Activation Mechanisms of Mafosfamide and the Role of Thiols in Cyclophosphamide Metabolism

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cis-Mafosfamide (*cis*-**5**) (ASTA Z7557), a stable analogue of *cis*-4-hydroxycyclophosphamide (*cis*-**2**), undergoes rapid decomposition in aqueous phosphate buffer or plasma at pH 7.4 and 37 °C. The reaction kinetics of *cis*-**5** are complex, and *trans*-mafosfamide (*trans*-**5**) and *cis*-**2** are produced and subsequently disappear over the course of the reaction. The rates of decomposition of *cis*-**5** as well as *cis*-**2** were much faster in plasma than in buffer. The cis-trans isomerization of *cis*-**5** occurred by a specific-base-catalyzed process via iminocyclophosphamide (**8**) as a transient intermediate. In contrast, formation of *cis*- and *trans*-mafosfamide (**5**) from *cis*-**2** and MESNA (sodium 2-mercaptoethanesulfonate) proceeded by an acid-catalyzed process via the hemithioacetal intermediate (**6**). The significance of these findings with respect to cyclophosphamide metabolism is discussed.

Cyclophosphamide (**1**), one of the most widely used anticancer agents, is a prodrug and requires metabolic activation to elicit its activity. After initial hydroxylation by mixed-function oxidase, the resulting 4-hydroxycyclophosphamide (**2**) undergoes ring opening to aldophosphamide (**3**), followed by spontaneous generation of the ultimately cytotoxic phosphoramide mustard (**4**) and acrolein by β-elimination.[1-3] *cis*-**2** in aqueous buffer establishes a pseudoequilibrium mixture consisting of *cis*-**2**, *trans*-**2**, the aldehyde **3**, and its hydrate in the approximate ratio of 48:33:5:14.[4-6] It is apparent that the 4-hydroxy compound **2** is an important circulating metabolite of **1** and plays a critical role as an intermediate in both activation and deactivation processes.[7]

Considerable interest was generated from the reports[8,9] that sulfhydryl compounds could significantly stabilize the alkylating capacity of the cyclophosphamide metabolites via the reversible formation of 4-(alkylthio)cyclophosphamide derivatives. However, there is a controversy over whether these 4-thio derivatives indeed represent "deactivated" analogues, since several derivatives have been shown[10] to undergo relatively rapid hydrolysis ($t_{1/2}$ = 4-17 min at pH 7, 37 °C). Recently, Zon et al.[5] investigated the effect of sulfhydryl compounds, i.e., *N*-acetyl-L-cysteine, on the formation, distribution, and kinetics of *cis*-**2**, *trans*-**2**, the aldehyde **3**, its hydrate, and the sulfhydryl conjugates by using ^{31}P NMR spectroscopy. It was concluded that *cis*-**2** underwent slow ring opening to **3** (and its hydrate) followed by facile formation of hemithioacetal, which then cyclized to 4-thiocyclophosphamide adducts.

Mafosfamide (**5**) was recently introduced as a stable derivative of 4-hydroxycyclophosphamide (**2**). Its physicochemical characterization has been reported, and its stability in aqueous buffer is highly dependent upon pH.[11] Mafosfamide (**5**) has undergone extensive preclinical evaluation of toxicity and therapeutic efficacy.[12-17] In general, the antitumor activity of **5** is similar to that of the parent compound **1**, but it was also marginally effective against the cyclophosphamide resistant P388 line.[18,19] Myelosuppression was the predominant toxicity in rodents, although **5** showed less severe myelo- and urotoxicity than **1**.[18,20] Phase I clinical studies have shown[21] that **5** causes severe local toxicity presumably due to the high concentration of the 4-hydroxy compound **2** and its subsequent toxic metabolites.

We describe here our studies of the hydrolysis of *cis*-**5** and *cis*-**2** under a variety of conditions to determine the activation mechanisms of mafosfamide (**5**) and the possible role of thiols in cyclophosphamide metabolism.

Results and Discussion

Hydrolyses of **5** and **2** were monitored by ^{31}P NMR spectroscopy, and the intermediates were quantitated by peak intensity (see Experimental Section for details); a representative spectrum is shown in Figure 1. *cis*-**5** rapidly equilibrated with *trans*-**5** in phosphate buffer (100 mM, pH 7.4, 37 °C). An equilibrium cis-trans ratio of 1.15 ± 0.05 was reached within 5 min (Figure 2A) and the ratio remained constant throughout the 2.5-h time course. The equilibrium ratio was independent of buffer structure and concentration, pH, and ionic strength. The relative rates of appearance of *cis*-4-hydroxycyclophosphamide (*cis*-**2**) and *trans*-**2** from **5** suggested that the formation of *cis*-**2**

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(1) Friedman, O. M.; Myles, A.; Colvin, M. *Adv. Cancer Chemother.* **1979**, *1*, 143-204.
(2) Stec, W. J. *Organophosphorus Chem.* **1982**, *13*, 145-174.
(3) Zon, G. *Prog. Med. Chem.* **1982**, *19*, 205.
(4) Borch, R. F.; Hoye, T. R.; Swanson, T. A. *J. Med. Chem.* **1984**, *27*, 490.
(5) Zon, G.; Ludeman, S. M.; Brandt, J. A.; Boyd, V. L.; Ozkan, G.; Egan, W.; Shao, K.-L. *J. Med. Chem.* **1984**, *27*, 466.
(6) Borch, R. F.; Millard, J. *J. Med. Chem.* **1987**, *30*, 427.
(7) Powers, J. F.; Sladek, N. E. *Cancer Res.* **1983**, *43*, 1101.
(8) Hohorst, H.-J.; Drager, U.; Peter, G.; Voelcker, G. *Cancer Treat. Rep.* **1976**, *60*, 309.
(9) Draeger, U.; Peter, G.; Hohorst, H.-J. *Cancer Treat. Rep.* **1976**, *60*, 355.
(10) Peter, G.; Hohorst, H.-J. *Cancer Chemother. Pharmacol.* **1979**, *3*, 181.
(11) Niemeyer, U.; Engel, J.; Scheffler, G.; Molge, K.; Sauerbier, D.; Weigert, W. *Invest. New Drugs* **1984**, *2*, 133.
(12) Klein, H. O.; Wickramanayake, P. D.; Christian, E.; Coerper, C. *Invest. New Drugs* **1984**, *2*, 191.
(13) Alberts, D. S.; Einspahr, J. G.; Struck, R.; Bignami, G.; Young, L.; Surwit, E. A.; Salmon, S. E. *Invest. New Drugs* **1984**, *2*, 141.
(14) Zaharko, D. S.; Covey, J. M.; Horpel, G. *Invest. New Drugs* **1984**, *2*, 149.
(15) Berger, M. R.; Bedford, P.; Zeller, W. J.; Kaufmann, M. *Invest. New Drugs* **1984**, *2*, 181.
(16) Nowrousian, M. R.; Schmidt, C. G. *Invest. New Drugs* **1984**, *2*, 207.
(17) Gorski, A.; Korczak-Kowalska, G. *Invest. New Drugs* **1984**, *2*, 227.
(18) Pohl, J. Asta-Z-7557, Summary for Investigators. Preclinical Report, Asta-Werke, 1983.
(19) Atassi, G.; Hilgard, P.; Pohl, J. *Invest. New Drugs* **1984**, *2*, 169.
(20) Pohl, J.; Hilgard, P.; Jahn, W.; Zechel, H. J. *Invest. New Drugs* **1984**, *2*, 201.
(21) Bruntsch, U.; Groos, G.; Hiller, T. A.; Wandt, H.; Tigges, F.-J.; Gallmeier, W. M. *Invest. New Drugs* **1985**, *3*, 293.

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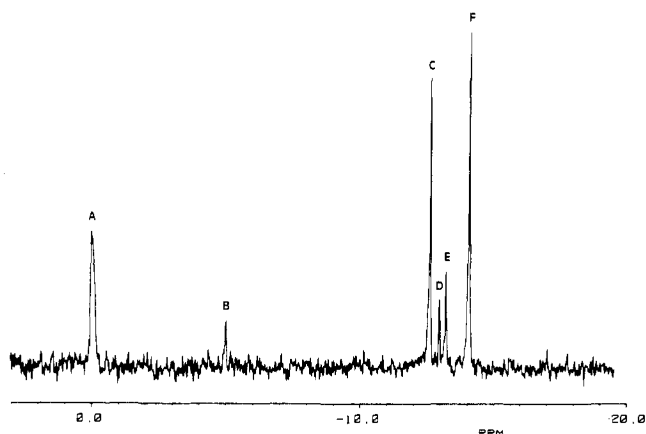


Figure 1. Partial display of a representative ^{31}P spectrum of *cis*-mafosfamide (*cis*-5) equilibrated in acetate buffer (100 mM, pH 5.55, 37 °C): (A) triphenylphosphine oxide (coaxial reference in toluene-d_8), (B) 3 and equivalents, (C) *trans*-5, (D) *trans*-2, (E) *cis*-2, (F) *cis*-5.

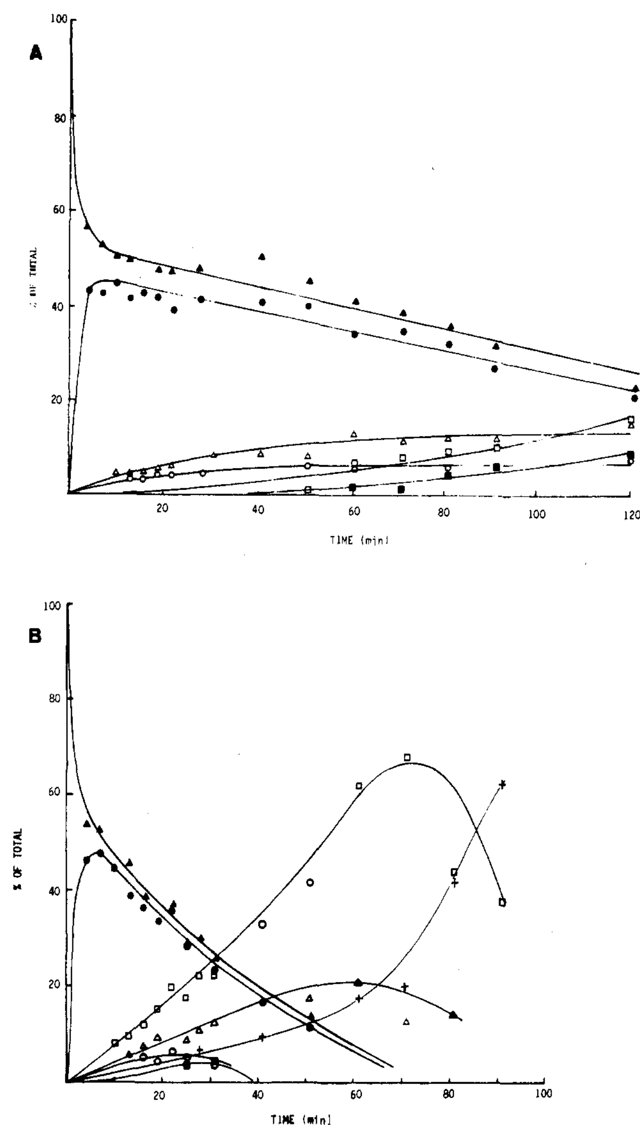


Figure 2. Reaction of *cis*-mafosfamide (*cis*-5) with phosphate buffer (100 mM, pH 7.4, 37 °C) (A) and with plasma (B): (▲) *cis*-5, (●) *trans*-5, (△) *cis*-2, (○) *trans*-2, (■) 3 plus equivalents, (□) 4, (+) 7.

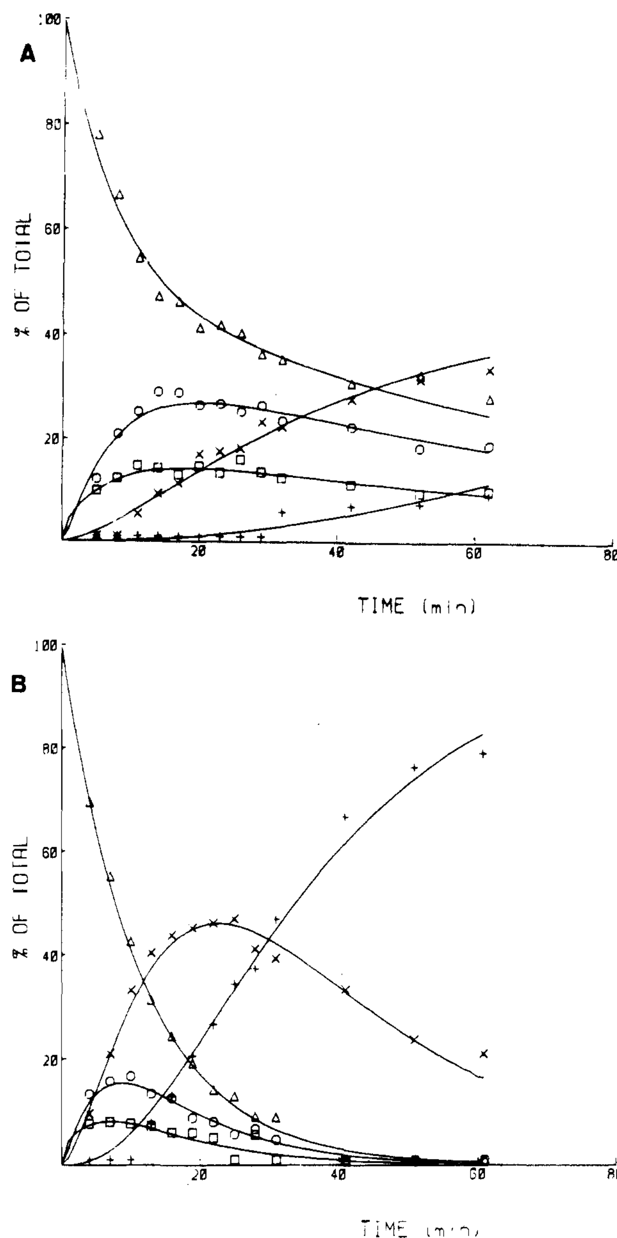


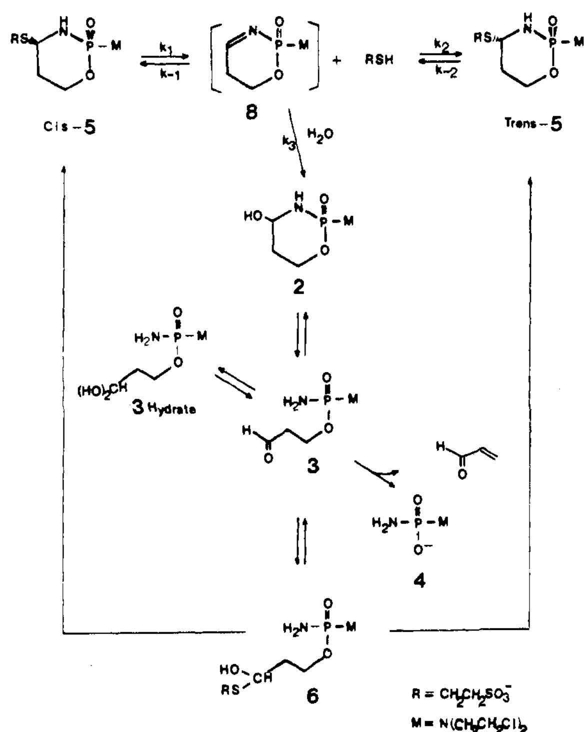
Figure 3. Reaction of *cis*-4-hydroxycyclophosphamide (*cis*-2) with phosphate buffer (100 mM, pH 7.4, 37 °C) (A) and with plasma (B): (△) *cis*-2, (○) *trans*-2, (□) 3 and its hydrate, (x) 4, (+) 7.

might be faster than *trans*-2. This was further supported by the observation that the overall decomposition pattern of *cis*-5 in the presence of *N*-ethylmaleimide (NEM) (to trap the liberated thiol) was virtually identical with that of *cis*-2. Aldophosphamide (3), its hydrate, or its hemithioacetal (6) were first observed at 80 min. Phosphoramide mustard (4) was not detected until 60 min when there was a sufficient amount of 2 generated from 5. This is consistent with the earlier observation that mafosfamide (5) did not lose alkylating capacity in buffered aqueous solution over a 30-min period.[22]

Having examined the hydrolysis reaction of 5 in aqueous buffer, it was of interest to investigate the reaction in human plasma and to compare this with reaction in aqueous buffer. The buffering capacity of plasma was maintained by the addition of noncatalytic concentrations of buffer. The rate of disappearance of *cis*- and *trans*-5 in plasma was much faster than in buffer with a concomitant enhanced appearance rate of phosphoramide mustard (4) and its products (7) (Figure 2B). However, the *cis*/

(22) Sladek, N. E.; Powers, J. F.; Grage, G. M. *Drug Metab. Dispos.* 1984, *12*, 553.

Scheme I



trans-**5** equilibration process was not affected. The conversion of *cis*-**2** to phosphoramide mustard (**4**) and its product **7** was also much faster in plasma than in buffer, and the generation of **4** and **7** in plasma was too fast for *cis*-**2** to establish "pseudoequilibrium" observed[5,6] in buffer systems (Figure 3). It is interesting to note that the in vitro plasma half-life of the equilibrium mixture extrapolated from Figure 3B ($t_{1/2}$ = 13.5 min) is essentially the same as that reported in vivo in the rat ($t_{1/2}$ = 14 min).[22]

In acetate buffer (50–200 mM, pH 5.25–5.90, 37 °C), where generation of phosphoramide mustard (**4**) from the 4-hydroxy compound **2** is negligible,[6] *cis*-**5** slowly equilibrated with *trans*-**5** and other metabolites. The equilibrium mixture consisted of *cis*-**5**, *trans*-**5**, aldehyde **3**/hydrate/hemithioacetal **6**, *cis*-**2**, and *trans*-**2** present in the approximate ratio 40:35:6:12:8. Addition of excess MESNA (5 equiv) to the reaction mixture prevented disappearance of **5** although it had no effect on the *cis*-/*trans*-**5** equilibration rate. In contrast, addition of excess NEM (5 equiv) to the reaction mixture trapped MESNA released from *cis*-**5** and thus accelerated its apparent disappearance. These results suggest that the reaction of **5** proceeds via iminocyclophosphamide (**8**); in the presence of excess MESNA, imine **8** is efficiently intercepted by the more nucleophilic thiol, thus preventing hydrolysis of **5** and resulting in the *cis*-/*trans*-**5** equilibration. On the other hand, in the presence of excess NEM, thiol is removed from the reaction and **8** is readily trapped by water to form 4-hydroxycyclophosphamide (**2**) (Scheme I).

These two modified reaction systems allowed us to study the rate of disappearance of *cis*-**5** more effectively. The kinetics were treated by using the steady-state approximation for the imine **8** as an intermediate and the following set of differential equations:

$$dC/dt = -k_1[C] + k_{-1}[I][S] \quad (1)$$

$$dT/dt = k_2[I][S] - k_{-2}[T] \quad (2)$$

$$dI/dt = k_1[C] + k_{-2}[T] - [I][S](k_{-1} + k_2) - k_3[I] \quad (3)$$

where C = *cis*-**5**, T = *trans*-**5**, I = **8**, and S = MESNA.

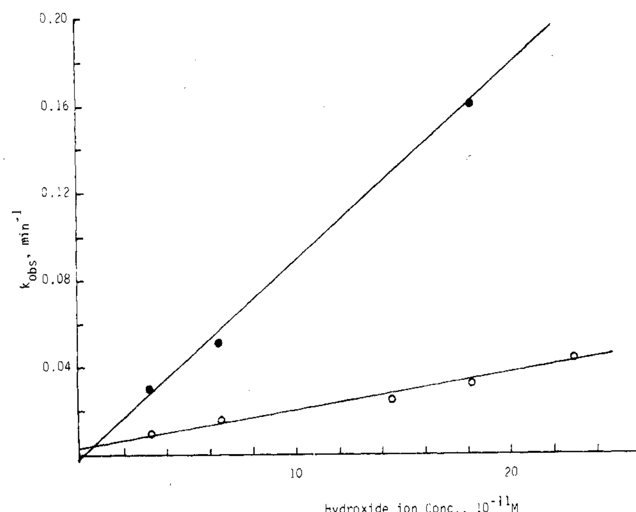


Figure 4. Effect of hydroxide ion concentration on the rate constant for the disappearance of *cis*-**5**, acetate buffer (100 mM, 37 °C), in the presence of 5 equiv of NEM (●) (r^2 = 0.996) or in the presence of 5 equiv of MESNA (○) (r^2 = 0.971).

Assuming $dI/dt = 0$ at steady state, solving eq 3 for [I] and substituting into eq 1 and 2, we obtain

$$dC/dt = [-k_1[C](k_3 + k_2[S]) + k_{-1}k_{-2}[S] \times [T]]/[k_3 + [S](k_{-1} + k_2)] \quad (4)$$

$$dT/dt = [k_1k_2[S][C] - k_{-2}[T](k_3 + k_{-1}[S])]/[k_3 + [S](k_{-1} + k_2)] \quad (5)$$

In the presence of excess NEM, [T] = [S] = 0 and eq 4 simplifies to $dC/dt = -k_1[C]$ as expected for a first-order reaction. Thus, the observed rate constant (k_{obsd}) in the presence of excess NEM gives k_1. In the presence of excess MESNA, reaction of the imine with water does not compete with MESNA, $k_3 = 0$, and we obtain the following simplified equations:

$$dC/dt = [-k_1k_2[C] + k_{-1}k_{-2}[T]]/[k_{-1} + k_2] \quad (6)$$

$$dT/dt = [k_1k_2[C] - k_{-1}k_{-2}[T]]/[k_{-1} + k_2] \quad (7)$$

As noted from eq 6 and 7, the reaction rates are independent of the MESNA concentration under these conditions. Figure 4 shows the effect of hydroxide ion concentration on the rate of disappearance of *cis*-**5**. The observed rate constant in the presence of excess NEM is equal to k_1, and the fractional decrease in k_{obsd} when measured in the presence of excess MESNA reflects the fraction of imine **8** that undergoes back-reaction to *cis*-**5**. From Figure 4, $k_{obsd} = k_1/5.59$, indicating that 82% of imine **8** returns to *cis*-**5** and 18% proceeds to *trans*-**5**:

$$k_{-1}/k_2 = 4.59 \quad (8)$$

And from the *cis*-/*trans*-**5** equilibrium ratio presented earlier, we have

$$k_{-1}k_{-2}/k_1k_2 = 1.15 \quad (9)$$

On the basis of our failure to observe **8**, an upper limit of about 0.02 is placed on the equilibrium constant K_1:

$$K_1 = k_1/k_{-1} \leq 0.02 \quad (10)$$

Now, all four rate constants can be estimated from the observed rate constant ($k_{obsd} = k_1$ for the reactions in the presence of NEM) by using eq 8–10. As an example, the estimated rate constants at pH 7.4 are k_1 = 4.1 min^{-1}, k_{-1} ≥ 204 min^{-1} M^{-1}, k_2 ≥ 45 min^{-1} M^{-1}, and k_{-2} = 1.0 min^{-1}.

The effects of pH and buffer concentration were examined in acetate and phosphate buffer (Table I). The rate

Table I. Rate Constants for the Apparent Rate of Disappearance of *cis*-Mafosfamide (*cis*-5) under Various Conditions at 37 °C ($r^2 > 0.970$)

buffer				
structure	concn, mM	ionic strength, μ	pH	k_{obsd}, min^{-1}
acetate	100	0.3	5.25	0.017
	100	0.3	5.55	0.023
	100	0.3	5.90	0.032
	50	0.3	5.90	0.029
	200	0.3	5.90	0.032
phosphate	50	1.0	5.55	0.035
	100	1.0	5.55	0.034
	200	1.0	5.55	0.033
acetate	100	1.0	5.85	0.043
phosphate	100	1.0	5.85	0.045
acetate + MESNA	100	0.3	5.25	0.009
(5 equiv)	100	0.3	5.55	0.016
	100	0.3	5.90	0.024
acetate + NEM (5	100	0.3	5.25	0.030
equiv)	100	0.3	5.55	0.050
	100	0.3	6.00	0.161
	100 (D_2O)	0.3	pD 6.00	0.040

of disappearance of *cis*-5 was first order with respect to hydroxide ion concentration but independent of buffer structure and concentration. Extrapolation to zero hydroxide ion concentration indicated that catalysis by water was negligible (Figure 4). Thus the reaction of *cis*-5 is specific-base catalyzed.

The initial disappearance rates of *cis*-5 were also studied in H_2O vs. D_2O (100 mM acetate buffer, pH 6.0, 37 °C); the observed rate constants were 0.16 and 0.04 min^{-1}, respectively, corresponding to a "solvent" isotope effect of approximately 4. An isotope effect of this magnitude is presumably a primary deuterium isotope effect resulting from prior rapid exchange of the N–H with solvent and reflects considerable N–H bond breaking in the transition state. An isotope effect of similar magnitude was observed in the base-catalyzed hydrolysis of 4-hydroperoxycyclophosphamide (**9**).[23] It is interesting to note that elimination of hydrogen peroxide from **9** exhibits general-base catalysis, whereas elimination of the thiol MESNA from **5** is specific-base catalyzed. The reaction of **5** may in fact be general-base catalyzed with a Brønsted slope β near unity, where the effects of buffer and solvent would not be detected. However it is unlikely that replacing HOO^- with $^-O_3SCH_2CH_2S^-$ would cause a change in β from 0.5 to 1. Alternatively, both reactions may be subject to the kinetically equivalent specific base–general acid catalysis, where the role of general acid contributes to the expulsion of the more basic leaving group (HOO^-) but not to the less basic thiolate anion.

Having established that hydrolysis of *cis*-mafosfamide (*cis*-5) proceeds via imine **8** by a base-catalyzed process, it was of interest to study the reverse reaction, i.e., the formation of **5** from 4-hydroxycyclophosphamide (**2**) with MESNA (see Scheme I). Reaction of *cis*-2 with phosphate buffer (100 mM, pH 7.4, 37 °C) in the presence of 1 equiv of MESNA generated not only the usual reaction products of *cis*-2 but also a small amount of both *cis*-5 and *trans*-5. Both isomers reached a maximum of approximately 6.5% of the total mixture in 13 min and subsequently disappeared by 22 min. At this pH, addition of excess MESNA resulted in a significant increase in ring-opened intermediates, although the generation of **5** and **4** did not appear to be affected. This indicates that the formation of hemithioacetal (**6**) by reaction of aldehyde **3** and MESNA is

(23) Borch, R. F.; Getman, K. M. *J. Med. Chem.* 1984, *27*, 485.

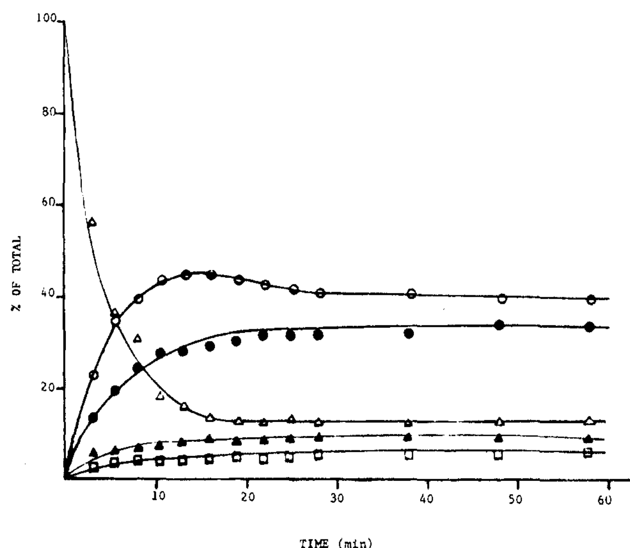


Figure 5. Reaction of *cis*-4-hydroxycyclophosphamide (*cis*-2) with acetate buffer (100 mM, pH 5.55, 37 °C), μ = 0.3, in the presence of MESNA (1 equiv): (△) *cis*-2, (▲) *trans*-2, (○) *cis*-5, (●) *trans*-5, (■) 3 and equivalents.

faster than the ring closure of **6** to either isomer of **5**, and the latter process is rate determining for the formation of **5** from **2** and MESNA in aqueous buffer at pH 7.4. Similarly, β-elimination from the aldehyde **3** to generate phosphoramide mustard (**4**) is considerably slower than the interconversions of **3**/**3** hydrate/hemithioacetal (**6**).

The formation of both *cis*-5 and *trans*-5 from *cis*-2 was much faster at lower pH (100 mM acetate, pH 5.25–5.90, 37 °C) and in the presence of MESNA (1 equiv) than from an identical reaction at pH 7.4. Approximately 30–35% of the total mixture was composed of *cis*-5 and *trans*-5 within 3 min (Figure 5). The formation of **4** from **3** by β-elimination is negligible[6] and the same equilibrium mixture was observed as for the reaction of *cis*-5 presented earlier. Furthermore, *cis*-2 was completely converted to both *cis*-5 and *trans*-5 (ratio 1.6:1) within 10 min in acetate buffer (100 mM, pH 5.55, 37 °C) and in the presence of excess MESNA (5 equiv); *trans*-2 was never observed. This indicates that the reaction of MESNA with *cis*-2 via aldehyde **3** to hemithioacetal **6**, followed by its ring closure to **5**, is faster than the ring closure of aldehyde **3** to **2**. Under identical reaction conditions, *cis*-5 equilibrated with *trans*-5 via imine **8** with $t_{1/2}$ = 17 min. However, addition of MESNA to **8** favors the formation of *cis*-5 over *trans*-5 by a factor of 4.59. The ratio of product stereoisomers observed in the reaction of **2** and MESNA is not consistent with that found in the addition of MESNA to **8**; this argues against the imine as an alternate intermediate in the formation of **5** from **2**.

Conclusion

In summary, these data suggest that, under model physiologic conditions, 4-alkylthio-substituted cyclophosphamide derivatives are activated by base-catalyzed elimination to form imine **8** as a transient intermediate. The imine may be trapped by the liberated thiol or by biological sulfhydryl containing compounds, viz., glutathione, to form thio adducts, or by water to form 4-hydroxycyclophosphamide (**2**). The hydrolytic product **2** then undergoes ring-opening reaction to aldehyde **3** followed by subsequent generation of the ultimate alkylating agent, phosphoramide mustard **4**. Under more acidic conditions, the elimination of **4** from **3** is suppressed, and **3** can react competitively with thiols to form hemithioacetal intermediates that subsequently cyclize to the 4-thio

adducts. This reaction may explain the bladder protective effect observed both in experimental animals[24,25] and patients[26,27] given 1 and MESNA and also in experimental animals[28] given 1 and agents that acidify the urine. The demonstration that 4-hydroxycyclophosphamide (**2**) can react with MESNA to form the cyclized thio adduct **5** suggests that 1, after bioactivation to **2**, may generate the imine **8** via cyclized thio adducts resulting from the reaction of **2** and biological thiols. This implies a biological role for the imine **8** as a common intermediary metabolite of both **5** and 1 in vivo.

Experimental Section

cis-Mafosfamide (*cis*-**5**) was kindly provided by Drs. J. Engel and U. Niemeyer. 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. ^1H 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 internal 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 pulse 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 of chemical shifts for *cis*-**5**, *trans*-**5**, **9**, *cis*-**2**, *trans*-**2**, **3**/**3** hydrate/**6**, and **4** are based on the chemical shifts of authentic samples and previously published data[4,6] are −13.7, −12.2, −13.5, −12.8, −12.5, −4.6, and −11.3 ppm, respectively. Aldehyde **3**, its hydrate, and its hemithioacetal **6** were not resolved under these conditions. However, reaction of propionaldehyde with MESNA (1 equiv of each) in D_2O was examined by ^1H NMR; the equilibrium mixture consisted of the free aldehyde, its hydrate, and its hemithioacetal in the approximate ratio of 1:2:8. A similar equilibrium ratio of **3**/**3** hydrate/**6** was assumed in the reaction of *cis*-**2** and MESNA. Acidity measurements were made on a Radiometer pH meter using a glass–calomel combination electrode; pD values represent the meter reading of D_2O solutions and are related to pH according to pH = pD + 0.4. Buffer solutions were maintained at 37 ± 0.1 °C by a VWR 1145 constant-temperature circulator. Water was purified with a Barnstead Nanopure II System.

***cis*-4-Hydroperoxycyclophosphamide (9)** was prepared in 33% yield as described elsewhere:[6] mp 106–107 °C dec (lit.[29] mp 107–108 °C); ^1H NMR (Me_2SO-d_6) 11.56 (1 H, s, OOH), 5.88–5.92 (1 H, m, NH), 4.92 (1 H, d of dd, J_{HP} = 25 Hz, H_4), 4.25–4.40 (1 H, m, H_{6a}), 4.00–4.20 (1 H, m, H_{6e}), 3.61–3.67 (4 H, m, CH_2Cl), 3.23–3.32 (4 H, m, NCH_2), 1.87–1.93 (2 H, m, H_5).

NMR Studies. Unless otherwise noted, NMR studies were carried out as described previously.[6] All ^{31}P NMR kinetics were conducted at 37 ± 2 °C. Solutions of *cis*-**2** were prepared immediately prior to use by dissolving the appropriate quantity of **9** in approximately 50–100 μL of methanol (<5% of final volume) and treating this solution with 4 equiv of dimethyl sulfide at 37 °C. The appropriate buffer (prewarmed to 37 °C) was then added to give a final concentration of 30–50 mM. Solutions of *cis*-**5** were also prepared immediately prior to use by addition of appropriate buffer to give a final concentration of 25–50 mM. Ionic strength of the buffer reaction mixtures for comparison study with plasma was adjusted by addition of NaCl to mimic that of plasma and a typical buffer mixture consisted of 1.7 mL of 100 mM phosphate buffer and 0.3 mL of D_2O. Buffer strength of the plasma reaction mixtures was adjusted by addition of concentrated phosphate buffer to be equivalent to that of buffer and a typical plasma mixture consisted of 1.6 mL of human plasma, 0.17 mL of 1 M phosphate buffer, and 0.23 mL of D_2O. The sample was then introduced into the preequilibrated spectrometer probe, and 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 heights of their respective phosphorus resonances.

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Registry No. **2**, 61903-29-5; **3**, 35144-64-0; **4**, 10159-53-2; *cis*-**5**, 84210-72-0; *trans*-**5**, 92745-18-1; **6**, 105639-84-7; **8**, 84489-09-8; **9**, 56922-83-9; MESNA, 19767-45-4.

(24) Freedman, A.; Ehrlich, R. M.; Ljung, B.-M. *J. Urol.* **1984**, *132*, 580.
(25) Brock, N.; Stekar, J.; Pohl, J.; Niemeyer, U.; Scheffler, G. *Arzneim.-Forsch.* **1979**, *29*, 659.
(26) Hows, J. M.; Mehta, A.; Ward, K.; Woods, K.; Perez, R.; Gordon, M. Y.; Gordon-Smith, E. C. *Br. J. Cancer* **1984**, *50*, 753.
(27) Link, H.; Neef, V.; Niethammer, D.; Wilms, K. *Blut* **1981**, *43*, 329.
(28) Sladek, N. E.; Smith, P. C.; Bratt, P. M.; Low, J. E.; Powers, J. F.; Borch, R. F.; Coveney, J. R. *Cancer Treat. Rep.* **1982**, *66*, 1889.
(29) Takamizawa, A.; Matsumoto, S.; Iwata, T.; Tochino, Y.; Katagiri, K.; Yamaguchi, K.; Shiratori, O. *J. Med. Chem.* **1975**, *18*, 376.