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4-HYDROXYCYCLOPHOSPHAMIDE ANHYDRO-DIMER: REVISED STRUCTURE OF THE FENTON OXIDATION PRODUCT OF CYCLOPHOSPHAMIDE[1]

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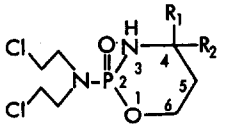
Cyclophosphamide (I),[2] an antitumor alkylating agent widely used in the treatment of various types of cancer, is thought to exert its cytostatic effect via biological oxidation at the C-4 position of its tetrahydro-2H-1,3,2-oxazaphosphorine ring.[3-5] Considerable effort has been directed to elucidate the active species of the alkylating agent, and some recent studies have revealed that C-4 hydroxylation is responsible for the activation of cyclophosphamide.[4,6-8] Most recently, Van Der Steen et al.[9] reported the formation of "4-hydroxycyclophosphamide" by the Fenton oxidation of cyclophosphamide. However, both the chemical and spectroscopic properties reported for their product differed significantly from those of our 4-hydroxycyclophosphamide (II)[8] which has been prepared by the deoxygenation of 4-hydroperoxycyclophosphamide (III). We wish to propose now that the structure of the Fenton oxidation product of cyclophosphamide should be revised to 4-hydroxycyclophosphamide anhydro-dimer (IV) for the following reasons.

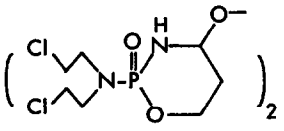


I, $R_1 = R_2 = H$

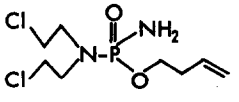
II, $R_1 = H$, $R_2 = OH$

III, $R_1 = H$, $R_2 = OOH$

V, $R_1, R_2 = O$



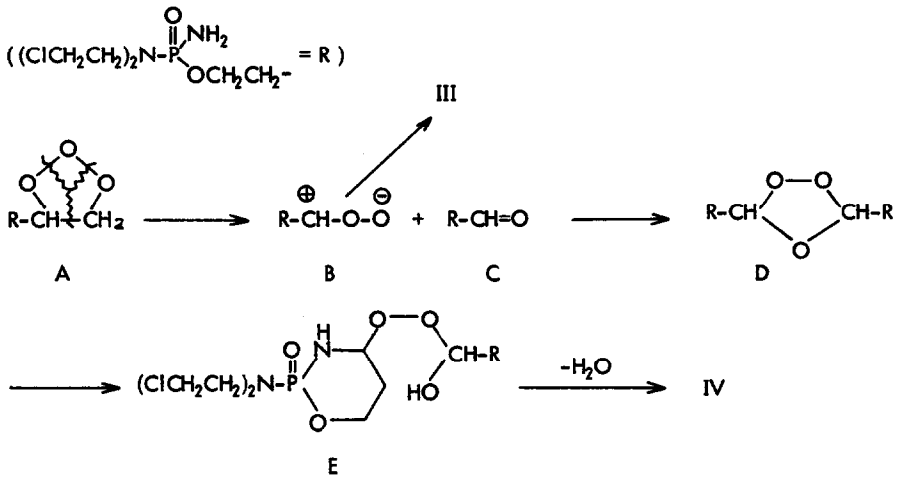
IV



VI

Ozonolysis of O-3-butenyl-N,N-bis(2-chloroethyl)phosphorodiamidate (VI) in aq. acetone yielded 4-hydroperoxycyclophosphamide (III) as a main product,[8] while in a less polar solvent such as chloroform or methylene dichloride, 4-hydroxycyclophosphamide anhydro-dimer (IV), mp 113-114° (with violent decomposition), was obtained in ca. 20% yield accompanied by a small amount of III (2%) after column chromatography on silica gel in AcOEt. Elemental analysis and molecular weight measurement[10] of IV agreed with the dimeric formula $(C_7H_{14}O_3N_2PCl_2)_2$ (Calcd: C, 30.45; H, 5.11; N, 10.15; P, 11.22; Cl, 25.68; mol. weight, 552.16. Found: C, 30.61; H, 5.25; N, 10.08; P, 10.09; Cl, 25.60; mol. weight, 540.0), and both the PMR and IR spectra were also consistent with the assigned structure (PMR $\delta_{TMS}^{d_6\text{-DMSO}}$: 1.5-2.4 (2H, m, C_5-H_2), 2.9-3.9 (8H, m, $(ClCH_2CH_2)_2$), 4.0-5.6 (2H, m, C_6-H_2), 5.22 (1H, d of q, $J_{P,C_4\text{-}H}$ = 28.0 Hz, C_4-H), 6.30 (1H, t, $J_{P,NH}$ = 3.8 Hz, NH); IR ν_{max}^{Nujol} cm^{-1}: 3200, 1320, 1257, 1233, 1217, 1120, 1064, 990, 970, 900, 818).

Oxidation of cyclophosphamide (I) with a Fenton reagent[11] in phosphate buffer solution (pH 6.4) for 2 hours at 0-10° afforded 4-ketocyclophosphamide (V)[3,5] (11%) and a small amount of IV (3%) which was identical with the product obtained according to the procedure described by Van Der Steen et al. The ^{13}C-NMR spectrum[9] reported for the Fenton oxidation product can also account for the structure IV. The anhydro-dimer IV was found to be stable at 5° for over 3 weeks, while 4-hydroxycyclophosphamide (II) decomposed within 2 days at the same temperature, and a more remarkable difference between II and IV was found in their TLC mobility[12] clearly ruling out the possibility that the Fenton oxidation product is a diastereomer of II. The formation of IV by the ozonolysis of VI can be rationalized by the following mechanism. Cleavage of the primary ozonide A gives two fragments B and C. In polar medium, the nucleophilicity of the $-\overset{O}{\overset{\|}{P}}-NH_2$ group in zwitterion B is increased due to enhanced dissociation of the amidate protons, and formation of cyclic hydroperoxide III is thus favored. In less polar conditions, however, dissociation of the amidate protons is retarded, and the cyclization of B has to compete with recombination with C leading to the symmetrical ozonide D from which IV is produced by dehydration via E.



It was found that the anhydro-dimer IV was obtainable in good yield from III by the action of reducing agents such as $K_4Fe(CN)_6$ or $NaHSO_3$. Conversion of III to IV was also effected by alkali (Na_2CO_3) at room temperature. On the other hand, reduction of III with $Na_2S_2O_3$ at 0° resulted in a quantitative formation of 4-hydroxycyclophosphamide (II), while action of reducing ions such as Fe^{2+} ($FeSO_4$) or Cu^{+} (CuCl) upon III yielded 4-ketocyclophosphamide (V) quantitatively.

4-Hydroxycyclophosphamide anhydro-dimer (IV), as well as 4-hydroperoxycyclophosphamide (III), is considered to be essentially equivalent in its biological behavior to the active species of cyclophosphamide. The antitumor activity of IV was in fact found to be almost comparable with that of 4-hydroxycyclophosphamide (II) in in vitro experiment.[13]

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10. Determined by vapor pressure osmometry in $CHCl_3$ solution.

11. $FeSO_4$ and H_2O_2 were used in the mol ratio of 1 : 5.

12. The Rf values (silica gel-acetone) of II and IV were 0.1 and 0.6 respectively.

13. The ED_{50} (μg/ml) of IV and II against B-HeLa cells were 0.8 and 0.6 respectively.