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Activated Cyclophosphamide Anticancer Drugs: Molecular Structures of *cis*- and *trans*-4-Hydroperoxyisophosphamides[1]

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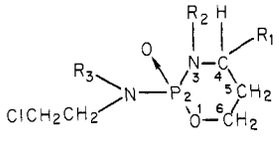
Molecular structures of two stereoisomers of 4-hydroperoxyisophosphamide (HPIPA) have been determined by single-crystal X-ray diffraction. These isomers are active cytostatic agents closely related to an active metabolite of the antitumor drug isophosphamide, an analogue of cyclophosphamide. Both isomers crystallize in monoclinic space group $P2_1/c$ with cell dimensions for *cis*-HPIPA of a = 8.999 (2), b = 8.743 (2), c = 17.078 (4) Å; β = 107.91 (2)°, and Z = 4 molecules per unit cell, and cell dimensions for *trans*-HPIPA of a = 15.184 (3), b = 10.345 (3), c = 18.205 (3) Å, β = 114.15 (1)°, and Z = 8. The structures were solved by direct methods and refined by anisotropic least squares to a discrepancy index R = 0.048 for *cis*-HPIPA and R = 0.065 for *trans*-HPIPA. In both isomers the 4-hydroperoxy group is situated axial to the ring. The phosphoryl oxygen atom is situated axial to the ring and, thus, cis to the C(4) oxygen in the *cis*-HPIPA isomer. In the *trans*-HPIPA isomer, the phosphoryl oxygen is equatorial to the ring and trans to the C(4) oxygen.

Cyclophosphamide (CPA, 1), one of the most extensively



1 (CPA), $R_1 = R_2 = H$; $R_3 = CH_2CH_2Cl$
2 (IPA), $R_1 = R_3 = H$; $R_2 = CH_2CH_2Cl$
3 (HPIPA), $R_1 = OOH$; $R_2 = CH_2CH_2Cl$; $R_3 = H$
4 (HPCPA), $R_1 = OOH$; $R_2 = H$; $R_3 = CH_2CH_2Cl$

used chemotherapeutic agents in the treatment of many types of cancer, has been the focus of research efforts aimed at understanding its mode of action and at developing analogues with improved function. One of the potentially clinically useful drugs that has resulted from this search is isophosphamide (IPA, **2**), which has one of the alkylating groups moved from the exocyclic nitrogen to the ring nitrogen.

The first step in the activation of CPA (and its analogues, including IPA) is monooxidation by the mixed-function oxidases of liver microsomes to form the 4-hydroxy derivative. The 4-hydroxy derivative is further metabolized by one of two competing pathways: (1) toxification, apparently by spontaneous chemical decomposition, to yield acrolein and phosphoramide mustard,[3] which is likely the ultimate cytostatic agent, or (2) detoxification by the enzymatic formation of 4-ketocyclophosphamide or carboxyphosphoramide. Thus, both formation of active metabolites of CPA's and detoxification (important for normal cells) involve enzymatic reactions at C(4); the stereochemistry at this position may therefore be an important consideration for improved specificity and selectivity in the design of cyclophosphamide analogues.

The chemistry and biochemistry of the 4-hydroxy and 4-hydroperoxy derivatives of IPA have been shown to be analogous to the corresponding derivatives of CPA: both compounds are cytostatically active; as with CPA, 4-hydroperoxy isophosphamide is readily converted in vivo to the 4-hydroxy derivative, but the 4-hydroperoxy compound is chemically the more stable of the two. It has been found that 4-hydroperoxyisophosphamide (HPIPA, **3**) can exist in two epimeric forms in acidic solution; the two forms have been identified as the major and minor products obtained in the ozonalysis synthesis of HPIPA.[4] Both epimers display in vitro and in vivo cytotoxic activity. We have previously determined the crystal structure of 4-hydroperoxycyclophosphamide (HPCPA, **4**)[5] and found that the mustard group in that molecule is situated equatorial and the hydroperoxy is situated axial on the heterocyclic ring. It is therefore of interest to determine the configuration at the phosphorus and C(4) atoms in the two active epimers of HPIPA. We now report the crystal and molecular structure determination of these two isomers as a further step toward understanding the relationship between stereochemistry and activity in this important group of drugs.

Experimental Section

The two IPA derivatives are hereafter referred to as *cis* and *trans*-HPIPA; this nomenclature describes the relationship between the phosphoryl oxygen and oxygen atoms attached to C(4), as revealed by the structure determination.

Crystals of *cis*- and *trans*-HPIPA were supplied by Dr. A. Takamizawa. The *cis*-HPIPA crystals were irregularly shaped, thick tablets, which were found by photographic methods to be

(1) Presented in part at the Symposium on the Metabolism and Mechanism of Action of Cyclophosphamide, London, July 10–12, 1975.
(2) (a) University of Washington. (b) University of Toronto.
(3) T. A. Connors, P. J. Cox, P. B. Farmer, A. B. Foster, and M. Jarman, *Biochem. Pharmacol.*, **23**, 115–129 (1974).
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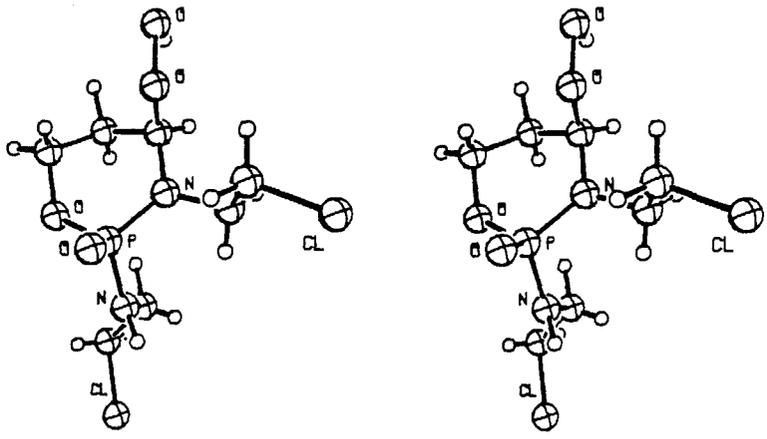

Figure 1. Stereoscopic drawing of *cis*-HPIPA showing the molecular conformation. The thermal ellipsoids of the non-hydrogen atoms are drawn at the 50% probability level.

Table I. Crystallographic Data for HPIPA

	cis-HPIPA	*trans*-HPIPA
mol. formula	$C_7H_{15}Cl_2N_2O_4P$	$C_7H_{15}Cl_2N_2O_4P$
M_r	293.09	293.09
size, mm	0.5 × 0.2 × 0.2	0.6 × 0.4 × 0.3 0.6 × 0.5 × 0.4
space group	$P2_1/c$	$P2_1/c$
a, Å	8.999 (2)	15.184 (3)
b, Å	8.743 (2)	10.345 (3)
c, Å	17.078 (4)	18.205 (3)
β, deg	107.91 (2)	114.15 (1)
volume, Å^3	1278.5	2609.5
temp, C	−5	−5
Z	4	8
density (calcd), g/cm^3	1.523	1.492
radiation	Mo $K\alpha$	Cu $K\alpha$
wavelength, Å	0.71069	1.5418
μ, cm^{-1}	6.34	56.8

triclinic with cell dimensions $a = 9.06$, $b = 9.09$, $c = 8.52$ Å, $\alpha = 110$, $\beta = 87$, and $\gamma = 106°$. However, when this compound was recrystallized from hot aqueous ethanol, monoclinic crystals were formed; these gave much higher quality X-ray diffraction patterns and were used for structure determination. Table I lists crystal and cell data for *cis*- and *trans*-HPIPA, determined by X-ray photographs and diffractometer measurements. In order to slow the moderate deterioration on exposure to X-radiation, all crystals were cooled to −5 °C by a stream of dry air during data collection. An automated four-circle diffractometer operating in the θ–2θ scan mode was employed to measure intensities. Stationary backgrounds were measured on both sides of each scan. For *cis*-HPIPA, Nb-filtered Mo radiation was used to measure all independent reflections having a $2\theta < 50°$, which corresponds to a minimum interplanar spacing of 0.84 Å. For *trans*-HPIPA, Ni-filtered Cu radiation was used to measure all reflections having a $2\theta < 100°$, which corresponds to a minimum interplanar spacing of 1.01 Å. In addition, reflections with $h = 0, 1$ were collected for $2\theta < 125°$. The remainder of the shell of data between 100 and 125° (2θ) was not collected due to the deterioration of the *trans*-HPIPA crystals.

For both compounds, three reflections, monitored periodically, showed a steady decline in intensity. When data collection was complete, the average monitor intensity had lost 17% of its initial value for *cis*-HPIPA, 36% for the first crystal of *trans*-HPIPA, and 52% for the second crystal. A quadratic decomposition curve, fit to a plot of monitor reflection intensity vs. reflection number, was used to calculate scale factors as a function of the serial order of data collection for all crystals. For *cis*-HPIPA, the 31 largest reflections were recollected at a lower X-ray tube current to eliminate any significant error due to saturation of the counter circuit. For *trans*-HPIPA, a correction ($\tau = 8.0 \times 10^{-8}$) was applied to all reflections to eliminate this error.

In each data set, only those reflections with intensity greater than twice their standard deviation were used in the structure refinement. For *cis*-HPIPA, 2256 reflections were measured and 1827 were used. For *trans*-HPIPA, 2713 reflections were measured and 2524 were used. An empirical absorption correction based on the variation in intensity of a reflection at $\chi = 90°$ as a function of ϕ was applied to the *trans*-HPIPA data set only. The relative transmission factors ranged from 1.0 to 0.54 for the first crystal and 1.0 to 0.49 for the second. The standard geometrical corrections were applied to both data sets, but no extinction corrections were made. Normalized structure amplitudes (E) were obtained by the Wilson plot method.

Structure Determination and Refinement. The *cis*- and *trans*-HPIPA molecular structures were solved by the multiple-solution tangent formula program, MULTAN.[6] For *cis*-HPIPA, the E map calculated from the phase set with the highest figure of merit (1.25) and the lowest residual (20.6%) revealed 12 of the 16 non-hydrogen atoms in the molecule, with the other four atoms subsequently determined in a difference map. The corresponding E map for *trans*-HPIPA (figure of merit = 1.20; residual = 29.9%) permitted the identification of 27 out of 32 non-hydrogen atoms, and the remaining five atoms were located in a difference map. With all heavy atoms in the model, the discrepancy factor, $R = \sum ||F_o| - |F_c||/\sum|F_o|$, was 0.31 for *cis*-HPIPA and 0.32 for *trans*-HPIPA.

The atomic positional and thermal parameters were refined by block-diagonal least squares, except for the final refinement, which was full matrix. The function minimized was $\sum w(|F_o| - |F_c|)^2$, initially with unit weights, but with statistical weights, $w = 1/\sigma_F^2$, used for final refinement. Atomic scattering factors for hydrogen[7] and the other atoms[8] were taken from the literature. Computations were done with the XRAY system.[9] Several cycles of refinement proceeded smoothly, except for an apparent disorder in the N(2A)–Cl(2A) chain of *trans*-HPIPA. C(8A) and C(7A) were each assigned two positions with occupancies of 0.5. Hydrogen atom positions were assigned by examination of difference-Fourier maps, except for the hydrogens on C(9) in *cis*-HPIPA, which were placed at calculated positions, and except for the hydrogens on C(8A) and C(7A) in *trans*-HPIPA, which were placed at calculated positions and not refined. In the final refinement of atomic parameters, heavy atoms were given anisotropic temperature parameters, except for C(8A) and C(7A), which were left isotropic as were hydrogen atoms. For *cis*-HPIPA, hydrogen thermal parameters were refined, and for *trans*-HPIPA, they were held fixed. The final R was 0.048 for *cis*-HPIPA and 0.065 for *trans*-HPIPA [omitting reflections with $I < 2\sigma(I)$]. Final atomic fractional coordinates and thermal parameters are available (see paragraph at the end of paper concerning supplementary material).

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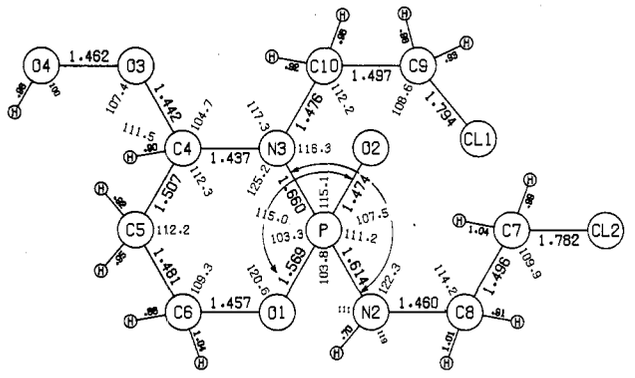


Figure 2. Atomic nomenclature, bond lengths (angstroms), and interbond angles (degrees) for *cis*-HPIPA. Estimated standard deviations are 0.006 Å for heavy atom bonds and 0.04 Å for bonds involving hydrogen atoms. Estimated standard deviations are 0.3° for heavy atom angles.

Results and Discussion

***cis*-HPIPA.** The three-dimensional molecular structure of *cis*-HPIPA is shown in Figure 1. The six-membered ring is in the chair conformation, and the configuration about the phosphorus atom has the chloroethylamine group equatorial and the phosphoryl oxygen axial. The hydroperoxy group at C(4) is situated axial to the ring and thus cis to the phosphoryl oxygen. This configuration at the phosphorus and at C(4) is the same as found in 4-hydroperoxycyclophosphamide (HPCPA).[5] Bond lengths and angles in *cis*-HPIPA are shown in Figure 2. The distance between the phosphoryl oxygen, O(2), and the C(4) oxygen, O(3), is 3.76 Å, slightly larger than the 3.54-Å length of the comparable distance in HPCPA. The spatial packing of the *cis*-HPIPA molecules is displayed in Figure 3. In the crystal the molecules are connected in a chain along the *a* direction by two intermolecular hydrogen bonds. Distances and angles for these hydrogen bonds are given in Table II.

***trans*-HPIPA.** Figure 4 shows the molecular structure of the two crystallographically independent molecules of *trans*-HPIPA. The only conformational dissimilarity between them is the orientation of the exocyclic chloroethylamine group, which differs considerably from one molecule to the other. In all other respects they appear identical. Structural features to note are: (a) the conformation of the ring is identical with HPCPA and *cis*-

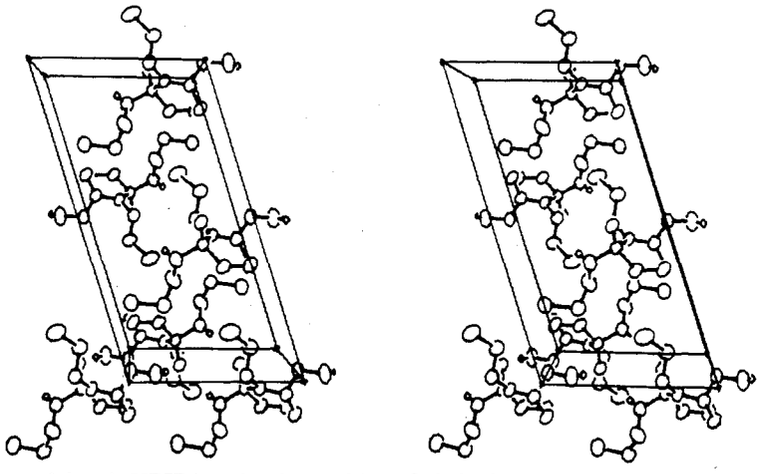


Figure 3. Stereoscopic diagram of the *cis*-HPIPA molecular packing. Only hydrogen atoms involved in hydrogen bonding are included. The origin is at the lower left hand corner with *a* to the right, *b* pointing away from the viewer; and *c* up.

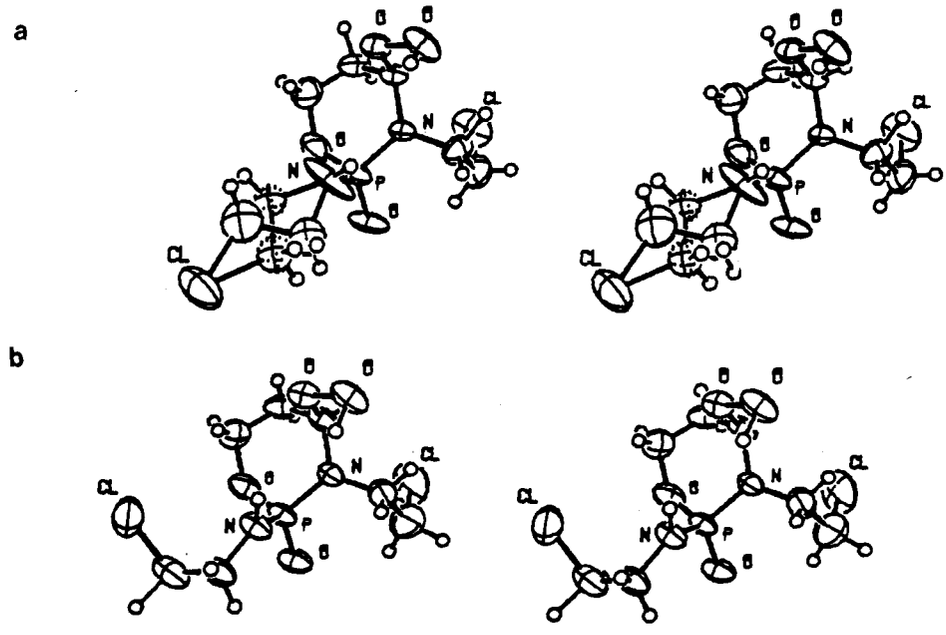


Figure 4. Stereoscopic drawings of the *trans*-HPIPA molecules showing the molecular conformations. The thermal ellipsoids of the the non-hydrogen atoms are drawn at the 50% probability level. The alternate positions for C(8A) and C(7A) are shown with dotted curves.

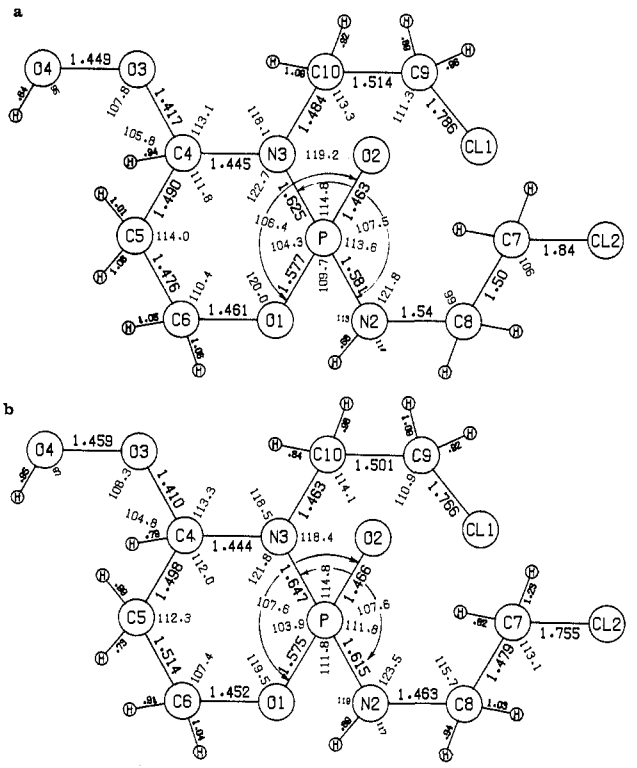


Figure 5. Atomic nomenclature, bond lengths (angstroms), and interbond angles (degrees) for the two crystallographically distinct *trans*-HPIPA molecules. Except for the distances and angles in the disordered chloroethyl group, estimated standard deviations are 0.009 Å for heavy atom bonds and 0.07 Å for bonds involving hydrogen atoms; estimated standard deviations are 0.6° for heavy atom angles. Bond lengths and angles involving the disordered atoms are averages of the values for the two orientations.

HPIPA; (b) the configuration at C(4) is hydroperoxy group axial, exactly as found in HPCPA and *cis*-HPIPA; and (c) the configuration at the phosphorus atom is phosphoryl oxygen atom equatorial and chloroethylamine group axial; the opposite configuration to that found for the other molecules studied. Thus, even though two stereoisomers

Table II. Hydrogen-Bond Distances (Angstroms) and Angles (Degrees) for HPIPA

	N(O)-H	H---O	N(O)---O	N(O)-H---O
cis-HPIPA[a]				
N(2)-H---O(2)^{i}	0.70	2.35	3.050	174
O(4)-H---O(2)^{ii}	0.96	1.80	2.749	172
trans-HPIPA[a]				
N(2A)-H---O(2B)^{iii}	0.68	2.33	2.954	153
O(4A)-H---O(2B)^{iii}	0.64	2.05	2.685	169
N(2B)-H---O(2A)^{iv}	0.89	2.13	2.958	155
O(4B)-H---O(2A)^{iv}	0.95	1.79	2.648	149

[a] Symmetry code: i = $1-x, -y, 1-z$; ii = $2-x, -y, 1-z$; iii = $1-x, 1-y, -z$; iv = $1-x, -y, -z$.

of HPIPA can be isolated, the significant finding from our studies is that they both maintain the same configuration at C(4); the position at which hydroxylation changes the inactive CPA compounds to activated species, and that configuration is hydroperoxy (or hydroxy) group axial to the ring. Since *cis*- and *trans*-HPIPA and HPCPA all display in vivo cytotoxicity and all have axial hydroxy substitution at C(4), it may also be that this configuration is a determining factor in the biological efficacy of these activated compounds.

Figure 5 shows the bond lengths and angles for the two molecules of *trans*-HPIPA. No significant differences between corresponding distances or angles were observed between the two molecules or between *trans*-HPIPA, *cis*-HPIPA, and HPCPA. Distances and angles in the disordered region of *trans*-HPIPA molecule A are not well determined and cannot be compared. The distance between the axial C(4) hydroxy oxygen atom and the equatorial phosphoryl oxygen atom is 4.73 Å in both *trans*-HPIPA molecules.

Figure 6 displays the three-dimensional packing arrangement of the two different *trans*-HPIPA molecules. The two molecules are stacked alternately (molecule A, then molecule B) in a hydrogen-bonded chain running parallel to the *b* direction. Hydrogen-bonding parameters are listed in Table II.

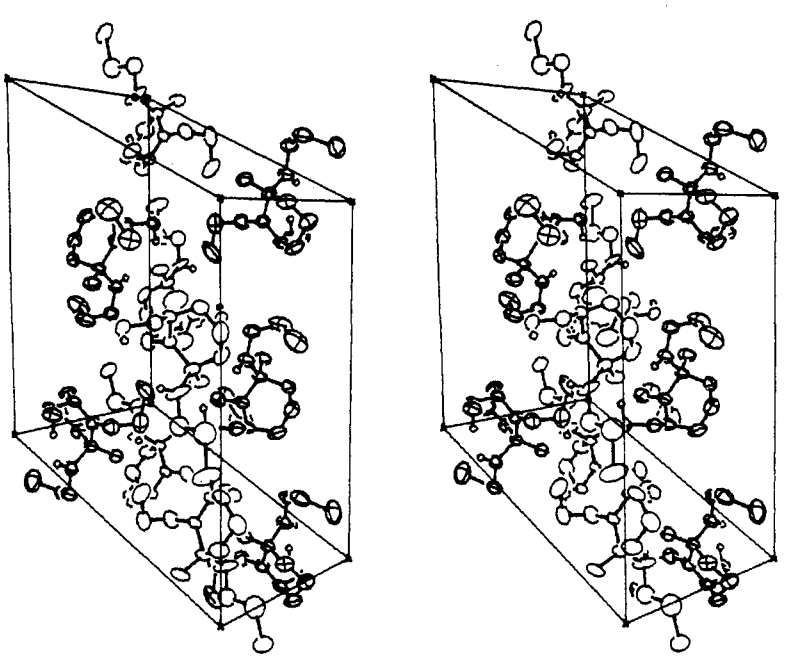
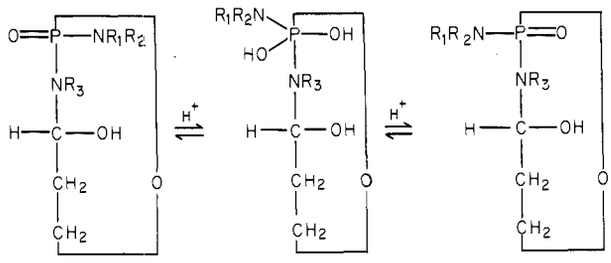
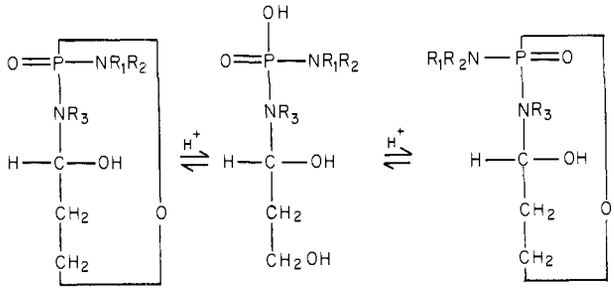


Figure 6. Stereoscopic diagram of the *trans*-HPIPA molecular packing. Only hydrogen atoms involved in the hydrogen bonding are included. Atoms of molecule A are represented by open ellipsoids. The origin is at the lower left hand corner with *a* forward and to the right, *b* pointing back and to the right, and *c* up.

Scheme I



Scheme II



It is not difficult to envision a mechanism of action for the acid-catalyzed interconversion of *cis*- and *trans*-HPIPA (or the comparable hydroperoxy or hydroxy isomers of CPA) with the retention of configuration at C(4) (Scheme I). Protonation of the phosphoryl oxygen atom, followed by nucleophilic attack by a water molecule, would lead to a dihydroxylated, pentacoordinate configuration at the phosphorous. Either stereoisomer could then be obtained, depending on which of the chemically equivalent hydroxy groups is removed in the subsequent dehydration.

A second, perhaps less likely, pathway; somewhat analogous to the well-known mechanism for mutarotation of sugars, could occur via protonation of the ester oxygen, leading to a short-lived, ring-opened intermediate that would recyclize with resultant random configuration at the phosphorus but with no change at C(4) (Scheme II). This second mechanism provides an alternate pathway for the decomposition of 4-hydroxycyclophosphamide to acrolein and phosphoramide mustard other than through an aldophosphamide intermediate. If scission of the N(3)–C(4) bond occurs to this ring-opened form of cyclophosphamide (possibly via an aldolase enzyme), with coincident aldehyde formation, the products would be phosphoramide mustard and $CH_2(OH)CH_2CHO$. The latter would immediately undergo dehydration to form acrolein. It is also of interest to note that the ring-opened intermediate is the 4-hydroxy derivative of cytotoxyl alcohol and, thus, would be expected to be a potent cytotoxic agent itself.

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Registry No. *cis*-3, 64858-36-2; *trans*-3, 59884-18-3.

Supplementary Material Available: Table of fractional atomic coordinates and anisotropic thermal parameters and tables of observed and calculated structure factors (21 pages). Ordering information given on any current masthead page.

Structures of Two Isomeric Bicyclic Derivatives of 4-Hydroperoxyisophosphamide

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Crystal structure determinations of C4-oxygen-substituted cytotoxic derivatives of the anticancer drug cyclophosphamide have all found the oxygen to be in the axial position, suggesting an inherent stability for this geometry. Recently, two isomeric bicyclic derivatives of 4-hydroperoxyisophosphamide (cyclized *cis*- and *trans*-HPIPA) have been obtained for which NMR coupling constants imply that the trans isomer has the C4-oxygen substituent in the equatorial position. Crystal structure determinations of both bicyclic compounds have now been performed. They show that the cis isomer has phosphoryl oxygen and C4-peroxy group both axial, similar to the conformation of the uncyclized HPIPA precursor and to the expectation based on NMR data; the trans isomer, however, has a phosphoryl oxygen equatorial, C4-peroxy group axial conformation, similar to its uncyclized HPIPA precursor but opposite in conformation at both positions to the NMR-based inferences. The oxazaphosphorinane ring in each isomer has a half-chair conformation, with the trans isomer probably flipping between two equally probable half-chairs; this disorder may account for the observed differences in the NMR C4-hydrogen coupling constants in the two isomers. The peroxy-containing ring adopts a chair conformation in both molecules.

Cyclophosphamide (CPA) is one of the most widely used drugs in the treatment of many types of cancer. CPA itself has little cytotoxic activity in mammalian cell cultures; there is considerable evidence that in vivo activation proceeds via hydroxylation at C4 of the 1,3,2-oxazaphosphorinane ring. Either 4-hydroxycyclophosphamide or a further degradation product, phosphoramide mustard, is generally believed to be the ultimate cancerotoxic selective agent. In either case, the synthesis of preactivated analogues of CPA is desirable both for enhancement of activity and for an understanding of the pathways of CPA activation and metabolism.

Takamizawa synthesized 4-hydroxy- and 4-hydroperoxycyclophosphamide and found that both have cytostatic activity;[2] determination of the crystal structure of the latter compound[3] revealed the configuration at the phosphorus atom to be phosphoryl oxygen axial and bis(chloroethyl)amine group equatorial, as found in a number of cyclophosphamide analogues, and the configuration at C4

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