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.

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

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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 $25.6° < 2\theta < 39.5°$ (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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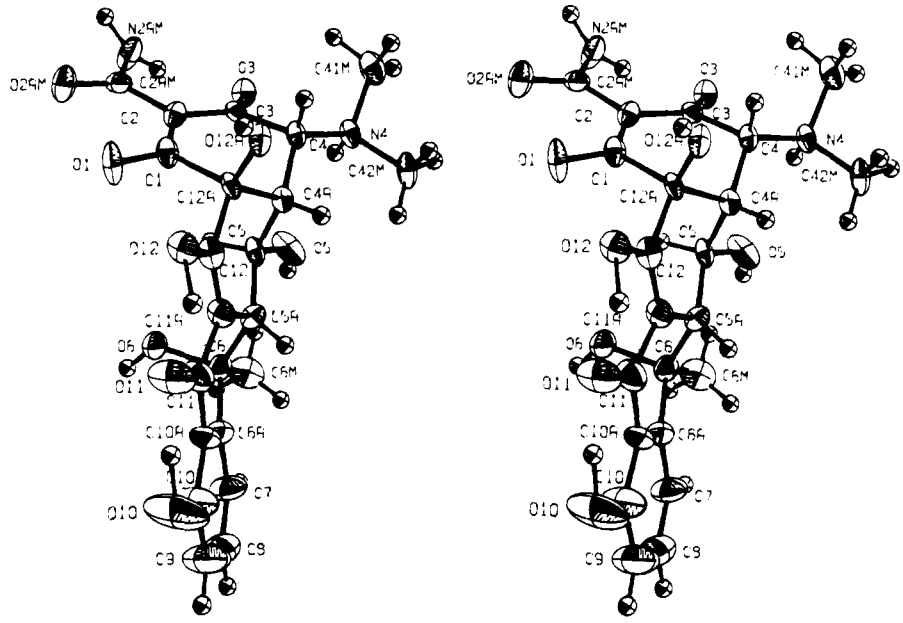

Figure 1. A stereoscopic projection[9] for the 4-epiOTC(±) molecule displaying the atom labels. C, N, and O atoms are depicted with ellipsoids representative of their refined thermal parameters; H atoms are drawn with arbitrarily reduced isotropic thermal parameters to reduce clutter.

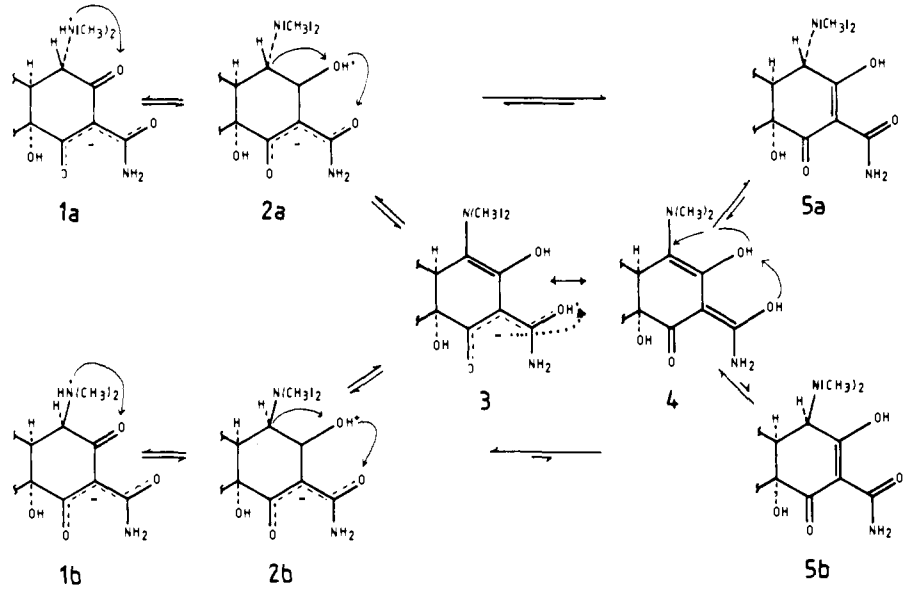

Figure 2. A schematic representation of possible species involved in the equilibrium between OTC and 4-epiOTC. Species 1 are the respective zwitterionic free bases, species 3 represents the convergence point of the epimerization mechanism, and species 5 are the respective nonionized free base forms. Only the A-ring portion of the tetracycline molecules has been drawn.

mode for which the scan range was 0.75°; the scan rate varied as a function of maximum peak intensity from 2.0 to 24.0° min^{-1}. Background radiation was measured on each side of the reflection center ($\Delta\omega$ = 1.0°) for one-half the total scan time. Three reference reflections, measured periodically, displayed neither systematic nor signifcant deviations in their average intensities. Of the 4551 unique reflections so measured ($\sin\theta/\lambda_{max}$ = 0.704 $Å^{-1}$), 2109 were classified as subjectively observed under the criterion $I > 3\sigma(I)$. Intensity data were corrected for Lorentz and polarization effects; absorption corrections were deemed not necessary.

Structure Determination and Refinement. The initial model was obtained by direct methods. All nonhydrogen atoms of the tetracycline molecule, of a dichloromethane molecule, and three water molecules were easily located in either the initial E map or in subsequent difference Fourier maps. The presence of a highly disordered fourth water molecule was also determined. This molecule is distributed over four sites within 2.3 Å contact distance; we arbitrarily set the occupancy of the four sites to total one and initially scaled individual occupancy factors to reflect the electron density contained in a difference Fourier map. Location of hydrogen atoms by difference Fourier techniques was only partially successful. Positions for those of the tetracycline molecule that were not so determined were calculated to conform to theoretical bonding geometry. All appropriate atomic coordinates, anisotropic temperature factors for nonhydrogen atoms, isotropic temperature factors for hydrogen atoms, and a single scale factor were refined by block-diagonal least-squares techniques. The population parameters of the oxygen atoms of the disordered water molecule were refined under the constraint that their occupancy total one. This refinement was carried out with isotropic temperature factors and was followed by refinement of anisotropic temperature factors with fixed population parameters. The block structure was such that parameters of one C, N, O, Cl, and any H atoms bonded to it were contained in one block; the parameters of the oxygen atoms of the fourfold disordered water molecule were contained in one block. A

Table I. Fractional Atomic Coordinates of Cl, O, N, and C Atoms Multiplied by 10^4

	x	*y*	*z*
C(1)	5635(6)	3694(9)	1985(9)
O(1)	6047(5)	3559(7)	1045(8)
C(2)	4933(6)	4606(9)	1931(9)
C(2am)	4617(6)	5452(10)	651(9)
N(2am)	3987(6)	6314(10)	681(8)
O(2am)	4918(5)	5343(7)	−420(7)
C(3)	4548(5)	4747(9)	3138(9)
O(3)	4062(4)	5645(7)	3343(7)
C(4)	4770(6)	3683(9)	4306(9)
N(4)	4407(5)	4058(8)	5544(8)
C(4M1)	3392(7)	3936(12)	5184(11)
C(4M2)	4770(7)	3283(10)	6861(10)
C(4a)	5765(5)	3355(9)	4660(8)
C(5)	6412(6)	4503(9)	5110(9)
O(5)	6093(5)	5304(7)	6077(8)
C(5a)	7363(6)	4028(9)	5652(9)
C(6)	8092(6)	5079(9)	5937(9)
C(6a)	9017(6)	4460(11)	6454(10)
O(6)	8022(5)	5659(9)	4545(8)
C(6M)	7955(8)	6127(11)	6967(13)
C(7)	9706(7)	5022(13)	7506(11)
C(8)	10579(8)	4487(15)	7843(13)
C(9)	10761(8)	3396(18)	7173(14)
C(10)	10084(8)	2834(14)	6093(14)
O(10)	10294(5)	1777(13)	5411(11)
C(10a)	9201(6)	3324(13)	5772(11)
C(11)	8487(7)	2682(12)	4711(11)
O(11)	8674(5)	1806(9)	3921(8)
C(11a)	7560(6)	3025(10)	4604(10)
C(12)	6913(6)	2452(9)	3566(10)
O(12)	7087(4)	1518(7)	2704(7)
C(12a)	5923(6)	2753(9)	3291(9)
O(12a)	5411(4)	1603(7)	2982(6)
Cl(1)	3034(2)	1755(0)	−1767(3)
Cl(2)	2685(2)	3347(4)	539(4)
C(DCM)	3505(8)	2445(13)	−43(13)
O(1w)	6667(9)	4469(12)	8870(11)
O(2w)	8441(11)	915(17)	8091(16)
O(3w,a)	8587(18)	3239(30)	495(28)
O(3w,b)	8281(40)	4159(45)	1100(53)
O(3w,c)	8937(26)	5447(51)	1106(41)
O(3w,d)	7887(33)	3627(58)	25(60)
O(4w)	596(13)	4214(14)	3186(20)

total of 487 parameters were refined with 3248 contributing reflections (observed data and those unobserved reflections with calculated intensities greater than subjective cutoff values) to give $R = 0.086$.

Results and Discussion

Although the crystals of 4-epiOTC·CH_2Cl_2·$4H_2O$ were not of as high quality as those used for structure determinations of free bases of the parent compound, the refined structural model clearly indicates that the tetracycline molecule is zwitterionic. A stereoscopic projection[9] of the molecular entity, henceforth 4-epiOTC(±), is presented with the applicable labeling scheme in Figure 1; atomic coordinates for nonhydrogen atoms are presented in Table I and bond distances in Table II.[10]

The bond distances and angles[10] for 4-epiOTC(±) are considerably less precise than those we have reported for the parent free bases but are entirely consistent with the structure of a tetracycline zwitterion. The conformation most closely resembles that we reported for the OTC(2−) divalent anion.[11] This observation, in conjunction with the observation by Mitscher[12] et al. that TC and 4-epiTC display the same conformation in basic but not acidic solution, is an indication that this is the solution conformation as well.

Considering the composition of the solvent system from which the crystals were grown, we find it intriguing that the conformation observed should be that representative of 4-epiOTC in aqueous solution. Dry solvents were used in preparation of the solvent system; crystallization was carried out in a closed container. The most likely source of water was the air-dried sample of 4-epiOTC, so it appears as though the zwitterion maintained its hydration sphere in the solvent system and that crystallization resulted as the hydrophobic character of the solvent system increased. The accompanying paper demonstrates that OTC free base behaves very differ-

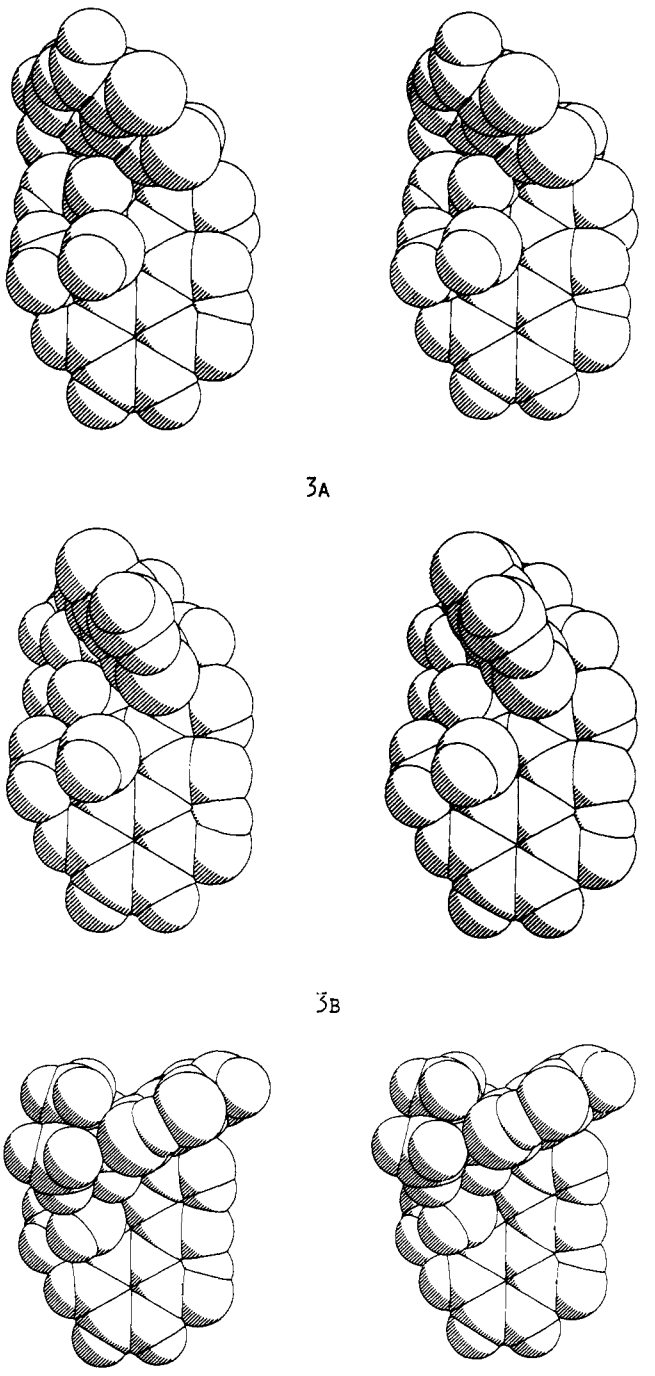

Figure 3. Stereoscopic projections of space-filling models for three oxytetracycline species. (3A) 4-epiOTC(±); (3B) OTC(±);[3] (3C) OTC(0).[4] The drawings are constructed[13] from crystallographic coordinates and represent accurate reproductions of observed conformations.

Table II. Bond Distances (Å) between C, N, and O Atoms with Estimated Standard Deviations[a]

C(1)-O(1)	1.241(13)	C(5a)-C(11a)	1.530(14)
C(1)-C(2)	1.425(13)	C(6)-C(6a)	1.525(13)
C(1)-C(12a)	1.560(12)	C(6)-C(6M)	1.519(16)
C(2)-C(3)	1.442(13)	C(6)-O(6)	1.446(12)
C(2)-C(2am)	1.487(12)	C(6a)-C(7)	1.392(14)
C(2am)-N(2am)	1.323(14)	C(6a)-C(10a)	1.408(17)
C(2am)-O(2am)	1.239(12)	C(7)-C(8)	1.413(17)
C(3)-O(3)	1.240(12)	C(8)-C(9)	1.363(22)
C(3)-C(4)	1.546(12)	C(9)-C(10)	1.396(17)
C(4)-C(4a)	1.523(12)	C(10)-C(10a)	1.411(15)
C(4)-N(4)	1.492(12)	C(10)-O(10)	1.355(19)
N(4)-C(4M1)	1.518(13)	C(10a)-C(11)	1.456(14)
N(4)-C(4M2)	1.482(11)	C(11)-C(11a)	1.450(14)
C(4a)-C(5)	1.538(12)	C(11)-O(11)	1.262(15)
C(4a)-C(12a)	1.532(12)	C(11a)-C(12)	1.353(12)
C(5)-O(5)	1.424(12)	C(12)-C(12a)	1.513(13)
C(5)-C(5a)	1.507(12)	C(12)-O(12)	1.343(12)
C(5a)-C(6)	1.536(13)	C(12a)-O(12a)	1.415(11)

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

ently in that an adjustment in the equilibrium between the zwitterionic and nonionized forms accommodates changes in the hydrophobic character of the solvent system.

The fact that crystals of OTC(0) were obtained from a warm toluene solution prepared from 4-epiOTC prompted us to look more closely at this system. We found that 4-epiOTC was initially nearly insoluble in toluene but that solution was eventually achieved. A TLC of the solvent that had been stirred for ca. 2 h with 4-epiOTC displayed (UV detection) no significant indication of the presence of a tetracycline. In contrast, when a portion of the solid phase was chromatogramed, the predominance of 4-epiOTC and a trace of OTC were observed. After ca. 48 h at 50 °C, a significant concentration of OTC was detected in the toluene, but no spot corresponding to 4-epiOTC was observed. A sample maintained at this temperature for 3 weeks was found to be devoid of residual solid and gave a chromatogram corresponding to nearly pure OTC.

We find these observations to be particularly relevant to the antibacterial activity of 4-epiOTC. They imply that 4-epiOTC undergoes reepimerization in hydrophobic environments thereby giving rise to OTC, the active species. We suggest that regeneration of the normal configuration is a direct result of the interrelationship between chemical structure (zwitterionic or nonionized) and conformation displayed by the TC free bases. Epimerization most likely proceeds via an intermediate C(3)-C(4) enol; the mechanism is summarized in Figure 2. The initial steps are the well-known acid-catalyzed enol formation (depicted as an intramolecular process but not intended to be confined to such) to give rise to common species **3**. Intermediate **3** is a convergence point suitable for generation of four species: OTC(±), 4-epiOTC(±), OTC(0), and 4-epiOTC(0). Our experiments with toluene were designed to favor the existence of the latter pair (**5a** and **5b** in Figure 2). The fact that only OTC(0) was found is strong evidence that a stable conformation for 4-epiOTC(0) does not exist.

In view of the significance of the interrelationships between chemical structure, configuration, conformation, and biological activity that are emerging for the TCs we consider it important that principal conformations be presented from various viewpoints. We have presented ORTEP[9] plots for each and now present stereoscopic projections of space-filling models, Figure 3, drawn from the X-ray coordinates.[13] The differences in the orientation of the A ring in the three structures are easily seen in these drawings. The drawing for OTC(0) particularly well illustrates the close intramolecular contact between the H(4) atom and the C-ring substituents at C(6). The concept that this conformation is inaccessible to 4-epiOTC(0) needs no further justification.

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Supplementary Material Available: Tabulated anisotropic temperature factors for C, N, O, and Cl atoms, fractional atomic coordinates and isotropic temperature factors for H atoms, selected bond and dihedral angles, and observed and calculated structure factors (24 pages). Ordering information is given on any current masthead page.

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