Synthesis and Antitumor Properties of Activated Cyclophosphamide Analogues

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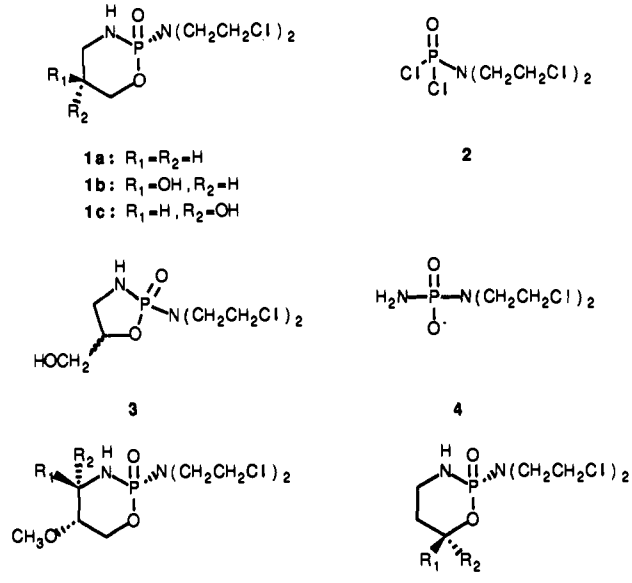
A series of 5- and 6-substituted cyclophosphamide analogues has been prepared, and their ^{31}P NMR kinetics of phosphoramide mustard (PDA) release and in vitro and in vivo cytotoxicity have been evaluated. *cis*-4-Hydroxy-5-methoxycyclophosphamide equilibrated very slowly and to a minor extent with the ring-opened aldophosphamide analogues in aqueous buffer; release of PDA was observed to a minor extent and only at high (1 M) buffer concentrations. This analogue was essentially inactive in vitro against L1210 and P388 leukemia cells. 6-Phenylcyclophosphamide and its 4-hydroperoxy derivative were potent inhibitors of blood acetylcholinesterase and were lethal at therapeutic doses in mice. In contrast, 4-hydroperoxy-6-(4-pyridyl)cyclophosphamide did not inhibit acetylcholinesterase and showed significant antitumor activity in vitro and in vivo against both wild-type and cyclophosphamide-resistant L1210 leukemia. The 4-hydroperoxy-6-arylcyclophosphamides were generally active in vitro against both wild-type and cyclophosphamide-resistant L1210 and P388 cells, and several analogues showed significant activity in vivo. Surprisingly, there was no correlation between antitumor activity in vitro and the rate of PDA release in aqueous buffer. Several compounds that showed essentially no release of PDA in aqueous buffer over several hours were highly cytotoxic to leukemia cells following a 1-h exposure in vitro. These results show that activated cyclophosphamide analogues substituted at the 6-position are not cross-resistant in these leukemia cell lines, and that a specific intracellular activation mechanism may be catalyzing PDA release in these analogues.

Introduction

Cyclophosphamide (**1a**) and its analogues are of continuing interest based upon their clinical efficacy and the possible contribution of their unique mechanisms of activation to therapeutic selectivity.[1,2] The mechanistic details of the activation process have been described,[3-5] and the role of aldophosphamide as the pivotal intermediate in both the activation[3-5] and enzymatic detoxication[6,7] processes has been demonstrated. The aldehyde moiety can serve as a substrate for metabolite inactivation by aldehyde dehydrogenase, which may provide a mechanistic basis for drug resistance.[6,7] The conjugated aldehyde acrolein that arises from aldophosphamide activation is a potent electrophile and the causative agent of the bladder toxicity associated with cyclophosphamide and its analogues.[8] Appropriate substitution of the 5- and 6-positions of 4-hydroxycyclophosphamide might improve therapeutic efficacy by generating a less electrophilic aldehyde product or by producing an aldehyde intermediate that is a poor substrate for tumor cell aldehyde dehydrogenase. We report here the synthesis, activation mechanisms, and antitumor evaluation of several analogues that further define the structural limitations of biologically active cyclophosphamide derivatives.

Chart I



Results and Discussion

Oxygen-Substituted Analogues. Upon the basis of our expectation that oxygen substituents at the C-5-position would diminish the electrophilicity of the corre-

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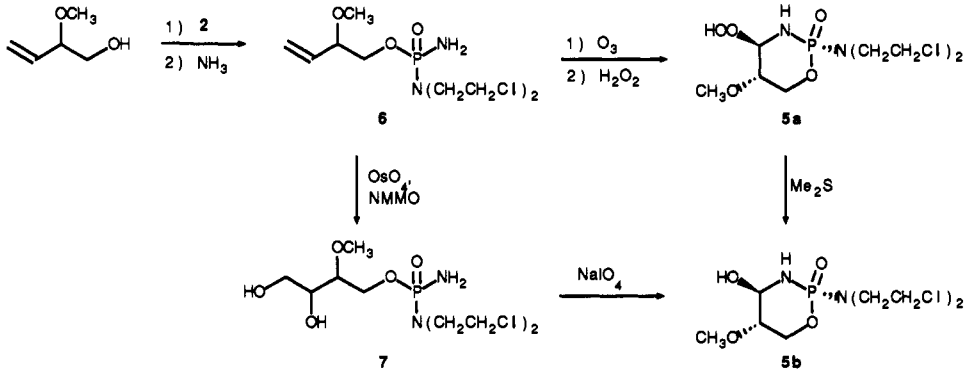
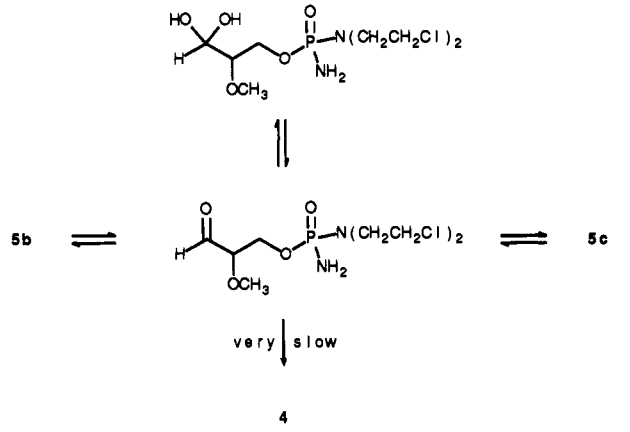
Scheme I



sponding unsaturated aldehyde and might decrease the affinity of the aldehyde intermediate for aldehyde dehydrogenase, 5-hydroxycyclophosphamide (**1b**, Chart I) was selected as the first synthetic target. Direct reaction of phosphoramidic dichloride **2** with 3-aminopropanediol afforded exclusively the two isomeric 5-membered ring hydroxymethyl oxazaphospholidines **3**; this assignment was based upon ^{31}P chemical shifts of +3.7 and +5.2 ppm for the isomers compared to ca. −13 ppm, which is typical of the 6-membered ring analogues.[3] Neither isomer of **3** showed significant activity against P388 leukemia in vivo (27% and 23% increased life span (ILS), 1 mmol/kg dose). Reaction of **2** with 2-(benzyloxy)-3-aminopropanol followed by removal of the protecting group afforded 5-hydroxycyclophosphamides **1b** and **1c** as a separable mixture of two isomers. The conformations of **1b** and **1c** were assigned by utilizing a combination of IR and NMR spectral correlations. The P=O stretching frequency occurs at 1207 and 1190 cm^{-1}, respectively, for the faster and slower eluting isomers **1b** and **1c**. Upon the basis of the correlation between equatorial P=O conformations and higher stretching frequency,[9,10] phosphoryl oxygen is assigned axial and equatorial in **1b** and **1c**, respectively. The absence of a *trans*-diaxial coupling constant for the C-5 proton in both **1b** and **1c** confirmed that the C-5-hydroxyl group was axial in both isomers. Unfortunately, both **1b** and **1c** were inactive against L1210 leukemia in vivo (ILS < 25%, 0.3–1 mmol/kg). It is not clear whether these compounds are not hydroxylated by the hepatic mixed function oxidase system, or whether the resultant 4,5-dihydroxy compound fails to undergo ring opening and/or elimination reactions to release phosphoramide mustard **4**.

4-Hydroperoxy-5-methoxycyclophosphamide (**5a**) was prepared as shown in Scheme I. The lithium salt of 2-methoxy-3-butenol was condensed with phosphoryl chloride **2** and the resulting phosphoramidochloridate reacted with ammonia to give **6**. Ozonolysis of the double bond and reaction of the ozonide with hydrogen peroxide[4] gave 4-hydroperoxy-5-methoxycyclophosphamide (**5a**). A single isomer was separated from the crude product by chromatography and crystallization. The 25-Hz H_{4e},P coupling constant is consistent with an axial orientation of the 4-hydroperoxy group,[12] and the small (<2.5 Hz) H_{6a},H_5 coupling indicates that the methoxy group is also in the axial position and trans to the hydroperoxy group. The phosphoryl oxygen is tentatively assigned the axial position upon the basis of the chemical shift of the H_{6a} proton (4.6 ppm), because the H_{6a} chemical shift is downfield for the axial as compared to the equatorial phosphoryl oxygen isomer (4.65 vs 4.25 ppm) in 4-hydroperoxycyclophosphamide.[12] The structure **5a** is therefore assigned to this isomer. Reduction of **5a** with triethyl phosphite or dimethyl sulfide afforded 4-hydroxy-5-methoxycyclophosphamide (**5b**). Alternatively, **6** was reacted with osmium tetroxide to give diol **7**, which was converted directly to a mixture of 4-hydroxy isomers **5b** and **5c** by periodate oxidation.

Scheme II



The catalytic activation of **5b** was examined in aqueous buffer by using ^{31}P NMR as described previously.[4] When **5a** was reduced with dimethyl sulfide and the product reacted in phosphate buffer (0.1 M, pH 7.4, 37 °C), the spectrum of **5b** appeared as a single peak at −14.4 ppm. Two new peaks subsequently appeared at −12.4 (**5c**, the *trans*-4-hydroxy isomer of **5b**) and −4.4 ppm (the aldehyde and/or its hydrate) (Scheme II).[4] The equilibrium com-

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position of 82:9:9 **5b**:**5c**:aldehyde/hydrate was attained by 80 min; this compares with an equilibrium composition of 46:34:20 reached within 20 min for *cis*-4-hydroxycyclophosphamide.[4] Phosphoramide mustard **4** and its solvolysis products were not detected by ^{31}P NMR at any time during the 3-h experiment. Addition of hydroxylamine (2.5 equiv) to the equilibrium mixture resulted in the gradual disappearance of **5b** and **5c** accompanied by an increase in the peak at −4.4 ppm (aldehyde + hydrate + oxime). Complete conversion to the oxime required 7 h; in contrast, similar trapping of 4-hydroxycyclophosphamide was complete within 10 min. In order to measure the amount of free aldehyde present in the equilibrium mixture, the equilibration was repeated in phosphate buffer/D_2O (pD 7.4, 37 °C) and examined by ^1H NMR. However, the aldehyde proton resonance could not be detected under these conditions. We conclude that the peak at −4.4 ppm represents the aldehyde hydrate, and that the aldehyde constitutes <1% of the total equilibrium mixture.

The equilibrium was then approached from the other direction by oxidative cleavage of diol **7** with periodate and immediate addition of the product to the same buffer. Diol **7** is a mixture of diastereomers; cleavage to the aldehyde and subsequent cyclization gave a mixture of the four 4-hydroxy-5-methoxy isomers in approximately equal amounts (−12.2, −12.4, −14.4, and −15.7 ppm), in addition to a broad peak (−4.5 ppm) for the isomeric aldehydes and/or hydrates. Both sets of equilibrating isomers reached steady state by 80 min, with an equilibrium composition of 43:7:11:26:13 for **5b**:**5c**:aldehydes/hydrates: isomeric **5b**:isomeric **5c**.

The equilibration was also investigated in HEPES buffer (1:4 acetone/0.1 M HEPES, pH 7.4, 37 °C). Experiments initiated either by dimethyl sulfide reduction of **5a** or by periodate cleavage of **7** showed no evidence of **4** or its solvolysis products when monitored for 20 h. When the buffer concentration was increased to 1.0 M, slow generation of **4** was observed. The half-time for conversion of **5b**/**5c** to **4** under these conditions was 740 min, compared to 15 min for 4-hydroxycyclophosphamide. It is interesting to note that the 5-methoxy isomer of **5b** was converted to **4** ca. 1.8 times faster than **5b** itself.

4-Hydroxy analogue **5b** was evaluated for cytotoxicity in vitro against L1210 and P388 cells in a clonogenic assay.[13] This compound was essentially inactive; concentrations required to reduce the clonogenic survival of L1210 and P388 cells by 2 orders of magnitude (LC_{99}) exceeded 1000 and 500 μM, respectively. These data indicate that cyclophosphamide analogues substituted with oxygen at the 5-position undergo ring opening and elimination at a rate that is too slow to achieve significant antitumor activity.

6-Phenylcyclophosphamide. The synthesis of 6-phenylcyclophosphamide was undertaken in the hope that the intermediate substituted aldophosphamides might be less susceptible to aldehyde dehydrogenase inactivation in drug-resistant cells. Phosphoramidic dichloride **2** was condensed with 3-amino-1-phenylpropanol to give *cis*- and *trans*-6-phenylcyclophosphamides **8a** and **8b**. The P=O stretching frequency of the faster eluting isomer (TLC) was higher than for the slower eluting isomer (1241 vs 1214 cm^{-1}), suggesting that the former has the phosphoryl oxygen equatorial. The ^{31}P chemical shifts of the two isomers (−14.3 and −11.3 ppm, respectively, $CDCl_3$) were also consistent with an equatorial phosphoryl oxygen in the faster eluting isomer. The small value of $J(H_6,P) < 2.5$ Hz for both isomers indicates that the phenyl group is equatorial in both **8a** and **8b**. These assignments were confirmed by a report of the crystal structure of **8b**, showing the diequatorial relationship between the phosphoryl oxygen and the phenyl substituent.[14]

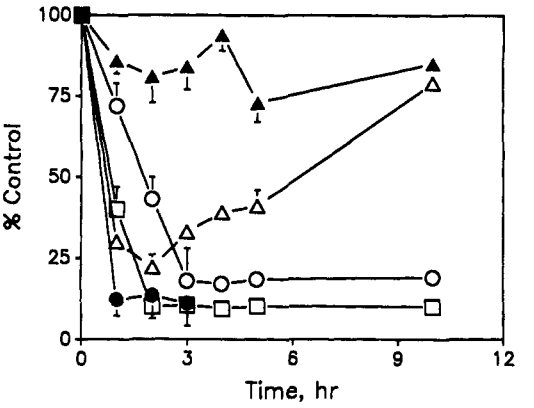
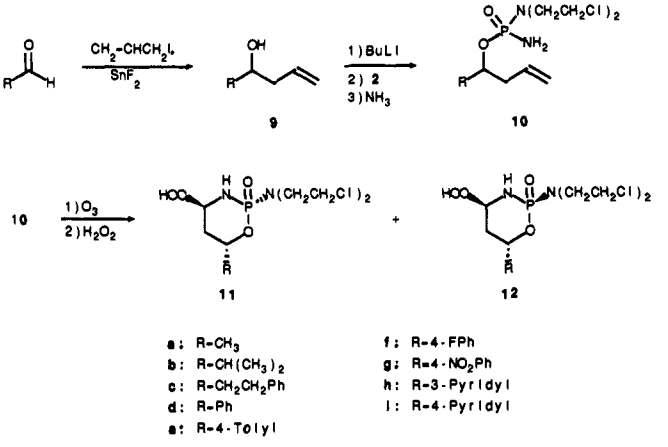


Figure 1. Inhibition of acetylcholinesterase in mouse blood following treatment with cyclophosphamide (CP) analogues: (●) *cis*-6-phenyl-CP **8a**, (○) *trans*-6-phenyl-CP **8b**, (□) 4-hydroperoxy-6-phenyl-CP **11d**, (▲) 4-hydroperoxy-6-(4-pyridyl)-CP **11i**, (△) cyclophosphamide **1a**.

Scheme III



The cis isomer **8a** was evaluated in vivo at an initial dose of 0.68 mmol/kg. All treated mice developed a syndrome characteristic of cholinesterase poisoning[15] that included lachrymation, muscular fasciculations, convulsions, and death within 5 h. In contrast, the trans isomer **8b** produced no signs of toxicity at doses up to 1.36 mmol/kg. Unfortunately, **8b** was also devoid of antitumor activity at this dose; the activation of this compound by hydroxylation at the 4-position has not been examined. Whole blood acetylcholinesterase activity was measured[16] in mice treated with **8a**, **8b**, or cyclophosphamide (a reversible inhibitor of acetylcholinesterase).[17] Cyclo-

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(17) Lalka, D.; Bardos, T. J. Cyclophosphamide, 2,2-dimethylaziridines and other alkylating agents as inhibitors of serum cholinesterase. *Biochem. Pharmacol.* **1975**, *24*, 455–462.

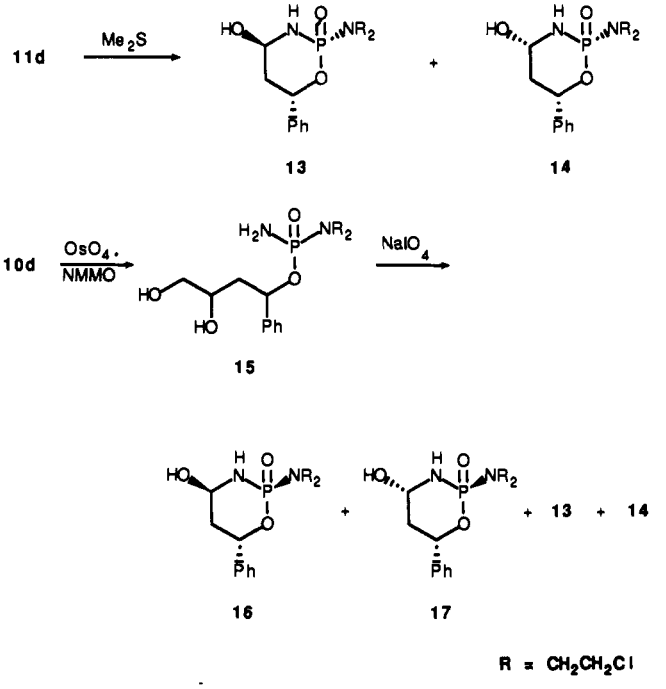
phosphamide exhibited transient inhibition of acetylcholinesterase activity as expected (Figure 1); although the toxicity of **8a** and **8b** in vivo were significantly different, the extent of whole blood enzyme inhibition was similar for the two isomers. Because death is associated with inhibition of brain cholinesterase,[18] the difference in lethality may be related to differential toxicity in the CNS. Given the toxicity of **8a** and the absence of antitumor activity of **8b**, these compounds were not investigated further. All subsequent synthetic studies were directed to the preactivated 4-hydroperoxy or 4-hydroxy analogues to circumvent the problem of initial hepatic activation and to allow evaluation of antitumor activity in vitro.

Activated 6-Substituted Cyclophosphamides: Chemistry. A series of 4-hydroperoxy-6-substituted cyclophosphamides was prepared as shown in Scheme III. The requisite aldehyde was reacted with allyl iodide/stannous fluoride to give butenyl alcohols **9a–g** in 50–95% yields. Poor yields of **9h** and **9i** were obtained in this reaction; hence these alcohols were prepared by addition of allyllithium to the corresponding pyridinecarboxaldehydes. Sequential reaction of alcohols **9** with butyllithium, phosphoramidic dichloride **2**, and ammonia afforded phosphorodiamidates **10a–i** as mixtures of isomers (50:50–65:35) in 50–90% yields. It is interesting to note that phosphorodiamidates **10** could not be isolated from alcohols **9** in which the substituent was *p*-methoxyphenyl or *p*-dimethylaminophenyl; elimination of the presumed phosphoramidate occurred to give the aryl butadiene as the only isolable product.

Phosphorodiamidates **10** were then ozonized and reacted with hydrogen peroxide to give 4-hydroperoxy-6-substituted analogues **11** in modest yields. Compounds **a–c** were each obtained as a 3:1 mixture of two inseparable diastereomers **11a–c** and **12a–c** following flash chromatography and crystallization; in the case of compounds **11d–i**, however, one of the four possible diastereomers was obtained by crystallization of the chromatographed product. Careful chromatography of **11d** afforded a pure sample of a second isomer **12d**, which had a ^{31}P chemical shift upfield compared to that of **11d**. The H_4–P coupling constants for all of the isolated analogues were >23 Hz, indicating that the hydroperoxy group was axial in all of these compounds. Similar analysis of the H_6–P coupling constants (all <2.5 Hz) confirmed that the substituent at the 6-position was equatorial for **11a–i**. The ^{31}P chemical shift for the major isomer of **11a–c** occurs downfield compared to the minor isomer, indicating that the major isomer has phosphoryl oxygen axial and cis to the hydroperoxy group. The ^{31}P chemical shifts for **11e–i** are also consistent with an axial disposition for the phosphoryl oxygen. On the basis of these spectroscopic data the major and minor isomers are assigned structures **11** and **12**, respectively. The selectivity presumably arises from more facile crystallization of **11** rather than from stereocontrol in the cyclization reaction, because all of the diastereomers are present in comparable amounts in the crude reaction products.

^{31}P NMR Experiments. 4-Hydroperoxy-6-phenylcyclophosphamide (**11d**) was reduced with dimethyl sulfide and the subsequent reactions (1:4 acetone/0.1 M HEPES, pH 7.4, 37 °C) monitored by ^{31}P NMR (Scheme IV). The initial reduction product **13** (–12.7 ppm) slowly equilibrated with its 4-hydroxy isomer **14** (–10.2 ppm) to give a final product ratio of 3:1 for **13:14**. Neither the aldehyde/hydrate nor the elimination product phosphoramide

Scheme IV



mustard (**4**) was detected by NMR over 3 h. In order to approach the equilibrium from the opposite direction, **10d** was converted to diol **15**; all four diastereomers were evident as separate resonances in the ^{31}P spectrum. Periodate cleavage of the diol and ^{31}P spectroscopy in the same buffer system initially showed the formation of the two isomeric aldehyde/hydrates (–5.9 and –6.3 ppm) which disappeared completely within 10 min. Four new resonances appeared, two of which corresponded to **13** and **14**, and two signals at –15.4 and –13.1 ppm (**16** and **17**, respectively). The latter resonances were also observed following reduction of the isomeric hydroperoxide **12d**. Equilibrium was reached within 1 h (**13:14** = 3:1, **16:17** = 2.3:1). All four resonances gradually diminished; although **4** was never detected, the degradation product of **4** appeared at –22.4 ppm. The combined half-life for the disappearance of all 4-hydroxy isomers was ca. 15 h; this slow rate is in striking contrast to the 45-minute half-life reported for the isomeric 4-hydroxycyclophosphamides.[4] In order to obtain faster rates for comparison purposes, hydroperoxides **11a–i** were reduced and the reactions carried out in 4:6 acetone/1.0 M HEPES, pH 7.4, 37 °C. In the presence of this higher buffer concentration, **11d** released phosphoramide mustard with a half-life of 92 min; the 3- and 4-pyridyl analogues **11h** and **11i** reacted at a comparable rate (Table I). The para-substituted phenyl analogues reacted approximately 2-fold more slowly, but analogues **11a–c**, having an alkyl moiety attached at the 6-position, reacted under these conditions with half-times in excess of 8 h. Given the relatively rapid equilibration of hydroxy isomers that occurs even at low buffer concentration, it may be concluded that ring opening is equally facile for 4-hydroxycyclophosphamide and its 6-substituted analogues, but the substituent significantly disfavors the aldehyde at equilibrium and retards the overall rate of elimination.

In Vitro Cytotoxicity. The 6-substituted 4-hydroperoxy analogues were evaluated for cytotoxicity against wild-type (/O) and cyclophosphamide-resistant (/CP) L1210 and P388 cell lines in a clonogenic assay, using a 1-h drug exposure. The drug concentration that produced a 2-log reduction in clonogenic survival (LC_{99}) was determined from the linear portion of the log survival vs con-

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Table I. Antitumor Activity of 4-Hydroperoxy-6-substituted-cyclophosphamides in Vitro

no.	substituent	$t_{1/2}$,[a] min	L1210/0,[b] μM	L1210/CP,[b] μM	RF[c]	P388/0,[b] μM	P388/CP,[b] μM	RF[c]
11	H	15	8	140	17	6	60	10
11a	CH_3	>500	39	180	4.6	26	69	2.7
11b	$CH(CH_3)_2$	>500	110	110	1.0	86	61	0.7
11c	$CH_2CH_2C_6H_4$	>500	13	9	0.7	10	5	0.5
11d	Ph	130	9	29	3.2	6	15	2.5
12d	Ph	92	20	26	1.3	16	21	1.3
11e	*p*-tolyl	240	5	6	1.2	3	3	1.0
11f	*p*-FC_6H_4	205	7	8	1.2	6	5	1.2
11g	*p*-$NO_2C_6H_4$	190	8	8	1.0	7	3	0.4
11h	3-pyridyl	105	36	56	1.6	33	34	1.0
11i	4-pyridyl	110	53	57	1.1	44	23	0.5

[a] Half-life for generation of 4 from the 4-hydroxy compounds in 4:6 acetone/1.0 M HEPES buffer, pH 7.4, 37 °C. [b] LC_{99} values determined against wild-type L1210 or P388 cells, or cyclophosphamide-resistant L1210 or P388 cells, as described in the text. [c] Resistance factor = (LC_{99} resistant)/(LC_{99} wild type).

Table II. Antitumor Activity of 4-Hydroperoxy-6-substituted-cyclophosphamides against L1210 Leukemia in Vivo

		% *T/C* (LTS)[a]			
no.	substituent	170 μmol/kg	340 μmol/kg	510 μmol/kg	680 μmol/kg
		Wild-Type L1210			
11	H	140	171	toxic	
11a	CH_3		138 (2/6)	toxic	
11c	$CH_2CH_2C_6H_4$		106	toxic	
11d	Ph	117	144	toxic	
11e	*p*-tolyl		150	175 (1/6)	175 (1/6)
11f	*p*-FC_6H_4		163	188 (1/6)	toxic
11g	*p*-$NO_2C_6H_4$		163 (1/6)	185 (2/6)	205 (4/6)
11h	3-pyridyl		131	toxic	
11i	4-pyridyl	130	150	160	190 (1/6)
cyclophosphamide		150	181	208 (2/6)	toxic
		Cyclophosphamide-Resistant L1210			
11	H	115	110	toxic	
11i	4-pyridyl	119	131	138	150
cyclophosphamide		105	95	110	toxic

[a] Increased survival determined as described in the text; LTS = 30-day survivors.

centration curves; the results are reported in Table I. For the wild-type cell lines, potency in the 6-alkyl substituent series decreases with increasing substituent size: H > CH_3 > $CH(CH_3)_2$. The substituted phenyl analogues **11d–f** are comparable in potency to 4-hydroperoxycyclophosphamide itself; no apparent effect of electronic contributions on cytotoxicity were noted in this series. The pyridyl derivatives were prepared in the hope that their increased polarity might circumvent the neurotoxicity observed with **11d**; although this objective was realized (vide infra), the potency of the pyridyl derivatives in vitro was diminished compared to that of the corresponding phenyl analogues. With the exception of **11a** and **11d**, all of these compounds were comparably cytotoxic to the wild-type and CP-resistant lines. Although both resistant cell lines have elevated levels of aldehyde dehydrogenase compared to the parent lines,[7] it is not known whether reduced susceptibility of these compounds to the enzyme accounts for the increased potency against CP-resistant cells.

In Vivo Activity. Antitumor activity was assessed with an intraperitoneal murine L1210 model and prolongation of survival as a measure of efficacy; the results are presented in Table II. The alkyl-substituted compounds **11a** and **11c** were not significantly active at lower dose and exhibited toxicity at higher doses. Substituted phenyl analogues **11d–g** showed good activity, although **11d** was lethal at doses >340 μmol/kg because of cholinesterase inhibition (vide infra). *p*-Nitrophenyl derivative was particularly effective, giving 4/6 long-term survivors at the highest dose. Although the 3-pyridyl analogue was toxic at doses >340 μmol/kg, the 4-pyridyl compound showed good antitumor activity with no lethal toxicity at 680 μmol/kg. The 4-pyridyl analogue was also evaluated against the cyclophosphamide-resistant cell line. 4-Hydroperoxycyclophosphamide was inactive against this line as expected; in contrast, **11i** showed moderate activity albeit less than in the sensitive cell line. The LD_{50} dose of **11i** was found to be ~1000 μmol/kg by using probit analysis and a 30-day survival time.

Inhibition of acetylcholinesterase activity was evaluated for the hydroperoxy analogues **11d–i** as described above. Compound **11d** was analogous to 6-phenylcyclophosphamide, inducing profound inhibition of blood enzyme activity and rapid death when given at a dose of 640 μmol/kg. In order to rule out a contribution of alkylating activity to this toxicity, the deschloro analogue of **11d** was administered. Enzyme inhibition and lethality of this analogue were identical with those of **11d**, confirming that the 6-phenyloxazaphosphorine moiety is the determinant of acetylcholinesterase inhibition. Surprisingly, analogues **11e–i** were devoid of enzyme inhibitory activity, and when these analogues were given at lethal doses the characteristic signs of cholinesterase toxicity were absent.

Conclusions

Cyclophosphamide analogues substituted with oxygen at the 5-position undergo ring opening and elimination at a negligible rate; the absence of significant antitumor activity was expected and is consistent with the kinetic data. However, the absence of a correlation between cytotoxicity in vitro and rate of phosphoramide mustard release in aqueous buffer for the 6-substituted analogues was unexpected. It is surprising that many of these analogues undergo activation with half-lives of several hours in aqueous buffer but are highly cytotoxic to tumor cells after a 1-h exposure time. The deschloro analogue of **11d** was pre-

pared and shown to be devoid of cytotoxicity in this assay, confirming that release of an alkylating phosphorodiamidate is essential for in vitro activity. These data argue in favor of an intracellular catalytic pathway, presumably enzymatic, that operates independent of the general base-catalyzed pathways documented previously. This catalysis may be similar to that observed for human serum albumin in the release of PDA from 4-hydroxycyclophosphamide.[13] It is apparent that the kinetics of phosphoramide mustard release in aqueous buffer are not predictive of cytotoxic potency for the 6-substituted cyclophosphamides.

Most of these compounds were more potent than 4-hydroperoxycyclophosphamide against the drug-resistant cell lines, and many analogues were essentially non-cross-resistant. Sladek and Landkamer have shown that inhibitors of aldehyde dehydrogenase restore the sensitivity of these resistant cell lines to oxazaphosphorines.[7] It is likely that substitution at the 6-position results in the formation of an aldophosphamide analogue that is a poor substrate for the enzyme. While most of these substituents enhance cytotoxic activity against resistant cells, the lethal neurotoxicity that presumably results from an increase in lipophilicity must be considered carefully in the selection of future analogues.

Experimental Section

Melting points were determined on a Kofler block and are uncorrected. Elemental analyses were carried out by Galbraith Laboratories, Inc., Knoxville, TN. Ultraviolet spectral kinetics were carried out on a Beckman DU-8 instrument fitted with an automatic sample changer and thermostated cell chamber using the DU-8 Kinetics Module. NMR spectra were recorded on a Bruker WP-270 SY instrument. ^1H chemical shifts are reported in ppm from Me_4Si (organic solvents) or $Me_3Si(CD_2)_2SO_3Na$ (D_2O); ^{31}P chemical shifts are reported in ppm from triphenylphosphine oxide in toluene-d_8 as coaxial reference. ^{31}P NMR kinetics were carried out as described previously.[4,13] Unless otherwise noted, all reactions were carried out under a nitrogen atmosphere, and organic extracts were dried over $MgSO_4$ and concentrated in vacuo.

***N,N*-Bis(2-chloroethyl)phosphoramidic Dichloride (2).** Bis(2-chloroethyl)amine hydrochloride (50 g, 0.28 mol) was suspended in phosphorus oxychloride (130 mL, 1.39 mol) and refluxed until complete dissolution occurred. The excess phosphorus oxychloride was removed by distillation at atmospheric pressure, and the residue was distilled to give 2 (67.5 g, 93%), bp 123–125 °C (0.6 mm). The distillate solidified to give a crystalline product, mp 57–59 °C (lit.[19] mp 54–56 °C).

2-[Bis(2-chloroethyl)amino]-5-(hydroxymethyl)-1,3,2-oxazaphospholidine 2-Oxide (3). A solution of 3-aminopropanediol (910 mg, 10 mmol) and triethylamine (4.8 mL, 30 mmol) in 50 mL of acetonitrile was added dropwise to a stirred solution of 2 (2.59 g, 10 mmol) in 50 mL of acetonitrile at 0 °C. Stirring was continued for 4 h at 0 °C, the solid was removed by filtration, and the filtrate was concentrated in vacuo. The residual oil (2.31 g, 88%) was applied to a RP-8 reverse-phase MPLC column and eluted with methanol/water (30:70). The fractions containing the separated isomers were combined, the methanol was removed in vacuo, and the resulting solution was lyophilized. The faster eluting isomer had mp 77–82 °C: ^1H NMR (DMSO-d_6) 4.65 (1 H, m), 4.1 (1 H, m), 3.9 (1 H, m), 3.65 (5 H, m), 3.25 (4 H, dt), 3.15 ppm (1 H, m); ^{31}P NMR ($CDCl_3$) +3.67 ppm. Anal. ($C_7H_{15}N_2O_3Cl_2P$) C, H, N. The slower eluting isomer had mp 105–107 °C: ^1H NMR (DMSO-d_6) 4.65 (1 H, m), 4.2 (1 H, m), 3.9 (1 H, m), 3.65 (5 H, m), 3.3 (5 H, m); ^{31}P NMR ($CDCl_3$) +5.21 ppm.

2-[Bis(2-chloroethyl)amino]-5-hydroxytetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (1b and 1c). A solution of 2 (7.21 g, 28 mmol) in dioxane (42 mL) was added dropwise to a stirred solution of 3-amino-2-(benzyloxy)-1-propanol (4.3 g, 23.7 mmol) and triethylamine (8.2 mL, 60 mmol) in 125 mL of dioxane at 0 °C. The suspension was stirred overnight at room temperature. Toluene (70 mL) was added and the resulting suspension filtered. The filtrate was concentrated, and the residue was dissolved in chloroform (200 mL) and washed with water (2 × 75 mL). The organic layer was dried and concentrated, and the resulting oil was purified by flash chromatography (acetone/CH_2Cl_2 3:1) to give the isomeric (benzyloxy)cyclophosphamides (3.37 g, 39% and 1.72 g, 20%).

To a solution of the faster eluting 5-(benzyloxy)cyclophosphamide (1.83 g, 5 mmol) in 15 mL of methanol and 5 mL of water was added 1.8 g of Pd/C. The reaction mixture was hydrogenated until the reaction was complete (TLC). The suspension was filtered through diatomaceous earth and concentrated. The residue was dissolved in acetone, filtered, and concentrated to an oil. Crystallization from acetone/hexanes afforded **1b** (871 mg, 63%): mp 99–100 °C; ^1H NMR (DMSO-d_6) 4.1 (1 H, m), 3.9 (1 H, m), 3.65 (5 H, m), 3.25 (4 H, m), 3.15 (1 H, m), 2.95 ppm (1 H, m); ^{31}P NMR (DMSO) −13.37 ppm. Anal. ($C_7H_{15}N_2O_3Cl_2P$) C, H, N.

Hydrogenolysis of the slower eluting isomer afforded **1c** (165 mg, 12%): mp 95–110 °C; ^1H NMR (DMSO-d_6) 4.2 (1 H, m), 3.9 (1 H, m), 3.65 (5 H, m), 3.3 (5 H, m), 2.95 ppm (1 H, m).

2-Methoxy-3-butenyl *N,N*-Bis(2-chloroethyl)phosphorodiamidate (6). A solution of 2-methoxy-3-buten-1-ol (10.2 g, 0.1 mol) in 90 mL of methylene chloride was added dropwise to a stirred solution of $POCl_3$ (9.3 mL, 0.1 mol) in 100 mL of methylene chloride at 5 °C. The solution was stirred for 1 h at 5 °C, warmed to room temperature, and stirred for 4 h. Bis(2-chloroethyl)amine hydrochloride (17.8 g, 0.1 mol) in 600 mL of methylene chloride was added, and the solution was cooled to 5 °C. Triethylamine (43 mL, 0.3 mol) was added dropwise over 15 min to the stirred solution, and the resulting suspension was stirred at room temperature overnight. The mixture was washed with KH_2PO_4 (10%, 3 × 300 mL), and the organic layer was dried and concentrated. The resulting oil was dissolved in methylene chloride (200 mL), saturated with ammonia at 5 °C, and placed in a freezer overnight. The solution was concentrated, and the residue was dissolved in ethyl acetate (100 mL) and filtered. The filtrate was concentrated and the residue purified by flash chromatography (acetone/CH_2Cl_2 3:1) to give 6 (12.5 g, 44%) as a clear viscous oil that solidified on standing at −20 °C: ^1H NMR ($CDCl_3$) 6.7 (1 H, m), 5.35 (2 H, m), 4.1 (1 H, m), 3.9 (2 H, m), 3.65 (4 H, t), 3.45 (4 H, dt), 3.35 ppm (3 H, s); ^{31}P NMR (DMSO) −7.17, −7.46 ppm (62:38). Anal. ($C_9H_{19}N_2O_3Cl_2P$) C, H, N.

2-[Bis(2-chloroethyl)amino]-4-hydroperoxy-5-methoxytetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (5a). Compound 6 (3.05 g, 10 mmol) was dissolved in acetone/water (30 mL, 3:1) and cooled to 0 °C. Ozone was passed through the solution for 30 min, and the solution was then flushed with oxygen to remove excess ozone. Acetone was added to restore the original volume, hydrogen peroxide (3 mL, 30%) was added, and the solution was stirred overnight at room temperature. Acetone was removed in vacuo and the mixture was extracted with chloroform (3 × 20 mL). The combined extracts were dried and concentrated. The residue was purified by flash chromatography (acetone/CH_2Cl_2, 3:7) and the product crystallized as a single isomer from ether/CH_2Cl_2 to give **5a** (572 mg, 18%): mp 107–108 °C; ^1H NMR ($CDCl_3$) 5.15 (1 H, ddd), 4.6 (1 H, ddd), 4.3 (1 H, ddd), 3.65 (4 H, t), 3.45 (3 H, s), 3.45 ppm (5 H, m); ^{31}P NMR ($CDCl_3$) −17.49 ppm. Anal. ($C_8H_{17}N_2O_5Cl_2P$) C, H, N.

2-[Bis(2-chloroethyl)amino]-4-hydroxy-5-methoxytetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (5b). Hydroperoxide **5a** (200 mg, 0.62 mmol) was dissolved in CH_2Cl_2 (0.5 mL), triethyl phosphite (160 μL, 0.9 mmol) was added, and stirring was continued for 10 min. Hexanes were added to the cloud point, and the solution was placed in the freezer. The crystalline product was collected and washed with ether/hexanes (1:1) to give **5b** (285 mg, 89%) as a single isomer: mp 96–98 °C; ^1H NMR (DMSO-d_6) 4.8 (1 H, ddd), 4.45 (1 H, ddd), 4.2 (1 H, ddd), 3.65 (4 H, t), 3.4 (3 H, s), 3.3 (4 H, m), 3.1 ppm (1 H, m); ^{31}P NMR (DMSO) −18.7 ppm. Anal. ($C_8H_{17}N_2O_4Cl_2P$) C, H, N.

2-Methoxy-3,4-dihydroxybutyl *N,N*-Bis(2-chloroethyl)phosphorodiamidate (7). Compound 6 (1.0 g, 3.2 mmol), *N*-

(19) Friedman, O. M.; Seligman, A. M. Preparation of N-phosphorylated derivatives of bis-β-chloroethylamine. *J. Am. Chem. Soc.* **1954**, *76*, 655–658.

methylmorpholine *N*-oxide (467 mg, 3.6 mmol), and 30 mg of osmium tetroxide were dissolved in acetone (5 mL) and water (1 mL) and stirred for 2 h. A mixture of sodium thiosulfate (0.4 g), 5 g of Florisil, and 5 mL of water was added, and stirring was continued for 10 min. The mixture was filtered through diatomaceous earth and the filter washed with a minimum volume of acetone. The filtrate was concentrated, adjusted to pH 2, saturated with NaCl, and extracted with ethyl acetate (3 × 20 mL). The combined extracts were dried, concentrated, and purified by flash chromatography (MeOH/EtOAc 1:9) to give **7** (700 mg, 63%) as a mixture of isomers: ^{31}P NMR (DMSO) −6.69, −7.01, −7.10 ppm (74:12:24).

***cis*- and *trans*-6-Phenylcyclophosphamide (8a and 8b).** Phosphoryl chloride **2** (5.31 g, 20.5 mmol) and 100 mg of 4-(dimethylamino)pyridine were dissolved in CH_2Cl_2 (100 mL) and cooled to 0 °C. A solution of 1-phenyl-3-amino-1-propanol (3.1 g, 20.5 mmol) and triethylamine (4.36 g, 43 mmol) in CH_2Cl_2 (30 mL) was added dropwise with stirring to the cooled solution over 30 min. The ice bath was removed and the solution stirred at room temperature overnight. The solution was filtered and evaporated at reduced pressure, and the residue purified by flash chromatography (EtOAc, R_f = 0.29 and 0.14). The faster eluting isomer was crystallized from CH_2Cl_2/Et_2O to give 1.14 g (16%) of **8a**: mp 124–127 °C; ^{1}H NMR (DMSO) 7.4 (5 H, m, phenyl), 5.3 (1 H, ddd), 5.15 (1 H, ddd), 3.7 (4 H, t), 3.3 (4 H, m), 3.2 (2 H, m), 1.85 ppm (2 H, m); ^{31}P NMR (DMSO) −14.57 ppm. Anal. ($C_{13}H_{19}N_2O_2Cl_2P$) C, H, N.

The slower eluting isomer was crystallized from CH_2Cl_2/hexanes to give 1.66 g (24%) of **8b**: mp 113–116 °C; ^{1}H NMR (DMSO) 7.35 (5 H, m), 5.9 (1 H, br d), 4.65 (1 H, br s), 3.7 (4 H, t), 3.4 (4 H, m), 3.25 (1 H, m), 3.15 (1 H, m), 1.85 ppm (2 H, m); ^{31}P NMR (DMSO) −11.30 ppm. Anal. ($C_{13}H_{19}N_2O_2Cl_2P$) C, H, N.

Butenyl Alcohols 9b–g. The synthesis of 1-*p*-tolyl-3-buten-1-ol (**9e**) is representative. *p*-Tolualdehyde (5.90 mL, 50 mmol), allyl iodide (5.5 mL, 60 mmol), and stannous fluoride (10.34 g, 66 mmol) were dissolved in 170 mL of DMI and stirred overnight at room temperature. Water (200 mL) was added, and the mixture was extracted with Et_2O (3 × 150 mL). The combined extracts were washed with saturated ammonium chloride (3 × 50 mL), dried, and evaporated. The residue was purified by flash chromatography (1:4 EtOAc/hexanes, R_f = 0.56) to give 7.5 g (93%) of **9e** as an oil: ^{1}H NMR ($CDCl_3$) 7.2 (4 H, dd), 5.8 (1 H, m), 5.15 (2 H, m), 4.65 (1 H, t), 2.5 (2 H, dd), 2.35 (3 H, s), 2.15 ppm (1 H, s).

1-(**2-Propyl**)-3-buten-1-ol (**9b**) was prepared as described to give 49% of **9b** as an oil after flash chromatography (1:4 EtOAc/hexanes, R_f = 0.63): ^{1}H NMR ($CDCl_3$) 5.85 (1 H, m), 5.15 (2 H, m), 3.4 (1 H, ddd), 2.3 (1 H, m), 2.1 (1 H, m), 1.7 (1 H, m), 1.6 (1 H, s), 0.9 ppm (6 H, d).

1-(**2-Phenethyl**)-3-buten-1-ol (**9c**) was prepared as described to give 86% of **9c** as an oil: ^{1}H NMR ($CDCl_3$) 7.2 (5 H, m), 5.8 (1 H, m), 5.15 (2 H, m), 3.65 (1 H, m), 2.8 (1 H, m), 2.7 (1 H, m), 2.3 (1 H, m), 2.2 (1 H, m), 1.75 (2 H, m), 1.7 ppm (1 H, s).

1-**Phenyl**-3-buten-1-ol (**9d**) was prepared as described to give 89% of **9d** as an oil after flash chromatography (2:3 EtOAc/hexanes, R_f = 0.6): ^{1}H NMR ($CDCl_3$) 7.3 (5 H, m), 5.8 (1 H, m), 5.15 (2 H, m), 4.75 (1 H, dd), 2.4 (1 H, m), 2.1 ppm (1 H, s).

1-(**4-Phenyl**)-3-buten-1-ol (**9f**) was prepared as described to give 94% of **9f** as an oil after flash chromatography (1:4 EtOAc/hexanes, R_f = 0.49): ^{1}H NMR ($CDCl_3$) 7.3 (2 H, m), 7.15 (2 H, m), 5.8 (1 H, m), 5.15 (2 H, m), 4.7 (1 H, t), 2.5 (2 H, m), 2.2 ppm (1 H, s).

1-(**4-Nitrophenyl**)-3-buten-1-ol (**9g**) was prepared as described to give 65% of **9g** as an oil after flash chromatography (1:4 EtOAc/hexanes, R_f = 0.36): ^{1}H NMR ($CDCl_3$) 8.2 (2 H, d), 7.55 (2 H, d), 5.8 (1 H, m), 5.15 (2 H, m), 4.85 (1 H, dd), 2.5 ppm (3 H, m).

1-(**3-Pyridyl**)-3-buten-1-ol (**9h**). 3-Pyridinecarboxaldehyde (5.36 g, 50 mmol) was dissolved in Et_2O (200 mL) and cooled to 0 °C. A solution of allyl lithium (50 mL, 1 M in THF, 50 mmol) was added dropwise to the stirred solution. Water (50 mL) was then added slowly, the layers were separated, and the aqueous layer was extracted with Et_2O (2 × 100 mL). The combined extracts was dried and evaporated to give a red soil. Purification by flash chromatography (EtOAc, R_f = 0.54) afforded 2.65 g (36%) of **9h** as a mixture of isomers: ^{1}H NMR ($CDCl_3$) 7.3–8.6 (4 H, pyridyl), 5.8 (1 H, m), 5.15 (2 H, m), 4.8 (1 H, t), 3.4 (1 H, s), 2.55 ppm (2 H, m).

1-(**4-Pyridyl**)-3-buten-1-ol (**9i**) was prepared exactly as described for **9h**. Flash chromatography (2:3 acetone/CH_2Cl_2, R_f = 0.48) afforded 4.69 g (63%) of **9i** as an oil: ^{1}H NMR ($CDCl_3$) 7.3–8.5 (4 H, pyridyl), 5.8 (1 H, m), 5.15 (2 H, m), 4.75 (1 H, m), 3.6 (1 H, s), 2.5 ppm (2 H, m).

Butenyl Phosphorodiamidates 10a–i. The synthesis of **10e** is representative. Alcohol **9e** (6.1 g, 37.6 mmol) and a trace of (phenylazo)diphenylamine were dissolved in 100 mL of THF and cooled to 0 °C. *n*-Butyllithium (24 mL, 1.6 M in hexanes) was added dropwise with stirring until the indicator turned purple. This alkoxide solution was then added dropwise to a solution of phosphoryl chloride **2** (9.74 g, 37.6 mmol) in 100 mL of THF at 0 °C. The solution was stirred for an additional 20 min at 0 °C. Ammonia gas was then bubbled through this solution at 0 °C for 15 min. The resulting suspension was filtered through diatomaceous earth, and the filtrate was evaporated to give a yellow oil. Flash chromatography (1:4 acetone/CH_2Cl_2, R_f = 0.59) afforded 12.34 g (90%) of **10e** as an oil: ^{1}H NMR ($CDCl_3$) 7.2 (4 H, dd), 5.7 (1 H, m), 5.3 (1 H, m), 5.1 (2 H, m), 3.65–3.1 (8 H, m), 2.7 (4 H, m), 2.35 ppm (3 H, s); ^{31}P NMR (DMSO) −7.84 and −7.95 ppm (60:40). Anal. ($C_{15}H_{23}N_2O_2Cl_2P$) C, H.

1-**Methyl**-3-butenyl ***N*,*N*-bis(2-chloroethyl)phosphorodiamidate (10a)** was prepared as described to give an 80% yield of product after flash chromatography (1:4 acetone/CH_2Cl_2, R_f = 0.59). An analytical sample was obtained by crystallization from CH_2Cl_2/Et_2O: mp 53–55 °C; ^{1}H NMR ($CDCl_3$) 5.8 (1 H, m), 5.1 (2 H, m), 4.55 (1 H, m), 3.65–3.4 (8 H, m), 2.8 (2 H, m), 2.35 (2 H, m), 1.3 (3 H, m); ^{31}P NMR (DMSO) −8.27 and −8.53 ppm (50:50). Anal. ($C_9H_{19}N_2O_2Cl_2P$) C, H, N.

1-(**2-Propyl**-3-butenyl) ***N*,*N*-bis(2-chloroethyl)phosphorodiamidate (10b)** was prepared as described to give an 86% yield of product as a white solid after flash chromatography (1:4 acetone/CH_2Cl_2, R_f = 0.55): mp 54–57 °C; ^{1}H NMR ($CDCl_3$) 5.85 (1 H, m), 5.1 (2 H, m), 4.35 (1 H, m), 3.65–3.4 (8 H, m), 3.3 (2 H, m), 2.4 (2 H, m), 1.95 (1 H, m), 0.9 ppm (6 H, m); ^{31}P NMR (DMSO) −8.24 and −8.33 ppm (55:45). Anal. ($C_{11}H_{23}N_2O_2Cl_2P$) C, H.

1-(**2-Phenethyl**-3-butenyl) ***N*,*N*-bis(2-chloroethyl)phosphorodiamidate (10c)** was prepared as described to give a 62% yield of product as an oil after flash chromatography (EtOAc, R_f = 0.5). An analytical sample was prepared by crystallization from Et_2O/hexanes: mp 64–66 °C; ^{1}H NMR ($CDCl_3$) 7.25 (5 H, m), 5.85 (1 H, m), 5.15 (2 H, m), 4.5 (1 H, m), 3.65–3.4 (8 H, m), 2.7 (4 H, m), 2.45 (2 H, m), 1.95 ppm (2 H, m); ^{31}P NMR (DMSO) −8.14 and −8.28 ppm (55:45). Anal. ($C_{15}H_{25}N_2O_2Cl_2P$) C, H.

1-(**1-Phenyl**-3-butenyl) ***N*,*N*-bis(2-chloroethyl)phosphorodiamidate (10d)** was prepared as described to give a 92% yield of product as an oil after flash chromatography (EtOAc, R_f = 0.4). An analytical sample was obtained by crystallization from Et_2O/hexanes: mp 64–66 °C; ^{1}H NMR ($CDCl_3$) 7.35 (5 H, m), 5.75 (1 H, m), 5.35 (1 H, m), 5.1 (2 H, m), 3.65–3.1 (8 H, m), 2.55–2.85 ppm (4 H, m); ^{31}P NMR (DMSO) −8.07 (acetone), −7.88 and −7.97 ppm (55:45). Anal. ($C_{14}H_{21}N_2O_2Cl_2P$) C, H.

1-(**4-Fluorophenyl**-3-butenyl) ***N*,*N*-bis(2-chloroethyl)phosphorodiamidate (10f)** was prepared as described to give a 68% yield of product as an oil after flash chromatography (1:4 acetone/CH_2Cl_2, R_f = 0.56). An analytical sample was prepared by crystallization from Et_2O/hexanes: mp 69–77 °C; ^{1}H NMR (DMSO) 7.35 (2 H, m), 7.05 (2 H, m), 5.7 (1 H, m), 5.35 (1 H, m), 5.1 (2 H, m), 3.65–3.15 (8 H, m), 2.8 (2 H, m), 2.67 ppm (2 H, m); ^{31}P NMR (DMSO) −7.75 and −7.80 ppm (60:40). Anal. ($C_{14}H_{20}N_2FO_2Cl_2P$) C, H.

1-(**4-Nitrophenyl**-3-butenyl) ***N*,*N*-bis(2-chloroethyl)phosphorodiamidate (10g)** was prepared as described to give a 69% yield of product as an oil after flash chromatography (2:3 acetone/CH_2Cl_2, R_f = 0.6). An analytical sample was prepared by crystallization from CH_2Cl_2/hexanes: mp 120–124 °C; ^{1}H NMR ($CDCl_3$) 8.25 (2 H, m), 7.55 (2 H, m), 5.7 (1 H, m), 5.5 (1 H, m), 5.1 (2 H, m), 3.7–3.25 (8 H, m), 3.0 (2 H, m), 2.65 ppm (2 H, m); ^{31}P NMR (DMSO) −7.42 ppm. Anal. ($C_{14}H_{20}N_3O_4Cl_2P$) C, H.

1-(**3-Pyridyl**-3-butenyl) ***N*,*N*-bis(2-chloroethyl)phosphorodiamidate (10h)** was prepared as described to give

a 57% yield of product as a separable mixture of isomers after flash chromatography (1:9 MeOH/EtOAc, R_f = 0.32 and 0.22): ^{1}H NMR (faster eluting isomer, $CDCl_3$) 8.6 (2 H, m), 7.5 (2 H, m), 5.75 (1 H, m), 5.40 (1 H, m), 5.10 (2 H, m), 3.45–3.20 (10 H, m), 2.65 ppm (2 H, m); ^{1}H NMR (slower eluting isomer, DMSO) 8.5 (2 H, m), 7.4 (2 H, m), 5.70 (1 H, m), 5.40 (1 H, m), 5.10 (2 H, m), 3.65–3.45 (10 H, m), 2.65 ppm (2 H, m); ^{31}P NMR (DMSO) −7.77 and −7.86 ppm (63:37). Anal. ($C_{13}H_{20}N_3O_2Cl_2P$) C, H.

1-(4-Pyridyl-3-butenyl) *N*,*N*-bis(2-chloroethyl)phosphorodiamidate (10i) was prepared as described to give an 83% yield of product as a separable mixture of isomers after flash chromatography (1:9 MeOH/EtOAc, R_f = 0.35 and 0.22): ^{1}H NMR (faster eluting isomer, DMSO-d_6) 8.60 (2 H, d), 7.25 (2 H, d), 5.70 (1 H, m), 5.40 (1 H, m), 5.10 (2 H, m), 3.45–3.25 (8 H, m), 2.95 (2 H, s), 2.60 ppm (2 H, m); ^{1}H NMR (slower eluting isomer, DMSO) 8.55 (2 H, d), 7.25 (2 H, d), 5.70 (1 H, m), 5.40 (1 H, m), 5.10 (2 H, m), 3.65–3.45 (8 H, m), 2.80 (2 H, s), 2.60 ppm (2 H, m); ^{31}P NMR (DMSO) −7.37 and −7.41 ppm (50:50).

4-Hydroperoxy-6-substituted-cyclophosphamides 11 and 12. The synthesis of **11d** and **12d** is representative. A solution of **10d** (3.0 g, 8.56 mmol) was dissolved in acetone/water (30 mL, 3:1), cooled to 0 °C, and ozonized for 30 min. Excess ozone was flushed from the solution, H_2O_2 (3 mL, 30%) was added, and the mixture was stirred overnight at room temperature. Most of the acetone was removed at reduced pressure, and the resulting mixture was extracted with $CHCl_3$ (3 × 20 mL). The combined extracts were dried and evaporated to give an oil. Crystallization from CH_2Cl_2/Et_2O/hexanes afforded diastereomerically pure **11d** (950 mg, 30%): mp 109–112 °C; ^{1}H NMR (DMSO-d_6) 11.7 (1 H, s), 7.4 (5 H, m), 6.15 (1 H, dd), 5.5 (1 H, ddd), 5.0 (1 H, dddd), 3.7 (4 H, t), 3.35 (4 H, m), 2.25 (1 H, m), 1.95 ppm (1 H, m); ^{31}P NMR (DMSO) −16.59 ppm. Anal. ($C_{13}H_{19}N_2O_4Cl_2P$) C, H, N.

The filtrate from the crystallization was evaporated and the residue crystallized from CH_2Cl_2/Et_2O to afford an additional 880 mg (58% overall) of a mixture of isomers. Flash chromatography (3:7 acetone/CH_2Cl_2, R_f = 0.40) afforded 180 mg of diastereomerically pure **12d**: mp 95–97 °C; ^{1}H NMR (DMSO-d_6) 11.8 (1 H, s), 7.4 (5 H, m), 6.5 (1 H, dd), 5.45 (1 H, ddd), 5.0 (1 H, dddd), 3.65 (4 H, t), 3.35 (1 H, m), 2.3 (1 H, m), 2.05 ppm (1 H, m); ^{31}P NMR (DMSO) −20.05 ppm. Anal. ($C_{13}H_{18}N_2O_4Cl_2P$) C, H, N.

2-[Bis(2-chloroethyl)amino]-4-hydroperoxy-6-methyltetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (11a and 12a) was prepared as described to give the product as an inseparable mixture of diastereomers after flash chromatography (2:3 acetone/CH_2Cl_2, R_f = 0.7). Crystallization from CH_2Cl_2/Et_2O/hexanes afforded a 78:22 mixture of **11a** and **12a** in 18% yield: mp 103–105 °C; ^{1}H NMR (major isomer, DMSO) 11.55 (1 H, s), 5.9 (1 H, m), 4.9 (1 H, dddd), 4.6 (1 H, ddd), 3.65 (4 H, t), 3.3 (4 H, m), 2.05 (1 H, m), 1.6 ppm (1 H, m); ^{1}H NMR (minor isomer, DMSO) 11.7 (1 H, s), 5.9 (1 H, m), 5.1 (1 H, dddd), 4.25 (1 H, m), 3.65 (4 H, t), 3.3 (4 H, m), 2.15 (1 H, m), 1.5 ppm (1 H, m); ^{31}P NMR (DMSO) −17.33 and −18.24 ppm (78:22). Anal. ($C_8H_{17}N_2O_4Cl_2P$) C, H, N.

2-[Bis(2-chloroethyl)amino]-4-hydroperoxy-6-(2-propyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (11b and 12b) was prepared as described to give the product as an inseparable mixture of diastereomers after flash chromatography (1:4 acetone/CH_2Cl_2, R_f = 0.4). Crystallization from CH_2Cl_2/Et_2O/hexanes afforded an 83:17 mixture of **11b** and **12b** in 14% yield: mp 83–85 °C; ^{1}H NMR (major isomer, $CDCl_3$) 5.15 (1 H, m), 4.7 (1 H, dd), 4.5 (1 H, m), 3.65 (4 H, t), 3.45 (4 H, m), 1.7–2.0 (3 H, m), 0.95 ppm (6 H, dd); ^{1}H NMR (minor isomer, $CDCl_3$) 5.25 (1 H, m), 4.85 (1 H, dd), 3.95 (1 H, m), 3.65 (4 H, t), 3.45 (4 H, m), 1.7–2.0 (3 H, m), 0.95 ppm (6 H, dd); ^{31}P NMR (DMSO) −16.58 and −17.31 ppm (83:17). Anal. ($C_{10}H_{21}N_2O_4Cl_2P$) C, H, N.

2-[Bis(2-chloroethyl)amino]-4-hydroperoxy-6-phenethyltetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (11c and 12c) was prepared as described to give the product as an inseparable mixture of diastereomers after flash chromatography (EtOAc, R_f = 0.5). Crystallization from Et_2O afforded a 74:26 mixture of **11c** and **12c** in 26% yield: mp 92–94 °C; ^{1}H NMR (major isomer, DMSO) 11.5 (1 H, s), 7.5 (5 H, m), 5.55 (1 H, dd), 4.9 (1 H, m), 4.45 (1 H, ddd), 3.65 (4 H, t), 3.3 (4 H, m), 2.65 (2 H, m), 1.5–2.2 (4 H, m); ^{1}H NMR (minor isomer, DMSO) 11.7 (1 H, s), 7.5 (5 H, m), 5.55 (1 H, dd), 5.05 (1 H, m), 4.1 (1 H, ddd), 3.65 (4 H, t), 3.3 (4 H, m), 2.65 (2 H, m), 1.5–2.2 ppm (4 H, m); ^{31}P NMR (DMSO) −16.59 ppm. Anal. ($C_{15}H_{23}N_2O_4Cl_2P$) C, H, N.

***cis*-2-[Bis(2-chloroethyl)amino]-4-hydroperoxy-6-(4-tolyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (11e)** was prepared as described in 6% yield after crystallization from CH_2Cl_2/Et_2O/hexanes: mp 100–102 °C; ^{1}H NMR (DMSO-d_6) 11.7 (1 H, s), 7.25 (4 H, m), 6.1 (1 H, dd), 5.5 (1 H, m), 4.95 (1 H, m), 3.7 (4 H, t), 3.35 (4 H, m), 2.3 (3 H, s), 2.25 (1 H, m), 1.95 ppm (1 H, m); ^{31}P NMR (DMSO) −16.27 ppm. Anal. ($C_{14}H_{21}N_2O_4Cl_2P\cdot0.5H_2O$) C, H, N.

***cis*-2-[Bis(2-chloroethyl)amino]-4-hydroperoxy-6-(4-fluorophenyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (11f)** was prepared as described in 7% yield after crystallization from CH_2Cl_2/Et_2O/hexanes: mp 102–105 °C; ^{1}H NMR (DMSO-d_6) 11.7 (1 H, s), 7.45 (2 H, m), 7.25 (2 H, m), 6.15 (1 H, dd), 5.55 (1 H, m), 5.0 (1 H, m), 3.7 (4 H, t), 3.35 (4 H, m), 2.25 (1 H, m), 1.95 ppm (1 H, m); ^{31}P NMR (DMSO) −16.24 ppm. Anal. ($C_{13}H_{18}N_2FO_4Cl_2P$) C, H, N.

***cis*-2-[Bis(2-chloroethyl)amino]-4-hydroperoxy-6-(4-nitrophenyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (11g)** was prepared as described in 12% yield after crystallization from CH_2Cl_2/Et_2O: mp 102–104 °C; ^{1}H NMR (DMSO-d_6) 11.8 (1 H, s), 8.3 (2 H, d), 7.7 (2 H, d), 6.25 (1 H, dd), 5.65 (1 H, m), 5.0 (1 H, m), 3.7 (4 H, t), 3.3 (4 H, m), 2.35 (1 H, m), 1.95 ppm (1 H, m); ^{31}P NMR (DMSO) −16.25 ppm. Anal. ($C_{13}H_{18}N_3O_6Cl_2P$) C, H, N.

***cis*-2-[Bis(2-chloroethyl)amino]-4-hydroperoxy-6-(3-pyridyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (11h)** was prepared as described and the crude product purified by flash chromatography (1:9 MeOH/EtOAc, R_f = 0.65). Crystallization from CH_2Cl_2/Et_2O afforded **11h** in 11% yield: mp 98–100 °C; ^{1}H NMR (DMSO-d_6) 11.7 (1 H, br s), 8.65 (1 H, s), 8.55 (1 H, d), 7.85 (1 H, d), 7.45 (1 H, dd), 6.2 (1 H, dd), 5.6 (1 H, m), 5.0 (1 H, m), 3.7 (4 H, t), 3.35 (4 H, m), 2.3 (1 H, m), 2.0 ppm (1 H, m); ^{31}P NMR (DMSO) −16.14 ppm. Anal. ($C_{12}H_{18}N_3O_4Cl_2P\cdot0.5H_2O$) C, H, N.

***cis*-2-[Bis(2-chloroethyl)amino]-4-hydroperoxy-6-(4-pyridyl)tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (11i)** was prepared as described in 9% yield after crystallization from acetone/CH_2Cl_2: mp 98–100 °C; ^{1}H NMR (DMSO-d_6) 11.8 (1 H, br s), 8.65 (2 H, d), 7.45 (2 H, d), 6.25 (1 H, dd), 5.55 (1 H, m), 5.0 (1 H, m), 3.7 (4 H, t), 3.4 (4 H, m), 2.35 (1 H, m), 1.85 ppm (1 H, m); ^{31}P NMR (DMSO) −16.07 ppm. Anal. ($C_{12}H_{18}N_3O_4Cl_2P\cdot0.5H_2O$) C, H, N.

Diol 15. Butenyl ester **10d** (1.09 g, 3 mmol), *N*-methylmorpholine *N*-oxide (467 mg, 3.6 mmol), and osmium tetroxide (1.22 mL, 2.5% in *t*-BuOH, 0.12 mmol) were dissolved in 5 mL of acetone and 1 mL of water and stirred at room temperature for 2 h. Sodium thiosulfate (0.38 g), Florisil (5 g), and 5 mL of H_2O were added, and the mixture was stirred for 10 min. The mixture was filtered through diatomaceous earth, the acetone was evaporated, and the resulting solution was adjusted to pH 2 with 1 N HCl. The resulting mixture was extracted with EtOAc (3 × 20 mL), the combined extracts were evaporated, and the residue was purified by flash chromatography (1:9 MeOH/EtOAc) to give diol **15** (828 mg, 67%) as a mixture of diastereomers; ^{31}P NMR (DMSO) −6.60, −7.11, −8.07, and −8.41 ppm (30:21:28:21 ratio).

Cytotoxicity Assays. Cytotoxic activity was evaluated in vitro by using 1-h drug exposure in a clonogenic assay as previously described.[13] LC_{99} values were obtained by linear regression of log survival vs drug concentration from at least three determinations and are calculated as the drug concentration required to reduce colony formation to 1% of control values. Standard errors are within ±10%. Resistance factors were calculated from the ratio of LC_{99} values for wild-type and cyclophosphamide-resistant cell lines.

Antitumor activity in vivo was determined by using standard NCI protocols for the L1210 line. Tumors were propagated in DBA/2 mice, and drug evaluations were carried out in $B_6D_2F_1$ mice. Mice were randomly assigned to groups of six and were injected ip with 10^5 L1210 cells; a suspension of drug in 2% (carboxymethyl)cellulose (0.2 mL) or vehicle alone was injected ip 24 h later. Survivors were counted daily for 30 days. Response is reported as % *T*/*C*, calculated as 100 × (treatment survival time)/(control survival time). Survival times of control mice were

in the range of 9-11 days for all experiments.

Cholinesterase Assay. Acetylcholinesterase activity in whole blood was determined as previously described.[16] Whole blood (20 μL) was collected by retroorbital puncture from $B_6D_2F_1$ mice and diluted into 10 mL of phosphate buffer (2 mM, pH 7.4, 37 °C) containing bromthymol blue (100 mg/L). The solution was divided into two portions; acetylcholine chloride (0.1 mL, 0.83 M) was added to one portion, and water (0.1 mL) was added to the other portion. An aliquot of each solution was transferred to a cuvette; the sample cuvette contained acetylcholine and the other was used as the reference cuvette in a thermostated spectrophotometer cell (37 °C). Net absorbance (620 nm) was measured at 1-min intervals for 20 min; absorbance change was linear over this interval, and enzymatic activity was determined from the rate of change in absorbance obtained from linear regression. Baseline activity was measured in each of three mice; drugs were suspended in 2% (carboxymethyl)cellulose and injected ip 1 h later. Inhibition of enzyme activity was determined from the ratio of enzyme activity following drug administration and the baseline level, with each animal serving as its own control.

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Synthesis, Activation, and Cytotoxicity of Aldophosphamide Analogues

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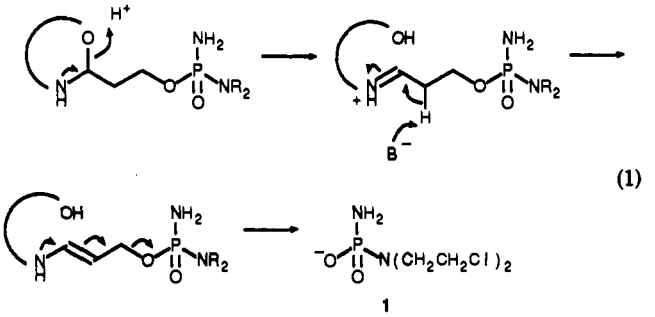
A series of perhydrooxazine analogues of aldophosphamide has been prepared, and their ^{31}P NMR kinetics and in vitro cytotoxicity have been evaluated. These compounds were developed on the basis of the idea that ring opening and tautomerization to an enamine intermediate might provide a mechanistic alternative to the β-elimination reaction for release of phosphoramide mustard. The 4,4,6-trimethyltetrahydro-1,3-oxazine moiety was selected on the basis of its rapid rate of iminium ion generation and relatively slow rate of hydrolysis. These analogues underwent phosphorodiamidate release by three distinct mechanisms: hydrolysis to aldophosphamide and subsequent β-elimination; cyclization to produce the 4-hydroxycyclophosphamides, which release phosphorodiamidate by ring opening and elimination; and tautomerization to the enamine with rapid expulsion of phosphorodiamidate. Kinetic studies demonstrated that hydrolysis to the aldehyde contributed minimally to the overall activation process and that the enamine pathway represented the major route of activation. For those analogues that could undergo cyclization, this pathway competed effectively with enamine release, and these analogues were essentially equivalent to their 4-hydroxycyclophosphamide counterparts in cytotoxicity. A series of tetra-N-substituted phosphorodiamidates that cannot undergo cyclization was prepared to explore the effects of cyclization on the cytotoxicity of these analogues. The tetrakis(chloroethyl)phosphorodiamidates were highly potent in vitro against both cyclophosphamide-sensitive and -resistant L1210 and P388 cell lines, and one of these analogues had significant antitumor activity against L1210 leukemia in vivo. These results demonstrate that the enamine mechanism provides a viable pathway for delivery of phosphorodiamidates and that this approach can be used to deliver phosphorodiamidates that are non-cross-resistant in cyclophosphamide-resistant cell lines.

Introduction

Cyclophosphamide and its analogues are important clinical agents in the treatment of cancer.[1] The development of drug resistance and the incidence of host toxicities prompted the development of a synthetic program directed at analogues designed to minimize these effects. We have reported the synthesis and evaluation of activated substituted cyclophosphamides that release phosphoramide mustard (PDA, 1) via β-elimination from structurally modified aldehydes.[2] Another approach, however, involves the synthesis of aldehyde analogues that may undergo activation by a different mechanism. In particular, we were attracted by the possibility that elimination of PDA could be driven by an enamine intermediate, generated from an appropriately substituted nitrogen derivative of the aldehyde (eq 1). Mechanistic precedent for this strategy exists in the well-established specific amine catalysis of carbonyl β-elimination reactions.[3] We



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report here the synthesis, activation kinetics, and antitumor activity of several aldophosphamide analogues and confirm that the enamine pathway provides a viable alternative for PDA release.

Results and Discussion

Aldehyde Derivatives. Efforts were directed initially to the identification of aldehyde analogues that would have

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