to 5-fluorovanillin (1a), identical with the material described above.

2-Fluoroisovanillin (2b). To 200 mg (1.28 mmol) of 2-fluoroveratraldehyde dissolved in 3 mL of methylene chloride and cooled to −78 °C was added 0.05 mL (1.08 mmol) of BBr_3 in one portion with rapid stirring. The solution was stirred at room temperature for 15 h, cooled to 0 °C, and treated with excess H_2O. Ether was added, and the organic layer was separated and extracted with 2 N NaOH. The alkaline extract was acidified with dilute HCl and extracted with ether, and the ether extract was dried (Na_2SO_4) and evaporated. The residue was purified by preparative TLC (silica gel GF; pentane–ether, 1:1, developed 3 times) to give 9 mg of pure 2-fluoroisovanillin (2b).

Enzymatic O-Methylation. Stock solutions (10 mM) of 3a–c and (±)-norepinephrine were prepared in 0.001 N HCl and stored at −4 °C until used. Enzymatic reaction mixtures were prepared in ice, containing the following components (final concentrations) in a final volume of 0.25 mL: catechol (2 mM); magnesium chloride (2.4 mM); *S*-adenosyl-L-methionine iodide (Sigma Chemical Co.) (0.1 mM); *S*-adenosyl-L-[*methyl*-^{14}C]methionine (New England Nuclear Corp., sp act. = 55 mCi/mmol), 0.1 μCi; freshly prepared dithiothreitol (2 mM); Tris buffer, pH 7 or 9 (25 mM); and partially purified COMT (calcium phosphate eluate step[8]), 4 mg. Reaction was initiated by the addition of the catechol, incubated at 37 °C for 30 min, and terminated by the addition of 5 N ammonium hydroxide (0.5 mL). Control reactions were carried by omission of (1) the catechol or (2) replacement of COMT by bovine serum albumin. These control mixtures did not yield components which interfered with the thin-layer separation of the final products (Scheme I). The efficiency of the ethyl acetate extraction was determined by extraction of standard mixtures of the respective *O*-methylbenzaldehydes. Quantitation was achieved by HPLC analysis [stationary phase; Waters μ-Bondapac C_{18} reverse phase; mobile phase; methanol/water, 1:2, containing PIC (0.005 M) (Waters; 1-heptanesulfonic acid)]. In all cases, base-line separations of the respective aldehydes was achieved. No significant differences between the meta and para isomers were found.

Periodate Oxidation. To the reaction mixture (above) was added freshly prepared 2% aqueous $NaIO_4$ (0.2 mL) with vigorous mixing at room temperature; after 5 min, a freshly prepared solution of aqueous 10% sodium metabisulfate (0.2 mL) was added, and the reaction was cooled in ice bath. Carrier amounts of the authentic aldehydes 1a and 2a, 1b and 2b, 1c and 2c, and vanillin and isovanillin were added to the appropriate tubes. The reaction mixtures were acidified by the dropwise addition of 5 N HCl (0.5 mL) with continuous cooling and extracted two times with ethyl acetate (10 mL). The extracts were clarified by centrifugation, transferred to conical tubes, and concentrated under a stream of N_2, and the final residue was dissolved in MeOH (0.025 mL).

Chromatographic Separation. The methanol solutions (above) were spotted on silica gel plates (Analtech, GF silica gel, 250 μm, 5 × 20 cm) and immediately developed in the appropriate solvent system.[16] The solvent system used for the products derived from 3a was petroleum ether–diethyl ether (1:1); for products from 3b, 3c, and (±)-norepinephrine, benzene–acetic acid (1 N)–*p*-dioxane (90:1:1) was used. The compounds were visualized by UV absorption, Gibbs reagent and scanning with a gas-flow TLC-scanner (Berthold LB2760, gas phase, methane). Quantitation was achieved by quantitative transfer of the silica gel (3-mm strips) and measurement of radioactivity by scintillation counting in hydroflor (National Diagnostic, Inc.). The mobility of the authentic aldehydes corresponded in each case to the mobility of the isotopically labeled products. The enzymatic conversion to O-methylated products was approximately 40% for each catecholamine.

5-Fluoro- and 5-Chlorocyclophosphamide: Synthesis, Metabolism, and Antitumor Activity of the Cis and Trans Isomers

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In seeking analogues of cyclophosphamide (1) having improved antitumor activity by virtue of accelerated formation of the cytotoxic metabolite phosphoramide mustard, cis and trans isomers of 5-fluoro- and 5-chlorocyclophosphamide (9, 10, 11 and 12, respectively) were synthesized by condensation of the appropriate 3-amino-2-halopropan-1-ol (13 or 26) with *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (14). The metabolism of the halocyclophosphamides by rat liver microsomes was stereoselective; the cis isomers (9 and 11) were poorly metabolized, whereas the trans isomers (10 and 12) were metabolized with efficiency comparable to that of cyclophosphamide. However, there was no evidence that the yield of phosphoramide mustard produced by the trans analogues was significantly greater than that from cyclophosphamide following microsomal 4-hydroxylation. Hence, the halogen substituents did not accelerate β-elimination of acrolein from the acyclic aldehydo tautomers. As expected, the poorly metabolized *cis*-5-fluoride (9) had little activity against the ADJ/PC6 tumor in mice. However, the *cis*-5-chloride (11) was as active as the trans isomer (12) and each had approximately half the therapeutic index of 1. The *trans*-5-fluoride (10) was much less active, having an ED_{90} value some 16-fold that of 1.

Cyclophosphamide (1, 2-[bis(2-chloroethyl)amino]-tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide) is one of the most widely used of the alkylating-type anticancer drugs. The drug itself has little, if any, cytotoxic activity and requires metabolic activation[1] presumably by the cytochrome P-450 system and mainly in the liver. The principle features of the metabolic profile[2] (Scheme I) involves the initial formation of 4-hydroxycyclophosphamide (2), which is assumed to equilibrate rapidly with the acyclic tautomer, aldophosphamide (3). Structures 2 and 3 can undergo further enzymatically mediated oxidation–dehydrogenation to give the weakly cytotoxic metabolites 4-ketocyclophosphamide (4) and carboxyphosphamide (5), respectively. In competition with the metabolic reaction 3 → 5 there is an apparently nonenzymatic β-elimination which yields phosphoramide mustard (PAM, 6) and acrolein (7). It is the reactive alkylating product PAM (6) which is now generally regarded as the main source of the cytotoxicity of cyclophosphamide.

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Scheme I. Metabolism Profile of Cyclophosphamide

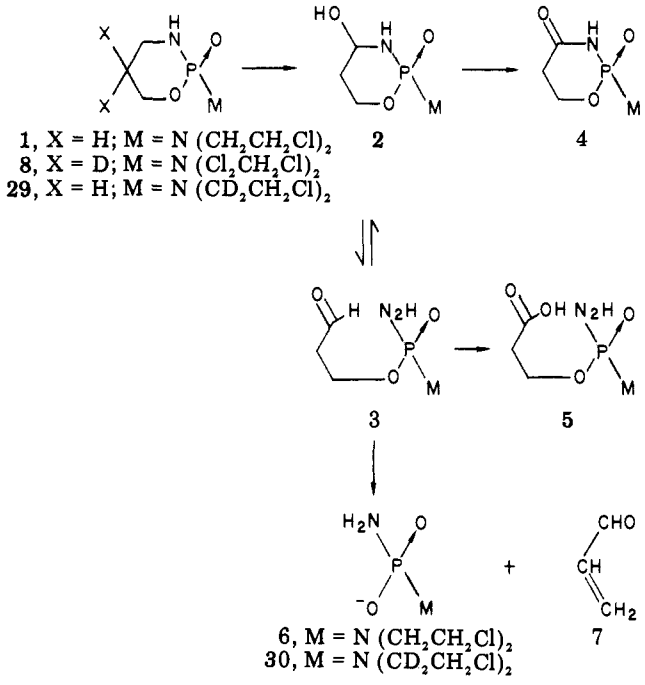


Table I. Screening against the ADJ/PC6 Mouse Plasma Cell Tumor[a]

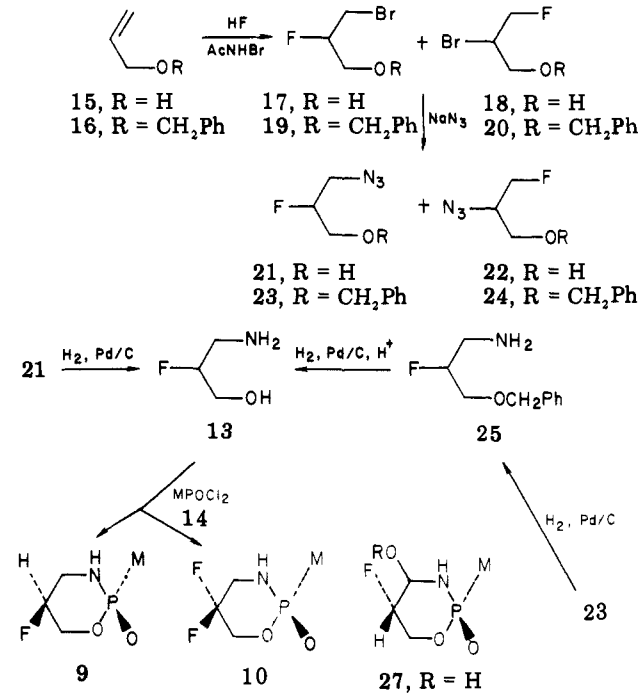
compd	LD_{50}, mg/kg	ED_{90}, mg/kg	TI
cyclophosphamide (1)	260	2.25	115.5
cyclophosphamide-5,5-d_2 (8)	700	12	58
5-fluorocyclophosphamide			
cis (9)	320	[b]	
trans (10)	320	37	8.9
5-chlorocyclophosphamide			
cis (11)	540	9.4	57
trans (12)	380	6.6	58

[a] LD_{50} = dose required to kill 50% of animals in group; ED_{90} = dose producing 90% reduction in tumor weight; TI = therapeutic index (LD_{50}/ED_{90}). [b] 88.3% inhibition at 320 mg/kg.

In seeking to verify the key role of PAM formation in the antitumor activity and cytotoxicity of cyclophosphamide, the 5,5-dideuterio derivative (8, cyclophosphamide-5,5-d_2) was studied. It was anticipated that whereas 5,5-dideuteration would not affect significantly the physicochemical properties and the rate of 4-hydroxylation, the operation of a primary kinetic isotope effect[3] (k_H/k_D) should retard the β-elimination reaction required for the formation of PAM from the derived dideuterioaldophosphamide. A substantial isotope effect (5.3) was observed, and in comparison with the parent drug (1), cyclophosphamide-5,5-d_2 (8) had a substantially lower potency as an antitumor agent. This finding is confirmed by the more complete antitumor test reported here (see Table I) which was made possible by the development of an improved synthesis[4] with resulting availability of adequate quantities of 8. Not only is 8 ~5-fold less potent than 1, as indicated by the ED_{90} values, but its therapeutic index is reduced by ~50% (Table I).

Thus, retardation of the β-elimination reaction 3 → 6 + 7 markedly and adversely affected the antitumor activity

Scheme II



of cyclophosphamide derivatives; therefore, it became of interest to explore the consequences of accelerating this reaction. In this context, we are investigating the effect of introducing metabolically stable electronegative substituents at position 5 of cyclophosphamide on the rate of the β-elimination reaction and on the antitumor activity. We now report on the cis (9) and trans isomers (10) of 5-fluorocyclophosphamide and on the corresponding 5-chlorides (11 and 12); the terms cis and trans refer to the relationship between the halogen substituent and the phosphoryl oxygen (see below).

Any increase in the rate of formation of PAM would involve a parallel increase for the accompanying acrolein derivative. Acrolein is responsible[5] for the urothelial toxicity of cyclophosphamide in vivo and it is unlikely that the 2-fluoroacrolein would be nontoxic. The toxic effect of cyclophosphamide-generated acrolein (and implicitly of its analogues) can be overcome in clinical practice by coadministering sodium 2-mercaptoethanesulfonate (Mesnum),[6] which reacts with acrolein by addition of the thiol group across the olefinic bond.

Results and Discussion

Synthesis of 5-Fluoro- and 5-Chlorocyclophosphamide. The route employed in the synthesis of 5-fluorocyclophosphamide (Scheme II) paralleled that normally used for cyclophosphamide derivatives[7] and involved the condensation of 3-amino-2-fluoropropan-1-ol (13) with *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (14). The addition of bromonium fluoride, generated[8] from *N*-bromoacetamide and hydrogen fluoride, to either allyl alcohol (15) or allyl benzyl ether (16) gave in each case a mixture of the two possible addition products, namely, 3-bromo-2-fluoropropan-1-ol (17) and 2-bromo-3-fluoro-

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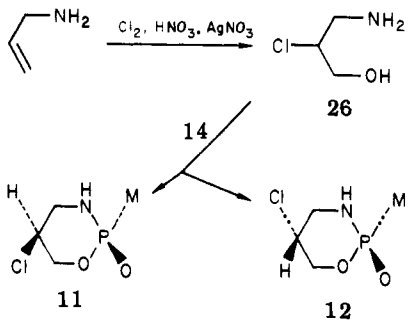
Scheme III



propan-1-ol (18) or benzyl 3-bromo-2-fluoropropyl ether (19) and the 2-bromo-3-fluoro isomer (20), with the anti-Markownikov products being the minor components.

Since the components of these mixtures were not readily isolable, each mixture was treated with sodium azide in *N,N*-dimethylformamide, and the resulting azidofluoro derivatives (21–24) were catalytically hydrogenated to the corresponding aminofluoro compounds, from which 3-amino-2-fluoropropan-1-ol (13) was readily isolated pure via the crystalline oxalate salt.

The structures of the cis (9) and trans (10) isomers of 5-fluorocyclophosphamide could not be assigned on the basis of NMR spectroscopy. The ^{19}F resonances were of little value due to extensive F, H, P coupling. In fluorocyclohexanes[9] a ^{13}C carrying an axial fluorine resonates at higher field (3.3 ppm) than when the substituent is equatorial. Also, the $^2J_{^{13}C,^{19}F}$ coupling is larger when the fluorine is equatorial ($J_{C,F_{eq}}$ = 172 Hz, $J_{C,F_{ax}}$ = 167 Hz). However, the corresponding data for C5 of the 5-fluorocyclophosphamides [cis isomer: δ 84.4 ($J_{C\text{-}5,F}$ = 178.5 Hz); trans isomer: δ 83.7 ($J_{C\text{-}5,F}$ = 179.6 Hz)] are not so clear cut but are consistent with axial and equatorial fluorines in the cis (9) and trans (10) isomers, respectively.

The structures of the two isomers of 5-fluorocyclophosphamide were established by X-ray crystallography. The details of this study will be published elsewhere,[10] but it may be noted here that in the crystal phase the isomer (mp 70–71 °C) of lower chromatographic mobility had a distorted chair conformation with fluorine and phosphoryl oxygen essentially equatorial. This isomer is designated trans (10) in accord with convention.[11] The crystal structure of the trans isomer contrasts with that reported[12] for cyclophosphamide in which the ring adopts a chair conformation with the phosphoryl oxygen axial.

The 5-fluorocyclophosphamide isomer (mp 56–58 °C) of higher chromatographic mobility existed in the crystal phase as a slightly distorted boat conformation with apical nitrogen and carbon atoms. The mustard group was boat-equatorial with the fluorine and phosphoryl oxygen in boat-axial positions. This isomer is designated[10] cis (9).

The route to 5-chlorocyclophosphamide (Scheme III) via the key intermediate 3-amino-2-chloropropan-1-ol (26) closely resembled that used[13] in the synthesis of the 5-bromo derivative. The structures of the crystalline cis (11) and trans (12) isomers of 5-chlorocyclophosphamide were assigned on the basis of ^{13}C NMR data. Thus, in chlorocyclohexane[9] when the chlorine is axial, the associated ^{13}C atom resonates at lower field than in the equatorial conformer (δ_{ax} 31.2, δ_{eq} 32.80). If this relationship is assumed to hold for the C5 in the oxazaphosphorine ring of 11 and 12, then (assuming P=O to be axial[12]) the isomer (11) of higher R_f value has the chlorine equatorial (^{13}C-5 resonance, δ 50.6) and, hence, the cis configuration, whereas the isomer (12) of lower R_f value has the chlorine axial (^{13}C-5 resonance δ 52.0) and, hence, the trans configuration. This proposed configuration of the isomer 12 has also been confirmed by X-ray crystallography.[14]

Metabolism and Antitumor Activity of 5-Fluoro- and 5-Chlorocyclophosphamides: Comparison with Cyclophosphamide. Two aspects of the metabolism of the cis (9) and trans (10) isomers of 5-fluorocyclophosphamide and of the corresponding 5-chlorides (11 and 12) were investigated relative to that of cyclophosphamide, namely, the susceptibility to metabolism by microsomes and the subsequent release of PAM (6). The metabolism by rat liver microsomes was markedly stereoselective. When 9 and 10 were coincubated for 50 min at 37 °C and the extents of metabolism of each component were determined using isophosphamide as internal standard (cf. the experiments with *cis*- and *trans*-4-methylcyclophosphamide),[15] the cis isomer 9 was scarcely metabolized (~4%), whereas ~46% of the trans isomer 10 was consumed. The low tumor-inhibitory activity of 9 (Table I) implies that it is metabolized inefficiently in the mouse. The trans isomer 10, though much more active than 9, was relatively ineffective compared with cyclophosphamide (Table I), the ED_{90} value being 17-fold higher for 10 than for 1. One explanation considered was a different route for microsomal metabolism, such as has been observed for one of the four stereoisomers of 4-methylcyclophosphamide, which was efficiently metabolized by rat liver microsomes but by hydroxylation of one of the chloroethyl side chains rather than at C-4 of the oxazaphosphorine moiety.[16]

However, metabolism of 10 appeared to follow the normal (Scheme I) pathway, since the major microsomal metabolite was 5-fluoro-4-hydroxycyclophosphamide (27), which was detected by TLC ($CHCl_3$–EtOH, 19:1; R_f 0.13, cf. 0.18 for 10) using acidic 2,4-dinitrophenylhydrazine and characterized by mass spectrometry (direct-insertion technique, source temperature 110 °C, ionizing voltage 70 eV). The mass spectrum contained, inter alia, peaks at m/z 245 ($[M - CH_2Cl]^+$, 5%), 227 ($[M - CH_2Cl - H_2O]^+$, 30%), and 74 (possibly $M^+\cdot$ for fluoroacrolein, 100%). On treatment with ethanol for 1 h at room temperature, a product was formed which gave a mass spectrum exhibiting an additional peak at m/z 273 (13% of base peak at m/z 74), presumably due to $[M - CH_2Cl]^+$ for the corresponding 4-ethoxy derivative 28.

Moreover, the intermediary hydroxy derivative yielded the active metabolite PAM (6) with an efficiency comparable to that of the 4-hydroxy derivative of 1. Thus, 10 and cyclophosphamide-d_4[17] (29) were coincubated (51 and 31%, respectively, metabolized during 50 min at 37 °C), and the ratio of PAM (6) to PAM-d_4 (30) was determined by GC-MS of the respective *N,N,O*-trimethyl derivatives

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using selected ion monitoring.[18] The peak height ratio for the respective $[M - CH_2Cl]^+$ ions for trimethylated **6** and **30** was 1:0.85 for a 1:1 (w/w) mixture of the synthetic compounds and reflected the presence of the trideuterated analogue of **6** as a minor component[17] in the sample of **30**. Hence, the ratios of 1:0.70 and 1:0.84 obtained from samples of **6** and **30**, isolated after incubation for 50 min and 2 h and 50 min of a mixture of **10** and **29** (of isotopic composition similar to that of synthetic **30**), implied that the active metabolite was produced from the intermediary metabolites of **10** and **29** with similar efficiency and that the lower potency of **10** compared to that of **1** in the antitumor test did not originate from differences in the extents of formation of PAM.

Analogous experiments were carried out on the corresponding 5-chlorides **11** and **12**. Again, the trans isomer **12** was much more efficiently metabolized by rat liver microsomes (41% consumed during 50 min) than was the cis isomer (8.7%). Equal quantities of PAM and PAM-d_4 were produced from a mixture of **12** and **29** during a 50-min incubation in which 48 and 23%, respectively, of each substrate was consumed. Because of *cis*-5-chloride **11** was somewhat more efficiently metabolized than the corresponding 5-fluoride **9**, the ratio of PAM/PAM-d_4 produced from a mixture of **11** and **29** during a 50-min incubation was also determined, and the value (0.33:1) showed the preponderance of the tetradeuterated analogue expected on the basis of the much more efficient metabolism of the parent compound. Surprisingly, the antitumor activities of **11** and **12** were virtually identical (Table I), though somewhat inferior, to that of the parent drug. It may be that the hepatic metabolism of the *cis*-5-chloride **11** is more efficient in the mouse. Alternatively, it should be noted that in any case **11** is likely to be more efficiently metabolized in vivo, where it is repeatedly exposed to hepatic metabolism during circulation, than in vitro. Since the corresponding 5-fluoride **9** is even less efficiently metabolized in vitro than is **11**, it is not surprising that its metabolism in vivo is still insufficient to elicit more than minimal tumor-inhibitory activity.

Thus, the introduction of fluorine or chlorine substituents at C-5 of the oxazaphosphorine ring of cyclophosphamide does not accelerate the rate of formation of PAM from the intermediary metabolites and, consequently, does not lead to improved antitumor activity. Studies are continuing with a view to rationalizing the differences in susceptibility to metabolism of the *cis*- and *trans*-5-halocyclophosphamides.

Experimental Section

NMR spectra (^{13}C, 15.09 MHz, Me_4Si; ^{19}F, 56.4 MHz, C_6F_6) were obtained with a Bruker WP60 and a Perkin-Elmer R12B spectrometer, respectively. TLC was performed using Merck Kieselgel G (0.25-mm layer) and column chromatography using Merck Kieselgel 7734. Melting points were determined using a Kofler hot-stage apparatus. Mass spectra were determined with an AEI MS-12 spectrometer. Gas chromatography was performed on a Perkin-Elmer F17 instrument using the conditions given below.

3-Amino-2-fluoropropan-1-ol (13). A. Allyl alcohol (**15**; 29 g, 0.5 mol) and *N*-bromoacetamide (69 g, 0.5 mol) were added in alternate portions during 40 min to a solution of anhydrous HF (65 mL) in dry Et_2O (100 mL) at −70 °C in a polythene container. The mixture was stirred for a further 2 h to allow the temperature to rise gradually to −10 °C and was then poured cautiously into 2.2 M K_2CO_3 (1 L) and extracted with Et_2O (500 mL). The extract was washed with saturated aqueous NaCl, dried ($MgSO_4$), and concentrated to give a pale amber-colored oil, which was distilled to give a mixture (22 g, 36%), bp 48–53 °C (0.5 mmHg), of 3-bromo-2-fluoropropan-1-ol (**17**) and 2-bromo-3-fluoropropan-1-ol (**18**).

The ^{19}F NMR spectrum of the mixture displayed two multiplets at δ −22.5 and −53.4 due to the 2- and 3-fluoride, respectively, in the ratio 7:1. 2-Fluoride: $J_{F,H\cdot2} = 47$, $J_{F,H\cdot1} = 20.2$, $J_{F,H\cdot3} = 22.4$ Hz. 3-Fluoride: $J_{F,H\cdot3} = 44.8$, $J_{F,H\cdot2} = 6.8$, $J_{F,H\cdot1} = 2$ Hz.

A solution of the foregoing mixture (7.8 g, 0.05 mol) in dry *N*,*N*-dimethylformamide (15 mL) was heated with sodium azide (6.42 g, 0.1 mol) at 115 °C for 4 h and was then poured into water and extracted with Et_2O. The extract was washed with saturated aqueous NaCl and H_2O, dried ($MgSO_4$), and concentrated to give a ~6:1 mixture (4.5 g, 76%) of 3-azido-2-fluoropropan-1-ol (**21**) and 2-azido-3-fluoropropan-1-ol (**22**): IR (liquid ν_{max} 3400 (OH), 2100 cm^{-1} (N_3); ^{19}F NMR δ −28.8 (m), −65 (m).

A solution of the foregoing mixture of azides (5 g, 0.042 mol) in MeOH (50 mL) was hydrogenated at 50 psi in the presence of 10% Pd/C (0.5 g) for 24 h and was then filtered and concentrated. A solution of the resulting oil (3.6 g) in EtOH (20 mL) was treated with a solution of oxalic acid (8 g) in hot EtOH (20 mL) to give the oxalate of 3-amino-2-fluoropropan-1-ol (**13**; 4.2 g, 60%): mp 144–145 °C (from EtOH–H_2O); NMR ^{13}C (D_2O, internal DSS) δ 93 (d, $J_{C,F}$ = 172 Hz, *C*HF), 63.9 (d, $J_{C,F}$ = 20 Hz, *C*HOH), 42.8 (d, $J_{C,F}$ = 21 Hz, *C*HNH_3), 168.2 (s, *C*OOH); ^{19}F (D_2O) δ −34.4 (septet).

A solution of the foregoing oxalate in water was passed through a column of Amberlite IRA-400 (HO^-) resin (50 g) and eluted with H_2O. Concentration of the eluate and removal of traces of H_2O by azeotropic distillation with C_6H_6 afforded 3-amino-2-fluoropropan-1-ol (**13**; 1.7 g, 77%): ^{19}F NMR ($CDCl_3$) δ −33.3 ($J_{H\cdot2,F}$ = 25.2, $J_{H\cdot3,F}$, $J_{H\cdot2,F}$ = 11.2 Hz).

B. Allyl benzyl ether (**16**; 18.5 g, 0.125 mol) and *N*-bromoacetamide (17.25 g, 0.125 mol) were added to an ethereal solution of HF (35 mL) as in part A. Workup of the reaction mixture gave a brown-colored oil (29.7 g), which was distilled to yield a colorless mixture (15.3 g, 49%), bp 108–110 °C (2 mmHg), of benzyl 3-bromo-2-fluoropropyl ether (**19**) and benzyl 2-bromo-3-fluoropropyl ether (**20**) in the ratio 7:1: ^{19}F NMR (internal C_6F_6) δ −22.0 (m, 2-fluoride), −56.6 (m, 3-fluoride).

Sodium azide (13.2 g, 0.2 mol) was stirred with a solution of the foregoing mixture (17.8 g, 0.07 mol) in *N*,*N*-dimethylformamide (120 mL) at 145 °C for 1 h. Workup as in part A gave a ~7:1 mixture (13.4 g, 89%) of 3-azido-2-fluoropropyl benzyl ether (**23**) and 2-azido-3-fluoropropyl benzyl ether (**24**): ^{19}F NMR δ −28.8 (m, 3-azide), −66.9 (m, 2-azide).

A solution of this mixture (13.4 g) in MeOH (130 mL) was hydrogenated at 60 psi in the presence of 5% Pd/C (0.6 g) for 1 h and was then filtered through Hyflo supercel and concentrated. To a solution of the thick syrupy residue in boiling EtOH (35 mL) was added a boiling solution of oxalic acid (9 g, 0.07 mol) in EtOH (35 mL) to give an oxalate salt (11 g, 59%), mp 133–137 °C. A suspension of the salt in MeOH was adjusted to pH ~11 by the addition of aqueous KOH. The mixture was diluted with H_2O and extracted with $CHCl_3$, and the extract was washed with saturated aqueous NaCl, dried ($MgSO_4$), and concentrated to give 3-amino-2-fluoropropyl benzyl ether (**25**; 6.5 g, 84%) contaminated with 6% of 2-amino-3-fluoropropyl benzyl ether: ^{19}F NMR δ −30.2 (m, 2-fluoride), 67.5 (m, 3-fluoride).

A solution of crude **25** (1.83 g, 0.01 mol) in MeOH (25 mL) was hydrogenated at 60 psi over 10% Pd/C (200 mg) in the presence of concentrated HCl (1.5 mL) and H_2O (3 mL) during 18 h and was then filtered and concentrated to dryness. A solution of the residue in H_2O was passed through a column of Amberlite IRA 400 (HO^-) resin and eluted with H_2O. Concentration of the eluate gave impure 3-amino-2-fluoropropan-1-ol (**13**; 0.443 g, 48%) which was purified via the oxalate salt as described in part A.

2-[Bis(2-chloroethyl)amino]tetrahydro-5-fluoro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (5-Fluorocyclophosphamide). To a stirred mixture of **13** (3.5 g, 0.037 mol), dry 1,2-dichloroethane (50 mL), and Et_3N (12.6 mL) at room temperature, *N*,*N*-bis(2-chloroethyl)phosphoramidic dichloride[19] (**14**; 9.4 g, 0.037

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mol) was added during 10 min. The solution was stirred for 18 h and then filtered and concentrated to dryness. A solution of the residue in $CHCl_3$ was washed with saturated aqueous NaCl and then H_2O, dried ($MgSO_4$), and concentrated to give an orange-colored oil (5.1 g, 50%). TLC ($CHCl_3$–MeOH, 3:1) and detection with the Epstein reagent[20] revealed two products, R_f 0.7 and 0.26.

Elution of the mixture from silica gel (200 g) with $CHCl_3$–MeOH (20:1) gave *cis*-5-fluorocyclophosphamide (**9**, 2.1 g), mp 42–44 °C (from acetone–water). This material was further purified by washing with 0.1 M HCl, and recrystallization from Et_2O gave material of mp 56–58 °C: mass spectrum, m/z 278 (M^+, 1.9), 243 ($[M - Cl]^+$, 2.0), 229 ($[M - CH_2Cl]^+$, 100); ^{13}C NMR ($CDCl_3$, internal Me_4Si) δ 45.0 (C-4), 84.4 (C-5), 69.8 (C-6), 48.6 (C-7,7′, CH_2NCH_2), 41.98 (C-8,8′, $2CH_2Cl$); $J_{4,P} = 2.5$, $J_{5,P} = 9.1$, $J_{6,P} = 8.0$, $J_{7(7'),P} = 4.03$, $J_{8(8'),P} = 3.0$ $J_{4,F} = 22.7$, $J_{5,F} = 178.5$, $J_{6,F} = 24.7$ Hz. Anal. ($C_7H_{13}Cl_2FN_2O_2P_2$) C, H, N.

Further elution with $CHCl_3$–MeOH (20:1) gave *trans*-5-fluorocyclophosphamide (**10**; 2 g), which was further purified by washing twice with 0.1 M HCl to remove basic impurities and further elution from silica gel with $CHCl_3$–EtOH (10:1). Recrystallization from Et_2O gave material of mp 70–71 °C: mass spectrum, m/z 278 (2.1), 243 (2.2), 229 (100); ^{13}C NMR δ 45.5 (C-4), 83.7 (C-5), 68.5 (C-6), 49.0 (C-7,7′), 42.4 (C-8,8′); $J_{4,P} = 2.3$, $J_{5,P} = 5.5$, $J_{6,P} = 7.0$, $J_{7(7'),P} = 4.7$, $J_{8(8'),P} = 0$, $J_{4,F} = 22.0$, $J_{5,F} = 179.6$, $J_{6,F} = 21.5$ Hz. Anal. ($C_7H_{13}Cl_2FN_2O_2P$) C, H, N.

3-Amino-2-chloropropan-1-ol (26). A solution of allylamine (19 g, 0.33 mol) in H_2O (350 mL) was adjusted to pH 6 with concentrated HNO_3 (~22 mL). A solution of $AgNO_3$ (56.7 g, 0.33 mol) in H_2O (150 mL) was added and a mixture of Cl_2 and air was passed into the well-stirred solution, which was cooled to 0–10 °C in ice. When the addition of Cl_2 (24 g, 0.33 mol) was complete (~31 h), the solution was stirred for 1 h at room temperature. AgCl was removed, and the filtrate was neutralized with Amberlite IR 45 (HO^-) resin and then concentrated. The residue (orange syrup) was diluted with H_2O and applied to a column of Amberlite IR 120 (H^+) resin (450 g). After washing with H_2O to remove nitrate, the title compound was eluted with 1 M HCl and the eluate was concentrated with repeated additions of MeOH. The crude chlorohydrin (24 g, ~50%) on storage overnight deposited the pure crystalline hydrochloride of **26** (2.73 g, 5.7%).

2-[Bis(2-chloroethyl)amino]tetrahydro-5-chloro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (5-Chlorocyclophosphamide). By a procedure essentially identical with that applied above to the fluoro analogue, **26**·HCl (2.07 g, 0.014 mol) was converted into an orange product (4.1 g, 97%) consisting of a mixture of two components, R_f 0.7 and 0.25 (TLC, acetone–$CHCl_3$, 3:1). Elution from silica gel (200 g, 10-mL fractions) with this solvent mixture gave in fractions 27–41 the cis isomer **11** (oil, 1.5 g), which solidified and was recrystallized from acetone–light petroleum (bp 60–80 °C) to give **11** (0.5 g): mp 92–94 °C; ^{13}C NMR ($CDCl_3$) δ 48.6 (C-4), 50.6 (C-5), 70.8 (C-6), 48.3 (C-7,7′, CH_2NCH_2), 42.0 (C-8,8′, $2CH_2Cl$); $J_{5,P} = 4.58$, $J_{6,P} = 7.4$, $J_{8(8'),P} = 1.8$ Hz. Anal. ($C_7H_{14}Cl_3N_2O_2P·H_2O$) C, H, N.

Further elution (fractions 46–57) yielded the trans isomer **12**, which similarly crystallized (0.5 g): mp 64–65 °C; ^{13}C NMR δ 48.2 (C-4), 52.0 (C-5), 71.0 (C-6), 48.7 (C-7,7′), 42.3 (C-8,8′); $J_{4,P} = 3.7$, $J_{5,P} = 5.5$, $J_{6,P} = 6.4$, $J_{7(7'),P} = 4.6$, $J_{8(8'),P} = 0$ Hz. Anal. ($C_7H_{14}Cl_3N_2O_2P·H_2O$) C, H, N.

Microsomal Metabolism. A. General Procedure. Rat liver microsomes were prepared as previously described,[21] and incubations were performed under oxygen in stoppered 25-mL flasks, shaken gently at 37 °C. Each flask contained microsomal suspension (1.5 mL, equivalent to 375 mg of rat liver), NADP (1.43 μmol), glucose 6-phosphate (30.6 μmol), $MgCl_2·6H_2O$ (24.6 μmol) and glucose-6-phosphate dehydrogenase (2.5 μL) made up to a total volume of 5 mL using 0.1 M Tris-HCl buffer (pH 7.4). The concentration of each individual substrate was 200 μg/mL. In control incubations, microsomes were inactivated by heating at 80 °C for 30 min. After incubation, duplicate samples (2 mL) were added to solutions (0.1 mL, 2 mg/mL) of isophosphamide (internal standard) in Tris-HCl buffer. Protein was precipitated by the addition of 1 mL each of aqueous 5% $ZnSO_4$ and saturated aqueous $Ba(OH)_2$. After centrifugation, each supernatant was extracted with EtOAc (3 mL). Each extract was dried (Na_2SO_4), and a portion (1 mL) was concentrated under a stream of N_2 in a 3-mL reacti-vial (Pierce Chemical Co., Rockford, IL). A solution of the residue in EtOAc (100 μL) and trifluoroacetic anhydride (100 μL) was heated for 20 min at 70 °C and then concentrated to dryness. The residue was dissolved in EtOAc (100 μL), and 1-μL samples were injected into a Perkin-Elmer F17 gas chromatograph fitted with a nitrogen detector and a glass column 4 m × 2 mm i.d.) packed with 3% OV-17 on Gas Chrom Q (100–200 mesh) and operating at a nitrogen pressure of 30 psi, injector temperature of 250 °C, and a column temperature of 240 °C. The retention times of the *N*-(trifluoroacetyl) derivatives of isophosphamide, cyclophosphamide, and *cis*- and *trans*-5-fluorocyclophosphamide were 5.4, 7.3, 7.3, and 6.3 min, respectively, and the retention times of the derivatives of *cis*- and *trans*-5-chlorocyclophosphamide were 7.3 and 8.6 min, respectively.

B. Comparative Metabolism. Parallel incubations of cyclophosphamide (**1**), the *trans*-5-fluoride (**10**), and of a cis/trans mixture (**9**/**10**) of 5-fluorocyclophosphamide were performed as described in part A. Incorporation of all three components into one reaction mixture was precluded because of the similar retention times of the *N*-trifluoroacetates of **1** and **9** under a variety of GC conditions. The amount of each substrate remaining after incubation for 50 min was assessed by GC (comparison of peak heights) after *N*-trifluoroacetylation as described above. The extents of metabolism of *cis*- (**11**) and *trans*-5-chlorocyclophosphamide (**12**) and **1** were similarly compared.

The products of metabolism of *trans*-5-fluorocyclophosphamide (**10**), incubated alone, were examined using a procedure similar to that described[1] for cyclophosphamide and isophosphamide except that protein was precipitated with $ZnSO_4$ and $Ba(OH)_2$ and not with EtOH.

C. Formation of Phosphoramide Mustard (PAM). Duplicate mixtures of **10** and cyclophosphamide-d_4 (**29**) were incubated with microsomes for 50 min as described in part A. The mixture of PAM (**6**) and its tetradeuterated analogue (**30**) was recovered from the duplicate aliquots (1 mL) of these incubates by selective adsorption onto AG1-X8 anion-exchange resin essentially by the procedure used[18] to recover a mixture of PAM and isophosphoramide mustard (i-PAM) from human plasma. Conversion into the respective *N,N,O*-trimethyl derivatives using methyl iodide–silver oxide–potassium carbonate and subsequent analysis by GC–MS were also carried out essentially as in the cited study, except that the multiple ion detector (Mass Spectrometry Services Ltd., Manchester U.K.) was set to monitor the ions $[M - CH_2Cl]^+$ at m/z 213 and 217 in the mass spectrum of the *N,N,O*-trimethyl derivatives of PAM (**6**) and PAM-d_4 (**30**), respectively. The selected ion recording traces for the products from the above procedure were compared with those given by a 1:1, w/w, mixture (1 mg) of **6** and **30** subjected to the same derivatization procedure. An essentially identical procedure was used to determine the ratio (20:6) produced from mixtures of **29** with either *cis*- (**11**) or *trans*-5-chlorocyclophosphamide (**12**) during a 50-min incubation with microsomes.

Antitumor Tests. Compounds were tested in parallel against the ADJ/PC6 plasma cell tumor in mice using the previously described protocol.[22] The results are shown in Table I.

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