3-carboxylic acid, 13368-86-0; 1,2,4-triazole-3-carboxaldehyde, 31708-25-5; 4-pyridazinecarboxylic acid, 50681-25-9; 5-pyrimidinecarboxylic acid, 4595-61-3; 4-pyrazinecarbonyl chloride, 19847-10-0; 6-hydroxy-3-pyrimidazinecarboxylic acid, 37972-69-3; 3,3-dimethyl-5-[(indol-2-ylcarbonyl)amino]-6-nitroindol-2-one, 120791-41-5; 3-quinolinecarboxylic acid, 6480-68-8; 4-quinolinecarboxylic acid, 486-74-8; 4-imidazol-1-ylbenzaldehyde, 10040-98-9; 4-acetylpyridazine, 50901-46-7.

Effects of N-Substitution on the Activation Mechanisms of 4-Hydroxycyclophosphamide Analogues

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The activation mechanisms of the N-substituted 4-hydroxycyclophosphamide analogues 4-hydroxyifosfamide (**2b**), 4-hydroxytrofosfamide (**2c**), and 3-methyl-4-hydroxycyclophosphamide (**2d**) were compared with that of the unsubstituted parent compound **2a**. The reaction kinetics of *cis*-**2b**, -**2c**, and -**2d** are qualitatively similar to those of **2a** in that they undergo ring opening to the respective aldophosphamide intermediates **3**, which can reclose to the *cis*- or *trans*-4-hydroxy isomers or undergo base-catalyzed β-elimination to generate the corresponding phosphoramide mustard products **4**. In contrast to the general acid catalysis observed for ring opening of **2a** and **2d**, the *N*-(chloroethyl)-substituted analogues **2b** and **2c** undergo specific base-catalyzed ring opening. This mechanistic difference was also illustrated by the rapid reaction of **2a** and **2d** with sodium 2-mercaptoethanesulfonate (Mesna) under acidic conditions to give the 4-(alkylthio)-substituted cyclophosphamide derivatives **5a** and **5d**. Compounds **2b** and **2c** did not react with Mesna to generate **5b** and **5c** under these conditions. Both the fraction of aldehyde/hydrate present at equilibrium and the cytotoxicity against L1210 cells in vitro decreased in the order **2c** > **2b** > **2a** > **2d**. The plasma-catalyzed acceleration of phosphoramide mustard generation previously reported for **2a** was also observed for these analogues.

Cyclophosphamide (**1a**; see Chart I), a widely used antitumor and immunosuppressive agent, is a prodrug that requires initial activation to 4-hydroxycyclophosphamide (**2a**) by the mixed-function oxidase system. The overall activation process and mechanistic details have been extensively studied and reviewed.[1-4] In aqueous solution, *cis*-**2a** undergoes acid-catalyzed ring opening to aldophosphamide (**3a**); this intermediate can recyclize to *cis*- or *trans*-**2a** or undergo base-catalyzed elimination to generate acrolein and the cytotoxic metabolite phosphoramide mustard (**4a**).[5-8] Generation of **4a** from the pseudoequilibrium mixture of **2a** and **3a** is also catalyzed by serum albumin[9,10] and possibly by 3′–5′ exonucleases.[4,11-13]

Numerous structural modifications have been carried out in an attempt to improve the drug's therapeutic index.[2-3,14] Cyclophosphamide analogues of particular clinical interest include mafosfamide (**5a**), ifosfamide (**1b**), and trofosfamide (**1c**). The mechanisms involved in the activation of **5a** were recently reported.[15] Compounds **1b** and **1c** are substituted with a 2-chloroethyl group at the 3-position, and the metabolic activation of these analogues is qualitatively similar to that of cyclophosphamide.[8,16-22]

Table I. Rate Constants for the Ring Opening of 4-Hydroxycyclophosphamide Analogues

compd	[phosphate], mM	pH	k_1, min^{-1}
cis-**2b**	100[a]	6.5	0.083
	100[a]	7.0	0.11
	100[a]	7.4	0.21
	100[b]	7.4	0.23
	150[b]	7.4	0.20
	200[b]	7.4	0.24
cis-**2c**	100[a]	6.5	0.023
	100[a]	7.0	0.049
	100[a]	7.4	0.10
	100[b]	7.4	0.11
	150[b]	7.4	0.11
	200[b]	7.4	0.12
cis-**2d**	100[a]	6.5	0.08[c]
	100[a]	7.0	0.05[c]
	100[a]	7.4	0.03[c]

[a] Ionic strength = 0.28. [b] Ionic strength = 0.6. [c] Upper limit estimated from plasma data.

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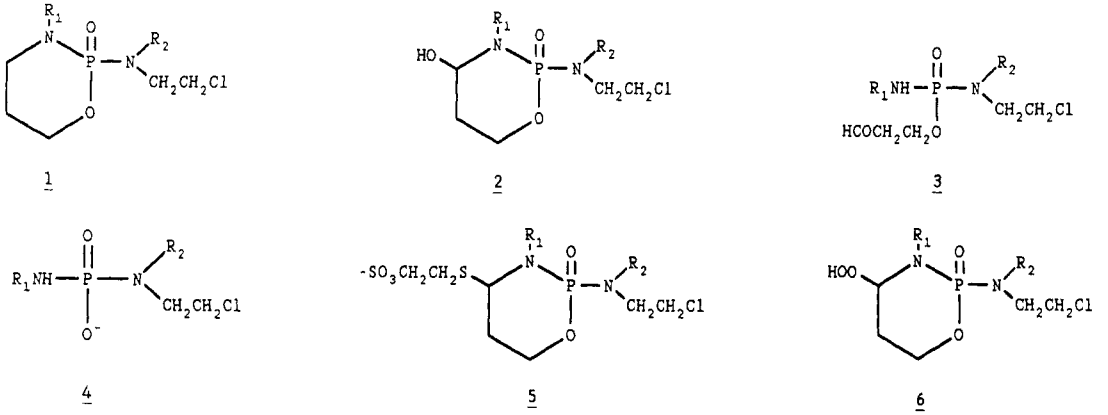
Chart I



Cpd	R_1	R_2
a	H	CH_2CH_2Cl
b	CH_2CH_2Cl	H
c	CH_2CH_2Cl	CH_2CH_2Cl
d	CH_3	CH_2CH_2Cl

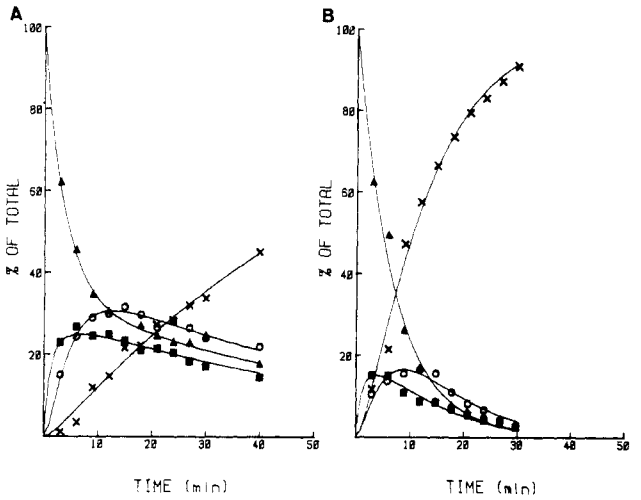


Figure 1. Activation of *cis*-4-hydroxyifosfamide (*cis*-**2b**) in (A) phosphate buffer (100 mM, pH 7.4, 37 °C) and (B) phosphate-buffered human plasma. Data points were measured from ^{31}P NMR line intensities; the solid lines represent the best-fit values calculated by the Simplex algorithm. (▲) *cis*-**2b**; (○) *trans*-**2b**; (■) **3b** + hydrate; (×) **4b** and its hydrolysis products.

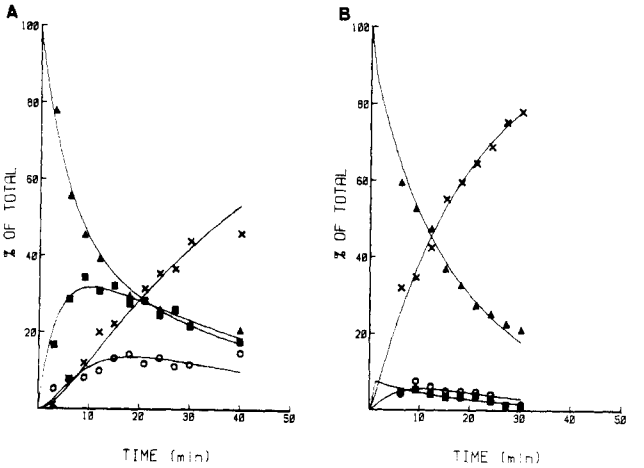


Figure 2. Activation of *cis*-4-hydroxytrofosfamide (*cis*-**2c**) in (A) phosphate buffer (100 mM, pH 7.4, 37 °C) and (B) phosphate-buffered human plasma. Data points were measured from ^{31}P NMR line intensities; the solid lines represent the best-fit values calculated by the Simplex algorithm. (▲) *cis*-**2c**; (○) *trans*-**2c**; (■) **3c** + hydrate; (×) **4c** and its hydrolysis products.

Cytotoxicity against L1210 leukemia in vivo appears to decrease in the order **1b** > **1c** > **1a**.[18-19] Ifosfamide has undergone Phase II trials and has demonstrated activity against a number of solid tumors, including some that are resistant to cyclophosphamide.[23-25] Hemorrhagic cystitis is dose-limiting for **1b**, but this toxicity has been modulated by coadministration of sodium mercaptoethanesulfonate (Mesna).[22,26-28]

As part of our mechanistic studies on the activation of cyclophosphamide and its analogues, we sought to determine the relationship between the N-3 substituent and the rates of drug activation and to correlate these rates with in vitro cytotoxic activity. We report herein the results of these studies.

Results and Discussion

Effect of Substitution on the Ring-Opening Reaction. The *cis*-4-hydroxy isomers of **2a–d** were prepared by in situ reduction of the respective 4-hydroperoxy derivatives **6a–d** with dimethyl sulfide. Kinetics were determined by Simplex analysis of ^{31}P NMR spectral data as described previously.[7,15] The kinetics of metabolite generation from **2b–d** (pH 7.4, 37 °C) are shown in Figures

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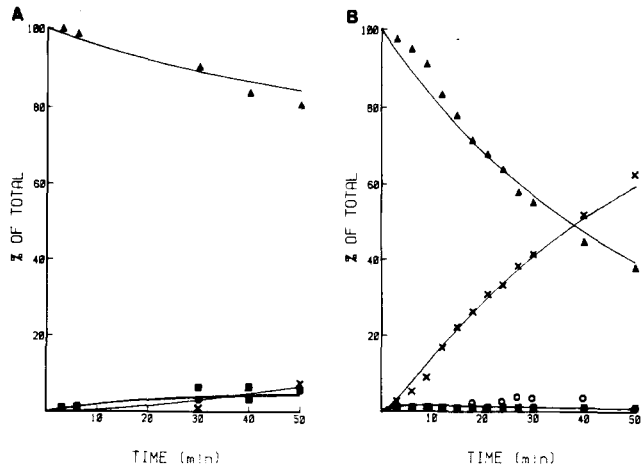
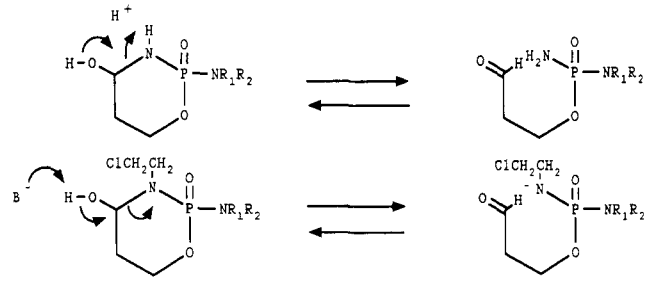


Figure 3. Activation of *N*-methyl-*cis*-4-hydroxycyclophosphamide (*cis*-**2d**) in (A) phosphate buffer (100 mM, pH 7.4, 37 °C) and (B) phosphate-buffered human plasma. Data points were measured from ^{31}P NMR line intensities; the solid lines represent the best-fit values calculated by the Simplex algorithm. (▲) *cis*-**2d**; (○) *trans*-**2d**; (■) **3d** + hydrate; (×) **4d** and its hydrolysis products.

1–3, respectively; data shown in the A panels were obtained in 100 mM phosphate buffer and those in the B panels in phosphate-buffer human plasma.[10] Disappearance of the *cis*-4-hydroxy derivatives and the production of phosphoramidates are qualitatively similar for **2a–c**. In contrast, the *N*-methyl derivative **2d** reacts very slowly, and the rate of phosphoramidate generation from **2d** is markedly retarded compared to those the other analogues. The catalysis of phosphoramidate generation by human plasma reported for **2a**[10] is also observed for the other analogues; the accelerated release of **4** results from albumin-catalyzed elimination from the aldophosphamides **3** rather than from an increase in the rate of ring opening (vide infra).

Rate constants for the ring-opening reaction are summarized in Table I. At pH 7.4 (100 mM phosphate, ionic strength 0.28 μ), the order of ring opening for the cis isomers is **2b** > **2c** > **2a** > **2d** (rate constants 0.21, 0.10, 0.07,[7] and 0.03 min^{-1}, respectively), indicating that electron-withdrawing groups accelerate and electron-donating groups retard the rate of ring opening. Identical ring-opening rates were observed in both buffer and plasma for **2b** and **2c**, confirming that plasma was not catalyzing this step. It should be noted that, because of the exceedingly slow disappearance of **2d** in buffer, an accurate rate constant for ring opening of this compound could not be obtained. Thus it was necessary to estimate the rate constants for ring opening from the corresponding plasma reactions. The observed pH dependence of the rate constants was unexpected, however, and this suggests that a change in substituent on N-3 can change the catalysis of the ring-opening reaction. We have reported that *cis*-**2a** undergoes ring opening via general-acid (or the kinetically indistinguishable specific-acid–general-base) catalysis;[7] similarly, ring opening of *cis*-**2d** appears to be acid-catalyzed. In contrast, the reactions of **2b** and **2c** are independent of buffer concentration but proceed faster at *higher* pH, suggesting that ring opening may be specific base catalyzed for these compounds. The rationale for this change in mechanism is shown in Scheme I. In the absence of an electron-withdrawing group on N-3, protonation is required to facilitate bond breaking in the rate-limiting step. The presence of an electron-withdrawing group, however, may stabilize the phosphoramide anion enough to promote bond breaking without protonation. Further evidence for this change in catalysis comes from the reaction of the **2b–3b** equilibrium mixture with 5 equiv

Scheme I



of Mesna (100 mM acetate buffer, pH 5.2). Reaction of **2a** under these conditions generates mafosfamide **5a** via acid-catalyzed cyclization of the aldophosphamide hemithioacetal.[15] In contrast, the equilibrium mixture of **2b–3b** generates predominantly the hemithioacetal of the aldophosphamide analogue (cis:trans:aldehyde/hydrate/hemithioacetal ratio 14:17:69 after 50 min), but this intermediate does not undergo detectable acid-catalyzed cyclization to the 4-(alkylthio) derivative **5b**. Essentially identical results were obtained for **2c**, except that the new equilibrium in which the hemithioacetal predominates was reached more slowly than for **2b**. This failure to undergo cyclization presumably results from the decreased nucleophilicity of the phosphoramide nitrogen when substituted with the electron-withdrawing chloroethyl group. However, the *N*-methyl derivative **2d** under the same conditions gave an 85% conversion to a 3:1 mixture of *cis*- and *trans*-*N*-methylmafosfamide (**5d**), confirming that the failure of the chloroethyl compounds to cyclize is not the result of further substitution on the ring nitrogen. It is interesting to note that, in contrast to mafosfamide **5a**, *N*-methylmafosfamide (**5d**) is stable indefinitely in aqueous buffer (pH 4.9–9.5, 37 °C). Addition of the thiol-trapping agent *N*-ethylmaleimide (5 equiv) has no effect on this stability, confirming that elimination of Mesna from **5d** does not occur. This is consistent with the mechanism previously proposed for activation of mafosfamide involving base-catalyzed elimination via the imminium species.[15,28]

Effect of Substitution on the Equilibrium Composition of Metabolites. Reaction of the *cis*-4-hydroxy derivatives **2a–d** in acetate or phosphate buffer (pH 4.9–7.4) generally established a pseudoequilbrium of *cis*-**2**, *trans*-**2**, and aldehyde **3** + hydrate within approximately 20 min; the ratios of these metabolites then remained constant for the duration of the experiment. It should be noted that the ^{31}P resonances for *cis*-**2b** and its corresponding phosphoramidate **4b** overlap, so the equilibrium composition at pH >6.5 was estimated by correction for the quantity of **4b** produced; accurate values were obtained at lower pH, conditions where generation of **4b** was not observed. The pseudoequilibrium composition for each drug was essentially independent of pH and buffer as noted previously for **2a**.[7] However, significant differences in metabolite composition were noted for the different drugs (Table II). 4-Hydroxyifosfamide (**2b**) underwent facile reversible ring opening with rapid ring closure to *trans*-**2b**, giving a pseudoequilibrium composition in which *trans*-**2b** predominated over the cis isomer, in contrast to the 1.5:1 cis:trans ratio reported for **2a**. The fraction of aldehyde **3b** + hydrate was approximately 1.5-fold higher than that observed for aldophosphamide **4a** and represented 29% of the equilibrium mixture. 4-Hydroxytrofosfamide (**2c**) rapidly formed the aldehyde **3c**, but cyclization to *trans*-**2c** was relativly slow. The same 1.5:1 equilibrium ratio of cis:trans metabolites was seen for **2a** and **2c**, but the quantity of aldehyde **3c** + hydrate was

Table II. Equilibrium Metabolite Composition of Cyclophosphamide Analogues

drug (buffer, pH)[a]	*cis*-2, %	*trans*-2, %	3, %
a[b] (acetate, pH 4.3–5.3)	46 ± 3	34 ± 2	20 ± 2
a[b] (phosphate, pH 5.3–7.0)	49 ± 4	33 ± 2	18 ± 2
b (acetate, 4.9–5.5)	32 ± 2	38 ± 3	31 ± 2
b (phosphate, 6.5–7.4)	35 ± 3	38 ± 3	27 ± 2
c (acetate, 4.9–5.5)	35 ± 2	27 ± 2	38 ± 4
c (phosphate, 7.4)	40 ± 4	22 ± 5	38 ± 4
d (acetate, 4.9–5.5)	86 ± 4	7 ± 3	7 ± 3

[a] Data are the average of at least four separate experiments. [b] Taken from ref 7.

2-fold higher than that observed for the cyclophosphamide metabolite **3a** (38% vs 19% of the pseudoequilibrium mixture, respectively). The most dramatic effect of N-substitution was found with *cis*-4-hydroxy-*N*-methylcyclophosphamide (**2d**). Ring opening was slow for this compound, and only 5–10% each of *trans*-**2d** and -**3d** were formed at equilibrium. Although approach to equilibrium was slow at pH 7.4, and generation of the phosphoramidate **4d** competed significantly at this pH, equilibrium values were finally obtained at pH below 5.5 where based-catalyzed elimination of **3d** did not occur.

The most important effect of phosphoramide substitution on metabolite composition is related to the increasing fraction of aldophosphamides **3a–d** present with greater substitution of electron-withdrawing groups. This presumably results from the inductive effects of the chloroethyl groups decreasing the nucleophilicity of the phosphoramide nitrogen, thereby retarding attack on the aldehyde and slowing the rate of cyclization. This inductive effect should also favor the expulsion of phosphoramidate from the carbinolamides **2a–d**, thus accelerating the ring-opening reaction. The aldophosphoamide metabolites are clearly the pivotal intermediates in oxazaphosphorine drug activation,[7,10] and the rates of intracellular drug activation as well as enzyme-catalyzed detoxication may depend upon aldehyde concentration. The human plasma inactivation of the 4-hydroxy derivatives **2a–d** is a case in point; a 4-fold variation in plasma half-life is found in this series (Table III), and an excellent correlation ($r^2 = 0.99$) exists between the fraction of aldehyde **3a–d** present at equilibrium and the rate of plasma inactivation. This correlation further supports our previous suggestion that human serum albumin catalyzes the decomposition of **2a** by accelerating the expulsion of phosphoramide mustard (**4a**) from aldophosphamide.[10]

Effect of Substitution on the Rate of Elimination. The release of phosphoramidates **4b** and **4c** from the corresponding aldophosphamides was negligible at pH <5.5; the rate of phosphoramidate release from **3b** and **3c** increased with increasing pH and buffer concentration as noted previously for aldophosphamide.[7] Rate constants for the elimination reaction in phosphate buffer were obtained as described previously; these constants were plotted against the fraction of HPO_4^{2-} present at each pH to obtain the catalytic constant k_{cat} for this species.[7,10] The values obtained were 0.81 and 0.93 M^{-1} min^{-1}, respectively, for elimination from **3b** and **3c**, compared to the value of 1.13 M^{-1} min^{-1} reported for aldophosphamide (**3a**).[10] These results suggest that modification of the phosphoramidate moiety has little effect on the elimination reaction from the aldehyde metabolite. Similarly, the catalytic effects of albumin on the elimination rates from **3a–c** were essentially equivalent.

Aqueous Stability of Phosphoramidates. The phosphoramidates **4a**, **4b**, and **4d** were prepared, and their half-lives were determined in aqueous buffer (pH 7.4, 37 °C; 100 mM HEPES for **4a** and **4d**, 200 mM phosphate for **4b**) by measuring the rates of disappearance with ^{31}P NMR. The electron-donating methyl group increases the rate of solvolysis of **4d** compared to **4a** (Table III), presumably by increasing the rate of aziridinium ion formation. The presence of the methyl group also increased the nucleophilicity of the phosphoryl oxygen as shown by the fact that the intramolecular O-alkylation product accounted for >30% of the final mixture from **4d**.[29] In contrast, ifosphoramide mustard **4b** was approximately 4-fold more stable than **4a** in aqueous buffer, reflecting a slower rate of cyclization for this compound. The solvolysis of **4b** was markedly reduced at elevated pH; the phosphoraziridine nitrogen of the cyclized intermediate must be protonated for nucleophilic attack on the ring to occur, and **4b** is deprotonated at pH >10. In fact, reaction of **4b** with aqueous base at pH 12 afforded the bis(aziridinyl) phosphoramidate as the exclusive product; when the pH was reduced below 10, rapid solvolysis occurred. Although the role of extracellular phosphoramidate in the cytotoxicity of the oxazaphosphorines is controversial,[3,21] it is interesting to note that the toxicity of these compounds to L1210 cells in vitro is inversely related to half-life in aqueous buffer (Table III).

Table III. Metabolite Half-Life and Cytotoxicity for Cyclophosphamide Analogues

compd	half-life,[a] min: buffer	half-life,[a] min: plasma[b]	% aldehyde	LC_{99}, μM[c]
4a	18[d]			169 ± 6
4b	61[e]			70 ± 4
4d	12[d]			620 ± 120
2a + 3a	44[f]	14	19	12.1 ± 2.2
2b + 3b	50[f]	10	29	12.6 ± 2.0
2c + 3c	40[f]	9	38	2.4 ± 0.6
2d + 3d	>200[f]	37	7	252 ± 17

[a] Obtained from linear regression of metabolite concentrations. [b] Human plasma containing 100 mM phosphate, pH 7.4. [c] Mean ± SD of at least three experiments carried out at five drug concentrations. [d] 100 mM Hepes, pH 7.4, 37 °C. [e] 200 mM phosphate, pH 7.4, 37 °C. [f] 100 mM phosphate, pH 7.4, 37 °C.

Effect of Substituent on Overall Stability and in Vitro Cytotoxicity. Although the rates of ring opening and the equilibrium compositions for the cyclophosphamide, ifosfamide, and trofosfamide metabolites are markedly different, the overall half-lives (and hence the rates of phosphoramidate generation) are essentially identical, both in aqueous buffer and in plasma (Table III). All three activated metabolites are also highly active against L1210 cells in vitro, although 4-hydroxytrofosfamide was significantly more active than the other metabolites in this system. Given that **2a** and **2b** release phosphoramidate at equivalent rates in aqueous buffer and that the phosphoramidate **4b** is over twice as potent as **4a** in vitro, it may be considered surprising that **2a** and **2b** demonstrate equivalent cytotoxicity in this system. Although there are a number of factors that may account for this apparent discrepancy, **2a** and **2b** release cytotoxic phosphoramidate intracellularly, and the discrepancy observed likely results from the difference in cytotoxicity between intracellular and extracellular delivery of the anionic phosphoramidate.[3,21] The presence of a methyl group on the ring nitrogen essentially abolished antitumor activity in this series, presumably because of the retarding effect on the rate of ring opening of **2d** and the acclerating effect on the inactivation of phosphoramidate **4d**. It should

(29) Engle, T. W.; Zon, G.; Egan, W. *J. Med. Chem.* **1982**, *25*, 1347.

be noted that modest antitumor activity has been reported for the parent compound *N*-methylcyclophosphamide (**1d**).[19] However, the first step in the activation of these compounds involves hydroxylation at a carbon atom adjacent to the oxazaphosphorine nitrogen. Although the C-4 position is the preferred site of attack in **1a–c**, attack at the methyl group of **1d** would result in demethylation to give **1a**. Thus the activity observed in vivo for **1d** may result from its conversion to cyclophosphamide rather than from generation and subsequent activation of the *N*-methyl-4-hydroxycyclophosphamide metabolite.

Summary

Several important conclusions may be drawn from this work. First, changes in oxazaphosphorine substitution alter significantly the rates of ring opening of the intermediates and the stabilities of the phosphoramidates, and these effects result from the electronic effects of the substituents. These substituent effects also change the mechanism and catalysis of the ring-opening reaction. Surprisingly, however, the separate effects for cyclophosphamide, ifosfamide, and trofosfame are compensatory, and the overall activation rates and cytotoxicities are very similar. Second, the ability of thiols to form 4-(alkylthio)-substituted derivatives **5** is highly dependent upon the N-3 substituent. For compounds containing electron-donating groups (e.g., hydrogen or methyl), the reaction proceeds smoothly under mildly acidic aqueous conditions. This reaction does not occur, however, when a chloroethyl group is appended to N-3. Thiols such as Mesna are known to provide protection to the bladder during ifosfamide administration. This implies that thiol interception of the equilibrium metabolites to generate **5** contributes little to the protective effects and that the major contribution arises via Michael addition to the bladder toxin acrolein. Acidification of the urine should also be important for reducing the bladder toxicity of ifosfamide and trofosamide, because for these compounds acidification reduces the rate of both the ring-opening and elimination steps. Finally, the results presented here imply that, in considering possible analogues for future sythesis, the choice of substituent at the N-3 position must be considered carefully if antitumor activity is to be retained.

Experimental Section

cis-4-Hydroxyperoxyifosfamide (*cis*-**6b**), *cis*-4-hydroperoxytrofosfamide (*cis*-**6c**), ifosphoramide mustard (**4b**), and 4-(2-sulfamoylethyl)thioifosfamide (ASTA 7623) (**5b**) were kindly provided by Dr. U. Niemeyer, Asta-Werke, Degussa Pharma Gruppe, Bielfeld, West Germany. Mesna and sodium phosphate were purchased from Sigma Chemical Co.; sodium acetate was purchased from Mallinckrodt. Other organic reagents and solvents were purchased from Aldrich Chemical Co. Human plasma was obtained from the Blood Bank, Strong Memorial Hospital, University of Rochester, Rochester, NY, and was frozen at −20 °C until use. Ozonolysis was performed with a Griffin-Technics Corp. ozone generator at an oxygen pressure of 1.5 psi, 130 V, and a 6.5 SCFH air flow. ^{1}H NMR spectra were recorded on an IBM WP-270 SY instrument using 5-mm sample tubes, a 4000-Hz spectral width, a 3-μs pulse width, and a 2-s repetition time. Chemical shifts are reported in parts per million from tetramethylsilane. ^{31}P NMR spectra were recorded on the same instrument equipped with an IBM-VSP multinuclear probe tuned for 109.368 MHz using 10-mm sample tubes, a 5000-Hz spectral width, a 10-μs pulse width, a 0.8-s repetition time, and 64 scans. Broad-band gated proton decoupling was used. Temperature was maintained at 37 ± 2 °C with an IBM VT1000 variable-temperature unit. Chemical shifts are reported in parts per million from 5% triphenylphosphine oxide in toluene-d_8 used as a coaxial reference. Assignments for the 4-hydroperoxy- and *cis*-4-hydroxycyclophosphamide derivatives **6a–d** and **2a–d** and the phosphoramidates **4a–d** are based on the chemical shifts of authentic samples and previously published data.[5,7] Assignments of ^{31}P chemical shifts for the other intermediates are based on the relative chemical shifts from the authentic cis isomers. The values obtained in aqueous buffer (pH 7.4) for the hydroperoxy, *cis*/*trans*-hydroxy, aldo, and phosphoramidate intermediates in each series are as follows: cyclophosphamide (**6a**, **2a–4a**), −13.5, −12.8/−12.5, −4.6, and −11.2 ppm, respectively; ifosfamide (**6b**, **2b–4b**), −12.7, −11.6/−12.0, −6.6, and −11.6 ppm, respectively; trofosfamide (**6c**, **2c–4c**), −12.7, −11.3/−12.5, −6.2, and −11.8 ppm, respectively; *N*-methylcyclophosphoramide (**6d**, 2–4d), −11.8, −10.6/−10.3, −4.2, and −9.6 ppm, respectively. Acidity measurements were made on a Radiometer pH meter using a glass calomel combination electrode. Buffer solutions were maintained at 37 ± 1 °C. Water was purified with a Barnstead Nanopure II system.

***cis*-4-Hydroperoxycyclophosphamide (6a)** was prepared in 33% yield as described elsewhere.[7,15]

***N*,*N*-Bis(2-chloroethyl)phosphoramidate (4a)** was prepared as described[30] and isolated as its cyclohexylamine salt in 61% yield: ^{31}P NMR ($CDCl_3$) −13.95 ppm.

***N*-Methyl-*cis*-4-hydroperoxycyclophosphamide (6d).** *n*-Butyllithium (7.33 mL, 11 mmol) was added dropwise to a solution of 3-buten-1-ol (0.72 g, 0.86 mL, 10 mmol) in dry THF (50 mL) at room temperature. Bis(2-chloroethyl)phosphoramide dichloride (2.59 g, 10 mmol) was added at 0 °C, and the mixture was stirred for 1 h at this temperature. Methylamine hydrochloride (0.68 g, 10 mmol) and triethylamine (2.02 g, 2.8 mL, 20 mmol) were added, and the mixture was stirred for 5 h at room temperature. The mixture was filtered and the filtrate evaporated in vacuo, resuspended in anhydrous diethyl ether, and filtered again. The filtrate was evaporated in vacuo to give *O*-(3-butenyl) *N*,*N*-bis(2-chloroethyl)-*N'*-methylphosphorodiamidate as an oil: ^{1}H NMR (DMSO-d_6) 5.7–5.9 (1 H, m, vinyl H), 5.1–5.2 (2 H, m, vinyl H), 3.7–3.8 (2 H, m, CH_2O), 3.5–3.7 (4 H, m, CH_2Cl), 3.3–3.5 (4 H, m, NCH_2), 2.5–2.7 (3 H, dd, NCH_3), 2.3–2.5 (2 H, m, CH_2CH_2O) ppm.

This crude product was dissolved in 50 mL of 2:1 acetone/water and ozonized for 15 min at 0 °C. Excess ozone was removed by passing nitrogen through the solution for 3 min. Hydrogen peroxide (30%, 3 mL) was added, and the reaction mixture was brought to room temperature and stirred overnight. After evaporation of the acetone in vacuo, the turbid aqueous mixture was extracted with methylene chloride (3 × 20 mL), the organic layer was dried over anhydrous sodium sulfate and filtered, and the filtrate was evaporated in vacuo to give crude *cis*-**6d** as a clear liquid. The liquid was diluted in 5 mL of methylene chloride, placed on a silica gel column (3 × 13 cm, 230–400 mesh) which was packed dry, and wetted with methylene chloride. The column was eluted with 1:3 acetone/methylene chloride under positive pressure. The fractions containing the desired compound were pooled and evaporated in vacuo to give a clear liquid that crystallized from acetone/anhydrous diethyl ether to give 373 mg (12.1% overall) of **6d**: mp 115–116 °C (lit.[31] mp 99–100 °C); ^{1}H NMR ($CDCl_3$) −11.77 (1 H, s, OOH), 4.85 (1 H, ddd, J_{HP} = 21 Hz, H_4), 4.28–4.48 (1 H, m, H_{6a}), 4.00–4.20 (1 H, m, H_{6e}), 3.66 (4 H, t, CH_2Cl), 3.20–3.38 (4 H, m, NCH_2), 2.64 (3 H, d, NCH_3), 2.05–2.2 (1 H, m, H_{5a}), 1.85–2.05 (1 H, m, H_{5e}); ^{31}P NMR (H_2O) −11.76 ppm.

***N*,*N*-Bis(2-chloroethyl)-*N'*-methylphosphorodiamidate (4d).** Triethylamine (3.04 g, 4.18 mL, 30 mmol) in 50 mL of toluene was added dropwise to a suspension of phenyl dichlorophosphate (3.17 g, 2.24 mL, 15 mmol) and bis(2-chloroethyl)amine hydrochloride (2.68 g, 15 mmol) in 150 mL of toluene at ice bath temperature. Then the mixture was refluxed for 2 h. After cooling to room temperature, the mixture was filtered, the filtrate evaporated in vacuo, and the resulting thick brown liquid was redissolved in 150 mL of THF. Methylamine hydrochloride (2.03 g, 30 mmol) was added to the solution, followed by dropwise addition of triethylamine (3.04 g, 4.18 mL, 30 mmol) in 50 mL of THF at 0 °C. The reaction was allowed to stir overnight. The mixture was filtered and the filtrate evaporated in vacuo to give

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a viscous liquid. The crude product was diluted in 10 mL of EtOAc/hexane (1:1) and applied to a silica gel column (230–400 mesh, 2.5 × 17 cm) packed with hexane and eluted with 1:2 EtOAc/hexanes. The fractions containing the desired compound were pooled and evaporated to give 2.32 g (50% yield) of *O*-phenyl *N*-methylphosphoramide mustard as a clear liquid: ^{31}P NMR ($CDCl_3$) −11.4 ppm.

A portion (1.24 g, 4 mmol) of this product was dissolved in 50 mL of absolute ethanol and hydrogenated over PtO_2 at 1 atm. After the reduction was complete, cyclohexylamine (1 equiv) was immediately added, and the mixture was stirred for 5 min. The reaction mixture was then filtered over Celite, and the filtrate was evaporated to dryness in vacuo. The product was precipitated by trituration with anhydrous diethyl ether to give 184 mg (14%) of **4d** as its cyclohexyamine salt: ^{31}P NMR ($CDCl_3$) −9.6 ppm.

^{31}P NMR Studies. Details of the NMR methods have been described previously.[5,15] All kinetics were carried out at 37 ± 2 °C. Solutions of *cis*-**2a**, **-2b**, and **-2d** were prepared immediately prior to use by dissolving the appropriate quantity of the corresponding hydroperoxide **6** in approximately 100 μL of methanol and treating this solution with 4 equiv of dimethyl sulfide at room temperature for 2–5 min. Solutions of *cis*-**2c** were also prepared the same way from **6c** except that **6c** was dissolved in 200 μL of 1:1 methanol/dimethyl sulfoxide to ensure proper dissolution in aqueous buffer. The appropriate buffer was then added to give a final concentration of 25–50 mM. The sample was then introduced into the preequilibrated spectrometer probe, and the spectra were acquired at varying intervals. Time points for each spectrum were taken at the midpoint of data acquisition. The FID spectra were stored on disk and subsequently processed by exponential multiplication with 2 Hz of line broadening, and relative concentrations of intermediates were determined from the peak areas of their respective phosphorus resonances.

In Vitro Cytotoxic Activity. A modification of the method of Chu and Fischer[32] was used; the details have been described previously.[10] Cultured mouse L1210 cells were obtained from EG&G Mason Research Institute, Tumor Bank, Worchester, MA. The 4-hydroperoxy drugs **6a–d** were treated with sodium thiosulfate (2.2 equiv) in 0.2 mL of prewarmed acetate buffer (100 mM, pH 5.3, 37 °C). A Vortex mixer was used to facilitate dissolution. The solution was allowed to stand at 37 °C for 5 min to allow complete reduction to the respective 4-hydroxy analogues, the Fischer's medium (0.84 mL) (Gibco Laboratories, Grand Island, NY) was added. Compounds **4a**, **4b**, and **4d** were dissolved directly in 1.04 mL of Fischer's medium. The solution was immediately sterilized by passage through a 0.22-μm Millipore filter, and 0.96 mL of Fischer's medium was added. This gave a 2-mL final volume of stock solution, which was maintained at 37 °C. L1210 cells in exponential growth were suspended in Fischer's medium to give 10-mL volumes at a final density of 2–3 × 10^5 cells/mL after addition of drug solution. Appropriate volumes of the drug/Fischer's medium stock solution (5–150 μL) were immediately transferred to the cell suspensions. The drug–cell suspensions were then incubated for 1 h at 37 °C. The cells were washed three times with 3 mL of supplemented Fischer's medium (containing 10% horse serum) and then resuspended in 5 mL of fresh medium. A 1-mL portion was used to determine the cell count (Coulter counter). From the remainder, a 5-mL suspension of cells was prepared at a density of 10^5 cells/mL, and between 10^2 and 10^5 cells were plated on soft agar and incubated at 37 °C. Colonies were counted after 10 days.

Determination of Rate Constants. Rate constants were determined by simultaneous fit of the experimental data points to the values calculated according to the differential equations used previously for the kinetic reactions of *cis*-**2a**.[7] A computer program based upon the Simplex algorithm[33] was used to optimize rate constants k_1–k_5 by nonlinear least-squares minimization.[7] Calculated data points were determined every 6 s for the first 10 min, every 30 s for the next 15 min, and every 1 min for the remaining time points.

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Registry No. *cis*-**2a**, 61903-29-5; *trans*-**2a**, 61903-30-8; *cis*-**2b**, 64858-43-1; *trans*-**2b**, 64858-44-2; **2b/3b** aldophosphamide hemithioacetal derivative, 120144-73-2; *cis*-**2c**, 120144-65-2; *trans*-**2c**, 120144-67-4; **2c/3c** aldophosphamide hemithioacetal derivative, 120144-74-3; *cis*-**2d**, 120144-66-3; *trans*-**2d**, 120144-68-5; **3a**, 35144-64-0; **3b**, 120144-70-9; **3c**, 120144-71-0; **3d**, 120144-72-1; **4a**, 88685-79-4; **4b**, 76174-16-8; **4c**, 120144-61-8; **4d**, 120144-62-9; *cis*-**5d**, 120144-63-0; *trans*-**5d**, 120144-69-6; **6a**, 56922-83-9; **6b**, 64858-36-2; **6c**, 64858-42-0; **6d**, 120144-64-1; 3-buten-1-ol, 627-27-0; bis(2-chloroethyl)phosphoramide dichloride, 127-88-8; *O*-(3-butenyl) *N*,*N*-bis(2-chloroethyl)-*N*′-methylphosphorodiamidate, 39800-38-8; phenyl dichlorophosphate, 770-12-7; bis(2-chloroethyl)amine hydrochloride, 821-48-7; *O*-phenyl *N*-methylphosphoramide mustard, 98650-18-1; bis(aziridinyl)phosphoramidate, 120144-75-4.

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