and immediately analyzing it by HPLC. Each experiment was carried out in duplicate.

Short-Term (<0.5 Half-Life) Kinetics. Because of the short time-point intervals desired (2–4 min) and the long time (8 min) required between HPLC injections, each data point required a separate experiment for determination. Thus, acetophenone was used as an internal standard and was introduced by addition to the stock solution of **5** at a concentration of 10 mM (final acetophenone concentration in the reaction solution was 0.5 mM). For each data point, 5 μL of stock solution was added to 95 μL of buffer solution at the desired temperature. HPLC analysis was carried out after the appropriate reaction time as described above. Initial peak heights were determined in triplicate for each run, and each data point was determined in duplicate. Peak heights were corrected for the reference peak height in all cases. Peak heights at time t were expressed as a fraction of initial peak height for all data calculations; rate constants were determined both by linear (short-term kinetics) and nonlinear (long-term kinetics) least-squares techniques.

Acknowledgment. This investigation was supported by PHS Grants CA 11198, CA 30348, and CA 34619, awarded by the National Cancer Institute, DHHS. We also thank Dr. Norman E. Sladek for his helpful suggestions and stimulating discussions during the course of this work.

Registry No. 1, 50-18-0; **5**, 56922-83-9; **6**, 61903-31-9; **8a**, 88685-77-2; **8b**, 88685-76-1; HPO_4^{2-}, 14066-19-1; Tris, 77-86-1; hydroxide, 14280-30-9; propionaldehyde, 123-38-6.

In Situ Preparation and Fate of *cis*-4-Hydroxycyclophosphamide and Aldophosphamide: ^1H and ^{31}P NMR Evidence for Equilibration of *cis*- and *trans*-4-Hydroxycyclophosphamide with Aldophosphamide and Its Hydrate in Aqueous Solution[1]

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cis-4-Hydroxycyclophosphamide (**2**) and aldophosphamide (**4**) were generated in aqueous phosphate or cacodylate buffer by dimethyl sulfide reduction of *cis*-4-hydroperoxycyclophosphamide (**8**) and by sodium periodate cleavage of 3,4-dihydroxybutyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate (**9**), respectively; the reactions of **2** and **4** were examined by ^1H and ^{31}P NMR. Within 30–60 min (pH or pD 7.0, 25 °C) the same pseudoequilibrium mixture was established in both reactions, with *cis*- and *trans*-4-hydroxycyclophosphamide (**2** and **3**), aldophosphamide (**4**), and its hydrate (**5**) present in the approximate ratio of 4:2:0.3:1. Structures of the intermediates were assigned unambiguously based upon analysis of the chemical shifts and coupling constants in the proton spectra determined in D_2O buffers, and the ^{31}P assignments followed by correlation of component ratios at equilibrium. Free energy differences of 0.4, 0.4, and 0.7 kcal/mol at 25 °C were estimated between **2**, **3**, **5**, and **4**, respectively, with **2** being the most stable. The aldehyde **4** reacted most rapidly with water to give hydrate **5**; cyclization of **4** to **3** occurred faster than to **2**, and the rate of cyclization to **2** was comparable to that for elimination to **6**. Compound **5** is formed much faster than **3** from the diol cleavage, but **5** and **3** are produced at comparable rates from **2**, suggesting that conversion of **2** to **3** can proceed by a mechanism other than ring opening. The rate of equilibration appears to be independent of buffer structure, indicating that bifunctional catalysis is not important in the ring-opening reaction. β-Elimination from **4** is rate limiting for the production of **6** and acrolein, and the rate for phosphate is 2- to 3-fold faster than for cacodylate under identical conditions. These results provide the first definitive evidence for the stability of the elusive aldehyde **4** in aqueous solution and for the existence of a preequilibrium among **2**–**5** prior to rate-limiting expulsion of phosphoramide mustard from **4**.

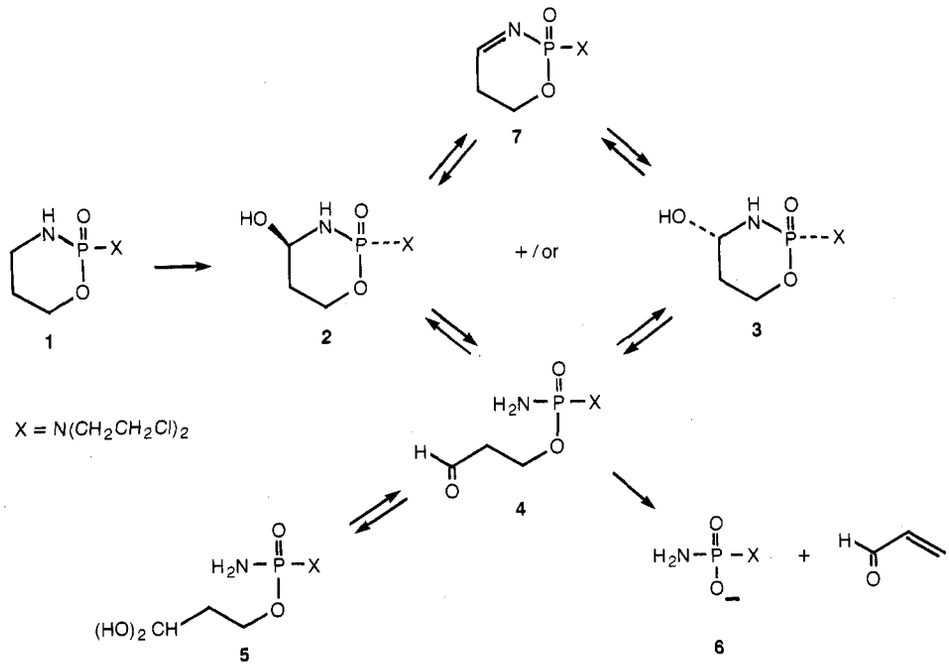
Cyclophosphamide (**1**) is a highly effective and extensively used agent for the treatment of many human malignancies. Its mechanism of action and the associated chemistry have been the subject of several recent reviews.[2-4] Activation of the prodrug **1** is initiated by hepatic P450 hydroxylation to produce one or both isomers[5] of 4-hydroxycyclophosphamide (**2** and/or **3**; see Scheme I). The 4-hydroxy compounds presumably undergo carbinol amide–amido aldehyde ring opening to generate aldophosphamide **4** which in turn undergoes base-catalyzed elimination to produce phosphoramide mustard (**6**) and acrolein. Intermediates **2**–**4** are susceptible to further oxidation, leading to 4-oxocyclophosphamide and carboxyphosphamide, respectively. These oxidations appear to represent the major pathway for drug detoxication in vivo. Although **4** is an obligatory intermediate in the activation sequence, efforts to isolate and characterize it have met with limited success.[6] The presence of hydrate **5** has been suggested,[6] but definitive evidence for its existence in equilibrium with **4** is lacking. Persuasive evidence has been presented for imine **7** as an intermediate in the aqueous reactions of 4-hydroperoxycyclophosphamide (**8**) and in the enzymatic oxidation of **1**;[7] recent data have confirmed

[†] University of Rochester.
[‡] University of Minnesota.

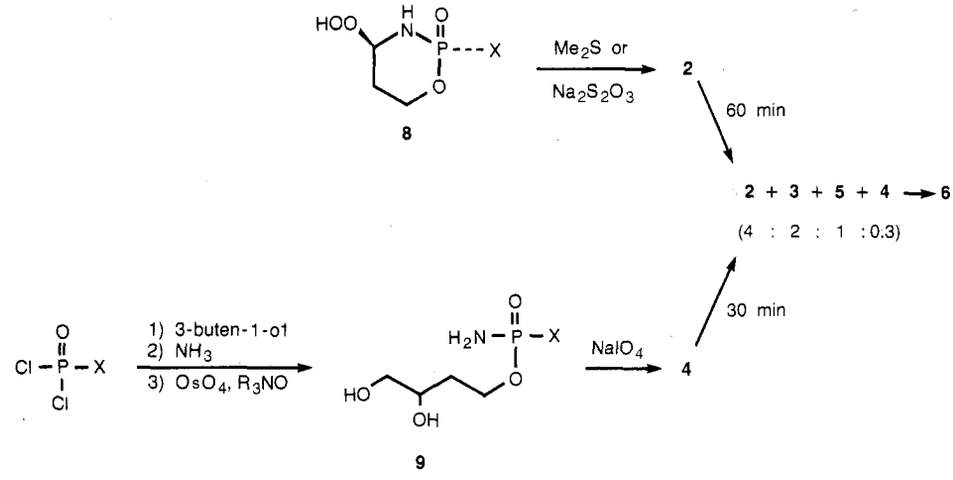
(1) A portion of this work was presented at the 74th meeting of the American Association for Cancer Research, San Diego, CA, May 25, 1983. See: Borch, R. F.; Swanson, T. A. *Proc. Am. Assoc. Cancer Res.* **1983**, *24*, 247.
(2) Friedman, O. M.; Myles, A.; Colvin, M. *Adv. Cancer Chemother.* **1979**, *1* 143–204.
(3) Stec, W. J. *Organophosphorus Chem.* **1982**, *13*, 145–174.
(4) Zon, G. *Prog. Med. Chem.* **1982**, *19*, 205.
(5) Cis and trans refers to the relationship between the substituent and the phosphoryl oxygen, according to the convention established by: Kinas, R.; Pankiewicz, K.; Stec, W. J. *J. Org. Chem.* **1977**, *42*, 1650.
(6) (a) Struck, R. F. *Cancer Treat. Rep.* **1976**, *60*, 317. (b) Myles, A.; Fenselau, C.; Friedman, O. M. *Tetrahedron Lett.* **1977**, 2475.
(7) Fenselau, C.; Lehman, J. P.; Myles, A.; Brandt, J.; Yost, G. S.; Friedman, O. M.; Colvin, O. M. *Drug Metab. Dispos.* **1982**, *10*, 636.

0022-2623/84/1827-0490$01.50/0

Scheme I



Scheme II



that **7** can be produced as a transient intermediate via general-base-catalyzed elimination of hydrogen peroxide from **8**, but **7** was not detectable spectroscopically.[8] Although it is generally agreed that the aziridinium ion derived from cyclization of **6** is the ultimate cytotoxic metabolite, controversy continues regarding extracellular **6**[10] vs. extracellular **2**/**3**/**4**[11] as the important circulating metabolite and the related question of **2**/**3**/**4** as obligatory transport forms of the drug. The basis for cyclophosphamide's oncotoxic specificity remains unknown, although hypotheses based upon selective metabolism and/or distribution of specific intermediates have been suggested.[1] Thus, the nature of the interconversions among **2**–**7** may be of considerable importance to cytotoxic specificity, especially insofar as differences in intracellular environment might alter these reactions and, ultimately, the intracellular activation of the drug. ^{31}P NMR has been used extensively to study several aspects of cyclophosphamide metabolism;[4,9] its major drawback, however, is the difficulty of making rigorous structural assignments for intermediates whose spectral features cannot be compared with those of authentic samples. Our major objective was to generate **2** and **4** in situ from synthetic intermediates and then utilize both ^{1}H and ^{31}P NMR to monitor the interconversions indicated in Scheme I. Although our initial plan was to utilize the proton spectra to provide specific assignments for the ^{31}P resonances, we have shown that ^{1}H NMR is very useful as a kinetic probe in its own right.

Results and Discussion

The penultimate compounds required for NMR studies were *cis*-4-hydroperoxycyclophosphamide (**8**) and the open-chain diol **9** (see Scheme II); reduction of the former should produce **2** stereospecifically, while periodate cleavage of the latter should generate aldehyde **4** directly. Thus, the fate of **2** and **4** could be examined separately, and reversible interconversion of **2** and **4** could be studied from both directions. The hydroperoxy derivative **8** was

(8) Borch, R. F.; Getman, K. M. *J. Med. Chem.*, preceding paper in this issue.
(9) Engle, T. W.; Zon, G.; Egan, W. *J. Med. Chem.* **1982**, *25*, 1347, and references cited therein.
(10) Struck, R. F.; Dykes, D. J.; Corbett, T. H.; Suling, W. J.; Trader, M. W. *Br. J. Cancer* **1983**, *47*, 15, and references cited therein.
(11) Powers, J. F.; Sladek, N. E. *Cancer Res.* **1983**, *43*, 1101.

prepared by ozonolysis of cyclophosphamide by a modification of the published procedure[12] and has been described elsewhere.[8] The ^1H spectrum of **8** in D_2O showed each ring proton clearly resolved, with all coupling constants, except those of the C-5 equatorial, readily assignable. Of particular interest was the apparent doublet of triplets centered at 5.17 ppm arising from the C-4 proton. Diol **9** was prepared as a 1:1 mixture of diastereomers in 61% yield by osmium tetroxide–*N*-methylmorpholine *N*-oxide oxidation[13] of the known[14] butenyl precursor. In a preliminary experiment, **8** was reacted at ~25 °C with excess aqueous sodium thiosulfate[15] and monitored by ^{31}P NMR. The resonance for **8** at −14.5 ppm[16] was replaced within minutes by a signal at −13.92 ppm, which we tentatively assigned to **2** on the basis of the expected stereospecific reduction of **8**. This resonance gradually decreased and was replaced by two new signals at −13.72 and −5.7 ppm. A minor peak (<10% of the total) was present at −16.12 ppm after the initial reduction and remained invariant. This peak was absent when the reduction was carried out with dimethyl sulfide (vide infra); so we assume that this peak represents a thiosulfate adduct, perhaps arising via competitive elimination–addition to **8**.[8] Subsequent reductions were carried out with dimethyl sulfide to avoid formation of this byproduct. We tentatively assigned the signal at −5.7 ppm to the trans 4-hydroxy compound **3**, based upon our initial observations of a new signal approximately 9-ppm downfield from **8** when **8** was reacted with Tris buffer.[17] Subsequent ^1H NMR spectra and further studies with Tris[8] showed that this tentative assignment was incorrect and that the signals at −13.72 and −5.7 ppm can be assigned to **3** and **4**/**5**, respectively.

Reaction of **8** dimethyl sulfide in cacodylate buffer (0.2 M, pH 7.0, ~25 °C) resulted in clean conversion of **8** to **2** (−14.5 to −13.9 ppm) within 10 min. Again the signal attributed to **2** gradually decreased and was replaced by new signals at −13.65 and −5.66 ppm; after 50 min, the relative intensities of these signals stabilized at a ratio of approximately 4:2:1 (−13.90/−13.65/−5.66 ppm), which remained constant as the intensities decreased over the next 2 h. After 90 min, a new signal was evident at −12.55 ppm, which we assign to **6** based upon subsequent addition of an authentic sample (as the cyclohexylamine salt). Over the next 90 min the signal at −12.55 ppm increased at the expense of the others, although the 4:2:1 ratio remained constant. A similar experiment carried out with phosphate buffer also produced the same constant ratio of these three resonances; formation of **6**, however, was evident at 50 min and increased approximately 2- to 3-fold faster with phosphate than with cacodylate.

Reduction of **8** was then examined by ^1H NMR; it was hoped that changes in the C-4 proton region of the spectrum (5.1–5.3 ppm) could be correlated with the steady-state composition observed in the ^{31}P spectrum. Addition of dimethyl sulfide to a solution of **8** in phosphate buffer (0.1 M in D_2O, pD 7.0, ~25 °C) resulted in a small (3 Hz) downfield shift in the C-4 proton resonances; a partial spectrum of the initial reduction product is shown in Figure 1A. The value of J_{H4-P} (24.5 Hz) confirms that the hydroxyl group has remained axial, and the chemical shifts and coupling patterns of the C-6 protons indicate that the phosphoryl oxygen is still axial in **2**.[18] After 30 min, the resonances from **2** had decreased, and a complex multiplet of new signals appeared (Figure 1B). At 60 min, the spectral changes were complete, and the spectral pattern shown in Figure 1C persisted for at least 2 h, albeit with progressively decreasing intensity. Spectral assignments were made based upon the work of Bentrude et al.[18] and were confirmed by the results of the diol cleavage experiment discussed below and by selective proton-decoupling experiments (data not shown). One set of new signals was

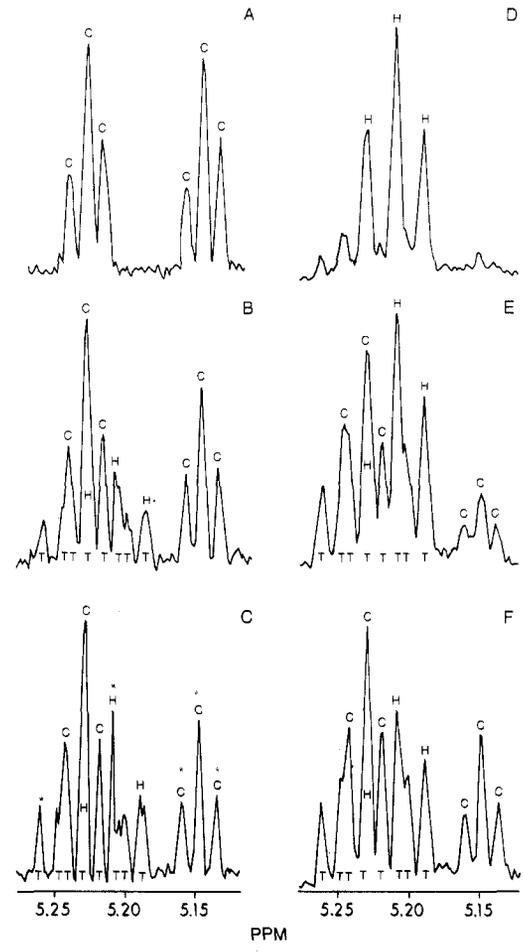


Figure 1. A–C. Partial ^1H NMR spectra (δ 5.1–5.3) after reduction of **8** excess dimethyl sulfide in phosphate buffer (0.1 M in D_2O, pD 7.0, ~25 °C): (A) 5 min; (B) 32 min; (C) 64 min. D–F. Partial ^1H NMR spectra after reaction of diol **9** with $NaIO_4$ (1.1 equiv) in cacodylate buffer (0.2 M in D_2O, pD 7.0, ~25 °C): (D) 5 min; (E) 18 min; (F) 36 min. C, T, and H represent the C-4 proton resonances of *cis*- (**2**), and *trans*-4-hydroxycyclophosphamide (**3**) and aldophosphamide hydrate, respectively. Asterisked peaks were used to determine product ratios.

(12) Peter, G.; Wagner, T.; Hohorst, J.-J. *Cancer Treat. Rep.* **1976**, *60*, 429.
(13) Van Rheenan, V.; Kelly, R. C.; Cha, D. Y. *Tetrahedron Lett.* **1976**, 1973.
(14) Takamizawa, A.; Matsumoto, S.; Iwata, T.; Tochino, Y.; Katagiri, K.; Yamaguchi, K.; Shiratori, O. *J. Med. Chem.* **1975**, *18*, 376.
(15) Takamizawa, A.; Matsumoto, S.; Iwata, T. *Tetrahedron Lett.* **1974**, 517.
(16) We have found HMPA to be a superior reference for these ^{31}P experiments. It occurs downfield from all resonances of interest and does not interfere with signals arising from hydrolysis products as occurs with H_3PO_4 references. It can be used as an internal standard for quantitative work and is far less sensitive than H_3PO_4 to changes in pH. $\delta_{H_3PO_4/H_2O} - \delta_{HMPA} = 26.1$ ppm.
(17) Low, J. E.; Borch, R. F.; Sladek, N. E. *Cancer Res.* **1982**, *42*, 830.
(18) Bajwa, G. S.; Chandrasekaran, S.; Hargis, J. H.; Sopchik, A. E.; Blatter, D.; Bentrude, W. G. *J. Am. Chem. Soc.* **1982**, *104*, 6385.
(19) Low, J. E.; Borch, R. F.; Sladek, N. E. *Cancer Res.* **1983**, *43*, 5815.
(20) Fedor, L. R.; Glave, W. R. *J. Am. Chem. Soc.* **1970**, *89*, 985.

a triplet centered at 5.21 ppm with J_{H_7H} = 5.9 Hz; the appearance of this resonance was accompanied by an overlapping doublet of triplets at 4.07 ppm, with J_{H-H} = 5.8 Hz and J_{H-P} = 6.1 Hz. These signals were identical with the initial product obtained 5 min after diol cleavage and are assigned to the aldehyde hydrate **5**. The other new resonances were consistent with an eight-line ddd system centered at 5.22 ppm and had coupling constants of 4.2, 5.5, and 12.8 Hz. The absence of a large (>20 Hz) H–P coupling and the presence of a 12.8-Hz H–H coupling confirmed that the C-4 proton is axial in this intermediate. The coupling constants of 5.5 (J_{H-P}) and 4.2 Hz (J_{H4-5e}) are in accord with this arrangement.[18] Thus, the eight-line system is assigned to the trans 4-hydroxy compound **3**, which is unique among the 4-hydroperoxy and 4-hydroxy diastereomers in adopting a preferred solution conformation with the C-4 oxygen substituent equatorial.[8] Estimation of the equilibrium composition of **2**, **3**, and **5** by integration of nonoverlapping peaks gave a ratio of approximately 4:2:1. Thus, the ^{31}P resonances can be assigned definitively as **2**, –13.90 ppm; **3**, 13.65 ppm; **5**, –5.66 ppm, consistent with those of Zon et al.[21]

The presence of aldehyde **4** in the equilibrium mixture was clearly discernible as a triplet (J = 1.5 Hz) centered at 9.73 ppm. The ratio of **2** to **4** appeared to be approximately 15:1, indicating that the equilibrium constant for **4** ⇌ **5** is approximately 3 in favor of the hydrate. The generation of acrolein was easily monitored by the appearance of a doublet (J = 7.6 Hz) at 9.48 ppm and the vinyl multiplet at 6.4–6.9 ppm. Acrolein represented approximately 15 and 35% of the product after 1 and 2 h, respectively, while the **2**/**3**/**4**/**5** ratio remained unchanged.

Thus, the reduction of **8** in phosphate buffer generates within 1 h a pseudoequilibrium mixture of **2**/**3**/**4**/**5** with the approximate composition 55:27:4:14. Elimination to **6** and acrolein proceeds at a rate slower than the establishment of the equilibrium. The same pseudoequilibrium ratio was noted when the reaction was carried out in cacodylate buffer (0.1 M, pD 7.0, ~25 °C), but acrolein accounted for approximately 5 and 20% of the reaction mixture after 1 and 2 h, respectively.

The diol **9** as a mixture of diastereomers was reacted with sodium metaperiodate (1.1 equiv) in cacodylate buffer (0.2 M, pH 7.0, ~25 °C) and monitored by ^{31}P NMR. After 5 min, the major peak at –5.7 ppm appeared essentially unchanged from **9**; two new signals appeared at –13.7 and –12.5 ppm, representing approximately 20 and 10% of the mixture, respectively. After 20 min, an additional resonance was apparent at –13.9 ppm, and by 40 min the peak ratios had stabilized at the same 4:2:1 ratio observed for the reduction of **8**. The reaction was repeated with periodate in cacodylate buffer (0.2 M in D_2O, pD 7.0, ~25 °C) and followed by ^1H NMR; the results are shown in Figure 1D–F. After 5 min, the triplet from hydrate **5** and the multiplet from **3** are clearly present, along with a minor amount of **2** (Figure 1D). The aldehyde resonance of **4** is also present at 9.72 ppm,[22] and the ratio of **4** to **5** is approximately 1:3. Acrolein respresents ~10% of the reaction mixture at this time. Spectra taken at 10 and 18 min show a progressive decrease in signals from **4** and **5** with corresponding increases in **2** and **3**; the 5.1–5.3-ppm region of the spectrum after 36 min is essentially identical with that of the 64-min spectrum from reduction of **8** (compare Figure 1C,F) and remains unchanged for at least 2 h. Acrolein accounts for ~30% of the product at 36 min.

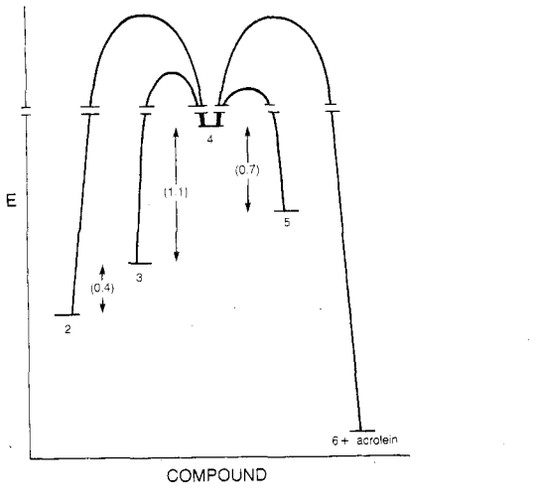


Figure 2. Energy diagram for interconversion of activated cyclophosphamide intermediates. Compounds **2**–**6** represent *cis*- and *trans*-4-hydroxycyclophosphamide, aldophosphamide and its hydrate, and phosphoramide mustard, respectively. Numbers in parentheses are estimates of energy differences, in kilocalories per mole, at 25 °C. Activation energies are estimates only and are intended to show relative magnitudes. See text for details.

These results demonstrate convincingly that **2**–**5** are in equilibrium and that this equilibrium is established faster from **4** than from **2** but in both cases at a rate that is competitive with the generation of **6**. Tentative conclusions may also be drawn regarding the energetics of the equilibrium, which are summarized Figure 2. First, free energy differences derived from the equilibrium composition indicate that **2** is the lowest and **4** the highest energy species present, with approximate values (in kilocalories per mole) of the differences shown. Second, estimates of relative reaction rates of **4** derived from the diol cleavage reaction indicate that the rates decrease in the product order **5** > **3** > **2** ≈ **6**; this is shown qualitatively in the activation energy curves of Figure 2. Cyclization to the trans compound **3** is clearly faster than to the cis **2**, and at pH ~7, cyclization to **2** occurs at a rate comparable to that for β-elimination to **6**. Third, hydrate **5** is formed much faster than **3** from the diol cleavage reaction, but the two products appear at comparable rates from **2**; this suggests the existence of a pathway from **2** to **3** that does not involve the aldehyde as an intermediate, perhaps it is via imine **7**.[8] The rate of acrolein formation is 2- to 3-fold faster in phosphate than in cacodylate buffer; this is in accord with the observation of general-base catalysis for acrolein generation from **2** as determined by UV spectroscopic methods.[19]

Conclusions

Correlation of ^1H and ^{31}P NMR spectra for the products of reduction of **8** and cleavage of **9** have provided definitive structural assignments to the ^{31}P resonances of intermediates **2**–**5**, as well as evidence for a pseudoequilibrium that is established prior to rate-limiting β-elimination to generate phosphoramide mustard **6** and acrolein. The approximate equivalence of phosphate and cacodylate in establishing this equilibrium from **2** indicates that bifunctional catalysis is not an important determinant for activation of **2** as suggested previously.[17] The apparent "stability" reported [17] for **2** in Tris buffer results from the formation of a Tris–**4** adduct, indicating that Tris is an effective catalyst for the ring-opening reaction.[8]

The mechanism consistent with the data presented in this and the preceding paper in this issue involves initial

(21) Zon, G.; Ludeman, S. M.; Sweet, E. M.; Egan, W.; Phillips, L. R. *J. Pharm. Sci.* **1982**, *71*, 443.

(22) Due to a typographical error, this value was erroneously reported as 8.5 ppm in ref 1.

hydroxylation of 1 to 2 and/or 3; the stereochemical consequence of this reaction is unlikely to be important in view of the subsequent equilibration that occurs. A pseudoequilibrium mixture of 2–5 is established via ring opening to aldehyde 4, with exchange of 2 and 3 also occurring via the transient intermediate 7. Ring opening of the 4-hydroxy isomers occurs both in the absence of buffer and with cacodylate, phosphate, and Tris buffers; the role of buffer structure and concentration on the associated reaction rates is currently under investigation. The rate-limiting step in drug activation involves β-elimination of 6 from 4, presumably via general-base catalysis, which is characteristic of eliminations involving weakly basic leaving groups.[20] The hydroxylated metabolites 2 and 3 can also undergo exchange reactions at C-4, presumably via elimination–addition of 7 or via addition and subsequent reclosure of 4; the stability of these exchange products will likely depend upon the basicity of the C-4 substituent and, hence, the ease with which they can undergo elimination to 7 and reenter the 2–5 equilibrium. These methods are currently being extended to the determination of possible intracellular factors that might affect oncotoxic specificity by alteration of the steady-state composition or reaction rates in this interesting metabolite system.

Experimental Section

Cyclophosphamide monohydrate and cacodylic acid and its sodium salt were obtained from Sigma Chemical Co.; other organic reagents were obtained from Aldrich Chemical Co. HPLC and preparative LC were carried out using the instrumentation and methods described elsewhere.[8] ^{31}P NMR spectra were recorded on a Nicolet NT-300 or IBM WP-270-SY instrument using 10-mm sample tubes, a 5000-Hz spectral width, a 12-μs pulse width, a 0.4-s pulse repetition time, and 400 acquisitions. Chemical shifts are reported in parts per million from 5% hexamethylphosphoramide (HMPA) in $CHCl_3$.[16] Low-power broad-band decoupling was used. ^1H NMR spectra were recorded with the same instruments using 5-mm sample tubes, a 5000-Hz spectral width, a 3-μs pulse width, a 2-s repetition time, and either 16 or 32 acquisitions. Improved sensitivity was obtained in some spectra by suppression of the residual HOD peak and/or the buffer peak using a 3000-Hz sweep width, a 5-μs pulse width, and homonuclear gated decoupling with the decoupler gated on for 3 s and off for 2 ms prior to acquisition. Chemical shifts are reported in parts per million from internal Me_4Si ($CDCl_3$) or 1-(trimethylsilyl)-propanesulfonate (D_2O). Acidity measurements were made on a Radiometer pH meter using a combination microelectrode; pD values represent the meter reading of D_2O solutions and are related to pH according to pH = pD + 0.4.

***cis*-4-Hydroperoxycyclophosphamide (8)** was prepared in 12–15% yields by ozonolysis of cyclophosphamide as described elsewhere:[8] ^1H NMR (D_2O) δ 5.17 (1 H, ddd, J_{HP} = 25.8 Hz, J_{5a} = J_{5e} = 3.5 Hz, H_4); ^{31}P NMR (D_2O) −14.52 ppm.

3,4-Dihydroxybutyl *N*,*N*-Bis(2-chloroethyl)phosphorodiamidate (9). To a stirred solution of $POCl_3$ (6.38 g, 41.6 mmol) in 21 mL of methylene chloride at −12 °C was added dropwise a solution of 3-buten-1-ol (3.0 g, 41.6 mmol) in 8 mL of methylene chloride. The mixture was stirred at −12 to −10 °C for 5 h. A solution of bis(2-chloroethyl)amine hydrochloride (7.43 g, 41.6 mmol) in 290 mL of methylene chloride was added in one portion. Triethylamine (12.6 g, 120 mmol) was added dropwise with stirring at −5 to −10 °C over 30 min. After stirring for an additional $2^1/_2$ h at this temperature, the mixture was filtered, and the filtrate was concentrated under reduced pressure to produce a yellow oil. The oil was taken up in ethyl acetate and filtered through a short silica gel column, eluting with hexane–ethyl acetate (3:2). The eluent was concentrated in vacuo and the resulting oil was dissolved in 25–30 mL of methylene chloride. The solution was saturated with ammonia at 0 °C, the cooling bath was removed, and stirring was continued for 1 h. The solution was again cooled and filtered, and the filtrate was concentrated in vacuo. This material was subjected to preparative LC[8] (Merck Si60 column, size A, 12 mL/min) in 1-g batches, with acetone as eluent. The desired fraction (t_R = 14.5 min) was collected and concentrated to give 3-butenyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate (510 mg), which solidified on standing at −20 °C. The total product obtained was 5.6 g (49% from $POCl_3$): mp 27–28 °C; ^1H NMR ($CDCl_3$) δ 5.79 (1 H, m, vinyl H), 5.13 (2 H, m, vinyl H), 4.05 (2 H, m, CH_2O), 3.53 (4 H, t, CH_2Cl), 3.39 (4 H, dt, NCH_2), 2.35 (2 H, q, CH_2CH_2O).

A solution of the butenyl compound (235 mg, 0.85 mmol) in 2 mL of acetone was added with stirring to a solution of *N*-methylmorpholine *N*-oxide (140 mg, 1.0 mmol) and osmium tetroxide (2.2 mg, 8.6 μmol) in 3 mL of water; stirring was continued at room temperature for 48 h. A mixture of sodium bisulfite (8 mg), Florisil (100 mg), and water (0.65 ml) was added. After stirring for 10 min, the mixture was filtered, the filtrate was adjusted to pH 7 with 1 N H_2SO_4, the acetone removed in vacuo, and the pH was adjusted to 2. The solution was saturated with NaCl and extracted with ethyl acetate (3 × 10 mL). The combined extracts were dried ($MgSO_4$) and filtered, and the filtrate was evaporated in vacuo to give 200 mg of a pale yellow oil. This product was purified by preparative LC (Merck Si60 column, 9:1 ethyl acetate–methanol, flow rate 5 mL/min) to give 9 as a colorless oil (160 mg, 61%), which was a 1:1 mixture of diastereomers: ^1H NMR (D_2O) δ 4.13 (2 H, dt, J_{HP} = 5.8 Hz, CH_2OP), 3.87 (1 H, m, $CHOH$), 3.71 (4 H, t, CH_2Cl), 3.50–3.65 (2 H, m, CH_2OH), 3.45 (4 H, dt, J_{HP} = 12.1 Hz, NCH_2), 1.91 (1 H, m, CH_2CH_2O), 1.76 (1 H, m CH_2CH_2O); ^{31}P NMR ($CHCl_3$) −7.58, −8.29 ppm (ca. 1:1 mixture of diastereomers); ^{31}P NMR (D_2O) −5.65 ppm (s).

NMR Studies. The appropriate buffer solutions were prepared in H_2O or D_2O as desired. For buffer solutions in D_2O, separate solutions of cacodylic acid and its sodium salt, or the mono- and dibasic salts of phosphoric acid, were prepared at the desired final concentration and were then mixed to achieve the desired pD. Solutions of 8 were prepared by adding the crystalline hydroperoxide to 0.5 (5-mm tubes) or 2.5 mL (10-mm tubes) of buffer to give a concentration of 5.8 mg/mL (20 mM). Solution was facilitated by brief high-speed vortexing. The solution was placed in the appropriate size NMR tube, and excess dimethyl sulfide was added rapidly (10 μL/mL of buffer solution). This was immediately vortexed at high speed for 2 min; because of the limited solubility of dimethyl sulfide in aqueous buffer, this step is crucial to achieve rapid and complete reduction. Spectra were then accumulated at selected intervals, and the FID spectra were stored on disk for later transformation and processing.

Experiments involving cleavage of 9 were carried out by preparing solutions of 9 in buffer (~20 mM) as described for 8 above. The reaction was initiated by the addition of 1.1 equiv of sodium metaperiodate, followed by high-speed-vortexing (1 min) to facilitate rapid dissolution. ^1H NMR indicated that cleavage of the diol was complete within 5 min. Spectra were then accumulated as described above.

Relative concentrations of reaction components were determined from peak integrals and should be regarded as estimates only. Spectra were taken over a 2-h period for all reactions.

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