product dissolved in EtOH was treated with excess of HBr, and the precipitated salt was crystallized from EtOH to give a dihydrobromide, **17**·2HBr: mp 263–265 °C; ^{1}H NMR δ 2.90 (s, NMe_2), 4.36 (s, C4′-CH_2), 7.68 (d, J = 8 Hz, H3′ and H5′), 7.92 (d, J = 8 Hz, H3 and H5), 8.10 (t, J = 8 Hz, H4), 8.18 (d, J = 8 Hz, H2′ and H6′). Anal. ($C_{23}H_{27}N_3$·2HBr), C, H, N.

2,6-Bis[4′-[[[2″-(dimethylamino)ethyl]amino]methyl]phenyl]pyridine Tetrahydrobromide (18·4HBr·2H$_2$O). A solution of *N,N*-dimethylethylenediamine (20 mL) in THF (50 mL) was stirred at 5 °C and treated dropwise with a solution of **34** (3.1 g, 7.5 mmol) in THF (25 mL). Then the mixture was stirred at 23 °C for 10 h. Workup as described above furnished 3.0 g (50%) of **18**·4HBr·2H_2O: mp 239–241 °C; ^{1}H NMR δ 2.80 (s, 4 Me), 3.06 (t, J = 6.8 Hz, CH_2), 3.19 (t, J = 6.8 Hz, CH_2), 4.00 (s, C4′-CH_2), 7.59 (d, J = 8 Hz, H3′ and H5′), 7.87 (d, J = 8 Hz, H3 and H5), 8.06 (t, J = 8 Hz, H4), 8.10 (d, J = 8 Hz, H2′ and H6′). Anal. ($C_{27}H_{37}N_5$·4HBr·2H_2O) C, H, N.

2,6-Bis[4′-[[(3″-aminopropyl)amino]methyl]phenyl]pyridine Tetrahydrobromide (19·4HBr). Treatment of a large excess of 1,3-diaminopropane with **34** in the manner described above and then followed by the same workup furnished **19**·4HBr in a 45% yield: mp 273–274 °C; ^{1}H NMR 1.70 (m, CCH_2C), 3.00 (m, CH_2), 3.10 (m, CH_2), 3.95 (s, C4′-CH_2), 7.58 (d, J = 8 Hz, H3′ and H5′), 7.85 (d, J = 8 Hz, H3 and H5), 8.02 (t, J = 8 Hz, H4), 8.10 (d, J = 8 Hz, H2′ and H6′). Anal. ($C_{25}H_{33}N_5$·4HBr) C, H, N.

2-[4′-[[[2‴-(Dimethylamino)ethyl]amino]methyl]phenyl]-6-(4″-methylphenyl)pyridine Dihydrobromide (20·2HBr·H$_2$O). A mixture of 2,6-di-*p*-tolylpyridine (1.9 g, 7.5 mmol), NBS (0.45 g, 3 mmol), benzoyl peroxide (20 mg), and CCl_4 was heated under reflux for 16 h and then cooled. The precipitated dibromo derivative **34** was filtered off, and then CCl_4 was removed under reduced pressure from the solution containing mainly a monobromo derivative, **35**. The residue was dissolved in THF (20 mL) and allowed to react with *N,N*-dimethylethylenediamine (10 mL) dissolved in THF (20 mL). Workup as described above was followed by silica gel chromatography (hexanes/Et_3N/EtOH 8:1:1) to give 0.70 g (20%) of **20** as an oil. A hydrobromide salt was prepared and crystallized from EtOH to give **20**·2HBr·H_2O: mp 234–235 °C; ^{1}H NMR δ 2.44 (s, C4″-Me), 2.79 (s, NMe_2), 3.06 (t, J = 6.8 Hz, CH_2), 3.18 (t, J = 6.8 Hz, CH_2), 4.00 (s, C4′-CH_2), 7.44 (d, J = 8 Hz, H3″ and H5″), 7.58 (d, J = 8 Hz, H3′ and H5′), 7.82 (d, J = 8 Hz, H3 and H5), 7.98 (d, J = 8 Hz, H2″ and H6″), 8.02 (t, J = 8 Hz, H4), 8.08 (d, J = 8 Hz, H2′ and H6′). Anal. ($C_{23}H_{27}N_3$·2HBr·H_2O) C, H, N.

Bleomycin and DNA Samples. Bleomycin-A2 and blenoxane were obtained from the Bristol-Myers Co. through the courtesy of Dr. William T. Bradner. Calf thymus DNA (Worthington Biochemical) was phenol extracted, dialyzed in pipes buffer (see below), and characterized as previously described.[17] High molecular weight DNA was used in the bleomycin amplification experiments and sonicated samples were used in the viscometric titrations and binding studies (800 ± 200 base pairs), and the NMR experiments (200 ± 50 base pairs). Commercial samples of synthetic DNA polymers (Pharmacia) were dialyzed in the pipes buffer before use. All stocks solutions were stored at −30 °C.

Methods. Procedures for viscometric titrations,[30] spectrophotometric binding,[17] NMR measurements,[31] and monitoring the bleomycin amplification by the viscometric method[9c,d] and HPLC[9b] have been recently presented. All bleomycin amplification experiments were conducted in pipes buffer (pH 7.00, [Na^+] 0.015 M) at 37 ± 0.1 °C.

Biological Assay. Biological effects of selected amplifiers were evaluated by using the following modification of the antibiotic/agar diffusion test.[32] Broth cultures of *Candida lipolytica* were surface inoculated onto 6-cm petri dishes containing 5 mL of 8% glucose, 0.67% yeast nitrogen base (Difco), and 2% agar (Difco). In control experiments 1.1-cm cellulose disks were treated with 50 μL of blenoxane (3.2×10^{-4} M stock solution) or 50 μL of a stock solution of an amplifier (3.2×10^{-3} M) and placed in the center of the inoculated plates. The plates were incubated in the dark at 22 °C for 48 h, after which time no inhibition zone was observed in each case. Then the experiments were conducted with the cellulose disks each containing 25 μL of the blenoxane solution and 25 μL of the amplifier solution. The inhibition zones were measured after 48 h of incubation at 22 °C in the dark.

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Chemically Stable, Lipophilic Prodrugs of Phosphoramide Mustard as Potential Anticancer Agents

Chul-Hoon Kwon,*,† Ki-Young Moon,† Nesrine Baturay,† and Frances N. Shirota‡

Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, Jamaica, New York 11439, and Veterans Affairs Medical Center, Minneapolis, Minnesota 55417. *Received May 21, 1990*

Benzyl phosphoramide mustard (**3**), 2,4-difluorobenzyl phosphoramide mustard (**4**), and methyl phosphoramide mustard (**5**) were examined as lipophilic, chemically stable prodrugs of phosphoramide mustard (**2**). These phosphorodiamidic esters are designed to undergo biotransformation by hepatic microsomal enzymes to produce **2**. The rate of formation of alkylating species, viz., **2**, from these prodrugs and their in vitro cytotoxicity toward mouse embryo Balb/c 3T3 cells were comparable to or better than that of cyclophosphamide (**1**). Preliminary antitumor screening against L1210 leukemia in mice, however, suggests that these prodrugs are devoid of any significant antitumor activity in vivo.

Cyclophosphamide (CP, **1**) is a widely used anticancer and immunosuppressive agent. Its chemistry and pharmacology have been reviewed by several investigators.[1-7] Cyclophosphamide is itself a prodrug that is activated by hepatic microsomal mixed-function oxidase (MFO) catalyzed C_4-hydroxylation. The resulting 4-hydroxycyclophosphamide (4-OH-CP) undergoes ring opening to aldophosphamide (Aldo), followed by generation of cytotoxic

† St. John's University.
‡ VA Medical Center.

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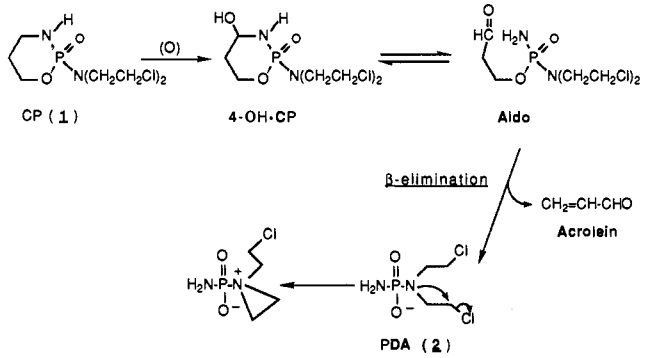
Scheme I

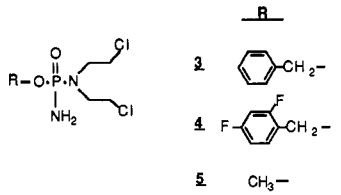


phosphoramide mustard (PDA, **2**) and acrolein by β-elimination (Scheme I).

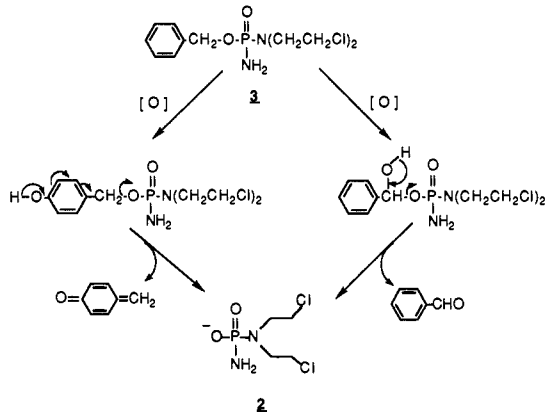
The cytotoxic activity of **1** is attributed to the aziridinium ion derived from **2**, the ultimate alkylating species that cross-links interstrand DNA.[8-10] Acrolein, a byproduct of β-elimination, is highly cytotoxic to cultured tumor cells,[12,13] but does not play a significant role in the anticancer activity of **1**.[11] Acrolein, however, is responsible for hemorrhagic cystitis, a side effect which is often dose-limiting in cyclophosphamide therapy.[14,15] In addition, acrolein has been shown to be embryotoxic in rabbits[16] and to cause denaturation of hepatic microsomal P-450.[17]

The reason for the high oncotoxic specificity of cyclophosphamide **1** is as yet not clear and remains to be elucidated. The identity of the circulating metabolite(s) which enters cells and ultimately exerts cytotoxic activity remains controversial. Considerable evidence has been presented supporting the hypothesis that the 4-OH-CP/Aldo species is the transport form of **1** and that generation of **2** occurs within cells sensitive to **1**.[2,7,18-23] Other data, however, suggest that the contribution of extracellular **2** may also be important, especially when the higher AUC value for **2** is considered.[24-28]

Chart I



Scheme II



One of the primary deactivation pathways of **1** is the oxidation of the 4-OH-CP/Aldo species to carboxyphosphamide by the enzyme aldehyde dehydrogenase (AlDH). Recent data suggest that the induction of AlDH may be responsible for the development of resistance to aldophosphamide (but not to phosphoramide mustard **2**) observed in certain L1210 cell lines.[29-31] Despite the assumption that the extracellularly generated **2** would not be readily accessible to the intracellular environment due to its anionic character at physiological pH (pK_a = 4.75), it has been shown that extracellularly delivered phosphoramide mustard derivatives are effective against many experimental tumors.[24] The recent finding that human serum albumin can accelerate the rate of formation of **2** from the 4-OH-CP/Aldo species[32] suggests that the extracellularly generated **2** may be important in cyclophosphamide chemotherapy. However, **2** is chemically unstable and possesses relatively short half-life ($t_{1/2}$ = 17.5 min in 100 mM HEPES buffer at 37 °C), limiting its effectiveness as a potential chemotherapeutic agent.

Properly designed bioreversible prodrugs of **2** may therefore serve as useful antitumor drugs with improved therapeutic index, especially against cyclophosphamide resistant cell lines. A limited number of ring substituted *O*-phenyl phosphoramide mustard derivatives have been examined as prodrugs of **2** with little success.[33] Phosphorodiamidic esters, i.e., benzyl phosphoramide mustard (**3**) (Chart I) have often been used as chemical precursors of the corresponding phosphoramide mustard derivatives,

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Table I. Microsomal Activation of Cyclophosphamide (1) and Phosphorodiamidic Esters

no.	pretreatment	μmol of mechlorethamine equiv/g of liver per h[a]	% dec
1	none	0.16 ± 0.02	0.7
	phenobarbital	0.51 ± 0.16	2.3
3	none	0.22 ± 0.13	1.0
	phenobarbital	0.26 ± 0.04	1.2
4	none	0.35 ± 0.17	1.6
	phenobarbital	0.27 ± 0.04	1.2
5	none	0.30 ± 0.04	1.3
		(0.31 ± 0.29)[b]	(1.4)
	phenobarbital	0.45 ± 0.05	2.0
		(0.61 ± 0.13)[b]	(2.7)

[a] Mean ± SE of at least three experiments. $p < 0.05$ for 1 and $p < 0.01$ for 5. [b] μmol of formaldehyde equivalent released/g of liver per h.

but little is known about their potential antitumor activities as prodrugs of phosphoramide mustard derivatives. For example, 3 may serve as a chemically stable, lipophilic prodrug of 2 based on the following postulated biooxidative activation pathways for the release of the cytotoxic 2 (Scheme II). Benzyl phosphoramide mustard 3 may undergo at least three different common oxidative metabolic processes in vivo, viz., (1) aromatic ring hydroxylation, (2) benzylic carbon hydroxylation, and (3) N-dealkylation. Ring hydroxylation (ortho or para) yields a transient 4-(or 2-)hydroxybenzyl phosphoramide mustard which should undergo further fragmentation to yield a quinone methide and 2. Such a 1,6-elimination mechanism is well documented.[34] Benzylic hydroxylation would produce a hemiacetal species which then spontaneously decomposes to benzaldehyde and 2. On the other hand, N-dealkylation would generate chloroacetaldehyde and a potential monofunctional alkylating agent after removal of the benzyl group via metabolism. This N-dealkylation process, which is known to take place on the chloroethyl side chain of cyclophosphamide, represents a deactivation pathway. The extent to which the above processes occur in vivo will determine whether 3 can be a useful prodrug of 2. We present data on the in vivo and in vitro activity of a selected group of phosphorodiamidic esters designed as chemically stable, lipophilic prodrugs of 2.

Results and Discussion

Benzyl phosphoramide mustard 3 was stable in aqueous buffer under physiological conditions (pH 7.4 and 37 °C) for at least 24 h. Alkylating activity was absent as determined by reaction with 4-(4-nitrobenzyl)pyridine (NBP). When 3 was incubated with rat hepatic microsomes (pH 7.4 and 37 °C), a time-dependent release of an alkylating species was observed (Table I). Compound 3 was devoid of any alkylating activity when incubated in the absence of oxygen (data not shown). These data suggest that 3 is oxidized, presumably by microsomal enzymes, to an alkylating species, i.e., 2. As stated earlier, 3 may also undergo N-dealkylation on the chloroethylamine side chain to produce chloroacetaldehyde. This metabolite is capable of reacting with NBP. It was therefore theoretically possible that all of the alkylating activity observed in the presence of NBP was due to liberated chloroacetaldehyde. The products of the microsomal incubation were also examined for benzaldehyde and its derivatives by HPLC (Table II). When 3 was incubated with the microsomal fraction for 20 min and analyzed by HPLC, a peak corresponding to the parent compound 3 [retention time (t_R) = 2.1], along with a peak (t_R = 23.5 min) corresponding to benzyl alcohol or possibly benzaldehyde, and three other peaks (t_R = 4.8, 5.4, and 14.7 min) were observed. One peak (t_R = 5.4 min) appeared to be 4-hydroxybenzaldehyde (t_R = 5.7 min). Analysis of the incubation mixture by TLC indicated the presence of 4-hydroxybenzaldehyde as well as benzyl alcohol and benzaldehyde. The presence of 4-hydroxybenzaldehyde was corroborated by the appearance of an HPLC peak (t_R = 3.2 min) when the incubation mixture was treated with semicarbazide, which was identical with an authentic sample of 4-hydroxybenzaldehyde semicarbazone. These data indirectly support the proposed bioactivation mechanism and indicate that 3 is a biooxidative prodrug of 2.

Table II. Microsomal Metabolites of Benzyl Phosphoramide Mustard (3) and 2,4-Difluorobenzyl Phosphoramide Mustard (4) Detected by HPLC and TLC

	metabolite (authentic sample)	
	HPLC (t_R, min)	TLC (R_f)
benzyl alcohol	23.5 (23.2)	0.40 (0.39)
benzaldehyde	23.5 (21.4)	0.69 (0.73)
4-hydroxybenzaldehyde	5.4 (5.7)	0.24 (0.22)
4-hydroxybenzaldehyde + semicarbazide	3.2 (3.8)	
2,4-difluorobenzyl alcohol	23.5 (23.3)	0.46 (0.44)
2,4-difluorobenzaldehyde	23.5 (23.7)	0.75 (0.75)
2,4-difluorobenzaldehyde + semicarbazide	6.8 (7.3)	

One of the possible metabolites of 3 is a quinone methide which may be toxic to the host cells. 2,4-Difluorobenzyl-PDA (4, Chart I) was designed to prevent the formation of this potentially toxic byproduct. The presence of two electron-withdrawing fluorine substituents would deactivate the aromatic ring and thus block ring hydroxylation. Compound 4 was tested for its ability to produce an alkylating species, i.e., 2 by metabolic activation. Preliminary studies indicated that 4 was stable in aqueous buffer (pH 7.4 and 37 °C) for at least 24 h and did not act as an alkylating agent as shown by the NBP assay. However, when incubated with the microsomal fraction, it liberated an alkylating species in a time-dependent manner (Table I). HPLC analyses, after incubation of 4 with microsomes, revealed a considerably simpler metabolic profile than that of 3. A peak with a retention time of 23.5 min was detected along with unchanged 4 (Table II). This retention time corresponded to 2,4-difluorobenzaldehyde and/or 2,4-difluorobenzyl alcohol. TLC analysis indicated the presence of both metabolites. In addition, when semicarbazide was included in the microsomal incubation mixture, the extract contained the parent molecule and a compound with t_R = 6.8 min which corresponded to an authentic sample of 2,4-difluorobenzaldehyde semicarbazone, indicating that 2,4-difluorobenzaldehyde was produced. These data support the hypothesis that 4 is a biooxidative prodrug of 2 which does not generate a potentially cytotoxic quinone methide byproduct.

The methyl ester of phosphoramide mustard (5, Chart I) has been tested in vivo against L1210 leukemia in mice[35] and showed only marginal activity [ILS (increase in life span) = 121%]. The lack of any significant antitumor activity was attributed to inefficient demethylation in vivo. As expected, 5 was stable in aqueous buffer (pH 7.4, 37 °C) and was devoid of any alkylating activity. However,

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Table III. Relative Cytotoxicities of Cyclophosphamide (1), Phosphoramide Mustard (2), and Phosphorodiamidic Esters to Balb/c 3T3 Cells

no.	LC_{50},[a] μM	no.	LC_{50},[a] μM
1	37.6 ± 4.8	4	19.6 ± 2.8
2	3.0 ± 0.1	5	16.5 ± 1.2
3	6.1 ± 0.8		

[a] Mean ± SE of at least three experiments carried out at five drug concentrations.

incubation of **5** with the microsomal fraction from rat liver generated an alkylating species in a time-dependent manner (Table I). The rate of formaldehyde production was measured to assess the extent of demethylation. This method is an indirect, but an efficient way to measure the rate of release of **2**. These results suggest that the principal alkylating species generated from **5** was **2**, and that the formation of an alkylating species from **5** in the presence of either untreated or phenobarbital-induced microsomes is equal to or greater than that from **1**. It is interesting to note that the metabolism of **5** was enhanced by phenobarbital pretreatment and that the rate of increase was similar to that from **1**. In contrast, both **3** and **4** did not show any significant enhancement of metabolism in the presence of phenobarbital-induced microsomes, suggesting that the enzymes involved in their biotransformation are not inducible by phenobarbital.

Having established that the above phosphorodiamidic esters are bioactivated by the liver microsomal enzymes, the in vitro cytotoxicity of these prodrugs toward mouse embryo BALB/c 3T3 cells was examined and compared with those of **1** and **2**. 3T3 Cells have been shown to possess cytochrome P-450 mixed function oxidase activity[36] and were selected to examine the cytotoxicity of drugs requiring P-450 activation. The results indicate that **2**, a compound that is active without metabolic intervention, was the most cytotoxic compound, followed by **3**, **5**, and **4**, with **1** being the least cytotoxic (Table III). Compound **3**, which is capable of producing a cytotoxic quinone methide, was three times as cytotoxic as **4**.

Preliminary antitumor activity of these phosphorodiamidic ester prodrugs was evaluated against L1210 leukemia in mice. None of the phosphorodiamidic esters increased the life span of animals innoculated with L1210 cells greater than 150%. Both **3** and **4**, however, showed some toxicity.

In summary, three chemically stable phosphorodiamidic esters have been prepared which were designed to undergo oxidative metabolism to produce **2** in vivo. Their ability to generate **2** as well as their cytotoxicity in vitro was comparable to or better than those of **1**. The lack of any significant in vivo antitumor activity of these prodrugs suggests that lipophilic prodrugs of **2** without tumor cell site selectivity may not be effective anticancer agents. This study further implicates the importance of the 4-OH-CP/Aldo intermediates in producing the high oncotoxic selectivity exhibited by **1**.

Experimental Section

Chemistry. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on a Varian EM-360 (60 MHz) spectrometer (Me_4Si as internal standard). Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, GA. Silica gel GF plates (250 μm, Analtech) were used for TLC. Silica gel (40 μM, Baker) was used for flash column chromatography. NBP spray was used for detection of potential alkylating compounds as follows: the plate was sprayed with 5% 4-(4-nitrobenzyl)-pyridine in acetone, heated at 100 °C for 5 min, and sprayed with 5% methanolic KOH. Alkylating agents are indicated by the appearance of a blue chromophore. Spectrophotometric analysis of alkylating activity was performed on a Milton Roy spectronic 301 spectrophotometer. All organic reagents and solvents were reagent grade and purchased from commercial vendors. Cyclophosphamide was purchased from Sigma Chemical Co. and used without further purification.

Bis(2-chloroethyl)phosphoramidic dichloride was prepared as described[37] in 84% yield.

Benzyl *N,N*-bis(2-chloroethyl)phosphorodiamidate (3) was prepared by a modification of the published procedure.[37] Benzyl alcohol (2.16 g, 2.07 mL, 0.02 mol) was added dropwise to a suspension of 0.8 g (0.02 mol) of sodium hydride, a 60% dispersion in mineral oil, in 70 mL of dry THF at ice-bath temperature with stirring over a 30-min period. The reaction was stirred at ice bath temperature for 3 h. The resulting gray sodium benzylate was added dropwise to a solution of bis(2-chloroethyl)phosphoramidic dichloride (5.0 g, 0.02 mol) in dry THF (50 mL) at ice-bath temperature. The reaction mixture was stirred for 4 h at this temperature and evaporated in vacuo to give a cloudy viscous liquid. This liquid was diluted with methylene chloride (50 mL) and ammonia was bubbled through the solution for 40 min. The resulting milky solution was evaporated in vacuo to give a white viscous liquid. The liquid was diluted with anhydrous diethyl ether (50 mL), the resulting precipitate was filtered off, and the filtrate was evaporated in vacuo to give a yellow liquid. The latter was applied to a silica gel column (40 μM; 5 × 15 cm) which had been packed dry and wetted with hexane. Flash chromatography using EtOAc under positive pressure (15 psi), gave a total of 40 25-mL fractions. The fractions, containing the desired compound, were pooled and evaporated in vacuo to give 0.75 g of a yellow oil. This oil was crystallized from hexane/diethyl ether to give 0.61 g of **3** (9.8% yield): mp 56–56.5 °C; TLC R_f = 0.27 in EtOAc; ^1H NMR ($CDCl_3$) δ 7.33 (5 H, s, C_6H_5), 5.00 (2 H, d, J = 8.2 Hz, $C_6H_5CH_2$), 3.00–3.70 [8 H, m, $N(CH_2CH_2Cl)$]. Anal. ($C_{11}H_{17}Cl_2N_2O_2P$) C, H, Cl, N.

2,4-Difluorobenzyl *N,N*-bis(2-chloroethyl)phosphorodiamidate (4) was prepared with 2,4-difluorobenzyl alcohol on a 0.02-mol scale as previously described for **3**, except that the eluting solvent for flash chromatography was EtOAc/hexane (2:1), to give **4** in 23.5% yield: mp 66–66.5 °C; TLC R_f = 0.36 in EtOAc; ^1H NMR ($CDCl_3$) δ 6.60–7.60 (3 H, m, $C_6F_2H_3$), 5.00 (2 H, d, J = 8.2 Hz, $C_6F_2H_3CH_2$), 3.00–3.70 [8 H, m, $N(CH_2CH_2Cl)_2$]. Anal. ($C_{11}H_{15}F_2Cl_2N_2O_2P$) C, H, Cl, N.

Methyl *N,N*-bis(2-chloroethyl)phosphorodiamidate (5) was prepared with methyl alcohol on a 0.02-mol scale as previously described for benzyl *N,N*-bis(2-chloroethyl)phosphorodiamidate. The product was crystallized directly from EtOAc/hexane without further purification to give **5** in 25.7% yield: mp 75.5–77.5 °C; TLC R_f = 0.19 in EtOAc; ^1H NMR ($CDCl_3$) δ 3.78 (3 H, s, CH_3), 3.10–3.70 [8 H, m, $N(CH_2CH_2Cl)_2$]. Anal. ($C_5H_{13}Cl_2N_2O_2P$) C, H, Cl, N.

***N,N*-Bis(2-chloroethyl)phosphorodiamidate (2)** was prepared as described[38] and isolated as its cyclohexylamine salt in 79.3% yield: mp 104–105 °C (lit.[38] mp 107–108 °C).

Stability Test under Physiological Conditions. Each incubation mixture contained 1 mL of 0.025 M sodium phosphate buffer (pH 7.4), 0.95 mL of distilled water, and 0.05 mL (2 μmol) of drug in EtOH in a final volume of 2 mL. Incubations were initiated by the addition of drug and terminated by immediately cooling in crushed ice. Blank controls contained ethanol instead of drug. Incubations were carried out at varying time intervals (0, 0.33, 0.67, 1, 2, 4, 20, and 24 h) in a water bath at 37 °C. Stability was determined by assaying for the release of an alkylating species using the NBP assay.

Determination of Alkylating Activity by NBP Assay. Alkylating activity was determined as described by Friedman and Boger[39] with slight modifications. A screw-capped, 20-mL

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round-bottomed test tube containing 1 mL of distilled water, 1 mL of 0.025 M sodium acetate buffer (pH 4.6), 0.5 mL of 5% NBP, and 1 mL of incubation sample was shaken and heated in a boiling water bath for 20 min. The sample was then immediately placed in an ice bath. To the cooled sample was added in succession 1 mL of acetone, 3 mL of ethyl acetate, and 1 mL of 0.25 N NaOH. The sample tube was thoroughly vortexed for 30 s and was allowed to stand for 1 min for complete phase separation. One milliliter of the upper phase was removed and the intensity of the blue chromophore measured at 542 nm. The color is stable for at least 3 min. The results were compared to a standard curve of alkylating activity prepared with mechlorethamine.

In Vitro Metabolism. (a) Microsomes. Male Sprague–Dawley rats (Taconic, Germantown, NY), weighing 201–225 g, were maintained ad libitum on water or on aqueous 0.1% sodium phenobarbital solution in place of their drinking water for 5 days. On day 6, all animals received regular water and were fasted overnight before sacrifice. After perfusion with 0.15 M KCl, the livers were removed and homogenized (25% w/v) in 0.15 M KCl. The homogenate was filtered through two layers of cheese cloth, the filtrate centrifuged at 9000*g* for 20 min, and the supernatant fluid removed. Microsomes were isolated from the latter by centrifugation at 105000*g* for 60 min. The microsomal pellet was suspended in 0.25 M sodium phosphate buffer, pH 7.4, to a final concentration corresponding to 270 mg wet weight of liver/mL and used immediately. All operations were carried out at 4 °C. Protein concentrations were determined by the biuret method (Protein Assay Kit, Sigma Chemical Co.) using bovine serum albumin as standard.

(b) Incubations. Incubation mixtures contained microsomes (100000*g* fraction) from PB-treated or naive rats corresponding to 270 mg of liver, test drug (1 mM), NADPH-generating system (consisting of 5 mM glucose-6-phosphate, 5 mM NADP, 0.8 mM $MgCl_2$) in 0.92 mL of 0.025 M sodium phosphate buffer (pH 7.4), and 5 units of glucose-6-phosphate dehydrogenase (500 units/3 mL of 0.005 M citrate buffer, pH 7.5) in a total volume of 2 mL. Incubations were carried out initially for varying time periods (0, 5, 10, 20, 30, and 40 min) or for 20 min in a shaking water bath at 37 °C. Incubations were initiated by addition of drug and terminated by addition of 2 mL of cold absolute EtOH. The precipitated proteins were removed by centrifugation (3000 rpm, 10 min). The supernatant was filtered through a syringe filter (0.45 μM, PTFG membrane) and the filtrate used to test for alkylating activity and to identify metabolites. Microsomes were heated at 100 °C for 5 min before use in control incubations.

(c) Identification of Metabolites by HPLC/TLC. An aliquot (5–15 μL) of the filtrate was applied to a silica gel plate (250 μm, Analtech) and the parent compound and metabolites eluted with EtOAc/hexane (1:4). HPLC studies were performed on a Waters 501 chromatograph equipped with a Waters Lambda-Max Model 481 detector; 5 μm Spherical C_{18} column (3.9 mm × 15 cm). Aliquots of the filtrate (5–15 μL) were injected and eluted with a solvent mixture [920 mL of water, 60 mL of MeOH, 20 mL of glacial acetic acid and 1.386 g of ammonium acetate (pH 3.2)] at a flow rate of 2.0 mL/min. The metabolites were detected at 254 nm (1.0 AUFS). All solvents were filtered through nylon-66 filters (0.45 μm, 47 mm).

(d) Determination of Formaldehyde. The rate of formaldehyde liberated from methyl *N,N*-(2-chloroethyl)phosphorodiamidate was determined as previously described,[40] with 5 equiv (10 μmol) of semicarbazide to trap the liberated formaldehyde.

In Vitro Cytotoxicity Assessment. Balb/c 3T3 mouse embryo fibroblasts (American Typical Culture Collection, Rockville, MD, ATCC #163) subcloned by this laboratory were used. Cell cultures were maintained at subconfluence in a 95% air, 5% CO_2 humidified atmosphere at 37 °C. The medium used for routine subcultivation as well as for experimental determinations was Dulbecco's Minimum Essential Medium (Gibco, Grand Island, NY) supplemented with 10% heat inactivated calf serum.

Log-phase cultures, fed 24 h prior to use, were disassociated from their growth substrate by scraping, and cells were disaggregated by repeated pipet aspiration. Cells were counted with a hemocytometer and the number of viable cells was determined by trypan blue dye exclusion.

Polystyrene tissue culture petri dishes (100 mm, Costar, Cambridge, MA) were seeded with 500 cells in 10 mL of media in the presence or absence of drugs or solvent in concentrations from 1 to 250 μM. Drugs were dissolved prior to use in EtOH/H_2O (1:1). Cultures were incubated in a 95% air, 5% CO_2 humidified atmosphere under standard conditions, undisturbed for 10–14 days. At this time, cultures were rinsed twice with Hank's Balanced Salt Solution (HBSS), fixed with 95% EtOH, and stained with 0.4% toluidine blue. Colonial growth was assessed by macroscopically counting the number of surviving colonies per dish. Results are reported as the number of colonies surviving chemical treatment per number of colonies in the solvent treated or untreated controls. The LC_{50} values were determined by semilogarithmically plotting the drug concentration versus cell viability as determined by the number of colonies surviving treatments. Each compound or control was prepared in triplicate and the series of experiments was repeated three separate times.

In Vivo Antitumor Evaluation. Preliminary antitumor activity of the phosphorodiamidic esters was evaluated by using male CDF1 mice (17–18 g) against lymphoid leukemia L1210 cells according to the standard NIH protocol 1.100 for testing anticancer drugs.[41] L1210 Cells were kindly provided by Dr. Norm E. Sladek, Department of Pharmacology, University of Minnesota, Minneapolis, MN. In order to determine the ability of the test compounds to increase the life span of the mice, animals were injected with 10^5 cells in 0.1 mL, ip, on day 1 and injected with drug, saline, or 2% carboxymethylcellulose on day 2. The survival rate of treated mice was compared with that of controls over a 30-day period. Cyclophosphamide (1.08 mmol/kg) served as a positive control. Two concentrations (0.36 and 1.08 mmol/kg) were used for the phosphorodiamidic esters. Three to five mice were used per dose per drug. The results are expressed as %ILS (percent increase in life span) as calculated by the following equation:

$$\%\,\mathrm{ILS} = \frac{\text{treated median day of death}}{\text{control median day of death}} \times 100$$

Deaths in the treated groups before or on day 8 were attributed to drug toxicity. Animals in the control groups (saline or CMC) surviving past day 18 were considered NT (no takes) and were not counted.

Registry No. **2**, 10159-53-2; **3**, 18228-80-3; **4**, 131068-52-5; **5**, 57154-91-3; benzyl alcohol, 100-51-6; benzaldehyde, 100-52-7; 4-hydroxybenzaldehyde, 123-08-0; 2,4-difluorobenzyl alcohol, 56456-47-4; 2,4-difluorobenzaldehyde, 1550-35-2.

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