# The Question of Chair-Twist Equilibria for the Phosphate Rings of Nucleoside Cyclic 3',5'-Monophosphates. <sup>1</sup>H NMR and X-ray Crystallographic Study of the Diastereomers of Thymidine Phenyl Cyclic 3',5'-Monophosphate

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Abstract: The diastereomeric cis and trans forms of thymidine phenyl cyclic 3',5'-monophosphate triesters 5 were synthesized starting with the corresponding cyclic N,N-dimethyl phosphoramide. X-ray crystallography unequivocally identified the trans diastereomer (PhO and 1-thyminyl, trans). <sup>1</sup>H NMR analysis shows the phosphate ring of cis-5 to be in the chair conformation with the PhO axial. trans-5 features a chair  $\Rightarrow$  twist phosphate ring equilibrium (acetone-d<sub>6</sub>) with  $J_{S'AP}$  (11.6 Hz) >  $J_{S'AP}$ (9.6 Hz). Use of reasonable assumed values of  ${}^{3}J_{HP}$  for the protons on C-5' in the chair and twist forms leads to the conclusion that about 60% of *trans*-5 is in the twist conformation 6b in acetone- $d_6$ , and a little over 40% of *trans*-5 populates 6b in CDCl<sub>3</sub>. Careful dissection of the free-energy components which contribute to the observed chair = twist equilibrium for trans-5 gives an estimated value for  $\Delta G^{\circ}_{c \to t}$  of only 2.2 kcal/mol. This represents the intrinsic resistance of the chair-form ring to conversion to the twist conformation with the PhO pseudoaxial and gives an upper limit value for the cyclic 3',5'-monophosphate itself. This very low value of  $\Delta G^{\circ}_{c \to t}$  is found in spite of the 5 kcal/mol of strain energy associated with the transoid fusion of the phosphate and deoxyribose rings. Clearly,  $\Delta G^{\circ}$  for the chair  $\rightarrow$  twist interconversion for a nucleoside cyclic 3',5'-monophosphate can be readily supplied by binding interactions within the active site of a phosphodiesterase or protein kinase. The apparent ease of chair-twist interconversion is reflected also in the X-ray structure in which the phosphate ring about phosphorus is flattened into a near half-chair conformation which approximates the barrier geometry from the chair  $\rightarrow$  twist interconversion. This appears to be a response to the propensity of the PhO to be axial (or pseudoaxial) and the intermolecular NH···O=P H-bonding interaction noted in the unit cell. (The internuclear N(3)-O(7) distance between the symmetry related molecules is only 3.004 Å.) The conformation of the deoxyribose ring of both cis- and trans-5, as determined from proton-proton coupling constants, is close to that of cTMP itself,  $_4E_{-4}T^3$ .

Cyclic nucleotides (nucleoside cyclic 3',5'-monophosphates) such as cAMP (1) and cGMP (2) play a central role in cell metabolism.<sup>1</sup> Thus, cAMP acts as a second messenger in its interaction



with protein kinases to trigger the phosphorylation of cellular enzymes resulting in the activation of various metabolic processes. Substrate structural requirements for the binding of cAMP to the regulator subunit of protein kinases<sup>2</sup> as well as to the phosphodieserases (PDE)<sup>2a,h,3</sup> which convert cAMP hydrolytically to 5'-AMP have been defined. The PDE hydrolysis stereochemistry at phosphorus is very predominantly inversion with enzymes from bovine heart and Baker's yeast.<sup>4</sup>

Dreiding molecular models show clearly that the phosphate ring of cAMP (and other cyclic nucleotides) can exist in the chair conformation 3 identified by <sup>1</sup>H NMR and X-ray crystallography



to be the conformation populated normally and also as a single, higher energy twist conformation 4. (Only a single twist form and no boat conformations appear to be accessible because of the strain and molecular distortion imparted to the system by the trans ribose/phosphate ring fusion.) The possible conformations of the phosphorus-containing ring following nucleophilic attack by appropriate functionality within the active site of a phosphodiesterase to form a pentacovalent phosphorus intermediate, and the possibility of either diequatorial or apical-equatorial attachment of that ring to phosphorus have been considered.<sup>3a,h,4a,5</sup> Formation of a pentacovalent adduct on coordination of cAMP with the regulatory subunit of a protein kinase also has been proposed by one group.<sup>5a,b</sup> Moreover, the premise has been set forth that the 1,3,2-oxaza- and perhaps the 1,3,2-dioxaphosphorinane ring of a pentacovalent phosphorus intermediate, if attached to phosphorus apical/equatorial, is preferably in a boat conformation.<sup>6</sup> However, the idea that the phosphate ring of cAMP might undergo a

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Table I. <sup>1</sup>H NMR Parameters for the Phosphate Rings of 5 and cTMP<sup>a</sup>

			J (Hz)							δ (ppm)				
compd	solvent	3'4'	4′5′a	4′5′b	5a'5'b	3'P	4′P	5'aP	5′bP	3'	4′	5′a	5′b	rms error <sup>b</sup>
cTMP <sup>a</sup> cis-5 trans-5 trans-5	$D_2O$ acetone- $d_6$ acetone- $d_6$ $CDCl_3^q$	9.2 9.3 <sup>c</sup> 9.4 <sup>e</sup>	10.6 10.7 10.4 10.3	4.7 4.5 6.0 5.8	-9.5 -9.3 -9.6 -9.7	1.7 0.9 0.8 $\sim$ 0.5	0.1 -0.7 -0.6 h	2.2 0.7 11.6 8.7	20.4 22.9 9.6 13.2	4.70 5.24 <sup>d</sup> 5.20 <sup>f</sup> 5.03	3.91 4.09 4.23 3.92	4.29 4.67 4.56 4.57	4.45 4.72 4.78 4.66	0.11 0.057

<sup>a</sup> From ref 13. <sup>b</sup> Root mean square error in line positions. <sup>c</sup> Probable errors in J, 0.032–0.040 Hz. <sup>d</sup> Probable errors in  $\delta$ , 0.020–0.032 ppm. <sup>e</sup> Probable errors in J (calculated by LAOCN3 program), 0.015-0.022 Hz. <sup>f</sup> Probable errors in δ, 0.011 ppm. <sup>g</sup> From first-order analysis of 4', 5'a, 5'b proton region. <sup>h</sup>Not discernible.

chair-to-twist  $(3 \rightarrow 4)$  interconversion on binding to a protein kinase, or prior to or concerted with formation of a twist-form pentacovalent adduct during PDE-catalyzed hydrolysis, seems not to have been examined. Key to these considerations is the free energy required to effect  $\mathbf{3} \rightarrow \mathbf{4} \ (\Delta G^{\circ}_{c \rightarrow t})$ .

In this paper we report the results of the study of the chair-twist equilibrium of the phosphate ring of a nucleoside cyclic phosphate triester, phenyl thymidine cyclic 3',5'-monophosphate 5. Mo-



nocyclic neutral 2-thio- and 2-oxo-1,3,2-dioxaphosphorinanes and those fused transoid to six-membered rings are known to populate twist conformations readily.<sup>7</sup> However, the 5 kcal/mol of strain accompanying the trans fusion of the rings of cAMP<sup>8</sup> makes the conformational properties of 5 uncertain a priori. The results of the present study show that 6b is highly populated by trans-5, and a careful dissection of  $\Delta G^{\circ}_{obsd}$  for **6a**  $\rightleftharpoons$  **6b** allows us to estimate that  $\Delta G^{\circ}_{c \to t}$  for  $\mathbf{3} \rightleftharpoons \mathbf{4}$  could indeed easily be supplied by binding forces operative within an enzyme active site either independent of, or concerted with, formation of a pentacovalent intermediate.

#### Results

Preparation of cis- and trans-5. Scheme I illustrates the method of preparation of adequate quantities of the cis and trans diastereomers (relationship of PhO to Thy) of the phenyl triester of thymidine cyclic 3',5'-monophosphate 5. The preparation of cis-5 is similar to the preliminary report of Baschang and Kvita.<sup>9</sup> As an improvement on their method, we isolate completely pure phosphoramidite 8 (trans/cis  $\approx 95/5$ ) by a simple column chromatography prior to its conversion to 9 and utilize for the oxidation of 9 and 10 a nonaqueous solution of  $N_2O_4$  rather than aqueous  $KMnO_4$ . (Pure 8 has proved to be a valuable intermediate



Figure 1. ORTEP perspective view of trans-5.

Scheme I



for the synthesis of a variety of derivatives of thymidine cyclic 3',5'-monophosphate.<sup>10</sup>) The N<sub>2</sub>O<sub>4</sub> oxidation of phosphites is known to proceed with retention of configuration at phosphorus.<sup>11</sup> Only the cis diastereomer of 9 was formed (as shown by <sup>31</sup>P NMR) which yielded cis-5 on oxidation. Use of o-nitrophenol and then  $N_2O_4$  oxidation yielded 11. Nucleophilic attack on 11 by PhONa displaced o-NO<sub>2</sub>C<sub>6</sub>H<sub>4</sub>ONa to give a 4:1 (trans:cis) mixture of diastereomers of 5 which were separated by MPLC to isolate pure trans-5.

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Table II. <sup>1</sup>H NMR Parameters for the 2'-Deoxyribose Rings of cTMP and 5

	J (Hz)							δ (ppm)					
compd	1'2'a	1′2′b	2'a2'b	2'a3'	2′b3′	3'4'	3'P	1'	2′a	2′b	3'	4'	rms error <sup>b</sup>
cTMP <sup>a</sup>	8.9	2.4	-13.3	10.8	8.0	9.2	1.7	6.30	2.59	2.50	4.70	3.91	
cis- <b>5</b>	8.9°	3.1	-13.6	10.3	8.2	9.3	0.9	6.43 <sup>d</sup>	2.72	2.70	5.24	4.09	0.035
trans-5	8.9 <sup>e</sup>	2.9	-13.2	10.4	8.3	9.4	0.7	6.47 <sup>f</sup>	2.67	2.66	5.20	4.23	0.045

<sup>a</sup> From ref 13. <sup>b</sup>Root mean square error in line positions. <sup>c</sup>Probable errors in J, 0.011-0.024 Hz. <sup>d</sup>Probable errors in δ, 0.004-0.010 ppm. \* Probable errors in J calculated by LAOCN3 program, 0.020-0.058 Hz. / Probable errors in  $\delta$ , 0.007-0.030 ppm.

		assume	d J (Hz)	·	% 6b b	ased on				
solvent	5a'P (6a)	5b'P (6a)	5a'P (6b)	5b'P (6b)	$\overline{J_{5a'P}(\text{obsd})}$	$J_{5b'P}(obsd)$	av %	$\Delta G^{\circ}{}_{\mathrm{obsd}}$	$\Delta G^{\circ}{}_{\mathrm{PhO}}$	ΔG° <sub>c→t</sub>
acetone- $d_6$	1.0	22.5	19.0	1.5	59	61	58	-0.18		
	1.0	22.5	20.5	0.5	54	59				
CDCl <sub>3</sub>	1.0	22.5	19.0	1.5	43	44	42	0.18	-2.0	2.2
	1.0	22.5	20.5	0.5	39	42				
acetone- $d_6$	1.5	23.5	19.0	1.5	59	63	60	-0.23		
Ū	0.5	23.5	20.5	0.5	56	60				
CDCl <sub>3</sub>	1.5	23.5	19.0	1.5	41	47	44	0.14	-2.0	2.1
5	0.5	23.5	20.5	1.5	41	45				

Conformational Analysis of the Phosphate Ring. A single-crystal X-ray structure determination identified the trans diastereomer (Figure 1). The relative <sup>31</sup>P chemical shifts for cis-5 ( $\delta$  -13.6, acetone- $d_6$ ) and trans-5 ( $\delta$  -11.8, acetone- $d_6$ ) also are in the order expected<sup>12</sup> for diastereomers with either a considerable population of chair conformer with PhO axial (7, upfield shifted) or with PhO equatorial (6, downfield shifted).

The primary interest of this study concerns the conformational aspects of the phosphate ring of cis- and trans-5. To this end an analysis of the chemical shifts and coupling constants for the protons of the phosphate and deoxyribose rings was carried out. At 300 MHz a first-order approximation analysis could be made with the guidance of the parameters known for  $cTMP^{13}$  itself. Refinement by use of the iterative LAOCN3 program led to the values in Table I. (See Experimental Section for details.)

The chair conformation 7 is assigned to the phosphate ring of cis-5. This conclusion is evident from the values for  $J_{5'aP}$  (0.7 Hz),  $J_{5'bP}$  (22.9 Hz), and  $J_{5'a4'}$  (10.7 Hz). These couplings are consistent with the antiperiplanar relation of  $H_{5'b}$  and P, the synclinal relation of  $H_{5'a}$  and P, and the predictions of the Karplus relationship which applies to such couplings.<sup>14</sup> The similarity of these couplings to those for the chair-form phosphate ring of cTMP (Table I) confirms the assignment of conformer 7 to cis-5. The extremely small 0.7-Hz value of  $J_{5'aP}$  for cis-5 suggests that it is completely in conformation 7.

By contrast, the values of  $J_{5'aP}$  (11:6 Hz) and  $J_{5'bP}$  (9.6 Hz) make it clear that conformation 6a is not the form predominantly populated by trans-5. Evidently, the conformational equilibrium **6a**  $\Rightarrow$  **6b** is operative. In **6b** H<sub>5'a</sub> has become pseudoequatorial, and  $H_{5'b}$  is pseudoaxial.  $J_{4'5'a}$  is unchanged in trans-5 compared to its value in cis-5 indicating that the  $H_{4'}CCH_{5'a}$  dihedral angle is similar in both chair 6a (7) and twist conformation 6b. Inspection of Dreiding molecular models of **6b** shows this to be true.

Although  $H_{5'a}$  and  $H_{5'b}$  are essentially interchanged in **6a** and **6b**, the sum of  $J_{5'aP} + J_{5'bP}$  is slightly less for *trans*-5 (21.2 Hz) compared to that for *cis*-5 (23.6 Hz). This means that  $J_{5'aP}$  for **6a** is not equal to  $J_{5'bP}$  for **6b**, and/or  $J_{5'bP}$  for **6a** is not equal to  $J_{5'aP}$  for **6b**. (More generally, the sums of the couplings are less for 6b compared to 6a.) In 6b the actual magnitudes of  $J_{5'aP}$  and

 $J_{5'bP}$  will depend on the degree of twisting of the phosphate ring. Indeed, Dreiding models show that the sum  $J_{5'aP} + J_{5'bP}$  for **6b** should be less than it is for 6a unless 6b is fully twisted. This would readily account for the observed reduced sum of the average of these couplings. Consistent with this is the slightly increased sum of  $J_{5'aP} + J_{5'bP}$  in CDCl<sub>3</sub> (21.9 Hz) in which the population of 6a is evidently increased.

One may readily estimate the mole fractions (N) of 6a and 6b populated from the observed couplings  $J_{5'aP}$  and  $J_{5'bP}$ . Thus

 $J_{5'aP}(obsd) = N(6a) \cdot J_{5'aP}(6a) + N(6b) \cdot J_{5'aP}(6b)$ 

 $J_{5'aP}(obsd) = (1 - N(6b)) \cdot J_{5'aP}(6a) + N(6b) \cdot J_{5'aP}(6b)$ 

therefore

$$N(6b) = \frac{J_{5'aP}(obsd) - J_{5'aP}(6a)}{J_{5'aP}(6b) - J_{5'aP}(6a)}$$
(1)

A similar derivation involving  $J_{5'bP}(obsd)$  gives a completely analogous equation. A reasonable value for  $J_{5'aP}$  (6a) is 1 Hz and for  $J_{5'bP}$  (6a), 22.5 Hz. These are close to those for cis-5 in conformation 7 (Table I). For 6b one may assume a somewhat reduced  $J_{5a'P}$  (6b) of 19 Hz and for  $J_{5b'P}$  (6b) also a slightly reduced value, perhaps 0.5 Hz. These values reflect the fact that the HCOP dihedral angle in 6b will be decreased for H<sub>5a'</sub> compared to that for the equatorial proton in 6a; while for  $H_{5b'}$  dihedral angle HCOP will be increased relative to the axial proton in 6a.

By use of the above and similar assumed  ${}^{3}J_{HP}$  values, eq 1, and the analogous equation based on observed  $J_{5'bP}$ , estimates of the percentage of 6b populated in solution can readily be made. As shown in Table III, the range of calculated percentages is not very sensitive to small changes in the assumed  ${}^{3}J_{HP}$  values. The use of combinations of coupling constants yields maximum ranges of estimated percentages. In acetone- $d_6$  about 60% of trans-5 is in the twist conformation. The twist population is reduced in CDCl<sub>3</sub> to a little over 40%.

Conformation of the Deoxyribose Ring. In Table II are listed the coupling constants for the 2'-deoxyribose rings of cis- and trans-5 along with those for cTMP for comparison purposes. The very close agreement of these values for all three compounds, especially those involving protons on C(1'), C(2'), and C(3'), establishes the close similarities of their conformations. The conformational freedom of the 2'-deoxyribose are shown of cyclic nucleotides is severely restricted by the transoid ring fusion. The conformation of the sugar ring of cTMP has been assigned on the basis of coupling constants to be held in the narrow  $_{4}E_{-4}T^{3}$ range of conformations.<sup>15</sup> Conformation <sub>4</sub>E is an envelope form with C(4') as a flap. The formation of the neutral phosphate

<sup>(12)</sup> See data compiled in the following: Maryanoff, B. A.; Hutchins, R.

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<sup>(15)</sup> Robins, M. J; MacCoss, M.; Wilson, J. S. J. Am. Chem. Soc. 1977, 99, 4660 and references therein.

Table	IV.	Crystal	Data	for	trans-5 <sup>a</sup>
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mol formula	C <sub>16</sub> H <sub>17</sub> N <sub>2</sub> O <sub>7</sub> P
mol wt	380.29
space group	$P2_12_12_1$ (no. 19) <sup>b</sup>
cell dimensns	
<i>a</i> , Å	10.241 (2)
b, Å	12.375 (3)
c, Å	13.725 (4)
V, Å <sup>3</sup>	1739.4 (8)
Z	4
$D_{\text{calcd}}$ , g cm <sup>-1</sup>	1.452
radiatn, Å	λ (Μο Κα) 0.71073
supplied power	40 kV, 20 mA
data colletn method	$\theta - 2\theta$ scan
scan speed, deg min <sup>-1</sup>	2.4
scan range $(2\theta)$ , deg	$[2\theta(K\alpha_1) - 1.0 \text{ to } 2\theta(K\alpha_2) + 1.0]$
ratio of total backgrd time to peak scan time	1.0
std reflens <sup>c</sup>	$(8,0,3);$ $(8,0,\overline{3});$ $(0,10,0);$
	$(2,1,\bar{1}\bar{0});(2,1,10)$
$2\theta$ limit, deg	3.0-50.0
no. of unique data <sup>d</sup>	3037

<sup>a</sup>The standard deviation of the least significant figure is given in parentheses. <sup>b</sup>The space group was unambiguously determined by the systematic absences; h00, h = 2n + 1; 0k0, k = 2n + 1; 00l, l = 2n + 1 (ref 25, 1969, vol. I, p 112.). <sup>c</sup>The standard reflections were monitored every 95 reflections and indicated no discernable decomposition of the crystal. <sup>d</sup>All data were used in the calculations.

Table V. Fractional Atomic Coordinates for trans-5<sup>a</sup>

atom	x	У	Z
P	0.1289 (1)	-0.1830 (1)	0.0440 (1)
O(5′)	0.0949 (3)	-0.1584 (3)	0.1525 (2)
C(5')	0.1741 (4)	-0.1099 (4)	0.2279 (3)
C(4')	0.2681 (3)	-0.0358(3)	0.1757 (3)
O(1')	0.3677 (3)	0.0034 (2)	0.2372 (2)
C(1')	0.4742 (3)	0.0359 (3)	0.1754 (3)
C(2')	0.4506 (4)	-0.0167 (3)	0.0752 (3)
C(3')	0.3425 (4)	-0.0947 (3)	0.0980 (3)
O(3′)	0.2558 (3)	-0.1171 (2)	0.0176 (2)
N(1)	0.5938 (3)	0.0066 (2)	0.2240 (2)
C(2)	0.6586 (4)	0.0855 (3)	0.2759 (3)
O(2)	0.6254 (3)	0.1798 (2)	0.2751 (2)
N(3)	0.7647 (3)	0.0493 (3)	0.3252 (3)
C(4)	0.8142 (4)	-0.0550 (3)	0.3292 (3)
O(4)	0.9134 (3)	-0.0749 (3)	0.3764 (3)
C(5)	0.7412 (4)	-0.1333 (3)	0.2741 (3)
C(6)	0.6352 (4)	-0.1001 (3)	0.2263 (3)
C(7)	0.7881 (5)	-0.2481 (4)	0.2723 (4)
O(6)	0.0186 (3)	-0.1255 (3)	-0.0168 (2)
C(8)	0.0635 (5)	0.0625 (4)	-0.0428 (3)
C(9)	0.0280 (6)	0.1692 (4)	-0.0273 (4)
C(10)	-0.0784 (7)	0.1952 (4)	0.0252 (4)
C(11)	-0.1521 (7)	0.1161 (5)	0.0618 (5)
C(12)	-0.1212 (6)	0.0095 (4)	0.0491 (5)
C(13)	-0.0124 (4)	-0.0160 (3)	-0.0020 (3)
O(7)	0.1335 (4)	-0.2975 (3)	0.0234 (3)

<sup>a</sup> Estd's are in parentheses.

triester and population of the twist conformation **6b** by *trans*-**5** has little effect on the conformation of the 2'-deoxyribose ring.

**Single-Crystal X-ray Structure.** The crystal data for *trans*-5 are listed in Table IV with the final atomic parameter compiled in Table V. An ORTEP perspective view of the molecule, along with the labeling scheme, is given in Figure 1. Bond lengths and selected bond angles are shown in Figure 2.

The conformation adopted by the thymine base in *trans*-5 is anti, an assignment based on the torsion angle about the glycosidic bond,  $\chi$ , C(2)-N(1)-C(1')-O(1'), of 262.1°.<sup>16</sup> There exists a hydrogen-bonding interaction between N(3)-H(3) of the thymine and the phosphoryl P=O(7) of a symmetry-related molecule such that chains of hydrogen-bonded molecules are formed (Figure 4).







Figure 3. Bond angles in trans-5.



Figure 4. ORTEP perspective view of *trans*-5 showing intermolecular hydrogen bonding between N(3)-H(3) and P=O(7).

The internuclear separation between N(3) and O(7) of the symmetry-related molecules is 3.004 Å.

In this crystal structure, the ribose ring of *trans*-5 adopts what can best be designated as a  ${}_{4}^{3}$ T or C(3') exo-C(4') endo twist conformation.<sup>17</sup> The C(4')-O(1')-C(1')-C(2'),  $\nu_0$ , and O(1')-C(1')-C(2')-C(3'),  $\nu_1$ , torsion angles are -16.6° and -11.3°, respectively. A  ${}_{4}^{3}$ T ribose ring conformation has also been noted for *cis*-thymidine 3',5'-cyclic methylphosphonate ( $\nu_0 = -16.0^{\circ}$ ,  $\nu_1 = -13.0^{\circ}$ ).<sup>18</sup> The  ${}_{4}^{3}$ T conformation is adjacent on the psuedorotational pathway for the 2'-deoxyribose ring to the  ${}_{4}E{}_{-4}T{}^{3}$ forms indicated by the <sup>1</sup>H NMR couplings.

The 1,3,2-dioxaphosphorinane ring of *trans*-5 is severely flattened at the phosphorus end. The conformation of the

<sup>(16)</sup> We follow here the most recent IUPAC recommendations for synanti designation, based on the dihedral angle C(2)-N(1)-C(1')-O(1') rather than C(6)-N(1)-C(1')-O(1'): Eur. J. Biochem. **1983**, 131, 9.

<sup>(17)</sup> The use of  $\nu_0$  and  $\nu_1$  instead of  $\tau_0$  and  $\tau_1$  also follows IUPAC recommendations, ref 16. The  ${}_{4}^{3}T$ ,  ${}_{4}E$ , etc. designations also are spelled out in ref 16. For the  ${}_{4}T^3$  form see: Sundaralingam, M.; Abola, J. J. Am. Chem. Soc. **1972**, *94*, 5070.

<sup>(18)</sup> Bentrude, W. G.; Sopchik, A. E.; Bajwa, G. S.; Setzer, W. N; Sheldrick, W. S. Acta Crystallogr., Sect. C: Cryst. Struct. Commun. 1986, C42, 1027.

phosphate ring is probably best described as a chaise lounge or half-chair (see also structure **18**) and may be thought of as approximating the transition structure for interconversion of the chair and twist conformations. The O(3')-P-O(5')-C(5') and O-(5')-P-O(3')-C(3') torsion angles of -15.4° and 27.8°, respectively, clearly show the large extent of flattening. The O(3')-P-O(5')-C(5') and O(5')-P-O(3')-C(3') torsion angles for the similar triester *trans*-benzyl 2'-acetyluridine cyclic 3',5'-monophosphate (**12**) are -49.1° and 49.3°, respectively.<sup>19</sup>



#### Discussion

Free-Energy Requirements for  $3 \rightarrow 4$ . *cis*-5 occupies the chair population as expected by the known axial preference of the phenoxy group.<sup>20</sup> This preference is strong enough to force the chair conformation 6a of trans-5 in part into conformation 6b with been observed for 2-phenoxy-2-oxo-1,3,2-dioxaphosphorinanes<sup>7c,d</sup> to which a six-membered ring is fused in transoid fashion at C(4)and C(5). However, in these unstrained systems, more flexibility is afforded the phosphate ring; Drieding models show that two twist forms and a boat are accessible. This potentially favors nonchair forms from an entropy standpoint more greatly than they are for trans-5 for which Dreiding models make it apparent that only a single twist conformation is available because of the strain and molecular distortion imparted by the transoid five- to sixmembered ring fusion. This strain has been estimated at 5 kcal/mol<sup>8</sup> and could intrinsically affect the relative energies of **6a** and **6b** and equilibrium **6a**  $\Rightarrow$  **6b**. It is therefore not a priori obvious that the chair  $\rightleftharpoons$  nonchair equilibria observed for other 2-oxo-1,3,2-dioxaphosphorinanes should be found in the cTMP phenyl phosphate ester trans-5.

The equilibrium  $6a \rightleftharpoons 6b$  can be dissected into two components



illustrated by the equilibrium shown involving 13, 14 and 15, i.e.,

 $\Delta G^{\circ}(\text{obsd}) = \Delta G^{\circ}_{\text{PhO}} + \Delta G^{\circ}_{c \to t}$ (2)



Figure 5. Diagramatic view of a nucleoside cyclic 3',5'-monophosphate enzyme bound in a twist conformation.

 $\Delta G^{\circ}_{PhO}$  is the driving force for reorientation of the phenoxy group from the equatorial to the axial position.  $\Delta G^{\circ}_{c \to t}$  corresponds to the free energy required to place the C(5) end of the ring opposite the pseudoaxial PhO in twist form 14. This is an estimate of the intrinsic free energy required for a chair  $\rightarrow$  twist interconversion in such rings in the absence of any driving force for such a reorientation and approximates the minimum free energy required to effect the conversion  $3 \rightarrow 4$ .

Since eq 2 is readily rearranged to eq 3

$$\Delta G^{\circ}_{c \to t} = \Delta G^{\circ}(\text{obsd}) - \Delta G^{\circ}_{PhO}$$
(3)

it follows that to obtain  $\Delta G^{\circ}_{c \to t}$  one needs only an estimate of  $\Delta G^{\circ}_{PhO}$  along with the measured  $\Delta G^{\circ}(obsd)$  for  $\mathbf{6a} \rightleftharpoons \mathbf{6b}, 0.2$ kcal/mol.  $\Delta G^{\circ}_{PhO}$  has been determined for  $13 \rightleftharpoons 14$  in CDCl<sub>3</sub> to be -2.0 kcal/mol.<sup>21</sup> From eq 3,  $\Delta G^{\circ}_{c \to t}$  is then about 2.2 kcal/mol (Table III). The reliability of this number, of course, depends on how well  $\Delta G^{\circ}_{PhO}$  for 13  $\approx$  14 is transferable to the cyclic nucleotide system. Nonetheless, it is clear that  $\Delta G^{\circ}_{c \to t}$  is a comparatively low number, well below the values of  $\sim$  5 kcal/mol for cyclohexane<sup>22</sup> and 8 kcal/mol for 1,3-dioxane.<sup>23</sup> It should be emphasized that as an estimate of the  $\Delta G^{\circ}_{c \to t}$  for  $3 \to 4, 2$ kcal/mol may be somewhat high. This is because the pseudoaxial oxygen of **6b** has a phenyl group attached which through steric or entropic factors may be of higher energy in the pseudoaxial position than is a phosphoryl oxygen. The six-membered rings of the trans-phenoxy cyclic phosphite of thymidine<sup>10a</sup> and the cis-Me<sub>2</sub>N cyclic 3',5'-phosphoramidate of thymidine<sup>10c</sup> also exist to a large extent in the twist conformation.

Clearly,  $\Delta G^{\circ}$  for  $3 \rightarrow 4$  can readily be supplied by binding forces within an enzyme active site. Most simply, a strong coordination with the pseudoaxial oxygen of 4 (Figure 5), possibly via a salt bridge, would essentially transform that functionality into a group analogous to OR. This alone would provide nearly sufficient driving force to effect  $3 \rightarrow 4$ . In this regard, negative charge on exocyclic oxygen on phosphorus appears to be necessary to cAMP binding of protein kinases I and II<sup>3cd,f</sup> but not so essential for the binding of cAMP to beef heart or slime mold phosphodiesterase.<sup>3a</sup> Stereospecificity of binding and protein kinase activation has been noted with diastereomeric cAMPS (phosphorothioate) analogues, the R<sub>p</sub> isomer (equatorial oxygen) being the more active.

Solvent Effects on  $6a \Rightarrow 6b$ . Solvent polarity within the enzyme cavity also may affect the stability of a twist-form complex such

<sup>(19)</sup> Depmeier, W.; Engels, J.; Klaska, K. H. Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem. 1977, 33, 2463. The thermal ellipsoids of Figure 1 for atoms O(5'), O(6), O(7), and perhaps phosphorus show that there is a certain amount of thermal motion in the crystal. However, the possibility that both chair and twist conformations are present in the crystal and lead to electron densities which result in the structure of Figure 1 (approximating the interconversion barrier conformation) can be readily discounted. The atom centers for O(6), O(7), and C(13) are separated by 1-2 Å in the two conformations (Dreiding models). However, the greatest rms amplitude of vibration, that for O(7), is only 0.43 Å. The largest anisotropy of vibration (longest/shortest rms amplitude) is not exceptional, 0.43/0.23. The direction of anisotropy for O(6) is contrary to that expected for the chair = twist motion, and C(13) shows evidence of very little thermal motion. The largest peak remaining any place in the difference Fourier map is only 0.38 electrons/Å<sup>3</sup>. Those near the phosphate ring atoms are even smaller. Thus, significant electron density not included in the thermal ellipsoids has not been excluded.

<sup>(20)</sup> For a summary of conformational preferences of substituents on phosphorus in 2-oxo-1,3,2-dioxaphosphorinanes see, ref 12.

<sup>(21)</sup> Majoral, J.-P.; Pujol, R.; Navech, J. C. R. Acad. Sci. Ser. C 1972, 274, 213.

<sup>(22)</sup> Squillacote, M.; Sheridan, R. S.; Chapman, O. L.; Anet, F. A. L. J. Am. Chem. Soc. 1975, 97, 3244.

<sup>(23)</sup> Clay, R. M.; Kellie, G. M.; Riddell, F. G. J. Am. Chem. Soc. 1973, 95, 4632.



phoryl dipole. Therefore, the net dipole moment of the phosphate ring is greater in 17, the form stabilized by polar solvent, than in 16 (6a) in which the ring and P=O/OPh dipoles largely cancel. Highly polar water molecules in the active site likewise could stabilize the twist conformation of Figure 5.

X-ray Structure. Severe ring flattening is rare in 1,3,2-dioxaphosphorinanes in general and has not been previously observed in cyclic nucleotides. cis-4,6-Dimethyl-2-oxo-2-triphenylmethyl-1,3,2-dioxaphosphorinane, 18, adopts a half-chair or chaise lounge conformation,<sup>24</sup> presumably because of steric interactions. Possible reasons for the ring flattening in trans-5 are several. As a result of the anomeric effect, the phenoxy substituent on phosphorus is axial-seeking, and this may contribute to the distortion. Triester 12 is not distorted in this way.<sup>19</sup> The PhCH<sub>2</sub>O, however, is less axial seeking than the more electronegative  $Ph\bar{O}^{,20}$ The observed hydrogen-bonding involving the phosphoryl oxygen may also be a factor as may crystal packing forces. The chairto-twist interconversion  $6a \rightarrow 6b$  for *trans*-5, as shown above, requires only about 2 kcal/mol. The distortion observed in Figure 1 suggests that the potential barrier for this interconversion, approximated by the deformed chair of Figure 1, also may be relatively low.

### Conclusions

trans-5 exists to a large extent in the twist conformation 6b, the population of **6b** being dependent upon solvent polarity. Dissection of the component free-energy changes (eq 3) involved in the chair  $\rightarrow$  twist interconversion for **6b** results in an estimate of 2.2 kcal/mol for  $\Delta G^{\circ}_{c \rightarrow t}$ . This very low value, similar to those for monocyclic six-membered ring phosphate triesters, applies in spite of the approximately 5 kcal/mol of strain energy attributable to the trans phosphate/deoxyribose ring fusion.  $\Delta G^{\circ}_{c \rightarrow t}$  measures the intrinsic free energy required for the chair  $\rightarrow$  twist conformational change which places the PhO pseudoaxial. In the conversion of the cyclic nucleotide diester from 3 to 4, a phosphoryl oxygen becomes pseudoaxial; hence, the 2.2 kcal/mol value may be in fact a little high for  $\Delta G^{\circ}$  for  $3 \rightarrow 4$ . Clearly,  $\Delta G^{\circ}$  for 3  $\rightarrow$  4 is well within what can be supplied by binding and solvent-substrate interactions within the active site of an enzyme. The X-ray data suggest that the barrier to this interconversion may be low as well. Whether there is any advantage to be gained from conversion of the phosphate ring of cAMP or cGMP to the twist conformation in concert with binding to the regulatory subunit of a protein kinase or prior to or concerted with phosphodiesterase-catalyzed hydration or nucleophilic attack by the enzyme itself is not evident. Finally, it is important to note that in as yet unpublished studies of neutral nucleotide cis-N-alkyl and N,N-dialkyl cyclic 3',5'-phosphoramidates, we find  $\Delta G^{\circ}(\text{obsd})$ for the chair  $\Rightarrow$  twist equilibrium analogous to  $6a \Rightarrow 6b$  to vary by only a few tenths of a kcal/mol on change of the nitrogen base from a pyrimidine to a purine or substitution of an  $\alpha$ -OH for hydrogen at the 2'-position of the sugar ring.

### **Experimental Section**

Materials and Chromatography. Thymidine was purchased from Sigma Chemical Co. and used as received;  $(Me_2N)_3P$  (Aldrich) was distilled before use. *p*-Dioxane was freshly distilled from CaH<sub>2</sub>. Acctonitrile was reagent grade. Reactions were routinely run under a dry nitrogen atmosphere. Medium-pressure liquid chromatography (MPLC) was performed on Merck silica gel 60 (230-400 mesh) on 15 mm × 1000 mm Altex columns at about 35 psi. Melting points are uncorrected. Elemental analyses were carried out by Galbraith Laboratories, Knoxville, TN.

**Spectral Analyses.** <sup>1</sup>H NMR spectra were recorded in the Fourier transform mode on a Varian SC-300 spectrometer. (Routine spectra were obtained on a Varian EM 390 instrument.) Coupling constants and computer simulations were measured at 300 MHz on 100-Hz expansions with a 32 K data base, acquisition time 5,459 s, accuracy approximately  $\pm 0.2$  Hz. <sup>31</sup>P NMR spectra were taken on a Varian FT-80A spectrometer at 32.2 MHz under proton decoupling conditions. <sup>31</sup>P shifts are reported in ppm downfield (+) or upfield (-) from external 85% H<sub>3</sub>PO<sub>4</sub>.

The assignments of proton chemical shifts to the phosphate and deoxyribose ring protons of 6 and 7 were ordered according to those of cTMP itself.<sup>13</sup> The assignment of  $H_4$ , could be confirmed by the presence of  $J_{4'5a'}$ ,  $J_{3'4'}$ , and  $J_{4'5'b}$  values close to those noted for other cyclic nucleotides and predictable from the expected geometry around C(4'). A negative sign for  $J_{4'P}$  better simulated the spectrum of  $H_{4'}$ .  $H_{5'a}$  and  $H_{5'b}$ were easily identified by their couplings to H4. Approximate coupling constants were determined in most cases by using the computer of a Varian FT-80A NMR spectrometer and its noniterative SIMEQ program starting with reasonable couplings predictable from the limited range of conformations available to the molecules. Proton  $H_{2^\prime a}$  and  $H_{2^\prime b}$  gave strongly coupled spectra for which first-order approximations were not possible. Parameters which gave simulated plots closely resembling the experimental spectra were iteratively refined with the LAOCN3 program on a VAX computer. Errors in line assignments were never more than the resolution of the spectrometer ( $\pm 0.2$  Hz). Long range couplings,  ${}^{5}J_{1P}$  $\simeq$  -0.5 Hz, were required to closely approximate the peak intensities. Because the LAOCN3 program can handle only seven spins, the eight-spin systems of 6 and 7 were divided into five-spin (H<sub>3'</sub>, H<sub>4'</sub>, H<sub>5'a</sub>, H<sub>5'b</sub>, and P) and six-spin ( $H_{1'}$ ,  $H_{2a'}$ ,  $H_{2b'}$ ,  $H_{3'}$ ,  $H_{4'}$ , and P) subsets.  $J_{3'4'}$ , a parameter common to both, was more accurately determined in the second set, and that value is reported.

trans-Thymidine 3',5'-Cyclic Phenyl Monophosphate, trans-5. cis-Thymidine 3',5'-cyclic o-nitrophenyl monophosphate (430 mg, 1.0 mmol) was dissolved in 10 mL of acetonitrile, and sodium phenoxide (130 mg, 1.1 mmol) was added. The resulting orange-red solution was stirred at room temperature for 3 h after which the solvent was removed under reduced pressure with gentle warming. The crude product was purified by MPLC (SiO<sub>2</sub>, ethyl acetate, 15 mm × 1000 cm column) to afford 115 mg (30% yield) of the pure mixture of diastereomers (4:1 trans/cis) as a brittle white foam. The trans diastereomer was isolated by MPLC (SiO<sub>2</sub>, 4:1 ethyl acetate/hexane): mp 194-197 °C; <sup>31</sup>P NMR (32.2 MHz, acetone- $d_6$ )  $\delta$  -11.8; <sup>1</sup>H NMR (300 MHz, acetone- $d_6$ )  $\delta$  7.60 (br s, 1 H, H<sub>4</sub>), 7.20-7.43 (m, 5 H, C<sub>6</sub>H<sub>5</sub>), 6.47 (dd, 1 H, H<sub>1</sub>), 5.20 (apparent q, 1 H,  $H_{3'}$ ), 4.78 (ddd, 1 H,  $H_{5'b}$ ), 4.56 (ddd, 1 H,  $H_{5'a}$ ), 4.23 (ddd, 1 H,  $H_{4'}$ ), 2.62–2.74 (m, 2 H,  $H_{2'a}$ , and  $H_{2'b}$ ), 1.85 (d, 3 H, 5-Me,  $J_{\rm HH} = 1.2$  Hz). Anal. Calcd for  $C_{16}H_{17}N_2O_7P$  (mixture of diastereomers): C, 50.53; H, 4.51; P, 8.14. Found: C, 50.70; H, 4.53; P, 8.26.

cis-Thymidine 3',5'-Cyclic o-Nitrophenyl Monophosphate, 11. trans-Thymidine 3',5'-cyclic N,N-dimethylphosphoramidite (2.0 g, 6.6 mmol) was dissolved in 30 mL of dry methylene chloride and stirred under a carbon dioxide atmosphere for 48 h. o-Nitrophenol (1.1 g, 7.9 mmol) was added, and the solution was stirred under carbon dioxide for another 24 h. The crude product without isolation was oxidized at -20 °C by the addition of a saturated methylene chloride solution of nitrogen dioxide until the reaction solution gained a persistent greenish color. Removal of the solvent in vacuo and purification by MPLC (SiO<sub>2</sub>, 20:1 chloroform/methanol) afforded 0.58 g (21% yield) of the product as a yellowish brittle foam: <sup>31</sup>P NMR (32.2 MHz, CDCl<sub>3</sub>)  $\delta$  -20.2; 'H NMR (90 MHz, acetone- $d_6$ )  $\delta$  7.3-8.2 (m, 6 H, C<sub>6</sub>H<sub>5</sub> and H<sub>4</sub>), 6.5 (dd, 1 H, H<sub>1</sub>), 5.3 (apparent q, 1 H, H<sub>3</sub>), 4.4-5.0 (m, 2 H, H<sub>5'a</sub>, and H<sub>5'b</sub>), 4.0-4.3 (m, 1 H, H<sub>4'</sub>), 2.6-2.9 (m, 2 H, H<sub>2'a</sub>, and H<sub>2'b</sub>), 1.8 (br s, 3 H, 5-Me).

trans-Thymidine 3',5'-Cyclic N,N-Dimethylphosphoramidite, 8. To a magnetically stirred suspension of thymidine (5.0 g, 21 mmol) in 0.5 L of dioxane was added hexamethylphosphorus triamide (4.3 g, 26 mmol). The mixture was warmed to 80 °C over a period of 30 min. After having been heated for 18 h, the then-clear solution was cooled to 50 °C, and the solvent was removed under reduced pressure to afford a white, dry foam. The foam was suspended in 40 mL of a 1:1 mixture of diethyl ether/ethyl acetate. The suspension was placed on a silica gel gravity column (2.5 cm × 25 cm) and rapidly eluted with the same solvent mixture, collecting the first 250 mL, to afford 2.4 g (38% yield) of 8 as a brittle white foam: <sup>31</sup>P NMR (32.2 MHz, C<sub>6</sub>D<sub>6</sub>)  $\delta$  144.9 (cis, 137.8), trans/cis ratio approximately 95:5 (<sup>31</sup>P NMR); <sup>1</sup>H NMR (300 MHz, acetone-d<sub>6</sub>)  $\delta$  7.55 (br s, 1 H, H<sub>4</sub>), 6.30 (dd, 1 H, H<sub>1</sub>), 4.36 (apparent q, 1 H, H<sub>3</sub>) 4.34 (ddd, 1 H, H<sub>5'b</sub>), 4.17 (ddd, 1 H, H<sub>5'a</sub>), 3.48 (ddd, 1 H, H<sub>4'</sub>), 2.66 (d, 6 H, NMe<sub>2</sub>, J<sub>HP</sub> = 9.2 Hz), 2.52 (ddd, 1 H, H<sub>2'a</sub>), 2.40 (ddd, 1 H, H<sub>2'b</sub>), 1.65 (d, 3 H, 5-Me, J<sub>HH</sub> = 1.3 Hz).

cis-Thymidine 3',5'-Cyclic Phenyl Monophosphate, cis-5. To cisthymidine 3',5'-cyclic phenyl phosphite, 9 (36 mg, 0.10 mmol), dissolved in 10 mL of methylene chloride at -20 °C was added dropwise a concentrated methylene chloride solution of nitrogen dioxide until a greenish

tint persisted. After the solution was allowed to warm to 0 °C, the solvent was removed under reduced pressure. Purification of the crude product by MPLC (SiO<sub>2</sub>, 20:1 chloroform/methanol) afforded 33 mg (88% yield) of cis-5 as a brittle white foam: mp 134-136 °C dec; <sup>31</sup>P NMR (32.2 MHz, acetone- $d_6$ )  $\delta$  –13.6; <sup>1</sup>H NMR (300 MHz, acetone- $d_6$ )  $\delta$  7.55 (br s, 1 H, H<sub>4</sub>), 7.22–7.49 (m, 5 H, C<sub>6</sub>H<sub>5</sub>), 6.43 (dd, 1 H, H<sub>1'</sub>), 5.24 (apparent q, 1 H, H<sub>3'</sub>), 4.63-4.79 (m, 2 H, H<sub>5'a</sub>, and H<sub>5'b</sub>), 4.05-4.13 (m, 1 H, H<sub>4'</sub>), 2.65-2.78 (m, 2 H, H<sub>2'a</sub>, and H<sub>2'b</sub>), 1.81 (d, 3 H, 5-Me,  $J_{\rm HH} = 1.2$  Hz).

cis-Thymidine 3',5'-Cyclic Phenyl Phosphite, 9. Phosphoramidite 8 (250 mg, 0.83 mmol) was dissolved in 20 mL of stirred methylene chloride under a dry CO<sub>2</sub> atmosphere. A few crystals of dimethylamine hydrochloride were added. After 24 h phenol (86 mg, 0.91 mmol) was added followed by further stirring for 24 h under CO<sub>2</sub>. Solvent removal in vacuo yielded crude 2 as a pale yellow brittle foam, which on purification by MPLC (SiO<sub>2</sub>, 20:1 chloroform/methanol) yielded 89 mg (36% yield) of 9 as the cis diastereomer, a brittle white foam: <sup>31</sup>P NMR (32.2 MHz, acetone- $d_6$ )  $\delta$  115.0; <sup>1</sup>H NMR (300 MHz, acetone- $d_6$ )  $\delta$  7.54 (br s, 1 H, H<sub>4</sub>), 7.0–7.2 (m, 5 H, C<sub>6</sub>H<sub>5</sub>), 6.28 (dd, 1 H, H<sub>1</sub>), 4.92–5.02 (m,  $1 H, H_{3'}, 4.71 (ddd, 1 H, H_{5'a}), 4.43 (ddd, 1 H, H_{5'b}), 3.80 (7, 1 H, H_{4'}),$ 2.51–2.58 (m, 2 H, H<sub>2'a</sub> and H<sub>2'b</sub>), 1.81 (d, 3 H, 5-Me,  $J_{HH} = 1.2$  Hz).

Collection and Reduction of X-ray Data. Crystals of trans-5, suitable for X-ray diffraction, were obtained by vapor diffusion of hexane into a solution of the compound in ethyl acetate. A well-formed crystal was mounted on a Syntex P1 auto diffractometer equipped with scintillation counter and graphite monochromated Mo K $\alpha$  radiation. The cell dimensions (Table IV) were obtained by a least-squares fit of  $2\theta$  settings of 15 reflections. The data were reduced to  $F_0$  and  $\sigma(F_0)$ . Lorentz and polarization corrections were applied to all reflections.

Solution and Refinement of Structure. The structure was solved by direct methods and was refined by full-matrix least-squares technique<sup>25</sup> by using neutral atom scattering factors<sup>26</sup> for all species. Hydrogen atoms were added in geometrically ideal positions. Anisotropic refinement converged at

and

$$R = \sum |F_{\rm o}| - |F_{\rm c}| / \sum |F_{\rm o}| = 0.053$$

$$R_w = \sum w^{1/2} |F_o - F_c| / \sum w^{1/2} |F_o| = 0.061$$

The weighting scheme was given by  $w = 0.2814 [\sigma^2(F_0) + 0.006 F_0^2]^{-1}$ . The final atomic parameters with their standard deviations are given in Table V.

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Registry No. trans-5, 108146-35-6; cis-5, 108146-36-7; trans-8, 66512-19-4; cis-9, 87970-09-0; 11, 108060-82-8; PhONa, 139-02-6; o-NO<sub>2</sub>C<sub>6</sub>H<sub>4</sub>OH, 88-75-5; (Me<sub>2</sub>N)<sub>3</sub>P, 1608-26-0; PhOH, 108-95-2; thymidine, 50-89-5.

Supplementary Material Available: Tables of hydrogen atom parameters, atomic thermal parameters, bond lengths, bond angles, and torsion angles (7 pages); table of structure factors (13 pages). Ordering information is given on any current masthead page.

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# Cu(II) Coordination Chemistry of Amine Oxidases: Pulsed EPR Studies of Histidine Imidazole, Water, and Exogenous Ligand Coordination

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Abstract: Pulsed EPR spectroscopy utilizing the electron spin echo envelope modulation technique was used to study the Cu(II) binding sites in porcine kidney and bovine plasma amine oxidases. For both proteins, two magnetically distinct histidyl imidazoles were identified as Cu(II) ligands. The assignment of water as another metal ligand is based on comparison with echo envelope data for a series of copper(II)-bipyridyl complexes where the individual effects of axial, equatorial, and ambient water can be differentiated. Anionic inhibitors of amine oxidases, such as cyanide and azide, are shown to bind directly to Cu(II), displacing equatorially bound water. Once these anions are bound, the distinction in magnetic coupling between the two populations of imidazoles is lost.

In recent years, considerable progress has been made toward understanding the active site structures and mechanisms of copper-containing amine oxidases. In addition to metal ions, these metalloenzymes also contain a covalently bound organic cofactor, believed to be methoxatin, pyrrolloquinolinequinone (PQQ) or a close analogue.<sup>2,3</sup> Two copper ions are bound per enzyme molecule, which is generally composed of two subunits with a total molecular weight of approximately 180 000.4.5 At least one copper and a single PQQ are required for enzyme activity,4-9 which is to catalyze the two-electron oxidative deamination of primary

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