Cluster plates (48 wells) were infected with 80 focus-forming units of MSV during 90 min. Medium was then replaced by 1 mL of fresh culture medium containing different concentrations of the test compounds. After 6 days of incubation at 37 °C, transformation of the cell cultures was monitored microscopically.

Determination of the cytopathic effect of HIV in human T-lymphocyte MT4 cells has been described.[6a,20] Briefly, MT-4 cells, subcultured 1 day before the start of the experiment, were adjusted to $5 \times 10^5$ cells/mL and infected with HIV (HTLV-III$_B$) at 400 $CCID_{50}$/mL. Then, 100 μL of the infected cell suspension was transferred to wells of a microtiter tray containing 100 μL of varying dilutions of the test compounds. After 5 days of incubation at 37 °C, the number of viable cells was recorded microscopically in a hematocytometer following the trypan blue exclusion procedure.

(20) Pauwels, R.; De Clercq, E.; Desmyter, J.; Balzarini, J.; Goubau, P.; Herdewijn, P.; Vanderhaeghe, H.; Vandeputte, M. *J. Virol. Methods* **1987**, *16*, 171–185.

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**Supplementary Material Available:** Tables of atomic parameters, bond lengths and bond angles, and hydrogen bonds (3 pages). Ordering information is given on any current masthead page.

# $^{31}$P NMR Studies of the Kinetics of Bisalkylation by Isophosphoramide Mustard: Comparisons with Phosphoramide Mustard

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$^{31}$P nuclear magnetic resonance spectroscopy was used to measure the p$K_a$ (4.28 ± 0.2) of isophosphoramide mustard (IPM) at 20 °C and to study the kinetics and products of the decomposition of IPM at a solution pH value of ca. 7.4 and at temperatures between 20 and 47 °C in the presence of nucleophilic trapping agents. At 37 °C, the half-life for the first alkylation was ca. 77 min and ca. 171 min for the second alkylation; these data may be compared with those for phosphoramide mustard (Engle, T. W.; Zon, G.; Egan, W. *J. Med. Chem.* **1982**, *25*, 1347), wherein the half-lives for the first and second alkylations are approximately the same (18 min). The rate of fragmentation of aldoifosfamide to IPM and acrolein was also studied by NMR spectroscopy (pH 7.0; 37 °C; 0.07 M phosphate); under the noted conditions, the half-life of aldoifosfamide was found to be ca. 60 min.

Cyclophosphamide (**1a**) is a bisalkylating agent that has been shown to be therapeutically useful against a broad range of human cancers. Of the multitude of structural analogues arising from **1a**, ifosfamide (**1b**) is among the most notably effective.[1-6] It is generally accepted that Scheme I includes the metabolic transformations of **1a** and **1b** which are of cytotoxic significance.[2,3] Oxidation (activation) of **1a** or **1b** leads to 4-hydroxycyclophosphamide/aldophosphamide (**2a**) or 4-hydroxyifosfamide/aldoifosfamide (**2b**), respectively. Fragmentation of these aldehydic metabolites (**2a** and **2b**) produces acrolein and the ultimate DNA alkylating agents, phosphoramide mustard (**3a**) and isophosphoramide mustard (**3b**).

Although the metabolic transformations of cyclophosphamide and ifosfamide are similar, structural differences between the two isomers affect the relative rates of the corresponding transformations, resulting in differences in pharmacokinetics, toxicities, and therapeutic efficacies.[5,6] In certain circumstances, ifosfamide is the superior antitumor agent. For example, while cyclophosphamide shows little activity against soft tissue sarcomas, ifosfamide, as a single drug or in combination chemotherapy, shows promise; moreover, ifosfamide is at times useful for the treatment of tumors that have become resistant to cyclophosphamide.[6-12]

The present study focuses on the comparative alkylating abilities of phosphoramide mustard and isophosphoramide mustard. Since the oncostatic effects of **3a** and **3b** are related to the extent to which they cross-link DNA (i.e., bisalkylate DNA), we considered it necessary to study, in detail, their respective alkylation kinetics. Half-lives for **3a** and **3b** have been reported;[13-16] however, bisalkylation

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(1) Zon, G. *Progr. Med. Chem.* **1982**, *19*, 205.
(2) Stec, W. J. *Organophosphorus Chem.* **1982**, *13*, 145.
(3) Friedman, O. M.; Myles, A.; Colvin, M. *Adv. Cancer Chemother.* **1979**, *1*, 143.
(4) Brock, N. *J. Cancer Res. Clin. Oncol.* **1986**, *111*, 1.
(5) Colvin, M. *Seminars in Oncol.* 1982, IX (No. 4, Suppl 1), 2.
(6) Brade, W. P.; Herdrich, K.; Varini, M. *Cancer Treat. Rev.* **1985**, *12*, 1.
(7) Pratt, C. B.; Horowitz, M.; Meyer, W. H.; Hayes, F. A.; Etcubanas, E.; Douglass, E.; Thompson, E. I.; Wilimas, J.; Green, A. A. *Proc. Am. Assoc. Can. Res.* **1986**, *27*, 179.
(8) Pratt, C. B.; Horowitz, M. E.; Meyer, W. H.; Etcubanas, E.; Thompson, E. I.; Douglass, E. C.; Wilimas, J. A.; Hayes, F. A.; Green, A. A. *Cancer Treat. Rep.* **1987**, *71*, 131.
(9) Marti, C.; Kroner, T.; Remagen, T.; Berchtold, W.; Cserhati, M.; Varini, M. *Cancer Treat. Rep.* **1985**, *69*, 115.
(10) Miser, J.; Kinsella, T. J.; Triche, T. J.; et al. *J. Clin. Oncol.* **1987**, *5*, 1191.
(11) Seeber, S.; Niederle, N.; Osieka, N.; et al. *Tumor Diagnostic Ther.* **1984**, *5*, 39.
(12) Pinkerton, C. R.; Rogers, H.; James, C.; Bowman, A.; Barbor, P. R. H.; Eden, O. B.; Pritchard, J. *Cancer Chemother. Pharmacol.* **1985**, *15*, 258.

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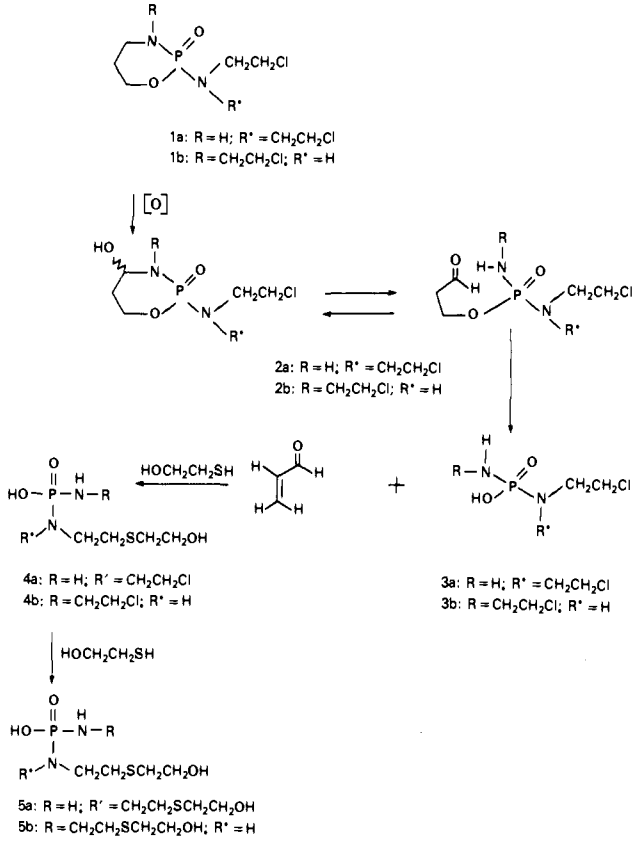
**Scheme I**




kinetics are available only for **3a**.[13,14] During the course of these studies, we also reinvestigated a previous claim,[13] which was subsequently challenged,[14] regarding the observation of a pseudostable aziridinium ion formed from **3a**. We now report the above-mentioned kinetic and product analyses, as well as a comparison of the rates at which **3a** and **3b** are generated from their precursor metabolites.

## Results

**Determination of Rate Constants for Consecutive Alkylation Reactions by 3b. Estimation of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for 3b.** $^{31}P$ NMR kinetic measurements with **3b** in the presence of excess 2-mercaptoethanol (used as trapping agent) were carried out at varying temperatures in 1 M 2,6-dimethylpyridine (lutidine) buffer, "pH" 7.4.[17] A stack plot displaying representative spectra obtained at 20 °C for the reaction of **3b** with thiol is shown in Figure 1. As can be seen in this figure, the gradual disappearance of the $^{31}P$ NMR signal for **3b** ($\delta$ 13.47, labeled as A) was accompanied by the steady growth of a new signal ($\delta$ 13.94, labeled as C), assigned to the expected bisalkylation product **5b** (Scheme I); the compound giving rise to this signal (C)

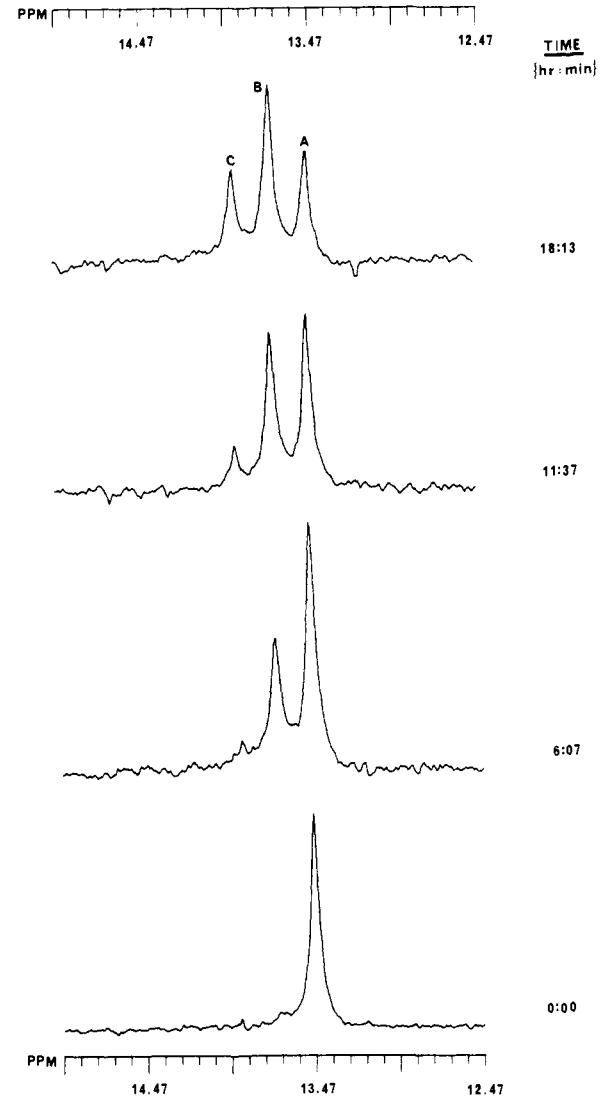



**Figure 1.** Stack plot of $^{31}P$ NMR spectra (121.5 MHz) of isophosphoramide mustard (conditions as in run no. 1 of Table I) as a function of time, showing the disappearance of starting **3b** (labeled as A in the figure) and the appearance of **4b** (labeled as B in the figure) and **5b** (labeled as C in the figure).

was isolated and its structure confirmed by $^{1}H$ and $^{13}C$ NMR spectroscopy and mass spectrometry (see the Experimental Section). A signal for a transient species ($\delta$ 13.7, labeled as B), kinetically associated with both A and C, was also observed and this was assigned to the monoalkylated intermediate **4b** (Scheme I), by analogy to the previously established alkylation chemistry of phosphoramide mustard (i.e., **3a** → **4a** → **5a**, Scheme I).[13]

It has been shown that in the reaction sequence **3a** → **4a** → **5a**, the formation of both **4a** and **5a** is preceded by an intramolecular cyclization of the chloroethylamido functionality, resulting in an aziridinium ion.[18] In the presence of nucleophiles, the aziridinium ions are short-lived and the sequence **3a** → **4a** → **5a** can be treated kinetically as simple, consecutive "first-order" reactions.[13,14]

The same kinetic analysis has been applied herein to the reaction of isophosphoramide mustard (**3b**) with 2-mercaptoethanol. Although the reaction is undoubtedly more complex (vide infra), intermediate species between **3b** and **4b** and between **4b** and **5b** were not observed, at least not in the presence of trapping agents. To the extent

(13) Engle, T. W.; Zon, G.; Egan, W. *J. Med. Chem.* **1982**, *25*, 1347.
(14) Watson, E.; Dea, P.; Chan, K. K. *J. Pharm. Sci.* **1985**, *74*, 1283.
(15) Brock, N.; Hohorst, H.-J. *Z. Krebsforsch.* **1977**, 88, 185.
(16) Boyd, V. L.; Robbins, J. D.; Egan, W.; Ludeman, S. M. *J. Med. Chem.* **1986**, *29*, 1206.
(17) For buffer solutions which were made with $D_2O$, the reported pH values correspond to meter readings to which 0.4 pH unit was added to correct for deuterium isotope effects (Lumry, R.; Smith, E. L.; Glantz, R. R. *J. Am. Chem. Soc.* **1951**, *73*, 4330); such corrected values are indicated in the text by enclosing the term pH in quotation marks. For buffer solutions that were made with 10% $D_2O$, the reported value is the meter reading; in these instances, the term pH does not appear in quotation marks.

(18) Colvin, M.; Brundrett, R. B.; Kan, M.-N. N.; Jardine, I.; Fenselau, C. *Cancer Res.* **1976**, *36*, 1121.

Table I. $^{31}P$ NMR Derived Kinetic Data for the Disappearance of Isophosphoramide Mustard (**3b**) and Phosphoramide Mustard (**3a**) under Various Reaction Conditions

| run no. | conditions | temp, °C | $k_1$, $\times 10^{-3}$ min$^{-1}$ | $k_2$, $\times 10^{-3}$ min$^{-1}$ |
|---|---|---|---|---|
| 1 | 11 mM **3b**; 1 M lutidine; "pH" 7.4; 63 mol equiv of 2-mercaptoethanol | 20 | 1.07 ± 0.021 (648 min)[a] | 0.63 ± 0.027 |
| 2 | 17 mM **3b**; 1 M lutidine; "pH" 7.4; 10 mol equiv of 2-mercaptoethanol | 27 | 2.36 ± 0.053 (294 min)[a] | 1.14 ± 0.037 |
| 3 | as in run no. 2 | 37 | 9.03 ± 0.33 (77 min)[a] | 4.05 ± 0.39 |
| 4 | as in run no. 2 | 47 | 34.30 ± 2.7 (20 min)[a] | 19.96 ± 1.07 |
| 5 | 17 mM **3b**; 1 M lutidine; "pH" 7.4; 10 mol equiv of sodium thiosulfate | 37 | 8.29 ± 0.25 (84 min)[a] | 4.29 ± 0.18 |
| 6 | 17 mM **3b**; 1 M phosphate; "pH" 9.4; 10 mol equiv of 2-mercaptoethanol | 20 | 1.2 ± 0.11 (575 min)[a] | |
| 7[b] | 17 mM **3a**; 1 M Tris; pH 7.4; 10 mol equiv of 2-mercaptoethanol | 37 | 37.7 ± 1.2 (18 min)[a] | 32.7 ± 1.7 |
| 8[c] | 50 mM **3a**; 1 M Tris sulfate; pH 7.4 | 38 | 37.1 ± 1.0 (19 min)[a] | |
| 9 | 12 mM **3a**; 1 M lutidine; "pH" 7.4; 50 mol, equiv of 2-mercaptoethanol | 20 | 2.16 ± 0.45 (321 min)[a] | 2.67 ± 0.75 |
| 10 | 17 mM **3a**; 1 M lutidine; "pH" 7.4; 10 mol equiv of 2-mercaptoethanol | 37 | 35.4 ± 2.90 (20 min)[a] | 44.3 ± 3.32 |
| 11 | as in 10 | 27 | 6.79 ± 0.02 (102 min)[a] | |

[a] Number in parentheses provides the half-life for the disappearance of starting compound. [b] Data taken from ref 13. [c] Data taken from ref 14.

that intermediary species are present, however, the interpretation of $k_1$ and $k_2$ must be modified. Thus, $k_1$ represents either the rate constant for the disappearance of **3b** (and this is independent of the presence of intermediates between **3b** and **4b**) *or* the composite rate constant for the production of **4b** from **3b**; similarly, in the presence of intermediate species between **4b** and **5b**, $k_2$ represents a composite rate constant for the production of **5b** from **4b**. Because intermediate species were not observed in the presence of trapping agents, then, to the extent that they are present, the rate constants for their conversion to **4b** and **5b** must be fast, and thus the composite rate constants closely approximate the rate constants for the interconversion of the observed species.

The rate constants $k_1$ and $k_2$ were experimentally derived from a least-squares fitting of the observed concentrations of **3b** (A), **4b** (B), and **5b** (C) to eq 1.

$$A(t) = A_0 \exp(-k_1 t) \tag{1a}$$

$$B(t) = [A_0 k_1/(k_2 - k_1)][\exp(-k_1 t) - \exp(k_2 t)] + B_0 \exp(-k_2 t) \tag{1b}$$

$$C(t) = A_0 + B_0 + C_0 - A(t) - B(t) \tag{1c}$$

In the above equations, $A_0$, $B_0$, and $C_0$ are the initial (time = "zero" spectrum) concentrations of A, B, and C. Equations 1a–c are the solutions[19] to the coupled set of differential equations, the kinetic model,[20] given by eq 2.

$$dA/dt = -k_1[A] \tag{2a}$$

$$dB/dt = k_1[A] - k_2[B] \tag{2b}$$

$$dC/dt = k_2[B] \tag{2c}$$

Least-squares fitting[21] of the $^{31}P$ NMR data (signal intensities of A, B, and C) to eq 1 resulted in values of $k_1$ and $k_2$ of (1.07 ± 0.02) × $10^{-3}$ min$^{-1}$ and (0.63 ± 0.03) × $10^{-3}$ min$^{-1}$, respectively at 20 °C. Rate constants obtained at other temperatures are given in Table I. Rate constants for **3a** were determined under similar reaction conditions; at 20 °C, it was found that $k_1$ = (2.16 ± 0.04) × $10^{-3}$ min$^{-1}$ and $k_2$ = (2.67 ± 0.07) × $10^{-3}$ min$^{-1}$. Rate data for **3a** in lutidine at other temperatures were also determined (see Table I), and these were in excellent agreement with values reported previously in other buffers [e.g., $k_1$ = 0.036 min$^{-1}$ and $k_2$ = 0.043 min$^{-1}$ in 1 M Tris, pH 7.4, 37 °C (nucleophile is 2-mercaptoethanol);[13] $k_1$ = 0.0433 min$^{-1}$ and $k_2$ = 0.031 min$^{-1}$ in 0.067 M phosphate, pH 7.4, 38 °C (nucleophile is water)[14]]. On the other hand, half-lives (derived from $k_1$ values) for **3a** (20 min) and **3b** (77 min) in lutidine at "pH" 7.4, 37 °C were significantly different from those (48 and 840 min, respectively) cited by Brock and Hohorst in 0.07 M phosphate, pH 7, 37 °C.[15]

Rate constants for the bisalkylation kinetics of **3b** (as well as **3a**) were also determined in lutidine buffer in the presence of a different trapping agent, viz., sodium thiosulfate ($Na_2S_2O_3$). Due to the overlap of $^{31}P$ NMR resonances, it was not as straightforward to determine concentrations of A, B, and C as when 2-mercaptoethanol was used. However, by means of either curve-deconvolution methods applied to the frequency-domain spectra (least-squares fitting[21] of the spectra to a sum of three Lorentzian signals), or resolution enhancement methods applied to the time-domain spectra (Lorentz–Gauss transformation[22]), it was possible to obtain relatively good estimates of the concentrations of A, B, and C as a function of time and, hence, estimates of $k_1$ and $k_2$. These values for the rate constants agreed (within experimental error) with those determined when 2-mercaptoethanol was used as the trapping agent. Structural identifications of the bisalkylated products obtained from the reactions of **3a** and **3b** with thiosulfate were made by $^{13}C$ and $^{1}H$ NMR (see the Experimental Section).

In order to be able to obtain reliable estimates of $k_1$ and $k_2$ at various temperatures (by means of the Eyring equation)[20] for the alkylation reactions of **3b**, the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were determined. Least-squares analyses of $k_1$ and $k_2$ as functions of temperature showed that both alkylation reactions possessed modest positive entropies, 12 ± 1.3 cal mol$^{-1}$ deg$^{-1}$ for the first alkylation reaction and 17 ± 6.8 cal mol$^{-1}$ deg$^{-1}$ for the second alkylation; the corresponding values of $\Delta H^{\ddagger}$ were 25.0 ± 0.4 and 27.0 ± 2.1 kcal/mol.

**Nontrapping Decomposition Kinetics and Product Analysis.** Under similar conditions to those used for the experiments described above (17 mM solutions of **3a** or **3b** in 1 M lutidine, "pH" 7.4), but in the absence of trapping agents, the chemistries of **3a** and **3b** were more complex. Nevertheless, it was possible to obtain reliable rate constants for the disappearance of the starting phosphoramide mustards. For both **3a** and **3b**, the respective

(19) The solution to eq 2a (eq 1a) may be substituted into eq 2b; eq 2b can be made "exact" through use of an integrating factor, $\exp(\sum k_2\, dt)$, and then solved (Ross, S. L. *Differential Equations*, 2nd ed.; John Wiley & Sons, Inc.: New York, 1974; pp 57–62) to yield eq 1b.

(20) Frost, A. A.; Pearson, R. G. *Kinetics and Mechanism*, 2nd ed.; John Wiley & Sons, Inc.: New York, 1953; pp 166–167.

(21) Knott, G. D. *Comput. Programs Biomed.* **1979**, *10*, 271.

(22) Ernst, R. R.; Bodenhausen, G.; Wokaun, A. *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*; Clarendon Press: Oxford, 1987; pp 330–332.

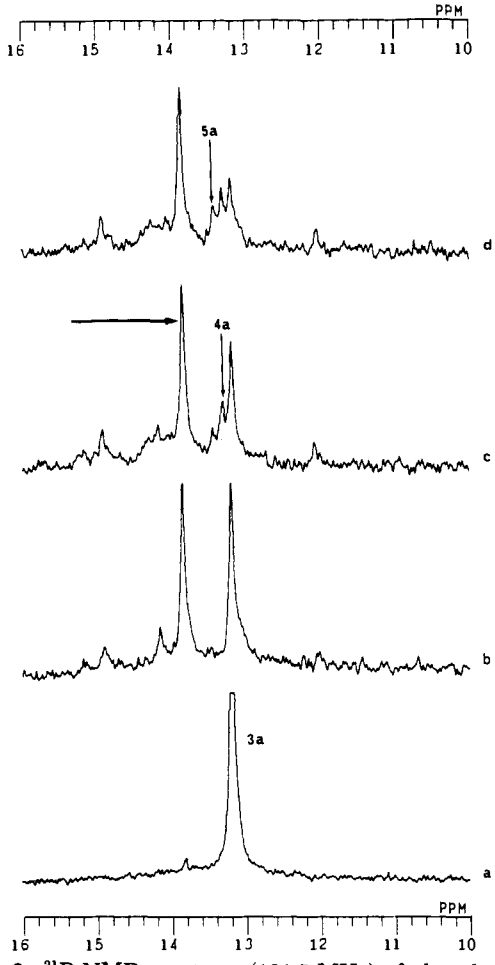



**Figure 2.** $^{31}P$ NMR spectrum (121.5 MHz) of phosphoramide mustard (**3a**) (37 °C; 1 M Tris; "pH" 7.4). (a) Spectrum taken at time = 0; note that the resonance for **3a** is off-scale, indicating the presence of few other phosphorus resonances. (b) Spectrum taken when approximately half of the starting material had decomposed. The new resonance (corresponding to that marked with an arrow in panel c) was that originally (see ref 13) thought to be an aziridinium ion. (c) Spectrum taken following the addition of excess mercaptoethanol (10 mol equiv), showing that the "aziridinium ion" (indicated by the arrow) is still present, and that **4a** and **5a** (see panel d) are being formed and trapped. (d) Spectrum showing the presence of **3a**, **4a**, and **5a**, as well as the reputed (see ref 13) "aziridinium ion" (indicated by the arrow in panel c).

values of $k_1$ (determined from $^{31}P$ NMR data acquired over ca. 1 half-life) were the same (within experimental error) as those obtained in the presence of trapping agents. Moreover, for **3b**, changing the solution pH value to 9.4 had no measurable effect of $k_1$; see Table I.

The decomposition of **3a** in lutidine produced a pattern of $^{31}P$ NMR signals quite similar to that previously obtained in either Tris[13] or phosphate[14] buffer: signals were observed corresponding to inorganic phosphate end products as well as to additional transient species associated with the hydrolysis of the 2-chloroethyl moieties. In separate experiments, solutions of **3a** (1 M lutidine or 1 M Tris as buffers; 37 °C, "pH" 7.4) were allowed to react for a period of time equal to 1 half-life (as determined by monitoring the reaction by $^{31}P$ NMR spectroscopy), at which point a 10-fold molar excess of 2-mercaptoethanol was added and the acquisition of $^{31}P$ NMR spectra continued (see Figure 2 for data acquired in Tris buffer; similar spectra were obtained in lutidine buffer). The following observations were made subsequent to the addition of 2-mercaptoethanol: (1) residual **3a** continued to disappear and its disappearance was associated with the production of **4a** and **5a**; (2) a resonance (indicated by the arrow in Figure 2), which had previously been assigned to the aziridinium ion produced by **3a**,[13] did *not* immediately disappear upon addition of 2-mercaptoethanol to produce either **4a** or **5a** or both but, instead, slowly reacted to produce other products.

Given the rapidity with which aziridinium species are trapped (vide supra), the species giving rise to the resonance indicated by the arrow cannot be the previously claimed[13] aziridinium ion intermediate; this species is, more likely, the hydrolysis product (**3c**: R = H; R′ = $CH_2CH_2OH$) suggested by Chan et al.[14]

**Comparison of p$K_a$ Values between 3a and 3b.** The $^{31}P$ NMR chemical shift of **3b**, in unbuffered water, was recorded as a function of the solution pH value; this chemical shift versus pH data was then fit to an appropriate form of the Henderson–Hasselbach equation.[13] In this manner, the p$K_a$ of **3b** was determined to be 4.28 ± 0.20, which is slightly lower than the reported range of values measured for **3a** (p$K_a$ = 4.6–4.8).[13,15,23,24]

**Solution Chemistry of 2a and 2b.** Since **3a** and **3b** are generated in vivo from **2a** and **2b**, respectively, the kinetics of these fragmentation reactions are also essential to an understanding of the comparative metabolisms of **1a** and **1b**. We have previously reported a comprehensive kinetic analysis of the interconversion of *cis*- and *trans*-4-hydroxycyclophosphamide (*cis*- and *trans*-4-OH-CP) with aldophosphamide (AP) and subsequent fragmentation of AP to form **3a**[25] and acrolein; this work has been substantiated by others.[26,27] While aggregate half-lives for 4-hydroxyifosfamide (4-OH-IF)/aldoifosfamide (AIF) have been cited in the literature,[15,28] the reported rates are divergent; direct observation of the individual metabolites was not possible by the methods used. We have used $^{31}P$ NMR spectroscopy to study the comparative solution chemistries of the metabolites of **1a** and **1b**.

As previously reported,[25] the rapid, stereospecific reduction of *cis*-4-hydroperoxycyclophosphamide (20 mM in 1 M lutidine, pH 7.4, 37 °C) with $Na_2S_2O_3$ (ca. 4 equiv) was observed by $^{31}P$ NMR spectroscopy to give *cis*-4-OH-CP ($\delta$ 12.22). The reversible stereomutation of the *cis*-hydroxy compound with its trans diastereomer ($\delta$ 12.42), through the intermediacy of AP (and its hydrate, AP·$H_2O$; $\delta$ 20.40), resulted in a pseudoequilibrium mixture of these metabolites. After ca. 15–20 min, the relative ratios of the interconverting species remained constant (*cis*-4-OH-CP:*trans*-4-OH-CP:AP/AP-$H_2O$ = 57:30:13); however, the absolute signal intensity for each species continually decreased as AP irreversibly fragmented to **3a** ($\delta$ 13.6). The apparent half-life for each intermediate metabolite was calculated, and to within experimental error, the values were identical [the average half-life for all species, collectively indicated by **2a**, was 38 ± 1 min (correlation coefficient, $r^*$ = 0.949, where $r^*$ is the average $r$ of the kinetic determinations)].

*cis*-4-Hydroperoxyifosfamide was deoxygenated with $Na_2S_2O_3$, as above, and this led to the production of *cis*-4-OH-IF ($\delta$ 13.41). The interconversion of *cis*-4-OH-IF

(23) Voelker, G.; Giera, H. P.; Jaeger, L.; Hohorst, H.-J. *Z. Krebsforsch.* **1978**, *91*, 127.
(24) Ulmer, W. *Int. J. Quantum Chem.* **1981**, *19*, 337.
(25) Zon, G.; Ludeman, S. M.; Brandt, J. A.; Boyd, V. L.; Ozkan, G.; Egan, W.; Shao, K.-L. *J. Med. Chem.* **1984**, *27*, 466.
(26) Borch, R. F.; Hoye, T. R.; Swanson, T. A. *J. Med. Chem.* **1984**, *27*, 490.
(27) Borch, R. F.; Millard, J. A. *J. Med. Chem.* **1987**, *30*, 427.
(28) Low, J. E.; Borch, R. F.; Sladek, N. E. *Cancer Res.* **1983**, *43*, 5815.

with *trans*-4-OH-IF (δ 13.09) and AIF (and, presumably, AIF·$H_2O$; δ 18.6) occurred rapidly such that within minutes a constant ratio of these metabolites was observed (51:38:11, respectively). As **3b** (δ 13.48) was generated, the absolute intensities of the signals for the intermediary metabolites decreased at the same apparent rate [the average half-life for each species, collectively described by **2b**, was 44 ± 4 min ($r^*$ = 0.97)].

Since previous half-lives for **2a** and **2b** were reported in 0.07 M phosphate, pH 7.0, 37 °C,[15,28,29] the above $^{31}P$ NMR experiments were repeated under these conditions (using 20 mM samples of the appropriate hydroperoxide and ca. 4 equiv of thiosulfate for the reduction). After ca. 15 min at 37 °C, *cis*-4-OH-CP (42%; δ 12.56), *trans*-4-OH-CP (37%; δ 12.79) and AP/AP·$H_2O$ (21%; δ 20.77) reached pseudoequilibrium; the average half-life of this mixture was 59 ± 3 min ($r^*$ = 0.97). For the metabolites of **1b** kinetic data were based on the disappearance of *trans*-4-OH-IF (δ 13.5) and AIF/AIF·$H_2O$ (δ 18.95); the $^{31}P$ signals for *cis*-4-OH-IF and **3b** were identical (δ 13.8). The average half-life of *trans*-4-OH-IF and AIF/AIF·$H_2O$ (ratio 69:31, respectively) was 62 ± 7 min ($r^*$ = 0.95). These NMR-derived kinetic data agree with those reported by others (half-life for both **2a** and **2b** = ca. 70 min, as determined by monitoring the release of acrolein by UV spectroscopy).[28,29] On the other hand, Brock and Hohorst reported half-lives of 180 min for 4-OH-CP and 542 min for 4-OH-IF;[15] however, these kinetics were measured by using the NBP colorimetric assay, which is rather unreliable.

## Discussion

A comparison of the alkylation kinetics of **3a** and **3b** reveals two significant differences. First, in the presence of trapping agents, the values of $k_1$ and $k_2$ are each considerably smaller for isophosphoramide mustard than for phosphoramide mustard. Second, whereas $k_1 \approx k_2$ for the alkylation reactions of phosphoramide mustard, $k_1$ is approximately twice $k_2$ for the alkylation reactions of isophosphoramide mustard.

The factor of two difference in $k_1$ and $k_2$ for isophosphoramide mustard is expected. In isophosphoramide mustard, there are two, independently reacting centers (2 × $NHCH_2CH_2Cl$), each of which can form the same monoalkylated species; a statistical factor of 2 is thus incorporated into $k_1$.[30] Thus, one expects monoalkylation by isophosphoramide mustard to proceed approximately twice as fast as, for example, the alkylation by (HO)P(O)($NHCH_2CH_2Cl$)($NHCH_2CH_3$). After undergoing one alkylation reaction, isophosphoramide mustard has only one remaining chloroethyl group that can react. Assuming that replacement of Cl with $SCH_2CH_2OH$ on the first arm has no effect on the reactivity of the $NHCH_2CH_2Cl$ grouping on the second arm, then $k_1$ and $k_2$ should differ only by a factor of 2, as is approximately observed. Similar kinetic behavior (wherein $k_1 \approx 2k_2$) is observed in formally analogous situations, as, for example, in the hydrolysis of the bisacetate of ethylene glycol.[30]

A similar 2-fold difference in rate constants is not observed in the alkylations by phosphoramide mustard. In phosphoramide mustard, there is only one nitrogen atom that can react to internally displace a chloride ion, and thus the two monoalkylation reactions are not mutually independent. Accordingly, with the assumption that replacement of Cl by $SCH_2CH_2OH$ on one arm does not affect the subsequent displacement of Cl on the other, $k_1$ should equal $k_2$, as is approximately observed.

In this study, we also observed that the rate of disappearance of isophosphoramide mustard was considerably slower than the rate of disappearance of phosphoramide mustard. The reason for this behavior is not entirely clear, particularly given the "statistical advantage" of the former compound and the observation that the $pK_a$ values for the two compounds are approximately the same. Presumably, it is simply differences in the electron densities of the respective reacting nitrogen atoms that determine the differences in the chloride displacement kinetics; in this regard, ab initio calculations, which could determine electron densities at nitrogen, would be most useful.

The alkylation reactions of **3a** and **3b** differ with regard to the nature of the initially formed aziridinium ion. Phosphoramide mustard gives rise to an aziridinium ion where, in addition to phosphorus, three carbon atoms are attached to nitrogen; isophosphoramide mustard gives rise to an aziridinium ion where, in addition to phosphorus, two carbon atoms and one proton are attached to the central nitrogen. The aziridinium ion formed from isophosphoramide mustard should deprotonate upon formation, the precursor phosphoramide being a weak base. Indeed, the alkylation kinetics at pH 9.4 were the same as at pH 7.4, suggesting, albeit very speculatively, that it is the "neutral aziridine" that is alkylated.

Although **3a** and **3b** differ in the details of their alkylation mechanisms, the measured $k_1$ values still represent the rate constants for the disappearance of the respective mustards, and these differ considerably. As the aziridinyl species deriving from **3b** is not present in readily observable concentrations (in the presence of trapping agents), we conclude that its lifetime is short, being readily intercepted by nucleophiles. As noted in the Results section, the nature of a trapping agent (mercaptoethanol, thiosulfate, hydroxyl ion) does not significantly alter the alkylation kinetics. This indicates that the rate-limiting process is the internal displacement of chloride ion by the mustard nitrogen, and not the subsequent alkylation reaction.

The differences in alkylation kinetics between isophosphoramide mustard and phosphoramide mustard are dramatic and ought to result in significant differences in in vivo DNA alkylation chemistry. Isophosphoramide mustard should survive longer within the cell cytoplasm and thereby have an increased probability of reaching the nucleus and alkylating DNA. Alternatively, the major cytotoxic event could be the fragmentation of **2a**/**2b** within the nucleus to generate the corresponding mustards, which then cross-link DNA. In any event, explanations of therapeutic differences between cyclophosphamide and ifosfamide must now consider the above-noted, and marked, differences in alkylation kinetics, particularly in view of the fact that both mustards (**3a** and **3b**) are generated, in the absence of enzymic interactions, at approximately the same rate from their precursor metabolites (**2a**/**2b**).

## Experimental Section

**NMR Spectra.** $^{31}P$ NMR spectra at 161.7 MHz were collected on a JEOL GX-400 spectrometer; $^{31}P$ NMR spectra at 36.23 MHz, $^{13}C$ NMR spectra at 22.5 MHz, and $^{1}H$ NMR spectra at 89.55 MHz were collected on a JEOL FX-90Q spectrometer. Data collection conditions were as described.[25,31] $^{31}P$ NMR chemical shifts refer to external 25% $H_3PO_4$ in $D_2O$; $^{1}H$ and $^{13}C$ NMR δ values refer

(29) Low, J. E.; Borch, R. F.; Sladek, N. E. *Cancer Res.* **1982**, *42*, 830.
(30) Pannetier, G.; Souchay, P. *Chemical Kinetics*; Elsevier Publishing Co., Ltd.: New York, 1967; p 186–191.
(31) Boyd, V. L.; Summers, M. F.; Ludeman, S. M.; Egan, W.; Zon, G.; Regan, J. B. *J. Med. Chem.* **1987**, *30*, 366.

to external sodium 3-(trimethylsilyl)propionate-2,2,3,3-$d_4$ (TSP) in $D_2O$ or tetramethylsilane in $CDCl_3$. Phosphoramide mustard (as the cyclohexylammonium salt) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. The synthesis of isophosphoramide mustard[32,33] and *cis*-4-hydroperoxycyclophosphamide[25] have been published.

**Mass Spectrometry.** Mass spectra were recorded with a Kratos MS-50 mass spectrometer (Manchester, England) fitted with an extended range magnet and equipped with a Kratos DS-55 data system and light beam oscillographic recorder. Fast atom bombardment mass spectra[34] were scanned from samples introduced directly into the ion source as colloidal suspensions in a glycerol sample matrix[35,36] (Alfa Products, Danvers, MA) placed on a copper sample probe-tip; bombardment of the sample was with a 25-$\mu$A beam of 8.5-keV xenon (Matheson, Dorsey, MD) fast atoms generated in a Saddle-Field neutral-beam gun[37] (Ion Tech Ltd., Teddington, England).

**$^{31}$P NMR Kinetic Studies. General Procedures.** NMR sample solutions of **3a** and **3b** were prepared immediately prior to their use by the addition of buffer to a glass vial that contained the compound of interest. In those studies where a trapping agent was used, excess 2-mercaptoethanol or sodium thiosulate was immediately added upon dissolution of **3a** or **3b** in the buffer. The pH of the resultant solution was adjusted as necessary (1 M HCl or NaOH) and the solution was transferred to a 10-mm NMR tube. The sample was allowed to thermally equilibrate for several minutes in the spectrometer probe prior to data collection. Over the course of each experiment, the pH varied by $\leq$0.2 pH unit.

The preparation as well as chemical and kinetic analyses of NMR samples of 4-hydroxycyclophosphamide/aldophosphamide (**2a**) have been reported.[25] The same procedures were used for studies of 4-hydroxyifosfamide/aldoifosfamide (**2b**).

***cis*-4-Hydroperoxyifosfamide.** Precursor *O*-(3-butenyl) *N,N'*-bis(2-chloroethyl)phosphorodiamidate was prepared according to the method of Takamizawa et al.[38] The crude material was purified [$R_f$ 0.60, $CHCl_3$–MeOH (9:1)] and then ozonated as previously described for *O*-(3-butenyl) *N,N*-bis(2-chloroethyl)-phosphoroamidate (precursor to *cis*-4-hydroperoxycyclophosphamide).[25] The product was obtained as white crystals (mp 94–96 °C) in 25% yield: $^1$H NMR (89.55 MHz, $CDCl_3$) $\delta$ 5.4–5.00 (m, 1 H, *H*COOH), 4.90–3.90 (m, 2 H, $CH_2O$), 3.90–3.00 (m, 9 H, 2 $NCH_2CH_2Cl$ and NH), 2.41–1.92 (m, 2 H, $CH_2CH_2O$); $^{13}$C NMR (22.49 MHz, $CDCl_3$) $\delta$ 90.32 (COO), 63.84 ($CH_2O$), 47.91 ($NCH_2$), 45.47 (d, $^2J_{CP}$ = 4.9 Hz, $NCH_2$), 43.36 ($CH_2Cl$), 42.19 (d, $^3J_{CP}$ = 3.7 Hz, $CH_2Cl$), 29.03 (d, $^3J_{CP}$ = 3.7 Hz, $CH_2CH_2O$); $^{31}$P NMR (36.23 MHz, $CDCl_3$) $\delta$ 8.89. Anal. ($C_7H_{15}Cl_2N_2O_4P$) C, H, N.

**Isophosphoramide Mustard Trapping Product. A. 2-Mercaptoethanol.** To a $H_2O/D_2O$ (8:2, 1.9 mL) solution of **3b** (31 mg, 0.14 mol) was added 2-mercaptoethanol (0.19 mL, 2.7 mmol). The pH was adjusted to 7.0 and this pH value was maintained throughout the course of the reaction by periodically adding base (1.0 M NaOH). After 7 h at 37 °C, the formation of trapping product **5b** was complete (as determined by $^{31}$P NMR spectroscopy). The reaction mixture was then lyophilized and the residual material was washed with $CHCl_3$ (3 × 5 mL). The remaining white solid (46% yield, corrected for NaCl contamination) was dissolved in $D_2O$ (0.4 mL) and analyzed by NMR spectroscopy: $^1$H NMR (89.55 MHz) $\delta$ 3.68 (t, $J$ = 6 Hz, 4 H, 2 $CH_2O$), 3.1–2.45 (m, 12 H, 2 $NCH_2$ and 2 $CH_2SCH_2$); $^{13}$C NMR (22.49 MHz) $\delta$ 63.1 (2 $CH_2O$), 43.6 (2 $NCH_2$), 35.9 (2 $SCH_2CH_2O$), 35.8 (d, $^3J_{C-P}$ = 7 Hz, $NCH_2CH_2S$). The negative atom FAB mass spectrum (glycerol matrix, 8.5-keV xenon atoms) displayed an ion peak at $m/z$ 303 [$^-$OP(O)(NHCH$_2$CH$_2$SCH$_2$CH$_2$OH)$_2$]; the $m/z$ 303 peak was the most intense in the mass spectrum. Other ions were present at reduced intensity (10–20%) relative to that at $m/z$ 303, but were not readily interpretable as simple fragmentations of $m/z$ 303.

**B. Sodium Thiosulfate.** Isophosphoramide mustard (31 mg, 0.14 mmol) was dissolved in 1 M phosphate buffer (2.4 mL) and to this was added sodium thiosulfate (0.56 mmol). The "pH" of the solution was maintained at 7.4 by periodically adding 1 M NaOH over the 17 h reaction time (37 °C). The final product [($^-$O)P(O)(NDCH$_2$CH$_2$S$_2$O$_3^{2-}$)$_2$] was analyzed in situ by NMR: $^1$H NMR (89.55 MHz) $\delta$ 3.2–3.0 (m); $^{13}$C NMR (22.49 MHz) $\delta$ 43.68 (2 $NCH_2$), 39.51 (d, $^3J_{CP}$ = 7 Hz, 2 $SCH_2$).

**Phosphoramide Mustard Trapping Product. A. 2-Mercaptoethanol.** Phosphoramide mustard (44 mg of the cyclohexylammonium salt, 0.14 mmol) was reacted at 37 °C for 6 h with 2-mercaptoethanol and was then purified (lyophilizing twice) as described above for isophosphoramide mustard. The residual white solid (**5a**) was dissolved in $D_2O$ and analyzed by NMR: $^1$H NMR (89.55 MHz) overlapping multiplets at $\delta$ 3.8–3.4, 3.4–3.0, and 3.0–2.5, plus resonances ($\delta$ 2–1) for cyclohexylamine; $^{13}$C NMR (22.49 MHz) $\delta$ 63.0 (2 $CH_2O$), 49.1 (d, $^2J_{CP}$ = 6 Hz, 2 $NCH_2$), 36.1 (d, $^3J_{CP}$ = 4 Hz, 2 $NCH_2CH_2S$), 36.0 (2 $SCH_2CH_2O$), and signals for cyclohexylamine [$\delta$ 53.1, 33.1 (2 C), 27.1, and 26.6 (2 C)].

**B. Sodium Thiosulfate.** Phosphoramide mustard (44 mg of the cyclohexylammonium salt, 0.14 mmol) was dissolved in 1 M phosphate buffer (1.5 mL) and to this was added sodium thiosulfate (0.56 mmol). The "pH" was maintained at 7.4 over the course of the reaction by adding aliquots of 1 M NaOH periodically, as needed. The reaction mixture was kept at 37 °C for 12 h and the final product [($^-$O)ND$_2$P(O)N(CH$_2$CH$_2$S$_2$O$_3^{2-}$)$_2$] was analyzed in situ by NMR spectroscopy: $^1$H NMR (89.55 MHz) broad multiplet at $\delta$ 3.6–2.8 and resonances for cyclohexylamine (2.1–0.8); $^{13}$C NMR (22.49 MHz) $\delta$ 49.21 (d, $^2J_{CP}$ = 5 Hz, 2 $NCH_2$), 36.75 (2 $CH_2S$), and signals for cyclohexylamine [53.11, 33.23 (2 C), 27.11, and 26.62 (2 C)].

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(32) Ludeman, S. M.; Boyd, V. L.; Regan, J. B.; Gallo, K. A.; Zon, G.; Ishii, K. *J. Med. Chem.* **1986**, *29*, 716.
(33) Struck, R. F.; Dykes, D. J.; Corbett, T. H.; Suling, W. J.; Trader, M. W. *Br. J. Cancer* **1983**, *47*, 15.
(34) Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tyler, A. N. *J. Chem. Soc., Chem. Commun.* **1981**, 325.
(35) Surman, D. J.; Vickerman, J. C. *J. Chem. Res., Synop.* **1981**, *6*, 170.
(36) Martin, S. A.; Costello, C. E.; Bieman, K. *Anal. Chem.* **1982**, *54*, 2362.
(37) Ligon, W. V., Jr. *Int. J. Mass Spectrom. Ion Phys.* **1982**, *41*, 205.
(38) Takamizawa, A.; Matsumoto, S.; Iwata, T.; Makino, I. *Chem. Pharm. Bull.* **1977**, *25*, 1877.