9] as indicating that tetracycline derivatives devoid of the 5-oxy substituent also adopt the latter conformation. The recent crystal-structure determination for *N-tert*-butyl-6-deoxy-6-demethyl-8-methoxytetracycline[10] (N-*t*-Bu-6-DO-6-DM-8-MOTC) has shown this to be the case.

It has been proposed[2] that the biological activity of the tetracycline antibiotics may involve the participation of both molecular species depending upon their interconversion in the thermodynamic environments of aqueous and lipid phases. The possible biological significance of the nonionized free base has recently been questioned.[11] In view of the extensive accumulation of structural and solution data, plus reported clinical testing, it is our judgment that the dual-conformation biological activity concept is a valid working hypothesis. Two lines of evidence offer continuing support for this viewpoint. The first is that Terada and Inagi[12] have demonstrated by ultraviolet and infrared spectroscopy that tetracycline free base, TC(0), is nonionized in the organic solvents ethanol, chloroform, and 1-octanol. The second, which complements these results, involves circular dichroism data presented herein for OTC free base in solution. The analysis clearly demonstrates that the two molecular species are in aqueous/organic solvent-dependent conformational equilibrium. Furthermore, the two molecular forms are readily interconvertible and this interconversion occurs at physiological temperatures.

Experimental Section

Anhydrous OTC free base, OTC(0), was prepared as described previously.[2] Solutions were typically prepared shortly before the spectral determinations and were protected from light. The solvent system was ethanol/water prepared from USP grade absolute ethanol (Rossville Gold Shield, IMC Chemical Group, Inc.) and distilled, demineralized water. Solvent composition ranged from pure alcohol to pure water. Fresh stock solutions in the pure solvents were prepared gravimetrically as needed and intermediate solvent compositions were obtained by accurate dilution of the ethanol solutions using class A volumetric flasks and lambda pipets. Crystalline OTC·2H$_2$O, in which the molecules are unambiguously[2] in the zwitterionic form, OTC(±), was prepared from anhydrous OTC by crystallization from aqueous methanol. The material thus obtained was found to be crystallographically identical with the OTC·2H$_2$O described earlier.[2]

Circular dichroism (CD) spectra were obtained with a Cary Model 60 spectropolarimeter and its CD accessory, Model 6001. Conventional procedures were used and the solutions were equilibrated in cylindrical fused-silica cells (22-mm o.d., 1–10-mm path lengths) for at least 0.5 h at ambient temperature (22 °C) before spectra were measured. Selected samples, including those in absolute ethanol, were remeasured the following day and were found to be reproducible. This confirmed that equilibrium conditions obtained. Molecular ellipticities, $[\theta]$, in deg cm^2/dmol are given by $[\theta] = \theta^\circ\, M/10lC$ where θ°, M, l, and C are the measured ellipticity in degrees, the gram-molecular weight, the path length in cm, and concentration in g/cm^3, respectively.

Results and Discussion

A series of CD spectra from OTC(0) in ethanol/water solutions of varying composition is presented in Figure 1. Spectra from solutions with ≤50% ethanol do not significantly differ from the one shown for pure water which, in turn, is similar to that reported[5] for OTC in aqueous acid. This spectrum clearly corresponds to the zwitterionic form OTC(±) and is characteristic of the state of the chromophore that is associated with the specific A-ring conformation of this species.

The spectrum obtained from the solution in absolute ethanol is strikingly different and, between 230 and 310 nm, shows Cotton effects with signs opposite to those found for the OTC(±) chromophore. As a check, a spectrum was obtained from OTC·2H$_2$O, dissolved without heating in absolute ethanol. As can be seen in Figure 2, the zwitterion form of this

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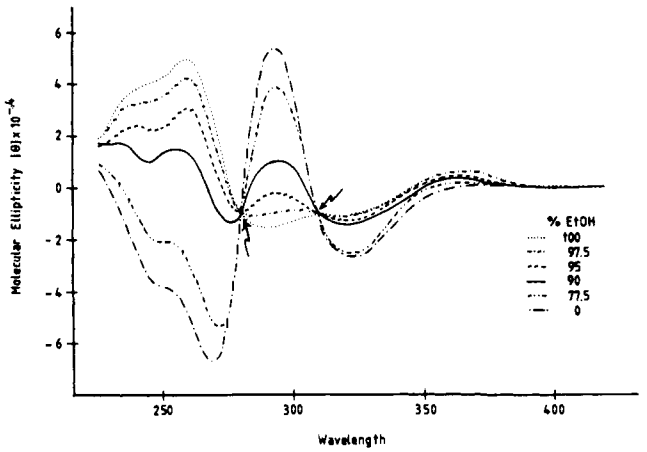

Figure 1. CD spectra for oxytetracycline free base in an EtOH/H_2O solvent system at various compositions. The solvent composition (v/v) is specified as % EtOH.

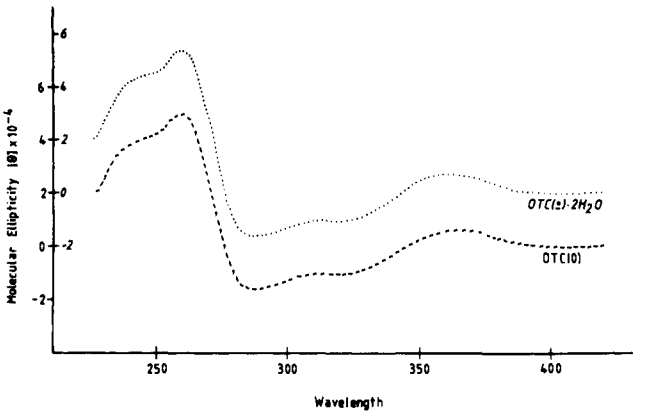

Figure 2. CD spectra obtained from absolute ethanol solutions of oxytetracycline free base prepared from crystalline anhydrous oxytetracycline and from oxytetracycline dihydrate. The ordinate scale has been displaced upward for the latter spectrum.

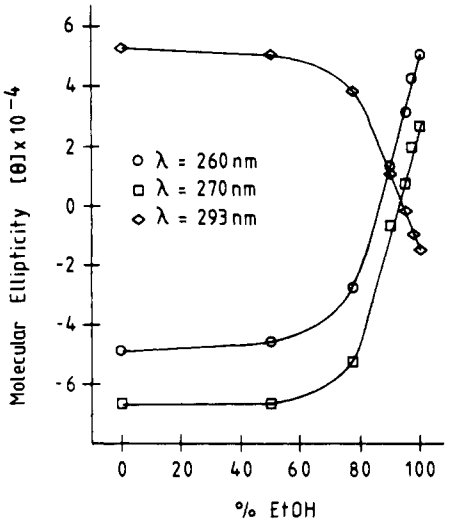

Figure 3. The molecular ellipticity (10^{-4}), θ, at constant wavelength as a function of % EtOH.

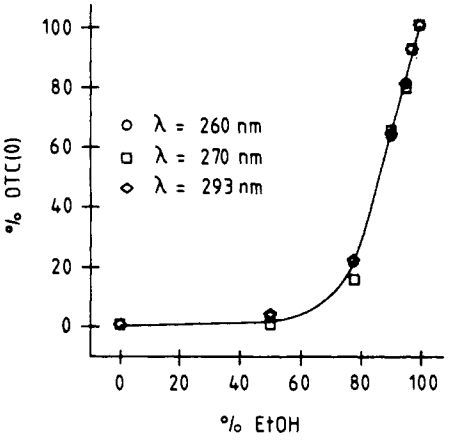

Figure 4. Calculated values of percent nonionized free base, OTC(0), as a function of the solvent composition. The values are estimated by linear interpolation of the curves in Figure 3.

sample completely disappears and the spectrum is identical with that found for the OTC(0) sample. We conclude that this spectrum is characteristic of the state of the chromophore that is induced by the A-ring conformation found in the crystal structures of OTC(0),[1,9] DAOTC(0),[7] and N-*t*-Bu-6-DO-6-DM-8-MOTC(0).[10]

The magnitudes of Cotton effects are well known to be solvent dependent for ketonic chromophores where equilibrium shifts are involved.[13] The systemic variation of the spectra in the mixed solvent systems (Figure 1) can be interpreted in terms of a solvent-dependent equilibrium between two conformers:

$$\mathrm{OTC(0)} \underset{\text{organic solvent}}{\overset{\text{aqueous or dilute acid}}{\rightleftharpoons}} \mathrm{OTC(\pm)}$$

The two well-defined isosbestic points at 281 and 311 nm are consistent with an equilibrium involving appreciable concentrations of only two absorbing species.[14,15] To examine the nature of this equilibrium, molecular ellipticities at the extrema at 260, 270, and 293 nm were recorded as a function of solvent composition as shown in Figure 3. Assuming additivity of the ellipticities of the two species, linear interpolation on these curves yields the percent nonionized free base at each solvent composition. The results, shown in Figure 4, indicate that the equilibrium constants, $K_{eq} = [\mathrm{OTC(0)}/[\mathrm{OTC(\pm)}]$, which can readily be obtained from the data, are solvent dependent but independent of wavelength.

Thus, the CD data provide a consistent picture of a facile interconversion of OTC(±) and OTC(0) in solution at room temperature. This interconversion is an equilibrium process that involves a drastic alteration of the conformation of the A ring and a concomitant change in the ionization state of the molecule. The two conformational states have been identified[2,7] with stable molecular geometries found in the crystalline state. It is clear that crystalline, zwitterionic OTC(±) dissolves in ethanol and converts directly to the un-ionized OTC(0). It is clear that, in ethanol/water solutions, crystalline OTC(0) establishes a dynamic equilibrium with the zwitterionic form. There is no basis to consider[11] that preparation of the anhydrous free base by azeotropic distillation at 80–100 °C conformationally locks the nonionic molecule in a form that is inaccessible under biological conditions.

It can be seen from Figure 4 that the equilibrium shifts rapidly toward the zwitterionic form with the addition of small amounts of water. Nevertheless, even after the addition of as much as 20% water (this figure would be much larger on a mole fraction basis), a substantial fraction of the molecules survive in the anhydrous form. In even more polar solutions with a higher water content, small but not inconsequential fractions of the nonionized form persist. In view of the enhanced solubility of this form in nonaqueous organic phases such as lipids, it is reasonable to suppose that low concentrations of the anhydrous form might be involved in a dynamic transport

mechanism in an interface phase at a lipid membrane in vivo.

The present data relate to equilibria in homogeneous solutions; one can infer the general features of the equilibrium partition of tetracycline antibiotics between two immiscible phases. Given an appropriate kinetic mechanism, it would appear that the hydrophilic/hydrophobic nature of the molecules would make it possible to permeate a sequence of polar and nonpolar barriers by structural and conformational changes. This conformational versatility of the tetracyclines can be expected to play a significant role in their mechanism of action and is probably related to their broad-based antimicrobial activity and membrane permeation.

Acknowledgment. This work was supported in part by the National Science Foundation through the Materials Science Center of Cornell University.

References and Notes

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Chemical-Structural Properties of Tetracycline Derivatives. 8. The Interrelationships between Oxytetracycline and 4-Epioxytetracycline

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Received April 16, 1979

Abstract: A structure determination for zwitterionic 4-epioxytetracycline, 4-epiOTC(±), has been carried out with a crystal of 4-epiOTC·$4H_2O$·CH_2Cl_2 maintained at 120 K. The space group is $P2_1$ with a = 15.423 (4) Å, b = 10.322 (3) Å, c = 9.626 (2) Å, and β = 104.88 (2)°; Z = 2. The tetracycline molecule displays a conformation similar to that of OTC(2−). The solvent system ($EtOH/CH_2Cl_2$, 1:1) was expected to give rise to a nonionized form of the free base if a stable conformation of this form were accessible to 4-epiOTC. Slow reepimerization of 4-epiOTC to the medicinally important parent OTC was found to occur in warm toluene; said reepimerization is attributed to the existence of a stable OTC(0) species which is favored by the hydrophobic character of the solvent. The in vitro and in vivo biological activity of 4-epiOTC is attributed to the production of the parent via reepimerization.

Introduction

The preceding paper in this series[1] presents strong evidence that the lipid-soluble tetracycline, TC, species is the nonionized free base. To date this species has been observed in several crystal structure analyses[2-5] to have a specific conformation. It was pointed out earlier[3] that said conformation is not accessible to 4-epiTCs because of steric hindrance. Nonetheless, 4-epiTCs display significant, though generally reduced, in vitro antibacterial activity and, in the case of 4-epiOTC, in vivo activity with *S. pyogenes* that is comparable[6] to that of OTC. If, as we propose, both a zwitterionic and nonionized form of the TC are required to support in vivo activity, it seemed necessary that a second conformation of the nonionized free base, one accessible to 4-epiOTC, exist. This report describes the results of our efforts to obtain crystalline nonionized 4-epiOTC and the implications of observations in the course of this study with respect to the mechanism of activity of 4-epiOTC.

Experimental Section

A sample of 4-epiOTC was prepared from OTC free base according to the method of McCormick et al.[7] Single crystals were obtained by dissolving a 100-mg sample in 20 mL of an ethanol/dichloromethane (1:1) solvent system. The solution was maintained at ca. 8 °C and the dichloromethane content slowly increased by vapor diffusion. After about 4 days at this temperature, crystals of marginal quality for a crystal structure determination were obtained. Attempts to grow crystals of higher quality have not been successful.

In the course of the crystallization experiments with 4-epiOTC, we applied the crystallization techniques we used to obtain high-quality single crystals of nonionized OTC free base;[3,4] that is, we attempted to grow crystals by slow evaporation of a warm toluene solution. The experiments yielded only crystalline OTC rather than 4-epiOTC. We subsequently monitored (by thin layer chromatographic (TLC) techniques) a 4-epiOTC/toluene system maintained at ca. 50 °C for a period of 3 weeks.

A single crystal of 4-epiOTC of parallelepiped morphology (0.5 × 0.5 × 0.1 mm^3), enclosed in a thin-walled glass capillary, was used for all crystallographic measurements. The space group was determined to be $P2_1$ from Buerger precession photographs. Lattice parameters (ca. 120 K) were determined by least-squares refinement[8] with automatically centered 2θ values (Syntex $P\bar{1}$ autodiffractometer equipped with a Syntex LT-1 low-temperature device) for 26 reflections in the angular range 25.6° < 2θ < 39.5° (Mo Kα, λ = 0.710 69 Å): a = 15.423 (4) Å, b = 10.322 (3) Å, c = 9.626 (2) Å, and β = 104.88 (2)°; Z = 2. Diffraction intensities were measured in an ω-scan

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