ceptor of the α-hydrogen removed from the amino acid substrate. (3) The α substrate hydrogen is most probably attached at C-5 in deazaFMNH_2 since the tritium in deazaFMNH_2 released from denatured intermediate remains nonexchangeable with solvent and less than 50% is lost during air oxidation. (4) Since deazaFMNH_2 has two potential hydrogens at C-5, either of which could be removed during reaction with methylamine, the observed incorporation of 85% of the tritium into *N*-methylglutamate indicates stereospecific enzymic reoxidation of deazaFMNH_2. (5) A similar hydrogen transfer appears likely with native enzyme since other aspects of catalysis by FMN and deazaFMN enzymes are similar even though the relative reaction rates are different.

Although the data clearly establish that the α hydrogen of the substrate is transferred to flavine, whether the reaction proceeds *via* hydride transfer or *via* a shielded proton transfer,[1b] cannot be presently determined. The data do show, however, that a proton transfer mechanism as proposed by Brown and Hamilton[8] is inapplicable since this mechanism requires hydrogen transfer to the 5 position of flavine prior to cleavage of the α-carbon–hydrogen bond of the substrate.[9]

(8) L. E. Brown and G. A. Hamilton, *J. Amer. Chem. Soc.*, **92**, 7225 (1970).

(9) Additional objections to the Brown–Hamilton mechanism are discussed in ref 5b and by P. Hemmerich and M. S. Jorns in "Federation of European Biochemical Society, Proceeding of the Meeting," Vol. 8, Academic Press, London, 1973, pp 95–118.

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Structure and Conformation of 4-Peroxycyclophosphamide. A Cytotoxic Oxidation Product of Cyclophosphamide

Sir:

Cyclophosphamide (2-bis(2-chloroethyl)aminotetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide) is used extensively as an antitumor agent.[1,2] The drug has little cytotoxic activity until it is activated in the liver by a mixed function oxidase of liver microsomes.[3,4] Several laboratories have been interested in isolating and synthesizing the active metabolites of cyclophosphamide.[3,5–8] There seems to be some ambiguity as to the identification of a biologically active Fenton oxidation product of the drug. Initially van der Steen, *et al.*,[5] identified this compound as *N*-hydroxycyclophosphamide on the basis of elemental analysis, ir and nmr spectroscopic data, and chemical reactivity of the compound with H_2O_2. However, these investigators, after comparing their ^{13}C nmr data with those of Struck and coworkers concluded that the compound was 4-hydroxycyclophosphamide.[5] In contrast, Struck, *et al.*,[9] presented evidence that this oxidation product is 4-peroxycyclophosphamide. To resolve this ambiguity, we determined the crystal structure of the compound.

Clear crystals, obtained from ethyl ether, were kindly provided by Dr. R. F. Struck.[10] The crystals are monoclinic, space group $P2_1/c$ with four $C_{14}H_{28}Cl_4N_4O_6P_2$ formula units per unit cell. Three-dimensional intensity data, which included 3986 independent reflections, were collected on an automated diffractometer by use of nickel-filtered copper radiation, a scintillation detector, and a θ–2θ scan technique. Three strong, medium-angle reflections (032, 025, 300), chosen as standards, were monitored periodically. Throughout data collection, the continuous and rapid decrease in the intensities of the standard reflections indicated that crystal decomposition was occurring. The three standards decreased at markedly different rates. When any one of the standards lost 33% of its initial intensity, a new crystal was mounted and data collection was continued. Seven different crystals were used to obtain a complete data set. The final crystal was used slightly beyond the 33% decay limit (37% decay for reflection 032, 38% for reflection 025, and 47% for reflection 300). Cell parameters for each crystal were determined by a least-squares analysis of the angular settings for six reflections. Among the seven independent determinations, the maximum variation in cell parameters was about 0.1%. Weighted-average values for the cell parameters are a = 11.206 (2) Å, b = 11.807 (2) Å, c = 19.202 (5) Å, and β = 103.94 (1)°. Intensity values were scaled by a least-squares procedure[11] in which the intensities of the standard reflections were used to calculate scale factors as a function of crystal exposure time.

A suitable trial structure was obtained by direct methods, with the use of the computer program MULTAN.[12] The structure was refined by least squares. Coordinates for hydrogen atoms were calculated by assuming tetrahedral coordination around the carbon atoms, trigonal coordination around the nitrogen atoms, and C–H and N–H bond distances of 1.0 Å. The hydrogen atoms were assigned the isotropic temperature factors of the heavy atoms to which they were bonded and were included in structure factor calculations but not in the least-squares refinement. As the refinement proceeded, new positions for the hydrogen atoms were calculated periodically. Initially refinement was terminated at an R index $(\Sigma||F_o| - |F_c||/\Sigma|F_o|)$ of 0.165 and a goodness-of-fit $([\Sigma w(F_o^2 - F_c^2)^2/(m - s)]^{1/2}$, where w is the weight given each reflection, m is the number of reflections used, and s is the number of parameters refined) of 4.49. At this stage, a difference Fourier map showed a residual peak of 1.9 e/Å^3 at a distance of 2.5 Å from atom C(10′), 3.2 Å from atom

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(4) D. L. Hill, W. R. Laster, Jr., and R. F. Struck, *Cancer Res.*, **32**, 652 (1972).

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(9) R. F. Struck, M. C. Thorpe, W. C. Coburn, Jr., and W. R. Laster, Jr., *J. Amer. Chem. Soc.*, **96**, 313 (1974).

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C(11′), 3.5 Å from the chlorine atom of a symmetry-related molecule and more than 3.6 Å from any other nonhydrogen atom. There were no other peaks in excess of 0.6 e/Å^3 except in the immediate vicinity of some chlorine atoms. It was also noted that atom Cl(12′) displayed unusually large thermal parameters. In a Fourier map that was phased with all atoms except those of the four chloroethyl side chains, the electron density at Cl(12′) was only about two-thirds that found at the positions of the other three chlorine atoms. These observations suggested that atom Cl(12′) might be disordered. Two positions, Cl(12′) and Cl(12A′), the latter corresponding to the residual peak, were assigned to this chlorine atom and population parameters for these two sites were allowed to vary when least-squares refinement was resumed. The population parameters, which were not constrained to a total of 1.0, converged to values of 0.556 (8) and 0.307 (9) for sites Cl(12′) and Cl(12A′), respectively. This result is consistent with the hypothesis that the chlorine atom occupies position Cl(12′) about two-thirds of the time and position Cl(12A′) about one-third of the time. Two feasible models for this disorder are (1) the C(10′)–C(11′)–Cl(12′) side chain was partially degraded during data collection, resulting in fragments that occupy the cavity around position Cl(12A′) or (2) the side chain assumes two or more conformations, perhaps under the effects of X-radiation, with the chlorine atom at site Cl(12′) or in the vicinity of site Cl(12A′).

The final R index, including all reflections, is 0.117 and the goodness-of-fit is 2.77. A final difference Fourier map shows no peaks or troughs exceeding 0.7 e/Å^3 in magnitude. The estimated errors in positional coordinates are about 0.002 Å for the two phosphorous atoms and 0.006 Å for other atoms of the two six-membered rings and their immediate substituents. In the chloroethyl side chains, the estimated errors in atomic positional coordinates are relatively large, ranging from 0.002 to 0.006 Å for chlorine atoms and from 0.01 to 0.03 Å for carbon atoms of the side chains, whereas the estimated error is 0.02 Å for position Cl(12A′).

Figure 1 depicts the chemical configuration, the conformation, and the heavy-atom thermal ellipsoids. As suggested by the data of Struck, *et al.*,[9] and of Takamizawa, *et al.*,[13] the compound is 4-peroxycyclophosphamide (4-hydroxycyclophosphamide anhydro dimer), with the two cyclophosphamide moieties joined by the peroxide linkage. The observed conformation is stabilized by two N–H--O intramolecular hydrogen bonds. It is likely that the low-field chemical shift of the NH protons found in the nmr experiment are a result of these bonds. Bond lengths and angles involving the central rings of the cyclophosphamide moieties and their immediate substituents are in agreement with the corresponding values reported for 4-ketocyclophosphamide.[14] The two C–O bonds of the peroxide linkage have lengths of 1.44 and 1.42 Å, respectively. As in hydrogen peroxide,[15] the O–O bond distance is 1.47 Å. The bond lengths within the chloroethyl side chain deviate greatly from their expected values. The C–Cl bond distances range from 1.47 to 1.94 Å with an average value of 1.77 Å, and the C–C and C–N bonds of the side chains range from 1.30 to 1.70 Å. These anomalous values are probably a consequence of the high degree of thermal motion and/or the extensive decomposition that occurred during data collection. Tables of atomic parameters and structure factors are included in the microfilm edition of this journal. See paragraph at end of paper regarding supplementary material.

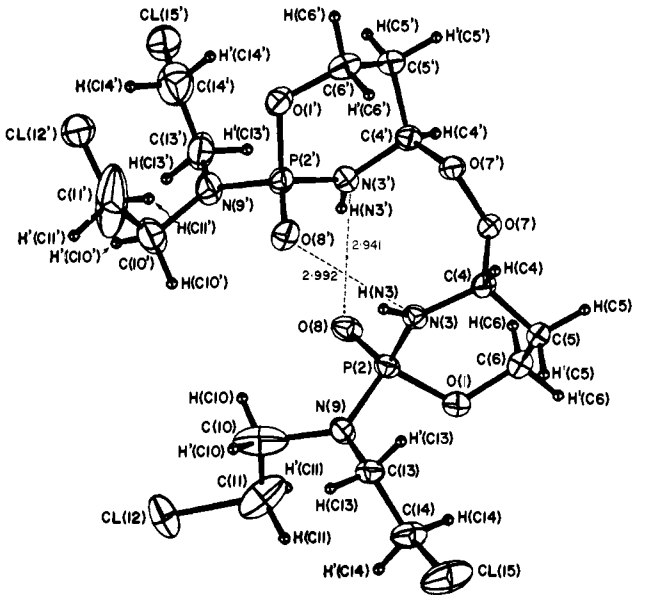

Figure 1. Conformation of 4-peroxycyclophosphamide. Nonhydrogen atoms are represented by thermal ellipsoids, which are defined by the principal axes of thermal vibration and are scaled to include 25% probability. The hydrogen atoms are represented by spheres of 0.1 Å radius. Only the major site of atom Cl(12′) is depicted. The donor–acceptor distances for the two intramolecular hydrogen bonds are shown.

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Supplementary Material Available. A listing of structure factor amplitudes and atomic parameters will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.00 for photocopy or $2.00 for microfiche, referring to code number JACS-74-4014.

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Cyclic Peptides. VIII. ^{13}C and ^{1}H Nuclear Magnetic Resonance Evidence for Slow Cis′–Trans′ Rotation in a Cyclic Tetrapeptide[1]

Sir:

Cis–trans isomerism has been observed about X-imino acid peptide bonds (*e.g.*, Gly-Pro) where rota-

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(1) For Cyclic Peptides, Part VII, see C. M. Deber and E. R. Blout, *Isr. J. Chem.*, in press.