STEREOCHEMISTRY OF ACTIVATED CYCLOPHOSPHAMIDE ANTITUMOR DRUGS: MOLECULAR STRUCTURE OF 4-HYDROPEROXYCYCLOPHOSPHAMIDE

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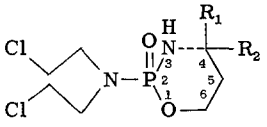
SUMMARY. The crystal and molecular structure of 4-hydroperoxycyclophosphamide, an active cytostatic agent closely related to an active metabolite of the antitumor drug cyclophosphamide, has been determined by X-ray crystallography. The configuration at the phosphorus atom is axial phosphoryl oxygen and equatorial dialkylamino group. The peroxide group attached to C_4 is axial and is thus cis to the phosphoryl oxygen. This same stereochemistry will occur for synthetic 4-hydroxycyclophosphamide, an equally potent cytostatic agent, and is likely for 4-hydroxycyclophosphamide produced by in vivo activation of cyclophosphamide.

INTRODUCTION

Cyclophosphamide (CPA) (I), an antitumor alkylating agent, is one of the most widely used agents in the treatment of many types of cancer. Though it is an effective antineoplastic agent against many tumors, CPA has virtually no cytotoxic activity against mammalian cell cultures (1); in vivo pharmacological activity requires conversion of CPA to alkylating substances by the mixed function oxidase system of liver microsomes (2). It is becoming increasingly clear that monooxidation at C_4 is responsible for activation of CPA with the first step being production of 4-hydroxycyclophosphamide (HCPA) (II) (3-5). It had been thought that HCPA was the active antitumor alkylating agent, but recent evidence (6,7) suggests that it undergoes decomposition to yield acrolein and phosphoramide mustard (PAM), with the latter being the ultimate cytostatic cyclophosphamide metabolite. We have previously determined the molecular structure of one of the metabolic end products of CPA, 4-ketocyclophosphamide (KCPA) (III) (8), but neither these results nor those for CPA (9) yield infor-

mation about the important configuration at C_4 of the hydroxylated antitumor species. Recently, Takamizawa et al. (5) synthesized HCPA and 4-hydroperoxycyclophosphamide (HPCPA) (IV). They found that both synthetic products exhibit pronounced in vitro and in vivo cytostatic activities and that HPCPA can be readily converted to HCPA by chemical and by biological reduction. HCPA is highly unstable and is not amenable to crystallographic studies so we have chosen to determine the crystal and molecular structure of HPCPA in order to elucidate the configuration about C_4 in these synthetic CPA derivatives. As they exhibit high cytostatic activity, it is likely that these compounds will have the same C_4 configuration as the active HCPA metabolite; thus stereochemical results for HPCPA may provide valuable aid in understanding steps in cyclophosphamide metabolic pathways.



I. CPA: $R_1 = R_2 = H$

II. HCPA: $R_1 = OH$; $R_2 = H$

III. KCPA: $R_1, R_2 = O$

IV. HPCPA: $R_1 = OOH$, $R_2 = H$

METHODS

Crystals of hydroperoxycyclophosphamide supplied by Dr. Akira Takamizawa were colorless plates which turned pale yellow and exhibited moderate decomposition in the X-ray beam. The crystals were monoclinic with cell dimensions at -5°C. of a = 14.229, b = 7.706, c = 11.891Å, β = 103.06°; space group $P2_1/c$ and density (assuming 4 molecules per unit cell) = 1.533 g cm^{-3}. X-ray reflection intensities were measured on an automated diffractometer with the use of MoKα radiation. Of the 2472 measured reflection intensities with interplanar spacings down to 0.84Å, 1957 had intensity above background and were used in structure refinement.

The structure was solved by direct methods. Positions of all chlorine, phosphorus, oxygen, nitrogen and carbon atoms were located on a three-dimensional

E map computed from the best set of phases generated by the program MULTAN. Refinement was by least squares with anisotropic thermal parameters for all non-hydrogen atoms. Positions for all 15 hydrogen atoms in the molecule were assigned from peaks on a difference Fourier map. Several additional cycles of least squares refinement of all atom positions with anisotropic thermal parameters for the heavy atoms and isotropic for the hydrogen resulted in a final discrepancy index R = 0.055.

DISCUSSION

Figure 1 is a stereoscopic drawing showing the three-dimensional molecular structure of 4-hydroperoxycyclophosphamide. The six-membered ring is in the chair conformation, and the configuration about the phosphorus atom has the bis(chloroethyl)amine group equatorial and the phosphoryl oxygen atom axial, the same as that found in KCPA (8) and in CPA (9). The major difference between these inactive compounds and activated HCPA and HPCPA is the hydroxy (or hydroperoxy) group at C_4. The significant finding in our structure determination of HPCPA is that this group is situated axial to the ring and thus is *cis* to the phosphoryl oxygen and *trans* to the bis(chloroethyl)amine group. The distance between the C_4 oxygen atom and the phosphoryl oxygen is 3.54Å; the same distance would, of course, pertain for synthetic HCPA.

The synthetic HPCPA, prepared by Takamizawa *et al.* (5) by ozonolysis of an appropriate open-chain compound, is a racemic mixture of *d* and *l* isomers,

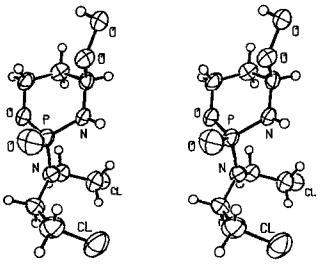


Figure 1. Stereoscopic drawing of 4-hydroperoxycyclophosphamide.

both of course having the C_4 oxygen axial and cis to the phosphoryl oxygen. HPCPA has also been produced by the Fenton oxidation of CPA and shown to be identical to synthetic HPCPA (10). In neither synthesis was an isomer with the C_4 oxygen in the equatorial position trans to the phosphoryl oxygen isolated. In addition, 4-peroxycyclophosphamide (PCPA) has been produced from the Fenton oxidation of CPA (10, 11, 12) and both halves of this dimer have the C_4 oxygen in the axial configuration and cis to the respective phosphoryl oxygens (13). As all of the synthetically prepared compounds, HCPA, HPCPA and PCPA, are essentially equivalent in biological behavior to the active species of cyclophosphamide, and all possess the configuration elucidated in our structure determination (Figure 1), it seems reasonable to suggest that this configuration is the most stable one for the C_4 hydroxylated cyclophosphamide compounds and may be the configuration of the 4-hydroxycyclophosphamide produced by in vivo activation of cyclophosphamide. Support for this latter suggestion comes from the reported (11) in vivo occurrence of PCPA identical to that obtained from the chemical reduction of synthetic HPCPA (12), although that result has not yet been duplicated (14).

As isomers of HCPA, HPCPA or PCPA with the C_4 oxygen atom trans to the phosphoryl oxygen do not result from either the ozonolysis synthesis of Takamizawa et al. or the Fenton oxidation of CPA, it is not known whether they would exhibit cytostatic activity or whether they could be degraded further to acrolein and phosphoramide mustard, the probable ultimate/active metabolites of cyclophosphamide.

In the absence of evidence for the existence of such isomers, it is reasonable to suggest that the configuration found in this structure determination --- axial C_4 oxygen, cis to the phosphoryl oxygen --- be maintained in the design of new pre-activated cyclophosphamide antitumor agents.

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