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SYNTHESIS OF ALDOPHOSPHAMIDE, A KEY CYCLOPHOSPHAMIDE METABOLITE[1]

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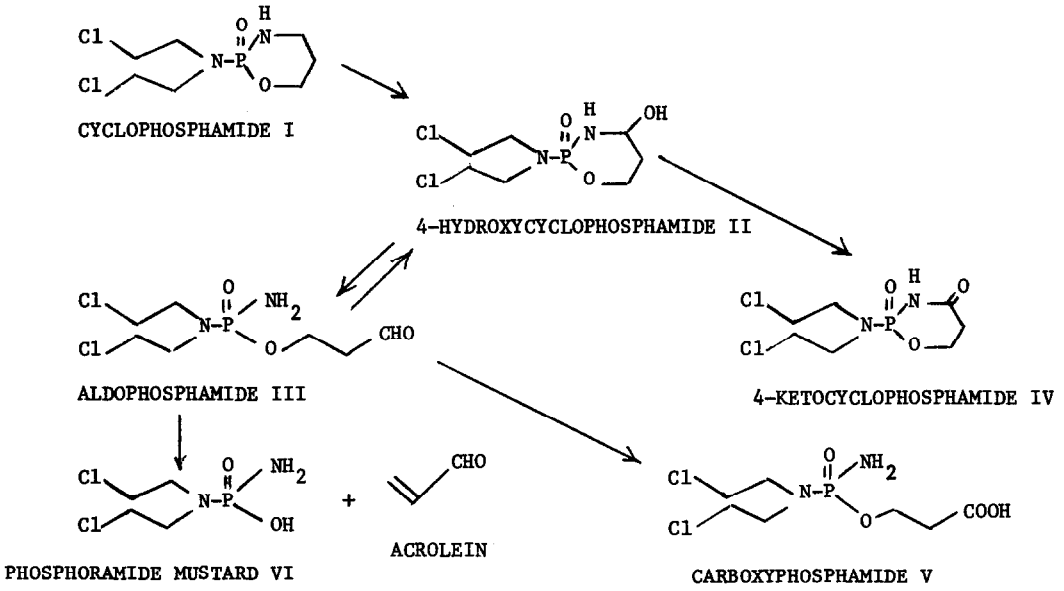
Cyclophosphamide, I, a latently cytotoxic alkylating agent widely used in the treatment of cancer, is now generally believed to exert its therapeutic effect through metabolic transformation in vivo to the phosphorodiamidic acid mustard, VI.[2] It appears to be oxidatively metabolized by liver microsomes to 4-hydroxycyclophosphamide, II,[3,4] the putative initial product, which can tautomerize to the open-chain aldehyde, aldophosphamide, III, from which the therapeutically effective phosphoramide mustard, VI,[5] would be released by β-elimination of acrolein.[6] Alternatively, 4-hydroxycyclophosphamide, II, and aldophosphamide, III, can be further oxidized to 4-ketocyclophosphamide, IV,[7] and carboxyphosphamide, V,[8] respectively, both of which are relatively non-toxic, therapeutically inactive excretion products. In fact, the comparative selectivity and therapeutic effectiveness of cyclophosphamide may be due to a balance between the enzymatic formation of these inactive metabolites, IV and V, and the spontaneous formation of the cytotoxic product, phosphoramide mustard, VI, in the tumor and in normal tissues of the host.[4,9a,b]

In contrast to the other principle metabolites which have been isolated or prepared synthetically and tested, aldophosphamide, III, the central intermediate in the now generally accepted scheme for cyclophosphamide metabolism, formulated below, has proven to be elusive in prior attempts at synthesis[10a,b] and definitive identification as a metabolite.[11a,b] In a recent study,[12] we demonstrated that aldophosphamide, which we had succeeded in preparing synthetically, is a very effective inhibitor of L1210 Leukemia in mice. In the present study, we report the method of synthesis of aldophosphamide and the identification of the product on the basis of qualitative tests, ir data and the formation of well characterized derivatives. In a companion communication,[13] we report positive detection of the presence of aldophosphamide in an incubation mixture of cyclophosphamide with mouse liver microsomes and in the serum of a patient treated with cyclophosphamide. The metabolically produced aldophosphamide, isolated from both these biologic media as the cyanohydrin derivative, was shown to be identical by mass spectral analysis to the synthetic aldophosphamide cyanohydrin reported here.



Our initial attempts to prepare aldophosphamide, III, by oxidation of the corresponding alcohol, hydroxyphosphamide, VII, under a variety of reaction conditions by the Fenton,[14] Seloxett[15] and Sarett[16] procedures gave in all cases a complex mixture of reaction products from which the desired aldophosphamide could not be isolated undecomposed. Oxidation with excess pyridinium chlorochromate in methylene chloride at room temperature[17] transformed VII initially to 4-ketocyclophosphamide, IV, (identified by ir and mass spectral analysis, melting point and elemental analysis) presumably via aldophosphamide and 4-hydroxycyclophosphamide. Carboxyphosphamide, V, as a possible alternative intermediate was ruled out since it was unchanged under the conditions of the reaction. Under milder oxidizing conditions with only 1.5 molar equivalents of pyridinium chlorochromate at -20°C, the reaction gave a single product as evidenced by a single alkylating (NBP)[18] and aldehyde positive (Purpald)[19] spot by TLC on silica gel. The product which proved to be the desired aldophosphamide was isolated as a clear syrup in approximately 10% yield by preparative thin layer chromatography on silica gel in the cold after preliminary precipitation of the chromium salts from the reaction mixture with ether. Two unresolved bands of almost identical R_f 0.71 with $CHCl_3$: C_2H_5OH (6:1) were obtained on analytical TLC, one of which is the product and the other most probably the tautomeric 4-hydroxycyclophosphamide, II. Although mass spectra by either chemical ionization or electron impact could not be obtained because of inadequate stability of the product, the ir data identified the product unambiguously. The spectrum showed the expected aldehyde absorption peak at 1720 cm^{-1} [10a]

identical to that of homoaldophosphamide prepared by us earlier[12] and different from that of acrolein, the possible decomposition product which absorbs at 1690 cm^{-1}. The intensity of the 1720 cm^{-1} band varied from sample to sample, presumably reflecting differences in the composition of the tautomers in the equilibrium mixture. The product is unstable even when stored at -10°C for periods of more than a few days. It decomposed rapidly at room temperature in phosphate buffer pH 6.9 with the evolution of acrolein which was trapped and identified as the 2,4-dinitrophenylhydrazone.

Condensation of III with semicarbazide gave a semicarbazone m.p. 127-128°C (Calc'd for $C_8H_{18}N_5Cl_2O_3P$: C, 28.75; H, 5.43; N, 20.96; Cl, 21.22. Found: C, 28.7; H, 5.4; N, 20.9; Cl, 21.1) the ir spectrum of which (ir_{Max}^{KBr} cm^{-1} 3315, 1675, 1590, 1450, 1180, 1140, 1070, 1010, 975, 945, 763) was virtually superimposable upon that of the semicarbazone of the homologous butyraldehyde ester, homoaldophosphamide.[12] Condensation of III with excess aqueous $NaHSO_3$ gave a mixture which was extracted with $CHCl_3$ to remove unreacted starting material and by-products. The adduct remaining in the medium was treated directly with NaCN to afford the cyanohydrin isolated as a clear syrup by preparative thin layer chromatography on silica gel $CHCl_3$: C_2H_5OH (6:1), R_f 0.50 (Calc'd for $C_8H_{16}N_3Cl_2O_3P$: C, 31.59; H, 5.30; N, 13.82; Cl, 23.31. Found: C, 31.8; H, 5.8; N, 12.4; Cl, 22.6). Ir spectrum (ir_{Max}^{NaCl} cm^{-1} 3330, 3000, 1560, 1445, 1345, 1220, 1110, 1085, 1040, 985, 748) and mass spectral analysis (M + H at m/e 304, 306 and 308 with relative intensities of 9:6:1 representing the combination of ^{35}Cl and ^{37}Cl at natural abundance; M + H - HCN m/e 277, 279, 281 with the same relative intensities 9:6:1) were entirely consistent with this structural assignment.

The starting hydroxyphosphamide, VII, was prepared by sequential reaction of $POCl_3$ with propane-1,3-diolmonobenzylether nor HN2 and ammonia followed by catalytic debenzylation with Pd/H_2. The product, VII, purified by chromatography on silica gel was obtained as a clear syrup (Calc'd for $C_7H_{17}N_2Cl_2O_3P$: C, 30.12; H, 6.14; N, 10.04; Cl, 25.40. Found: C, 30.2; H, 6.1; N, 10.2; Cl, 25.4).

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APPENDIX

cyclophosphamide (NSC-26271) 2H-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl) amino] tetrahydro-, 2-oxide

phosphoramide mustard (NSC-69945) phosphorodiamidic acid, N,N-bis(2-chloroethyl)-

carboxyphosphamide (NSC-145124) phosphorodiamidic acid, N,N-bis(2-chloroethyl)-,2-carboxyethyl ester

4-hydroxycyclophosphamide (NSC-196562) 2H-1,3,2-oxazaphosphorine-4-ol, 2-[bis(2-chloroethyl) amino] tetrahydro-2-oxide

aldophosphamide (NSC-254687) phosphorodiamidic acid, N,N-bis(2-chloroethyl)-, 3-oxopropyl ester