# NMR Spectroscopic Studies of Intermediary Metabolites of Cyclophosphamide. A Comprehensive Kinetic Analysis of the Interconversion of *cis*- and *trans*-4-Hydroxycyclophosphamide with Aldophosphamide and the Concomitant Partitioning of Aldophosphamide between Irreversible Fragmentation and Reversible Conjugation Pathways

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Multinuclear ($^{31}P$, $^{13}C$, $^{2}H$, and $^{1}H$) Fourier-transform NMR spectroscopy, with and without isotopically enriched materials, was used to identify and quantify, as a function of time, the following intermediary (short-lived) metabolites of the anticancer prodrug cyclophosphamide (**1**, Scheme I): *cis*-4-hydroxycyclophosphamide (*cis*-**2**), its trans isomer (*trans*-**2**), aldophosphamide (**3**), and its aldehyde–hydrate (**5**). Under a standard set of reaction conditions (1 M 2,6-dimethylpyridine buffer, pH 7.4, 37 °C), the stereospecific deoxygenation of synthetic *cis*-4-hydroperoxycyclophosphamide (*cis*-**12**, 20 mM) with 4 equiv of sodium thiosulfate ($Na_2S_2O_3$) afforded, after ~20 min, a "pseudoequilibrium" distribution of *cis*-**2**, **3**, **5**, and *trans*-**2**, i.e., the relative proportions of these reactants (57:4:9:30, respectively) remained constant during their continual disappearance. NMR absorption signals indicative of "iminophosphamide" (**8**) and enol **6** were not detected (<0.5–1% of the synthetic metabolite mixture). A computerized least-squares fitting procedure was applied to the individual $^{31}P$ NMR derived time courses for conversion of *cis*-**2**, **3** plus **5** (i.e., "**3**"), and *trans*-**2** into acrolein and phosphoramide mustard (**4**), the latter of which gave an expected array of thiosulfate S-alkylation products (e.g., **16**) and other phosphorus-containing materials derived from secondary decomposition reactions. This kinetic analysis gave the *individual forward and reverse rate constants* for the apparent tautomerization processes, viz., *cis*-**2** ⇄ "**3**" ⇄ *trans*-**2**, as well as the rate constant ($k_3$) for the irreversible fragmentation of **3**. The values of $k_3$ at pH 6.3, 7.4, and 7.8 were equal to 0.030 ± 0.004, 0.090 ± 0.008, and 0.169 ± 0.006 $min^{-1}$, respectively. Replacement of the $HC(O)CH_2$ moiety in **3** with $HC(O)CD_2$ led to a primary kinetic isotope effect ($k_H/k_D$ = 5.6 ± 0.4) for $k_3$. The *apparent half-lives* ($\tau^*_{1/2}$) for *cis*-**2**, "**3**", and *trans*-**2** under the standard reaction conditions, at "pseudoequilibrium" (constant ratio of *cis*-**2**/"**3**"/*trans*-**2**), were each equal to ~38 min, which is considerably shorter than the widely cited colorimetrically derived half-lives reported by earlier investigators. The values of $\tau^*_{1/2}$ for *cis*-**2**, "**3**", and *trans*-**2** were affected by pH in the same manner as that found for $k_3$ but were relatively insensitive to the presence of either $K^+$, $Na^+$, $Ca^{2+}$, or $Mg^{2+}$. The presence of certain primary amines led to marked decreases in $\tau^*_{1/2}$ and, in some cases, the formation of acyclic adducts of aldehyde **3**. The relatively stable adduct formed from **3** and tris(hydroxymethyl)aminomethane (Tris) at pH 7.4 and 37 °C gave rise to a $^{31}P$ NMR signal that other investigators have mistakenly ascribed to **2**. $^{31}P$ NMR spectroscopy was also used to examine, in considerable detail, the manifold effects of *N*-acetyl-L-cysteine upon the chemistry of **2**, "**3**", and **4**, which featured the formation of a mixture of diastereomeric, acyclic thiohemiacetal conjugates (**26**) and a coexistent mixture of the four possible diastereomeric 4-thiocyclophosphamide conjugates (**27**). In the presence of an initially 2-fold molar excess of *N*-acetyl-L-cysteine at pH 7.4, the spectroscopic observation of the continual appearance of **4** and its S-alkylation products was in opposition to the prolonged "alkylating capacity" that has been reported by other investigators, who used 4-(*p*-nitrobenzyl)pyridine (NBP) as a colorimetric reagent. The hydrolysis of **26**/**27** was also studied by a $^{31}P$ NMR spectroscopic method that employed *N*-ethylmaleimide to scavenge both the unincorporated *N*-acetyl-L-cysteine and the sulfhydryl compound that was released from **26**/**27**. $^{13}C$ and $^{2}H$ NMR studies of the decomposition of *cis*-**2**-*4*-$^{13}C$ and *cis*-**2**-*5,5*-$d_2$ revealed, via spectra of the isotopically labeled acrolein fragments [$H^{13}C(O)CH{=}CH_2$ and $HC(O)CD{=}CH_2$], that essentially all of this urotoxic material was in rapid, reversible equilibrium with thermodynamically favored adducts at pH 7.4, 37 °C. These findings represented a caveat for metabolic and toxicological investigations of "acrolein".

Cyclophosphamide (**1**) is a widely used drug for the treatment of human cancers.[1] The metabolism, pharmacokinetics, and mechanisms of action of **1**, which have been extensively studied and reviewed,[2-8] continue to receive considerable attention,[9-42] especially with regard to the elucidation of reasons for cyclophosphamide's oncostatic selectivity. The central features of cyclophosphamide metabolism are depicted in Scheme I,[43] wherein dashed arrows designate hypothetical transformations in vivo; a variety of other metabolites that are known[44] have been excluded for the sake of simplicity.

It is generally accepted that liver microsomal oxidation ("activation") of **1** produces 4-hydroxycyclophosphamide (**2**) and that subsequent formation of aldophosphamide (**3**) leads to acrolein and phosphoramide mustard (**4**), the latter of which functions as a highly reactive alkylating agent. A detailed understanding of the multifacet mechanism of selective cytotoxicity must contend with a for-

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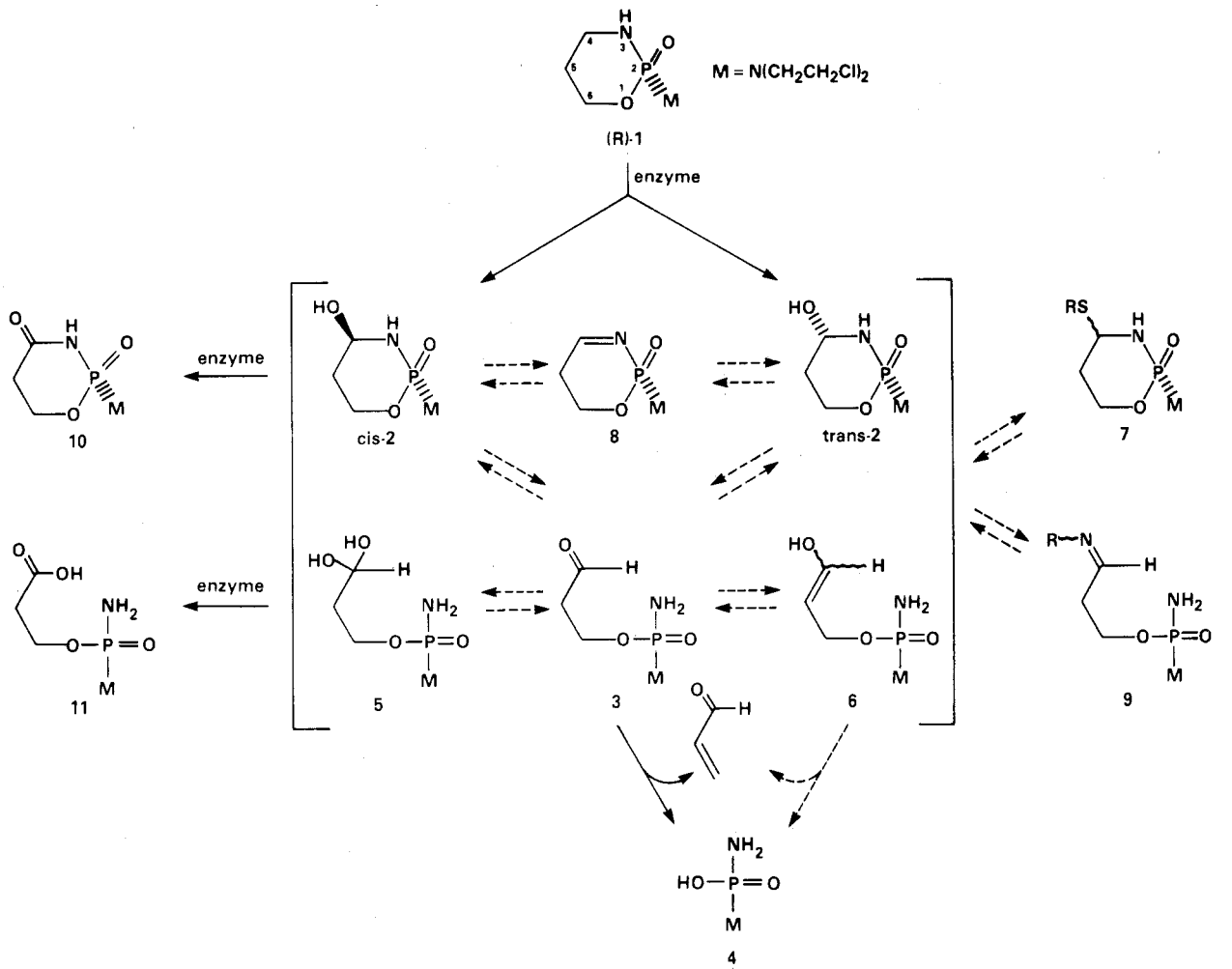
Scheme I




midable array of intervening chemistry that is still poorly defined. For example, the kinetics and thermodynamics for the presumed interconversion of *cis*-**2**, **3**, and *trans*-**2** have not been measured, although circumstantial evidence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has led to the suggestions[45] that 3 is much less stable than 2 and that no equilibrium exists between 2 and 3 at room temperature or above. Similarly, there is no conclusive information regarding possible equilibria between aldehyde 3, hydrate 5,[45] and enol 6[46] or equilibria between *cis*-2, 5, and *trans*-2, which are not shown in Scheme I. The equilibria for 3 and 5 are more than academically interesting when one considers that in vivo factors that influence the kinetics and thermodynamics of the aldehydo hydration could modulate, in effect, the location and rate of release of 4 and acrolein from 3. This point is perhaps more apparent in the context of sulfhydryl compounds that reportedly[47,48] "deactivate" and transport 2/3 by the reversible formation of 4-thiocyclophosphamide conjugates (7). "Iminophosphamide" (or "iminocyclophosphamide", 8) has been discussed,[4,49,50] reportedly identified,[50b] and recently criticized[6] as a possible intermediate in these chemical transformations, which might also lead to bimolecular counterparts of 8, viz., Schiff-base conjugates of aldophosphamide (9).[7] All of these reversible processes, as well as the irreversible fragmentation of 3 (or 6[46]) into acrolein and 4, and the alkylation chemistry of 4 could, in principle, be influenced by pH and metal ions; however, there have been few detailed studies[32,35,51,52] of these factors. Considerably more information, both biochemical[11] and stereochemical,[53-56] has been obtained for the enzymatic "detoxification" of 2/3 that produces the urinary metabolites 4-ketocyclophosphamide (10) and carboxyphosphamide (11), although the importance of these processes and related stereochemical[7,57,58] phenomena is unclear at this time.[6,7]

Friedman, Myles, and Colvin[4] have reviewed some possible bases for the oncostatic selectivity elicited by cyclophosphamide, viz., the relative roles of reactive metabolites in tissue distribution, the influence of presumed sulfhydryl conjugates, and the differential rates of oxidative detoxification of 2/3 (i.e., formation of 10/11) in normal as compared to cancerous cells. A more recent addition to this list of theories concerns the toxicogenic influence of 3′-5′ exonucleases on the rates of decomposition of 2 and 7.[6,59] In view of the unresolved chemical questions that were noted above, it is apparent that further insight into selectivity mechanisms requires a better understanding of the fundamental chemistry and kinetic behavior of cyclophosphamide's intermediary (short-lived) metabolites. We have addressed this problem by the extension of our NMR studies[10,20,32,42,60] of cyclophosphamide to synthetically derived samples of 2/3. The goals of this initial work were to structurally characterize, in an unambiguous manner, the activated metabolites of cyclophosphamide that are present in aqueous solutions, to develop kinetic models for the reversible and irreversible chemistry associated with these intermediary metabolites, and to systematically examine, in a quantitative fashion, chemical factors that may affect the reactivity patterns for these activated metabolites. While the presently described studies were restricted to cell-free aqueous reaction media that are only remotely related to a biological milieu, we believe that the results nevertheless provide reliable benchmarks for the design of future experiments. In addition, our results indicate that several previously published[35,47] aspects of the chemistry of 2/3 must be reconsidered.

## Results and Discussion

**Synthesis.** The synthesis of various precursors to 2[61-67]

(43) Throughout the present report, only the *R* configuration at phosphorus is shown for 1 and other structures that have an asymmetric center at phosphorus [for X-ray crystallographic determinations of the absolute configurations of (+)-(*R*)-1 and (−)-(*S*)-1, see: Karle, I. L.; Karle, J. M.; Egan, W.; Zon, G.; Brandt, J. A. *J. Am. Chem. Soc.* **1977**, *99*, 4803; Adamiak, D. A.; Saenger, W.; Kinas, R.; Stec, W. J. *Z. Naturforsch.* **1977**, *32C*, 672]. The descriptors "cis" and "trans" refer to the relative stereochemistry of the phosphoryl oxygen and the substituent at the C-4 position, as is the usual convention [for example, see: Camerman, A.; Smith, H. W.; Camerman, N. *Cancer Treat. Rep.* **1976**, *60*, 517]. Unless specified otherwise, the application of these stereochemical descriptors to cyclic molecules connotes a hypothetical time-averaged "planar" ring system, rather than a specific geometry in which the reference groups occupy axial or equatorial positions.

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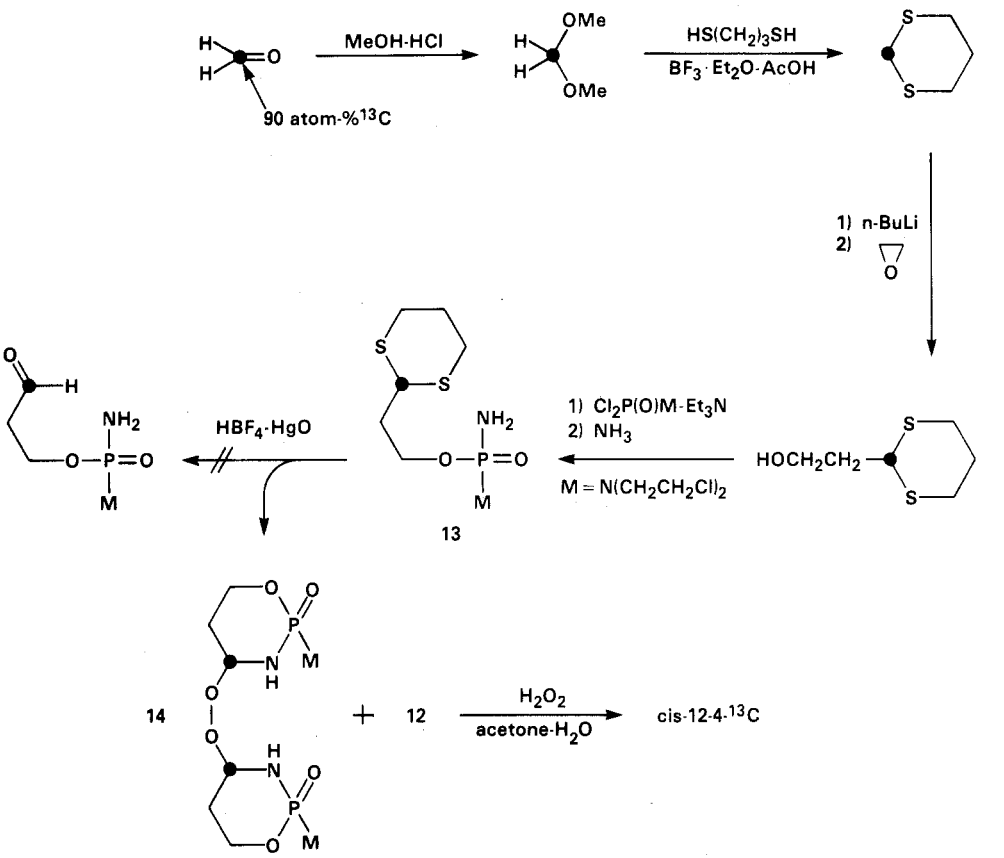
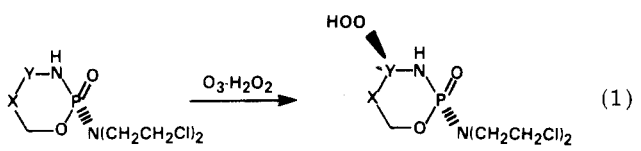
Scheme II




and **3**[45,68] has been studied extensively. Oxidation of **1** with $O_3$–$H_2O_2$,[65] which is known from X-ray crystallography[69] to give a cis diastereomer of 4-hydroperoxycyclophosphamide (*cis*-**12**), was therefore used to convert **1**-*5,5-d*$_2$[70] into *cis*-**12**-*5,5-d*$_2$ in an isolated yield of 4% (eq 1). The isotopic




(1)

**1**, X = Y = $CH_2$
**1**-5,5-$d_2$, X = $CD_2$, Y = $CH_2$
**1**-4-$^{13}$C, X = $CH_2$, Y = $^{13}CH_2$

cis-**12**, X = $CH_2$, Y = CH
cis-**12**-5,5-$d_2$, X = $CD_2$, Y = CH
cis-**12**-4-$^{13}$C, X = $CH_2$, Y = $^{13}$CH

purity (98 atom % $^2$H) and location of the deuterium labels in the hydroperoxide product were established by $^1$H and $^{13}$C NMR spectroscopic analysis; diastereomeric purity (>95%) was established by $^{31}$P NMR spectroscopic analysis. The cis 1,3-diaxial relationship between the $C_4$ OOH and P=O groups in **12**, which would serve as the "anchor" compound for assignment of relative stereochemistry, was based on a comparison of the magnitude of the solution-state spin–spin coupling between the phosphorus and $C_4$ H. The value of $^3J_{HP}$ (25.9 Hz) that was measured for the $C_4$ H in the hydroperoxide product at 20 °C in $CDCl_3$ was equal, within the experimental error limits, to the corresponding value (24.5 Hz) reported by Takamizawa and co-workers[64] for *cis*-**12** at 38 °C in dimethyl-$d_6$ sulfoxide. These findings, which are in accord with an equatorial $C_4$ H,[30,64] imply that the necessarily cisoid[69] $C_4$ OOH and P=O groups jointly occupy the axial positions in the weighted time-averaged structure of *cis*-**12** in both solvents, granted that adventitious acid-catalyzed stereomutation at phosphorus did not occur.[6,64,71]

Relatively small-scale reactions of $O_3$–$H_2O_2$ with ring-carbon labeled **1**-*4*-$^{13}$*C* were unsatisfactory due to the low yields of *cis*-**12**-*4*-$^{13}$*C*.[71] Fortunately, we were able to obtain the latter material as the "salvage product" of an errant reaction sequence (Scheme II) that had been investigated initially as a route to **3** having a $^{13}$C-enriched aldehyde group. Conventional synthetic chemistry was used to incorporate the isotopically enriched carbon (90 atom % $^{13}$C) of formaldehyde into structure **13** as a masked aldehyde moiety. Unlabeled **13** had been previously reported by Struck,[45] who was unable to find evidence for the formation of aldophosphamide upon treatment of this material with $BF_3$·$Et_2O$.[45] The same result was obtained in our labora-

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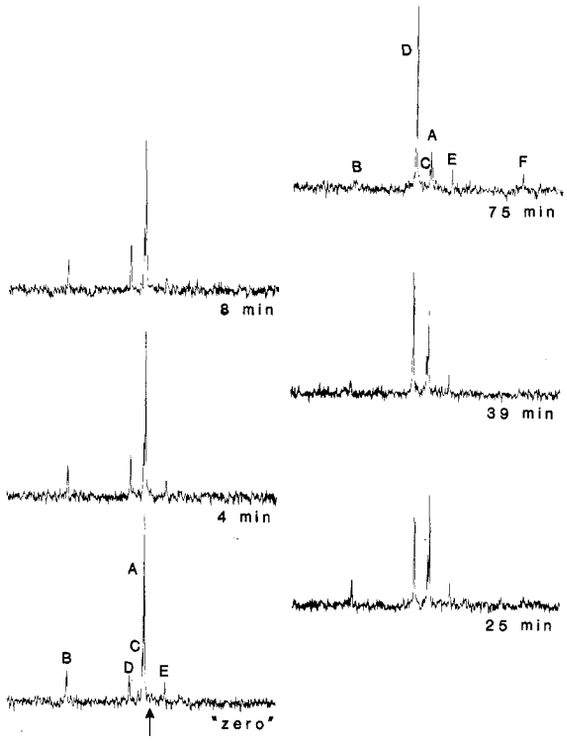



**Figure 1.** Partial displays of 36.23-MHz $^{31}P$ NMR time-averaged spectra (with NOE) recorded for the decomposition of *cis*-**2** (20 mM initial concentration) in 1 M 2,6-dimethylpyridine buffer at pH 7.4 and 37 °C. The designated times refer to the start of data acquisition (100 pulses, 2-s pulse recycle time), with "zero" time corresponding to 2 min after placement of the 10-mm sample tube in the spectrometer probe [~15 min after dissolution and thiosulfate deoxygenation of the *cis*-**12** precursor, which has a chemical shift (δ 11.51) indicated by the arrow]; A = *cis*-**2** (δ 12.22), B = **3**/**5** (δ 20.40), C = *trans*-**2** (δ 12.42), D = **4**/**16** (δ 13.6–13.8), E = **17**, and F = product(s) derived from the hydrolysis of **4**/**16**.

tory, and a large variety of alternative deprotection methods also failed to produce aldehyde **3**. However, a modified version of a relatively new deprotection procedure[72] with $HBF_4$–HgO was applied to unlabeled **13** and afforded a crude-product extract that contained a mixture of the diastereomers of unlabeled 4-peroxycyclophosphamide (**14**) and a relatively small amount of **12**, based on $^{31}P$ NMR chemical-shift comparisons with authentic samples. Since we were unable to separate these products by silica gel chromatography, they were reacted directly with $H_2O_2$ in aqueous acetone and were monitored by $^{31}P$ NMR spectroscopy in an attempt to observe the conversion of the peroxy dimer into **12**. This oxidation method and subsequent crystallization gave *cis*-**12** in 3% yield, relative to unlabeled **13**. A sample of **13** was similarly converted (8% yield) into *cis*-**12**-*4*-$^{13}C$, which featured $^1H$-decoupled $^{13}C$ and $^{31}P$ NMR doublets ($J \cong 3$ Hz) due to the 2-bond coupling between C-4 and phosphorus. Repeated efforts to prepare aldehyde **3** by the method of Myles et al.[68] were unsuccessful.[71]

In our hands, pure samples of crystalline *cis*-**12** were usually obtained in only 2–4% yields by the oxidation of **1** with $O_3$–$H_2O_2$;[65] however, more reproducible results and 20–40% yields of *cis*-**12** were obtained by the use of Takamizawa's method[61,63] for the oxidative cyclization of the 3-butenyl precursor $CH_2$=$CHCH_2CH_2OP(O)(NH_2)M$. Numerous synthetic approaches to **8** were unsuccessful and have been described elsewhere.[7] We were also unable to

(72) Degani, I.; Fochi, R.; Regondi, V. *Synthesis* **1981**, 51.

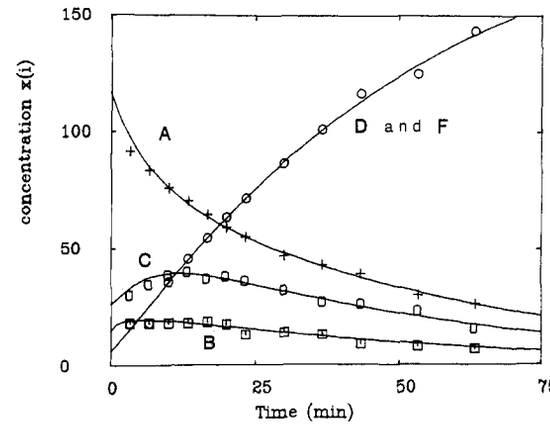
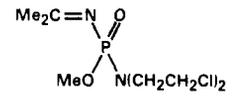


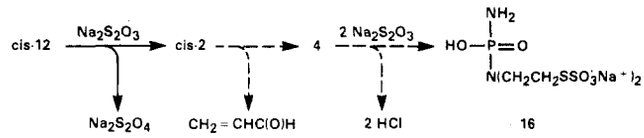

**Figure 2.** $^{31}P$ NMR derived time courses for the reaction system described in the legend to Figure 1. The ordinate values refer to relative concentrations (peak heights) of the *i*th reaction component (either A, B, C, or D plus F), and the indicated times are relative to the start of data acquisition. As detailed under Experimental Section, the smooth curves represent the least-squares fit to the data (the symbols shown).

prepare **15** as an acyclic model compound for **8**, starting with $Me_2C$=NH and $Cl_2P(O)M$.[71]




15

**NMR Spectroscopic Identification of "Activated" Metabolites of Compound 1. Phosphorus Spectra.** The usual advantages that obtain for $^{31}P$ NMR spectroscopy,[73,74] and our previous experience with the multinuclear NMR characteristics of cyclophosphamide, indicated that phosphorus would be the nucleus of choice for the preliminary identification of the reactive metabolites presented in Scheme I; corraborative information could then be sought by appropriate $^{13}C$, $^2H$, and $^1H$ NMR experiments. Early $^{31}P$ NMR studies[10] of **2**/**3** employed samples that were obtained by the deoxygenation of *cis*-**12** with $Ph_3P$ in an organic solvent, followed by extraction of the product(s) into an aqueous buffer; however, the reproducibility and efficiency of the extraction were relatively poor, especially with regard to monitoring the early stages of the metabolite reactions. These problems were solved by the reaction of either a solution (20 mM) or suspension of *cis*-**12** in 1 M 2,6-dimethylpyridine ("lutidine") buffer with a 4-fold molar excess of sodium thiosulfate[62] ($Na_2S_2O_3$) at pH 7.4 and 25–37 °C. Under these conditions, the thiosulfate ion caused rapid, stereospecific (vide infra) deoxygenation of *cis*-**12** and also served as a nucleophilic trapping agent for **4** via sequential S-alkylation reactions to give product **16** (eq 2); the expected[32,51] conversion of

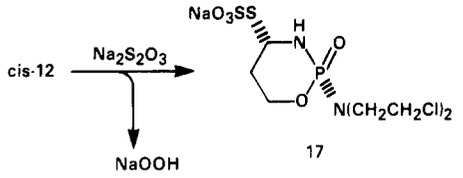



(2)

(73) O'Neill, I. K.; Richards, C. P. In "Annual Reports on NMR Spectroscopy"; Webb, G. A. Ed.; Academic Press: New York, 1980; pp 134–236.

(74) Quin, L. D. In "The Heterocyclic Chemistry of Phosphorus: Systems Based on the Phosphorus–Carbon Bond"; Wiley-Interscience: New York, 1981; pp 196–271.

4 ($\delta$ ~13.6) into 16 ($\delta$ ~13.8) was verified by control spectra, which were obtained by the reaction of the cyclohexylammonium salt of 4 with $Na_2S_2O_3$. The "zero-time" $^{31}P$ NMR spectrum (Figure 1) recorded at 37 °C ~15–20 min after the addition of $Na_2S_2O_3$ thus showed that the *cis*-12 starting material at $\delta$ 11.51 was not detectable and that the absorption for 4/16 was accompanied by four signals at $\delta$ 20.40, 12.42, 12.22, and 10.01. There was no evidence for the formation of 4-ketocyclophosphamide (10, $\delta$ ~9.00). The time courses for the relative concentrations (signal intensities) of the reactants are presented in Figure 2. The intensity of the signal at $\delta$ 10.01 (~5% of the total signal intensity) was invariant and is not shown in Figure 2; this absorption was ascribed tentatively to the competitive formation of 17 during the deoxygenation reaction (eq 3). The data presented in




(3)

Figure 2 revealed that after ~20–30 min the reaction components seen at $\delta$ 20.40, 12.42, and 12.22 reached a constant proportionality equal to 13:30:57, respectively, while their combined intensity decreased gradually with the concomitant growth of signal intensity for 4/16. In the absence of $Na_2S_2O_3$ (i.e., $Ph_3P$ deoxygenation of *cis*-12), the time courses for the signals at $\delta$ 20.40, 12.42, and 12.22 were essentially identical with those given in Figure 2; furthermore, the signal attributed to 17 was not detectable, and 4 gave rise to a characteristic array of hydrolysis products.[32] From chemical-shift comparisons to model compounds,[42] the transient intermediates that led to 4 were tentatively identified as "acyclic material", seen at $\delta$ 20.40, and C-4 oxidized "cyclic material", seen at $\delta$ 12.42 and 12.22. Since the signal at $\delta$ 12.22 corresponded to the *initial detectable* product of thiosulfate deoxygenation of *cis*-12, this resonance absorption was ascribed to *cis*-2 as a working hypothesis. It followed that the signal at $\delta$ 12.42 could have arisen from either a conformer of *cis*-2, a diastereomer of *trans*-2, or imine 8. The assignment of the signal at $\delta$ 12.42 to *trans*-2 was based on the following set of arguments. A low energy barrier (~5 kcal/mol) has been calculated[7] for the interconversion of the mobile[60b] chair-like conformers of 1, which implied that the conformers of *cis*-2 (and *trans*-2) at 37 °C would likely undergo rapid interconversion on the NMR time scale and thus give rise to a weighted time-averaged signal. The possibility of rapid stereomutation at phosphorus in 2 was discounted, since this epimerization process presumably requires catalysis by, for example, a strong acid, as found[71] for *cis*-12 and $PhSO_3H$ in $CDCl_3$ solvent, as well as analogous reaction systems.[6,64] That the signal at $\delta$ 12.42 arose from 8 was ruled out by $^1H$ and $^{13}C$ NMR spectroscopic data (vide infra). In this connection it should be mentioned that the hydrolytic stability of $PhCH{=}NP(O)Ph_2$ has previously been cited[6] as evidence against the likely intermediacy of 8. The absence of a detectable $^{31}P$ NMR signal for 8 in the mixture of reactants derived from *cis*-12 indicated that if 8 was present, it was a very minor component, i.e., <0.5–1% of the metabolite distribution.

**Proton Spectra.** The reaction mixture derived from deoxygenation of *cis*-12 with thiosulfate ion was next examined by $^1H$ NMR spectroscopy to obtain, in particular, further information about the "acyclic material" that had given rise to a relatively weak phosphorus signal at $\delta$ 20.40. It was not possible to use 1 M lutidine as a buffer because of its intense proton absorptions; consequently, an unbuffered solution of *cis*-12 (34 mmol) in $D_2O$ (1.5 mL) was treated with $Na_2S_2O_3{\cdot}5H_2O$ (4 equiv), and the $^{31}P$ NMR spectrum was monitored at 27 °C until the relative intensity of the signal at $\delta$ 20.40 reached a maximum value, viz., 12% of the total $^{31}P$ spectral intensity after ~60 min. The corresponding $^1H$ NMR spectrum was then obtained over the next 50 s of reaction. A low-intensity triplet ($^3J_{HH} \cong 1.5$ Hz) at $\delta$ 9.7 was indicative of the $HC(O)CH_2$ moiety in 3; however, enolic absorptions for 6 were not discernible in the range of $\delta$ 5.5–6.0.[75a] It was not possible to observe the $HC(OD)_2CH_2$ moiety in hydrate 5 at $\delta$ ~5, since there was an intense *H*OD absorption that obscured this spectral region; on the other hand, indirect evidence for the existence of 5 was obtained in the following manner. Spectra recorded at 27 °C for 0.1 M propanal [$HC(O)CH_2CH_3$] in $D_2O$ solvent, with and without $Na_2S_2O_3$ (2 equiv), revealed that this model compound gave rise to a slow-exchange equilibrium between the free aldehyde [$HC(O)CH_2CH_3$, $\delta$ 9.7, $^3J_{HH} \cong 1.2$ Hz] and its hydrate [$HC(OD)_2CH_2CH_3$, $\delta$ 4.9, $^3J_{HH} = 5$ Hz] and that the ratio of free aldehyde to its hydrate was approximately 40:60. Comparison of the integrated signal intensity of the aforementioned aldehydic proton in 3 to the integrated signal intensities of other assignable proton absorptions led to the calculation[75b] of an approximately 30:70 ratio of 3 to 5, which was chemically reasonable in view of the equilibrium distribution found for propanal. If the two-site chemical exchange[76] between propanal and its hydrate was also representative of the dynamic behavior of 3 and 5, then the observation of 1.5-Hz three-bond coupling for $HC(O)CH_2CH_3$ gave ~2 $s^{-1}$ as an upper limit, at 37 °C, for the hydration and dehydration rate constants for 3 $\rightleftarrows$ 5, which was in accord with kinetic data reported[77] for an aldehydo sugar. These estimated relative concentrations and reaction rates for 3 and 5 implied that their individual phosphorus and carbon signals should be resolvable, which was confirmed by subsequent NMR experiments (vide infra).

**Deuterium Spectra.** The 46-MHz $^2H$ NMR spectrum of 1-*5,5-d*$_2$ in $^2H$-depleted water showed two, broadened ($w_{1/2} \approx 5$ Hz), overlapped signals at $\delta$ ~2 for the diastereotopic[78] deuterium nuclei; a relatively weak absorption was seen at $\delta$ ~5 due to residual $^2H$ in the water solvent. An essentially similar spectrum was recorded for *cis*-2-*5,5-d*$_2$ immediately after its generation from *cis*-12-*5,5-d*$_2$ and $Na_2S_2O_3$ in 1 M lutidine buffer in $^2H$-depleted water at pH 7.4 and 37 °C. The decomposition of this sample

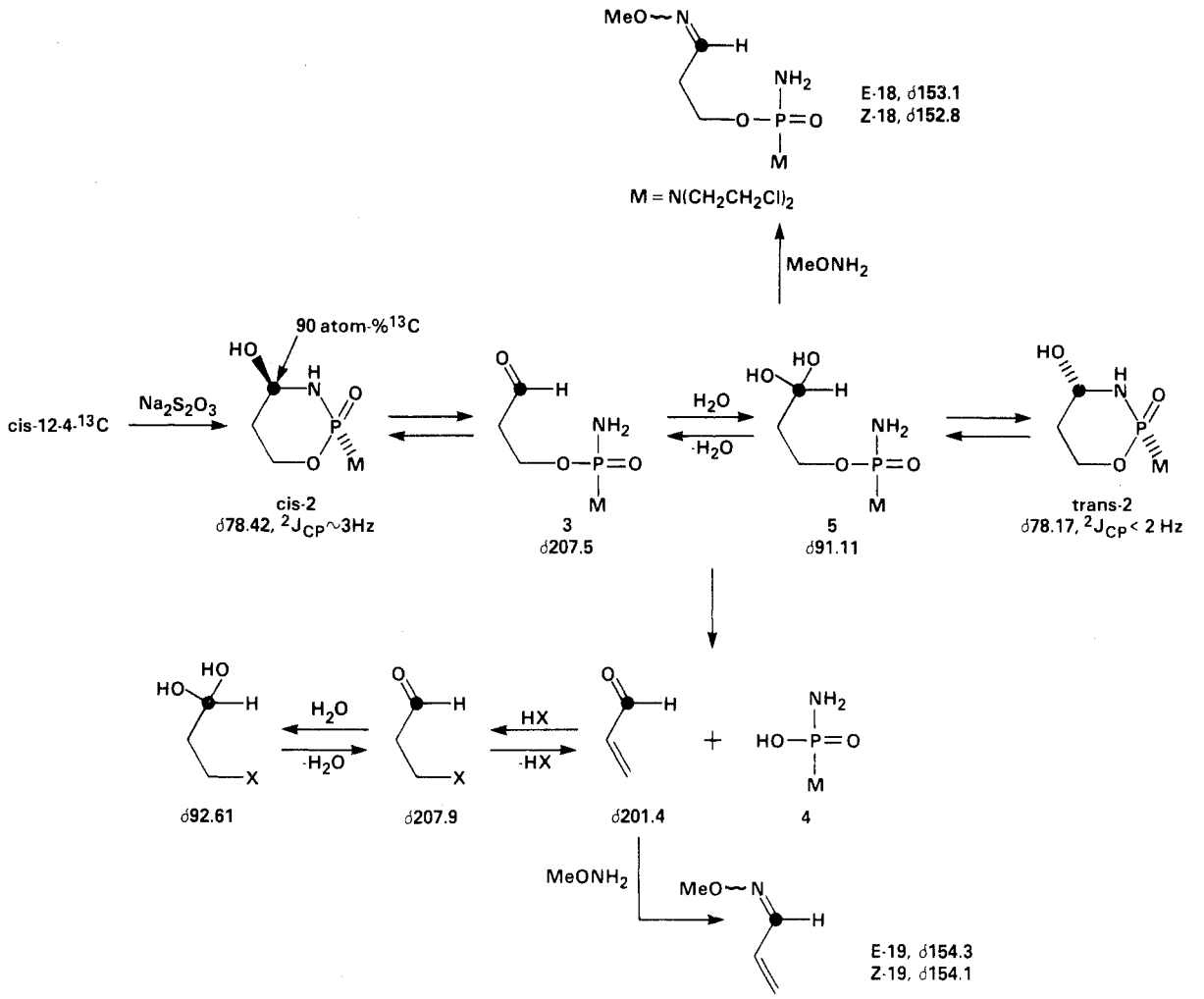
(75) (a) For aldehydic enol ethers, see: Gilbert, J. C.; Weerasooriya, U. *J. Org. Chem.* **1983**, *48*, 448. (b) Relative signal intensities ($I$) were measured by the cut and weigh method. After 12.5% conversion (i.e., [3 + 5]/[2 + 3 + 5] = 0.125), $I$ = 4 units for $HC(O)$ in 3, and $I$ = 208 units for the $CH_2CH_2O$ protons in 2, 3, and 5 at $\delta$ 1.5–2.5; hence, $I$ = 208 (0.125) = 26 units for $CH_2CH_2O$ in 3 plus 5. Subtraction of the contribution due to 3 gave $I$ = 26 – 4(2) = 18 units for $CH_2CH_2O$ in 5, or I = 18/2 = 9 units per proton in 5. Thus, [3]/[5] = 4:9 = 31:69. A second spectral accumulation and signal integration also gave a final ratio of 31:69.

(76) Sandstrom, J. In "Dynamic NMR Spectroscopy"; Academic Press: New York, 1982; pp 77–92, and pertinent references therein.

(77) From reported [Risley, J. M.; Van Etten, R. L. *Biochemistry* **1982**, *21*, 6360] kinetic data at 23 °C for the tautomerization of D-erythrose in water at equilibrium, we estimated that the pseudo-first-order rate constants for hydration of this aldehydo sugar and dehydration of its hydrate were 15 and 1.5 $min^{-1}$, respectively, at 37 °C. For a recent and detailed kinetic study of structure–reactivity effects in the hydration of benzaldehydes, see: McClelland, R. A.; Coe, M. *J. Am. Chem. Soc.* **1983**, *105*, 2718.

(78) Mislow, K.; Raban, M. *Top. Stereochem.* **1967**, *1*, 1–38.

Scheme III




was monitored periodically for 24 h (Figure 3); however, extensive overlap among the resonances upfield from water precluded their reliable assignment, at 46-MHz, to the various reaction components. On the other hand, it was evident from the spectra that abstraction of a deuteron from the $HC(O)CD_2$ moiety in **3**-$d_2$ led to growth of the water resonance and that the resultant fragmentation to give acrolein-$\alpha$-$d$ [$HC(O)CD{=}CH_2$] did not lead to detectable accumulation of this byproduct, which would have been seen as a signal at $\delta$ ~6–7. From the $^2H$ NMR signal integrations, it was also evident that more than 1 equiv of $D^+$ was transferred to the water solvent, which revealed that the stable vinylic C–D bond in acrolein-$\alpha$-$d$ was transformed into a relatively labile C–D bond in material derived from the acrolein fragment. The fate of the acrolein was clarified by $^{13}C$ NMR studies that are described in the next section.

**Carbon Spectra.** Sodium thiosulfate was used for the deoxygenation of *cis*-**12**-4-$^{13}C$ (20 mM) in 1 M lutidine buffer at pH 7.4, and the resultant reaction mixture was monitored by NMR spectroscopy with the use of a broad-banded, tunable probe, which allowed the accumulation of $^{13}C$ and $^{31}P$ spectra, in an alternating fashion, for the same sample. Spectra at 25 °C were acquired with continuous $^1H$ decoupling and a relatively rapid pulse recycle time; consequently, the relative carbon and phosphorus signal intensities were compared qualitatively. The initial data acquisition period (0–30 min) gave a $^{13}C$ spectrum that featured a doublet for *cis*-**2** ($\delta$ 78.42, $^2J_{CP} \cong 3$ Hz), which was the major reaction component; the observation of $^{13}C$–$^{31}P$ coupling required that the rate of interconversion of *cis*-**2** and **3**/**5** was $<\sim 3\ s^{-1}$. In addition, there was an apparent singlet for *trans*-**2** ($\delta$ 78.17, $^2J_{CP} <$ 2 Hz) and two singlets that were characteristic of the labeled positions in aldehyde **3** ($\delta$ 207.5) and hydrate **5** ($\delta$ 91.11) (cf. Scheme III); the relative ratio of **3** to **5** was approximately 27:73. The aldehydic carbon in acrolein ($\delta$ 201.4) was not detected in this spectrum, although a minor unidentified reaction component ("A", $\delta$ 92.61) was present and had a chemical shift indicative of a hydrated aldehyde group. The chemical shifts for the C-4 positions in *cis*- and *trans*-**2** were close to the values reported for C-4 in model compounds,[79a] while the signal assignments for **3** and **5** were based on literature data for aldehydo sugars,[79b] as well as measurements obtained for propanal under similar conditions: $\delta$ 212.2 for $HC(O)CH_2CH_3$ and $\delta$ 95.24 for $HC(OH)_2CH_2CH_3$. Further support for the metabolite signal identifications followed from the observation that the relative $^{13}C$ signal intensities for *cis*-**2**, **3** plus **5**, and *trans*-**2** (70:10:20, respectively) were roughly equal to the corresponding $^{31}P$ signal distribution (60:12:28) that was measured during the next data acquisition period (35–45 min). This $^{31}P$ NMR spectrum also confirmed the larger two-bond $^{31}P$–$^{13}C$ coupling in *cis*-**2** ($^2J_{PC} \cong 3$ Hz) as com-

(79) (a) In an organic solvent, $\delta$ ~87 has been reported for C-4 in **12** and its anhydro dimer [Struck, R. F.; Thorpe, M. C.; Coburn, W. C., Jr.; Laster, W. R., Jr. *J. Am. Chem. Soc.* **1974**, *96*, 313]. (b) An aqueous solution of D-[1-$^{13}C$]threose gave rise to signals at $\delta$ ~206 and ~91 for the aldehydo carbon and its hydrated form, respectively [Serianni, A. S.; Pierce, J.; Huang, S.-G.; Barker, R. *J. Am. Chem. Soc.* **1982**, *104*, 4037].

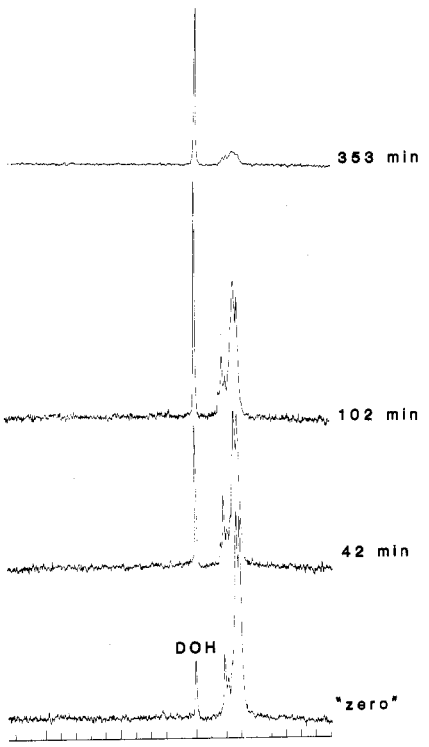



**Figure 3.** 46-MHz $^2H$ NMR time-averaged spectra recorded for the decomposition of *cis*-**2**-*5,5-d*$_2$ (20 mM initial concentration) in 1 M 2,6-dimethylpyridine buffer ($^2H$-depleted water) at pH 7.4 and ~20 °C. The designated times refer to the start of data acquisition (200 pulses, 1-s pulse recycle time), with "zero" time corresponding to ~15 min after dissolution and thiosulfate deoxygenation of the *cis*-**12**-*5,5-d*$_2$ precursor; DOH appears at $\delta$ 4.8; scale = 1 ppm/division. The top spectrum is shown with a relative amplitude of 0.25.

pared to *trans*-**2** (apparent singlet, $^2J_{PC} < 2$ Hz). The subsequent $^{13}C$ spectrum (45–75 min), which represented the "equilibrium" distribution of *cis*-**2**/**3** plus **5**/*trans*-**2** (59:11:30), revealed that (1) the relative ratio of **3** to **5** was 21:79, (2) the proportion of unknown component "A" ($\delta$ 92.61) had increased, (3) free acrolein was now barely detectable [HC(O)CH=CH$_2$, $\delta$ 201.4] above the noise level, and (4) an unknown aldehydo species ("B", $\delta$ 207.9) was present, based upon the chemical shift of the carbon label. Aside from the additional absorptions due to lutidine,[80] byproduct **17**,[81] and contaminant **10** ($\delta$ 178.3, C-4), there were no $^{13}C$ signals for either enol **6** [H(HO)C=CH, $\delta$ 140–150, estimated range] or imine **8** (HC=N, $\delta$ 140–180, estimated range). The next $^{13}C$ data acquisition period (90–120 min) gave a spectrum in which the proportions of "A", "B", and free acrolein had increased, relative to **2** plus **3**/**5**, which thus linked the appearance of "A" and "B" with the fragmentation of **3** into acrolein and phosphoramide mustard (**4**). Control studies (see Experimental Section) failed to define the exact nature of aldehyde B and aldehyde hydrate A; however, it was clearly established that A and B were related to acrolein by *reversible* chemistry: addition of excess *O*-methylhydroxylamine ($CH_3ONH_2$) to the metabolite mixture after ~50% fragmentation (125–135 min; based on $^{31}P$ NMR data) afforded a $^{13}C$ NMR spectrum that showed the presence of near equimolar amounts of aldophosphamide *O*-methyl oxime[42] (**18**) and acrolein *O*-methyl oxime (**19**), which were both ~2:1 mixtures of *E*/*Z* isomers that had the chemical shifts given in Scheme III. Unlabeled counterparts of **18** and **19** were

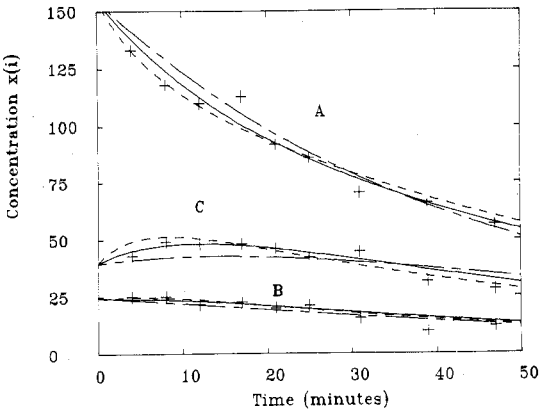
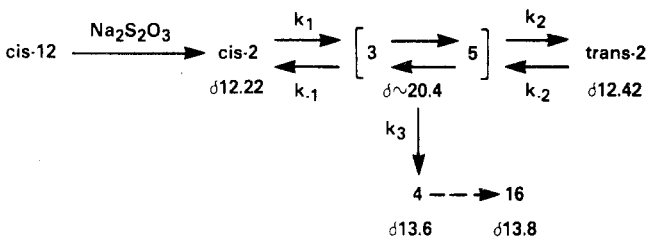



**Figure 4.** Calculated curves for the reaction system described in the legend to Figure 1; $^{31}P$ NMR derived data points are indicated by the symbol "+". The ordinate values refer to relative concentrations (peak heights) of the *i*th reaction component (either A, B, or C), and the indicated times are relative to the start of data acquisition. As detailed under Experimental Section, the solid curves were obtained by simultaneously fitting the experimental data to the integrated kinetic expressions and represent the unrestrained "best-fit" (smallest sum of squares) values for the rate constants $k_i$. The short-dashed and short-long-dashed curves were respectively obtained by the use of $2k_1$ and $0.5k_1$ as constants and calculating new values for the remaining rate constants, $k_{-1}$, $k_2$, and $k_{-2}$.

independently prepared by separate reactions of $CH_3ONH_2$ with **2**/**3**[42] and acrolein, respectively; the *E* and *Z* stereochemical assignments for the diastereomers of **18** and **19** were based on reported structure–spectrum correlations.[42,82] The relatively rapid conversion of acrolein into aldehyde B and hydrate A accounted for the previously described $^2H$ NMR results concerning the near absence of acrolein-$\alpha$-*d* and the loss of its deuterium, e.g., enolization of aldehyde B. The completely reversible nature of this "postfragmentation" chemistry was in accord with the fact that stable derivatives of acrolein are isolable via incubations of **2**/**3** in the presence of a trapping agent.[46,83] Other investigators[35] have stated that the acrolein produced from **2**/**3** may "polymerize", but no details were given.

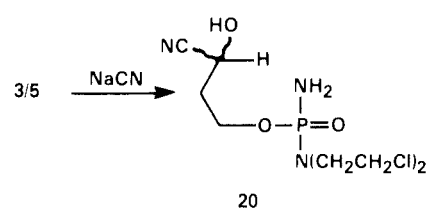
**Kinetic Analysis of "Activated" Metabolites of Compound 1. Measurement of Individual Rate Constants.** The multinuclear NMR studies described above indicated that (1) the *approach* to a pseudoequilibrium distribution of *cis*-**2**, **3**/**5**, and *trans*-**2** was measurable by $^{31}P$ NMR spectroscopy at pH 7.4 and 37 °C, (2) the relative ratio of aldehyde **3** to hydrate **5** was approximately 20:80 during the pre- and postequilibrium periods ($^{13}C$ NMR data), (3) there were no detectable amounts of enol **6** and imine **8** throughout the overall reaction, and (4) the use of excess $Na_2S_2O_3$ to deoxygenate *cis*-**12** and then trap **4** by S-alkylation reactions did not interfere with the kinetics of **2** and **3**/**5**.[84] A simplified representation of the pertinent chemistry is given by eq 4, wherein the chemical-shift values refer to the signals of the reaction components

(80) $^{13}C$ NMR: $\delta$ 159.2, 141.4, 123.9, 24.91.
(81) A signal at $\delta$ 69.78 was ascribed to the C-4 position in **17**.
(82) Karabatsos, G. J; Hsi, N. *Tetrahedron* **1967**, *23*, 1079. Karabatsos, G. J.; Taller, R. A. *Tetrahedron* **1968**, *24*, 3347.
(83) Alarcon, R. A. *Anal. Chem.* **1968**, *40*, 1704. Alarcon, R. A. *Cancer Treat. Rep.* **1976**, *60*, 327.
(84) The time courses shown in Figure 2 were not significantly influenced by either exclusion of the 4-fold molar excess of $Na_2S_2O_3$ or the use of an 8-fold molar excess of this reagent (<10% changes in relative rates and pseudo-equilibrium distributions). In contrast to these observations for $S_2O_3^{2-}$, it has been reported[35] that $HPO_4^{2-}$ catalyzes the decomposition of **2**.




(4)

that were observed by $^{31}P$ NMR spectroscopy at 36.23 MHz. While the overlapped signals for the approximately 20:80 mixture of **3** ($\delta$ 20.35) and **5** ($\delta$ 20.40) were partially resolved at higher magnetic field strength (121.51 MHz),[85] it was possible to treat this mixture of **3** and **5** as a single kinetic component. The same simplification holds for fragment **4**, its mono S-alkylation product, and **16**. Consequently, the rate constants given in eq 4 were used to define the kinetic features of interest, and the resultant set of differential equations (see Experimental Section) were simultaneously fit to the 36.23-MHz $^{31}P$ NMR data by a least-squares procedure using the MLAB facilities of the NIH;[86] possible signal intensity differences due to differential Overhauser effects, as well as differential relaxation rates, were neglected.[87] The least-squares curves (Figure 4, *solid* lines) agreed well with the experimental data points; however, $k_1$, $k_{-1}$, $k_2$, and $k_{-2}$ (Table I, runs 1 and 2) had high dependency values[86] (~0.99) as compared to the dependency value (~0.83) for $k_3$. These findings indicated that the "best-fit" (least-squares) value for $k_3$ was reliable, whereas the best-fit values for the four pseudoequilibrium rate constants might be incorrect due to their interdependence. Evidently, the precision of the $^{31}P$ NMR data particularly during the initial segments of the reaction was not suitable for an unambiguous fit. In order for the *absolute* error limits for $k_1$, $k_{-1}$, $k_2$, and $k_{-2}$ to be evaluated, each best-fit rate constant ($k_i$) was separately multiplied by a factor ($n$), and the value of $nk_i$ was held constant while new values for the other rate constants ($k'_j$) were calculated by the least-squares fitting procedure. The results shown in Figure 4 demonstrated that multiplication of $k_1$ by $n = 2$ and $n = 0.5$ gave calculated curves that were *visually* compatible with the data points, although the sum of squares was slightly higher in each case. On the other hand, multiplication by $n = 10$ and 20 gave calculated curves (Figure 5) that were, by inspection, incompatible with the data points, especially those for *trans*-**2** at the *onset* of "equilibration". These findings indicated the need for highly accurate measurements, particularly at the early stages of the metabolite interconversion for establishing precise values for $k_1$, $k_{-1}$, $k_2$, and $k_{-2}$. Our conclusion that the values of the equilibrium rate constants were reasonable and probably reliable to within factors of 5 was supported by the deoxygenation of *cis*-**12** with $Na_2S_2O_3$ and then the immediate addition of 4 equiv of NaCN, which is known[50] to trap aldehyde **3** (or hydrate **5**) by formation of a relatively stable cyanohydrin derivative (**20**, eq 5).




(5)

**20**

Under these reaction conditions at pH 7.2 and 37 °C, the $^{31}P$ NMR absorption signal for *cis*-**2** ($\delta$ 12.22) was gradually converted into an apparent singlet assigned to either one or both of the diastereomers of **20** ($\delta$ 20.37), which in turn exhibited an apparent half-life of ~3 days during its decomposition to give trapping product **16**. There was no detectable formation of *trans*-**2** ($\delta$ 12.42); however, two signals ($\delta$ 10.91 and 11.68) that were presumably due to *cis*- and *trans*-4-cyanocyclophosphamide[50b] grew in intensity as the overall reaction proceeded to completion. These findings were in accord with ring opening of *cis*-**2** to give **3/5**, which was converted to **20** without appreciable "leakage" to *trans*-**2**. The apparent unidirectional conversion of *cis*-**2** into **20** was therefore treated as two sequential "first-order" reactions (*cis*-**2** $\xrightarrow{k'}$ **3/5** $\xrightarrow{k''}$ **20**) with rate-limiting ring opening ($k' < k''$[NaCN]). A least-squares fit of the $^{31}P$ NMR data gave $k' = 0.056 \pm 0.005$ min$^{-1}$, which was close to the $0.060 \pm 0.006$ min$^{-1}$ value for $k_1$ that was measured in the absence of NaCN (Table I, average value for runs 1 and 2). To the extent that this independent measurement of $k_1$ established the magnitude of the rate constant for the ring opening of *cis*-**2**, then the values of $k_{-1}$, $k_2$, and $k_{-2}$ are likewise reliable (vide supra).

**Kinetic Isotope Effects.** The kinetic isotope effects that result from ring deuteration of 1 have been used to study various aspects of cyclophosphamide metabolism.[37,46,56,70] It was therefore of interest to employ *cis*-**12**-*5,5-d*$_2$ for the first *direct* measurement of kinetic isotope effects associated with eq 4. In addition to mechanistic information, this specifically deuterated precursor of **2/3** would provide information about enol **6**. The deoxygen-

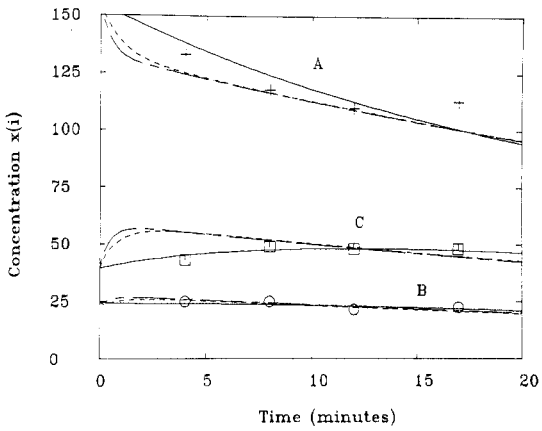



**Figure 5.** Calculated curves, as described in the legend to Figure 4, for the rate constants $k_i$. The solid curves represent the unrestrained "best-fit" (smallest sum of squares) values of $k_i$, whereas the short-dashed and short-long-dashed curves were respectively obtained by the use of $10k_1$ and $20k_1$ as constants and calculating new values for the remaining rate constants, $k_{-1}$, $k_2$, and $k_{-2}$.

(85) The 121.51-MHz $^{31}P$ NMR spectra for the thiosulfate-derived reaction mixture also revealed the presence of a minor component (~1–2%) that gave rise to a signal at $\delta$ 20.16. This component, which was unreactive (invariant signal intensity) and was formed during the deoxygenation of *cis*-**12** with $Na_2S_2O_3$, may be due to the reductive conversion of **2** to "alcophosphamide",[44] $HO(CH_2)_3OP(O)(NH_2)M$, which has a phosphorus chemical shift near that of **3/5**.

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

(87) Comparative $^{31}P$ NMR measurements at 121.51-MHz with gated $^1H$ decoupling, to suppress nuclear Overhauser effects, and a longer pulse recycle time gave an approximately 51:15:34 pseudoequilibrium distribution for *cis*-**2**, **3** plus **5**, and *trans*-**2**, respectively, which was in reasonably good agreement with the 57:13:30 distribution measured at 36.23 MHz. At this lower magnetic-field strength there was a negligible decrease (<10%) in the *total* signal intensity at the end of the reaction ([**2/3**] = 0), whereas the apparent loss of magnetization for the spectra recorded at 121.51 MHz was substantially greater (~50%), in the absence of the precautions mentioned above.

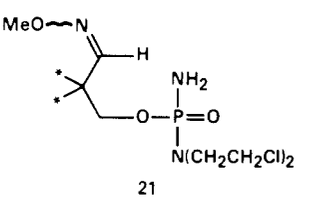
Table I. $^{31}P$ NMR Derived Rate Constants for Reactive Metabolites of Cyclophosphamide (1) under Various Conditions at 37 ± 2 °C[a]

| run no. | conditions | av | rate constants,[b] min$^{-1}$ $k_1$ | $k_{-1}$ | $k_2$ | $k_{-2}$ | $k_3$ |
|---|---|---|---|---|---|---|---|
| 1 | pH 7.4, 1 M lutidine | | 0.066 ± 0.017 (0.992) | 0.19 ± 0.08 (0.998) | 0.52 ± 0.18 (0.988) | 0.24 ± 0.08 (0.998) | 0.081 ± 0.006 (0.835) |
| 2 | pH 7.4, 1 M lutidine | | 0.053 ± 0.012 (0.987) | 0.14 ± 0.06 (0.996) | 0.36 ± 0.13 (0.984) | 0.17 ± 0.06 (0.995) | 0.098 ± 0.008 (0.834) |
| | | av[c] | 0.060 ± 0.006 | 0.17 ± 0.03 | 0.44 ± 0.08 | 0.21 ± 0.04 | 0.090 ± 0.008 |
| 3[d] | pH 7.4, 1 M lutidine | | 0.045 ± 0.010 (0.990) | 0.13 ± 0.03 (0.999) | 0.31 ± 0.08 (0.990) | 0.14 ± 0.03 (0.999) | 0.016 ± 0.001 (0.775) |
| 4 | pH 6.3, 1 M lutidine | | 0.14 ± 0.06 (0.997) | 0.26 ± 0.13 (0.999) | 0.38 ± 0.19 (0.996) | 0.25 ± 0.13 (0.999) | 0.027 ± 0.001 (0.674) |
| 5 | pH 6.3, 1 M lutidine | | 0.13 ± 0.07 (0.995) | 0.23 ± 0.13 (0.999) | 0.34 ± 0.20 (0.995) | 0.22 ± 0.13 (0.999) | 0.034 ± 0.002 (0.767) |
| | | av[c] | 0.14 ± 0.01 | 0.24 ± 0.02 | 0.36 ± 0.02 | 0.24 ± 0.02 | 0.030 ± 0.004 |
| 6 | pH 7.8, 1 M lutidine | | 0.22 ± 0.20 (0.999) | 0.71 ± 0.75 (0.999) | 1.61 ± 1.87 (0.999) | 0.67 ± 0.75 (0.999) | 0.175 ± 0.017 (0.890) |
| 7 | pH 7.8, 1 M lutidine | | 0.20 ± 0.09 (0.995) | 0.73 ± 0.37 (0.999) | 1.60 ± 0.91 (0.995) | 0.67 ± 0.37 (0.999) | 0.163 ± 0.015 (0.909) |
| | | av[c] | 0.21 ± 0.01 | 0.72 ± 0.01 | 1.60 ± 0.01 | 0.67 ± 0.01 | 0.169 ± 0.006 |

[a] Except as noted, precursor *cis*-**12** (~20 mM) was deoxygenated with $Na_2S_2O_3$ (~80 mM) in 2,6-dimethylpyridine ("lutidine") buffer, which was adjusted to the indicated pH by addition of HCl; ΔpH ≤ 0.2 after complete reaction; see text for $^{31}P$ NMR spectroscopic details. [b] The rate constants are defined in eq 4 and were calculated by a five-parameter least-squares fitting procedure; the error limits for $k$ in each run refer to standard error limits; the dependency values are given in parentheses (see text for details). [c] Plus and minus values refer to the precision error for duplicate runs. [d] The starting material was *cis*-**12**-*5,5-d*$_2$.

ation of *cis*-**12**-*5,5-d*$_2$ with $Na_2S_2O_3$ under the standard reaction conditions (1 M lutidine, pH 7.4, 37 °C) and subsequent acquisition of $^{31}P$ NMR spectra afforded a set of best-fit rate constants (Table I, run 3). The error limits for $k_1$, $k_{-1}$, $k_2$, and $k_{-2}$ precluded an evaluation of the usually[88a] (but not always[88b]) small "steric isotope effects" expected for the pseudoequilibrium steps, which were not significantly affected by the multiple isotopic substitution at the C-5 position. However, the value of 5.6 ± 0.4 for the kinetic isotope effect on $k_3$ (cf. Table I, runs 1 and 2 vs. run 3) was consistent with a primary effect for rate-determining removal of a proton that is adjacent to a carbonyl group;[89-91] no correction was made for the *concurrent*, comparatively small (<~1.15) secondary kinetic isotope effect of the remaining α-deuterium.[92] It should also be noted that the inherently diastereotopic $H^a$ and $H^b$ nuclei in the $HC(O)CH^aH^bCH_2$ moiety in **3** must give rise to different kinetic isotope effects; however, this point can be neglected, since $(k_H/k_D)_{obsd} = (k_{H^a} + k_{H^b})/(k_{D^a} + k_{D^b})$ i.e., the ratio of observed rate constants leads to a cancellation of the reactivity difference between the a- and b-type nuclei.

"Wash out" of deuterium from labeled **3**, via exchange between enol **6** and the $H_2O$ solvent, could have decreased the magnitude of the kinetic isotope effect that was measured by $^{31}P$ NMR spectroscopy; consequently, a sample of *cis*-**2**-*5,5-d*$_2$ was allowed to undergo ~50% fragmentation under the same conditions used for the kinetic studies. *O*-Methylhydroxylamine was then added to trap quantitatively the "unreacted" **2**, **3**, and **5** as the *E* and *Z* diastereomers of **21**. The $^1H$ content at the




**21**

$^2H$-labeled carbon (*) was measured by proton NMR spectroscopy (300 MHz) by a comparison of the spectral regions at δ 2.5–2.6 (allylic) and 3.7–3.8 (methyl), and it was found that there was <~5% deuterium wash out (i.e., no detectable proton incorporation). This result showed that, *if* fragmentation of **3** proceeded through enol **6** (or its conjugate base), then the rate of return to partially protonated **3** was slow relative to the velocity of the forward reaction. In addition, one can rule out the possibility of a relatively facile interconversion of **3** and enol **6** wherein the enol is a *cul-de-sac* in the reaction manifold.

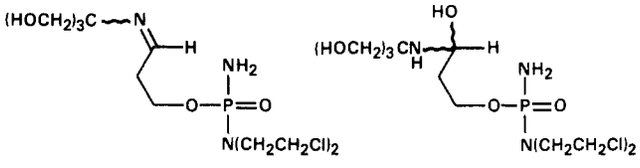
**pH and Buffer Effects.** It was not apparent to us how the pH of the reaction medium would affect the values of the four pseudoequilibrium rate constants ($k_1$, $k_{-1}$, $k_2$, and $k_{-2}$), whereas the kinetic isotope effect found for $k_3$ implied that the fragmentation step should be accelerated by increased pH, and decelerated by lowered pH, until the onset of acid-catalyzed P–N bond cleavage.[32,60d] The use of 1 M lutidine buffer at 37 °C led to a rather narrow (but biologically relevant) pH range that could be studied; at pH >~8 the initially homogeneous reaction solution became biphasic, and at pH <~5 the buffering capacity would be insufficient for extensive formation of **4**, which generates 2 equiv of HCl. Table I lists the results obtained by simultaneously fitting $^{31}P$ NMR data for kinetic runs at either pH 7.4 (runs 1 and 2), 6.3 (runs 4 and 5), or 7.8 (runs 6 and 7). Inspection of the results for $k_1$, $k_{-1}$, $k_2$, and $k_{-2}$ indicates that the reasonably good degree of precision (cf. average values) was underminded by disappointingly high standard errors, which precluded a meaningful

(88) (a) For example, see Anet, F. A. L.; Basus, V. J.; Hewett, A. P. W.; Saunders, M. *J. Am. Chem. Soc.* **1980**, *102*, 3945. Aydin, R.; Gunther, H. *Angew. Chem.* **1981**, *93*, 1000. (b) For example, see: Goldstein, M. J.; Pressman, E. J. *J. Am. Chem. Soc.* **1981**, *103*, 6533.
(89) March, J. In "Advanced Organic Chemistry", 2nd ed.; McGraw-Hill: New York, 1977; pp 536–537.
(90) Miller, D. J.; Saunders, W. H., Jr. *J. Org. Chem.* **1982**, *47*, 5039.
(91) Leadlay, P. F.; Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5617.
(92) Scheppele, S. E. *Chem. Rev.* **1972**, *72*, 511, and pertinent references therein.

analysis of the influence of pH on these pseudoequilibrium rate constants. By contrast, the relatively low (±5–10%) standard errors for $k_3$ allowed one to conclude that the rate of fragmentation was accelerated by an increase in the pH of the solution (~6-fold increase in $k_3$ for pH 7.8 vs. pH 6.3).

Sladek et al.[52] have recently noted their unpublished results which indicate that the conversion of **2** to acrolein and **4** is subject to general-base catalysis. That the sterically hindered nitrogen in lutidine may catalyze the fragmentation of **3** was suggested by kinetic measurements in 0.5 M lutidine at pH 7.4 and 37 °C, which gave a value for $k_3$ (0.068 ± 0.003 $min^{-1}$) that was somewhat less than the value of $k_3$ (0.090 ± 0.008 $min^{-1}$) listed in Table I for 1 M lutidine.[93] The influence of the concentration of lutidine on the pseudoequilibrium rate constants[94] was not clear, for the reasons mentioned above.

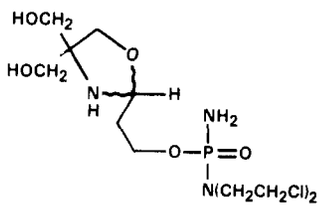
A fundamentally different kind of influence that a buffering agent may have on the chemistry of **2**/"**3**" was evident in our preliminary kinetic studies of **2**/"**3**" that employed tris(hydroxymethyl)aminomethane [Tris, $H_2NC(CH_2OH)_3$].[71] In short, it was found by $^{31}P$ NMR spectroscopy that the overall rate of conversion of **2** to **4** in 0.5–1 M Tris buffer at pH 7.4 was significantly slower than the rate measured in lutidine buffer. $^{31}P$ NMR spectra, which were subsequently recorded for the deoxygenation of *cis*-**12** (20 mM) with $Na_2S_2O_3$ in 1 M lutidine (pH 7.4, 37 °C), in the presence of various amounts of added Tris, revealed that the pseudoequilibrium distribution of "acyclic" ($\delta$ 20.40) to "cyclic" ($\delta$ 12.42 plus 12.22) reactants changed from 13:87, in the absence of Tris, to 36:64 with 2 equiv of Tris, and further changed to 53:47 with 4 equiv of Tris; the latter conditions led to an ~2-fold decrease in the rate of production of **4**/**16**. These findings were consistent with competing formation of adduct(s) derived from aldophosphamide and Tris (**22–24**) that had



**22**



**23**



**24**

virtually the same chemical shift as **3** but was (were) more resistant to fragmentation (direct or stepwise), which would give **4**.[95] Acyclic structures **22–24** were indicated by the downfield $^{31}P$ resonance position; however, the Schiff base **22** was discounted by the absence of either proton or carbon signals for CH=N and the observation of a $^{13}C$ absorption at $\delta$ 91.76, which was instead consistent with either **23** or **24**. $^{31}P$ NMR spectroscopy has been used by Low et al.[35] to examine the decomposition of **12** (unspecified sterochemistry) in 0.5 M Tris buffer (pH 7.4, 37 °C), and the signal that we have now assigned to **23**/**24** was incorrectly ascribed by these investigators to **2** (unspecified stereochemistry).[35] Since their erroneous assignment was incorporated into mechanistic interpretations of UV spectroscopic data for the kinetics of the formation of acrolein and **4**, the reported[35] conclusions must be reconsidered. Our further investigation of the formation of adducts between **3**/**5** and primary amines is described in a following section.

**Measurement of Apparent Half-Lives.** The observations that aldehyde **3** and hydrate **5** could be treated as a single kinetic species (hereafter referred to as "**3**") and that interconversion of *cis*-**2**, "**3**", and *trans*-**2** gave rise to a pseudoequilibrium mixture implied that *each* of these reaction components could be characterized by an *apparent half-life*, $\tau^*_{1/2}$. Least-squares fits of *separate* "first-order" plots of the disappearance of *cis*-**2**, "**3**", and *trans*-**2** at "equilibrium" ($t$ > 15–30 min) in 1 M lutidine buffer (pH 7.4, 37 °C) gave, respectively, $\tau^*_{1/2}$ = 38.3 (correlation coefficient $r$ = 0.992), 37.2 ($r$ = 0.911), and 38.5 min ($r$ = 0.974), or an *average apparent half-life* ($\bar{\tau}^*_{1/2}$) equal to ~38 min. The results of a duplicate analysis indicated a precision error of ±5% (Table II, runs 1 and 2). Data obtained at pH 6.3 (run 3) and 5.5 (run 4) gave $\bar{\tau}^*_{1/2}$ = 123 ± 6 and 168 ± 23 min, respectively. Since the *ratio* of the pH-modulated values of $\bar{\tau}^*_{1/2}$ at pH 6.3 and 7.4 was equivalent, within the experimental error limits, to the corresponding *ratio* of $k_3^{-1}$ that was indicated by the five-parameter fitting procedure (cf. Table I), it was concluded that $\tau^*_{1/2}$ could be used as a readily determined kinetic parameter that was related to the apparent lifetime of cyclophosphamide's metabolites or conjugates under *pseudoequilibrium conditions*.

**Evaluation of Chemical Factors That Affect the Apparent Half-Lives of Compounds 2 and "3".** For comparative purposes, it was desirable to measure the values of $\tau^*_{1/2}$ for **2** and "**3**" under conditions that were essentially the same as those reported by Völker et al.[96] in their original kinetic study of synthetic **2**/"**3**", which employed a combination of analytical methods, viz., TLC separations, colorimetric measurement of alkylating activity toward 4-(*p*-nitrobenzyl)pyridine (NBP), and fluorometric measurement of acrolein by derivatization with 7-hydroxyquinoline. Völker et al.[96] were unable to distingish between *cis*- and *trans*-**2**, and they could not differentiate between **3** and **5**; however, they concluded that (1) $\tau^*_{1/2}$ = 80 min for "**3**" in 70 mM phosphate buffer at pH 7.0 and 37 °C, (2) the "equilibrium" between **2** and "**3**", which was defined by $K_{eq}$ = [**2**]/["**3**"], was equal to 1.69 under these reaction conditions, and (3) an ~30% *reduction* in the rate of acrolein release in the presence of $Ca^{2+}$ and $Mg^{2+}$ was due to metal ion complexation with either **2** or "**3**". Our $^{31}P$ NMR kinetic measurements at 37 °C with approximately the same initial concentration of **2**/"**3**" (23 mM) in 70 mM $K_2PO_4H$–$KPO_4H_2$ solution, with manual titration to maintain pH ~7.0, gave $\tau^*_{1/2}$ =

(93) For a review of hydrolysis reactions that include an example of the first-order dependency of rate on the concentration of 2,6-dimethylpyridine, see: Cox, J. R., Jr.; Ramsay, O. B. *Chem. Rev.* **1964**, *64*, 317.

(94) The values of the pseudoequilibrium rate constants measured in 0.5 M lutidine buffer at pH 7.4 and 37 °C did not differ, within the standard error limits, from those values obtained in 1 M lutidine (cf. Table I, runs 1 and 2): $k_1$ = 0.071 ± 0.008 $min^{-1}$; $k_{-1}$ = 0.18 ± 0.03 $min^{-1}$; $k_2$ = 0.34 ± 0.06 $min^{-1}$; $k_{-2}$ = 0.17 ± 0.03 $min^{-1}$.

(95) For a recent study of acid-catalyzed hydrolysis of Schiff bases, see: Kamogawa, H.; Mukai, H.; Nakajima, Y.; Nanasawa, M. *J. Polym. Sci., Chem. Ed.* **1982**, *20*, 3121.

(96) Völker, G.; Dräger, U.; Peter, G.; Hohorst, H. J. *Arzneim.-Forsch.* **1974**, *24*, 1172.

Table II. $^{31}P$ NMR Derived Pseudoequilibrium Distributions and Apparent Half-Lives for Reactive Metabolites of Cyclophosphamide (1) under Various Conditions at 37 ± 2 °C[a]

| run no. | conditions | pseudoequilibrium distribution[b] of *cis*-2/"3"/*trans*-2 | $\tau^*_{1/2}$,[c] min | | | |
|---|---|---|---|---|---|---|
| | | | *cis*-2 | "3" | *trans*-2 | $\bar{\tau}^*_{1/2}$, min |
| 1 | pH 7.4, 1 M lutidine | 56:14:30 | 38 (0.992) | 37 (0.911) | 38 (0.974) | 38 ± 1 |
| 2 | pH 7.4, 1 M lutidine | 58:12:30 | 38 (0.996) | 38 (0.862) | 37 (0.957) | 38 ± 1 |
| 3[d] | pH 6.3, 1 M lutidine | 42:24:34 | 117 (0.988) | 133 (0.958) | 120 (0.968) | 123 ± 6 |
| 4 | pH 5.5, 1 M lutidine | 38:30:32 | 133 (0.970) | 193 (0.978) | 178 (0.986) | 168 ± 23 |
| 5 | pH 7.0, 70 mM phosphate | 42:21:37 | 63 (0.995) | 56 (0.932) | 58 (0.970) | 59 ± 3 |
| 6[d] | pH 7.4, 0.5 M lutidine | 51:15:34 | 51 (0.972) | 54 (0.994) | 44 (0.978) | 50 ± 4 |
| 7 | pH 7.4, 0.5 M lutidine, 0.5 M NaCl | 48:18:34 | 38 (0.990) | 41 (0.997) | 37 (0.974) | 39 ± 2 |
| 8 | pH 7.4, 0.5 M lutidine, 0.5 M KCl | 50:18:32 | 41 (0.988) | 55 (0.863) | 35 (0.980) | 44 ± 8 |
| 9 | pH 7.4, 0.5 M lutidine, 0.5 M $MgCl_2$ | 52:16:32 | 28 (0.976) | 24 (0.952) | 33 (0.944) | 28 ± 3 |
| 10 | pH 7.4, 0.5 M lutidine, 0.5 M $CaCl_2$ | 52:16:32 | 30 (0.996) | 35 (0.890) | 33 (0.986) | 32 ± 2 |
| 11 | pH 7.4, 1 M lutidine, 2 equiv of Tris | 40:24[e]:36 | 41 (0.991) | 38[e] (0.990) | 42 (0.980) | 40 ± 2 |
| 12[d] | pH 7.4, 1 M lutidine, 4 equiv of Tris | 30:53[e]:17 | 56 (0.927) | 71[e] (0.983) | 51 (0.992) | 59 ± 8 |
| 13 | pH 7.4, 1 M lutidine, 4 equiv of *t*-$BuNH_2$ | 53:13:34 | 37 (0.998) | 48 (0.959) | 42 (0.959) | 42 ± 4 |
| 14 | pH 7.4, 1 M lutidine, 4 equiv of $H_2NCH_2CH_2OH$ | 59:13:28 | 21 (0.996) | 28 (0.895) | 27 (0.967) | 25 ± 3 |
| 15 | "pH" 7.2, 0.8 M lutidine, 20% $Me_2SO$ | 51:14:35 | 67 (0.990) | 72 (0.937) | 71 (0.987) | 70 ± 2 |
| 16 | same as run 15, + 4 equiv of Tris | 35:43[e]:22 | 67 (0.999) | 64[e] (0.997) | 76 (0.998) | 69 ± 5 |
| 17 | same as run 15, + 4 equiv of 4-nitroaniline | 53:14:33 | 58 (0.999) | *f* | 76 (0.972) | 67 ± 9 |
| 18 | same as run 15, + 4 equiv of aniline | 47:25[g]:28 | 44 (0.999) | 48[h] | 45 (0.997) | 46 ± 2 |
| 19 | same as run 15, + 4 equiv of 4-methoxyaniline | 51:27[i]:22 | 21 (0.942) | 15[j] | 19 (0.982) | 18 ± 2 |
| 20 | pH 7.4, 1 M lutidine, 2 equiv of L-Lys | 54:13:33 | 27 (0.995) | *f* | 30 (0.983) | 29 ± 2 |
| 21 | pH 7.4, 1 M lutidine, 2 equiv of *N*-Ac-L-Lys Me ester | 53:17:30 | 24 (0.977) | 22 (0.966) | 25 (0.986) | 24 ± 1 |
| 22 | pH 7.4, 1 M lutidine, 4 equiv of *N*-Ac-L-Lys Me ester | 56:13:31 | 25 (0.994) | 21 (0.834) | 20 (0.997) | 22 ± 2 |
| 23 | pH 7.4, 1 M lutidine, ~1 equiv of Lys residues[k] | 60:10:30 | 19 (0.998) | 20 (0.792) | 23 (0.975) | 21 ± 2 |

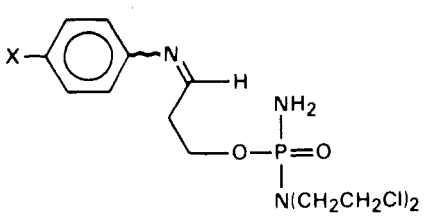
[a] In each run, precursor *cis*-12 (~20 mM) was deoxygenated with $Na_2S_2O_3$ (~80 mM) in 2,6-dimethylpyridine ("lutidine") buffer, which was adjusted to the indicated pH by addition of HCl; $\Delta pH \leq 0.2$ after complete reaction; see text for $^{31}P$ NMR spectroscopic details. [b] See text for details; the estimated error limits are ±5–20%. [c] Individual values of the apparent half-life (see text for details); the values in parentheses refer to the linear least-squares correlation coefficient; the estimated error limits are ±5–20%. [d] Data refer to two independent measurements. [e] Refers to "3" and the amine adduct with aldophosphamide. [f] Not determined. [g] Refers to an approximately 15:10 mixture of "3" and the amine adduct with aldophosphamide. [h] Average value for "3" [$\tau^*_{1/2}$ = 48 min (0.986)] and the amine adduct with aldophosphamide [$\tau^*_{1/2}$ = 37 min (0.981)]. [i] Refers to an approximately 14:13 mixture of "3" and the amine adduct with aldophosphamide. [j] Average value for "3" [$\tau^*_{1/2}$ = 14 min (0.927)] and the amine adduct with aldophosphamide [$\tau^*_{1/2}$ = 16.5 min (0.758)]. [k] Estimated value for lysyl residues in human serum albumin [HSA/*cis*-12 = 3:1 (w/w)].

56 min for "3", $\bar{\tau}^*_{1/2}$ = 59 ± 3 min for *cis*-2, "3", and *trans*-2, and $K_{eq}$ = 3.8 ± 0.4 (Table II, run 5), which indicated that the kinetic methods used by Völker et al.[96] provided relatively reliable results.[97] The reported[96] divalent metal ion stabilization effect was not evidenced by our $^{31}P$ NMR measurements for *cis*-2, "3", and *trans*-2 in 0.5 M lutidine (pH 7.4, 37 °C) that contained either no metal ion or 0.5 M (20-fold excess) NaCl, KCl, $CaCl_2$, or $MgCl_2$ and gave, respectively, $\bar{\tau}^*_{1/2}$ = 50 ± 4, 39 ± 2, 44 ± 8, 32 ± 2, and 28 ± 3 min (Table II, runs 6–10). Furthermore, kinetic runs 6–10 led to the same pseudoequilibrium distribution of *cis*-2, "3" and *trans*-2 (50:16:34), within a precision error of ±5–10%, which demonstrated that the metal ions did not significantly alter the equilibrium under these reaction conditions. The possibility of a relatively small rate *acceleration* caused by a large excess of $M^{2+}$ can be rationalized in terms of precedented

(97) The 312-min half-life for 2 → 4 reported by Hohorst et al.,[47a] and quoted by other investigators,[12,28] is much longer than the 59 ± 3 min value of $\bar{\tau}^*_{1/2}$ for 2 → 4 that we have measured unambiguously by $^{31}P$ NMR spectroscopy under the same reaction conditions. The half-life for 2/3 has also been determined[35] by measurement of the rate of production of acrolein, which gave an ~50-min value in 100 mM phosphate buffer at pH 7.0 and 37 °C.

catalysis mechanisms;[98] however, the use of only 1 or 2 equiv of $MgCl_2$ (data not presented) had virtually no effect on the apparent lifetime of *cis*-**2**.

The rapid condensation of **2**/"**3**" with Tris to give **23**/**24** under mild reaction conditions prompted a brief survey of other primary amines in order to gauge the feasibility of aldophosphamide adducts as possible metabolite conjugates. In 1 M lutidine buffer at pH 7.4 and 37 °C, the presence of 2 equiv of Tris had no significant influence on the value of $\bar{\tau}^*_{1/2}$ and only a small effect on the pseudoequilibrium distribution (Table II, run 11 vs. run 1); 4 equiv of Tris (run 12) increased the value of $\bar{\tau}^*_{1/2}$ ~1.5-fold and caused further perturbation of the distribution of reaction components. In contrast, the presence of 4 equiv of either *t*-$BuNH_2$ (run 13) or 2-aminoethanol (run 14) had no apparent stabilizing effect upon either the value of $\bar{\tau}^*_{1/2}$ or the distribution of reaction components. These findings and the fact that Tris is a weak base ($pK_a$ = 8.3) compared to *t*-$BuNH_2$ ($pK_a$ = 10.8) and 2-aminoethanol ($pK_a$ = 9.5) suggested that the basicity of a primary amino group may have been an important factor with regard to the formation and hydrolytic stability of an amine adduct with aldophosphamide. This possibility was examined with **2**/"**3**" in 1 M lutidine buffer (pH 7.4) that was diluted with dimethyl sulfoxide to solubilize 4 equiv of either 4-methoxyaniline ($pK_a$ = 5.3), aniline ($pK_a$ = 4.6), or 4-nitroaniline ($pK_a$ = 1.0). The ~80:20 v/v buffer–$Me_2SO$ mixture, which had an apparent pH of ≃7.2, was first used to reexamine the chemistry of **2**/"**3**" alone (Table II, run 15 vs. run 1) and in the presence of 4 equiv of Tris (run 16 vs. run 15). It was found that the value of $\bar{\tau}^*_{1/2}$ increased from 38 to 69 min in the buffer–$Me_2SO$ mixture, as expected,[51] and that $\bar{\tau}^*_{1/2}$ was *not* significantly altered by the presence of Tris in the buffer–$Me_2SO$ mixture, although the Tris-induced perturbation of the pseudoequilibrium distribution was still evident.[99] The results with 4-nitroaniline (run 17) were essentially the same as those obtained for **2**/"**3**" alone (run 15); however, aniline (run 18) and 4-methoxyaniline (run 19) led to ~1.5- and ~4-fold *reductions* in the value of $\bar{\tau}^*_{1/2}$, respectively. In addition, aniline and 4-methoxyaniline each led to the gradual appearance and then disappearance of a $^{31}P$ NMR signal (δ 22.0–22.3) that was near the absorption due to "**3**" (δ 22.1–22.2) and was therefore assigned tentatively to either the corresponding Schiff base derivative (**25**, X =




**25**

H, $OCH_3$) or its hydrated (hemiaminal) counterpart. The pseudoequilibrium mixtures derived from aniline and 4-methoxyaniline each had approximately 50:13:13:24 distributions of *cis*-**2**, "**3**", **25**, and *trans*-**2**, wherein $\tau^*_{1/2}$ for

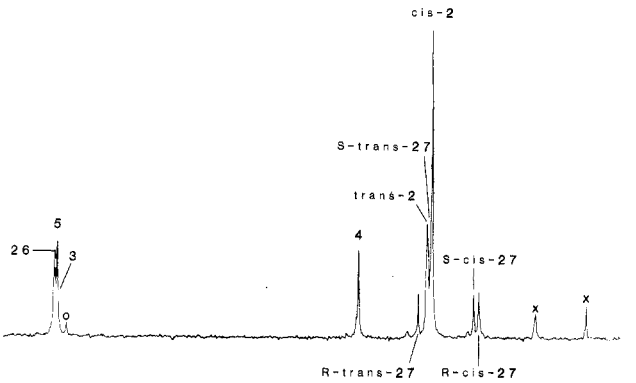



**Figure 6.** 121.51-MHz $^{31}P$ NMR time-averaged spectrum recorded ~45 min after the partial decomposition of racemic *cis*-**2** (20 mM initial concentration) in 1 M 2,6-dimethylpyridine buffer at pH 7.4 and ~20 °C in the presence of an initially 4-fold molar excess of *N*-acetyl-L-cysteine. See text and Scheme IV for signal assignments; the asterisks refer to unidentified side-reaction products. The assignments for *cis*- and *trans*-**27** are tentative; *R* and *S* refer to the absolute configuration at phosphorus.

"**3**" and **25** were roughly comparable.

Approximately 1.3- to 1.8-fold *reductions* in the value of $\bar{\tau}^*_{1/2}$ for **2**/"**3**" (Table II, run 1) were caused by the presence of L-lysine (2 equiv, run 20), its *N*-acetyl methyl ester (2–4 equiv, runs 21 and 22), and human serum albumin (~1 equiv of lysyl residues, run 23). However, none of these samples gave evidence for either an appreciable alteration of the metabolite distribution or an additional $^{31}P$ NMR signal indicative of a Schiff-base conjugate of aldophosphamide.[100a] Based on these findings and the aforementioned results for other amino-containing compounds, it was evident that mechanistic studies beyond the scope of the present work would be needed to reliably decipher how the chemistry of **2**/"**3**" is altered by primary amino groups—especially in multifunctional biological molecules.[100b]

Reports[47] concerning the "deactivation" of cyclophosphamide metabolites by sulfhydryl compounds (e.g., *N*-acetyl-L-cysteine[47b]) via the reversible formation of 4-thio derivatives (**7**, Scheme I) have led to considerable interest[4,23,26,28,39,47,48,66,101] in the biological significance of this chemistry. On the other hand, several synthetic representatives of **7** have been shown[66] to undergo relatively rapid hydrolysis ($\tau_{1/2}$ = 4–17 min at pH 7, 37 °C), which has led to questions[7] regarding the compatibility of such kinetic data with the sulfhydryl "deactivation" hypothesis.[47] $^{31}P$ NMR spectroscopy was therefore used to investigate the influence of *N*-acetyl-L-cysteine (R*SH) upon the formation, distribution, and kinetics of **2**/"**3**" and the sulfhydryl conjugates. Scheme IV is a partial representation of the expected reaction components; for the sake of convenience, the possible intermediacy of imine **8** is not shown, and only the 2*R*,4*R* enantiomer of racemic *cis*-**2** is shown as the point of entry into the reaction manifold.[102]

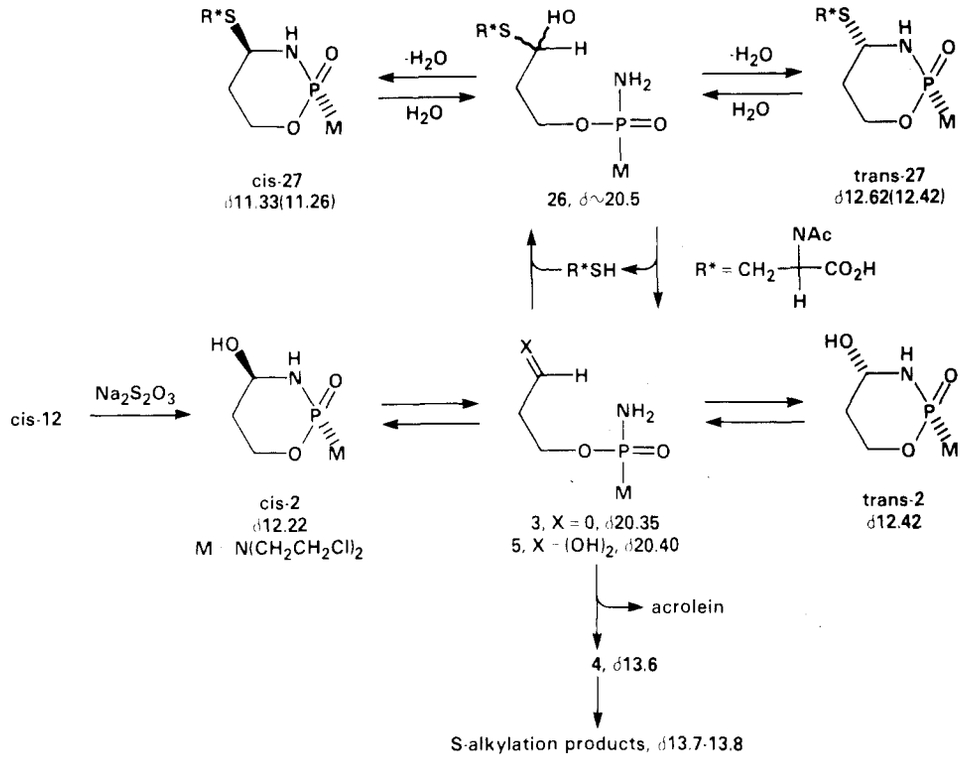
(98) For Lewis acid and template effects, see: Egan, W.; Schneerson, R.; Werner, K. E.; Zon, G. *J. Am. Chem. Soc.* **1982**, *104*, 2898, and pertinent references therein. For kinetic effects related to the dielectric constant of the reaction medium, see: Owen, W. R.; Stewart, P. J. *J. Pharm. Sci.* **1979**, *68*, 992.

(99) Organic cosolvents are known to alter hydrolysis rates. For example, see: Bonicamp, J. M.; Haake, P. *Tetrahedron Lett.* **1982**, *23*, 3127.

(100) (a) As a lead reference to nonenzymatic glycosylation (Schiff base formation) at ε-amino groups of lysine residues in proteins, see: Garlick, R. L.; Mazer, J. S. *J. Biol. Chem.* **1983**, *258*, 6142. (b) For a detailed study of the rates and equilibria of aldimine formation between pyridoxal 5′-phosphate and 1-aminohexane, see: Sanchez-Ruiz, J. M.; Rodriguez-Pulido, J. M.; Llor, J.; Cortijo, M. *J. Chem. Soc., Perkin. Trans. 2* **1982**, 1425.

(101) Asta-Werke A.-G. Belgian Patent BE 892 589; *Chem. Abstr.* **1983**, *98*, 53945*s*. Levy, L.; Vredevoe, D. L. *Semin. Oncol.* **1983**, *10* (Suppl. 1), 7 [see also other articles in this proceedings of a symposium on *N*-acetylcysteine as a chemoprotective adjunct].

Scheme IV




Deoxygenation of a solution of racemic *cis*-**12** (20 mM) in 1 M lutidine buffer (pH 7.4, ~20 °C) with 4 equiv of $Na_2S_2O_3$, followed by the immediate addition of 4 equiv of enantiomerically pure R*SH, gave, after ~45 min, a $^{31}P$ spectrum (Figure 6) in which the previously assigned signals for *cis*-**2**, "**3**", *trans*-**2**, and **4** (see Scheme IV for $\delta$ values) were accompanied by new signals due to the diastereomers of the acyclic thiohemiacetal **26** (two of the four possible diastereomers were partially resolved) and the diastereomers of **27** (three of the four possible diastereomers were completely resolved, and one diastereomer was partially obscured by the signal for *trans*-**2**). The downfield signals assigned to **26** were diagnostic of ring-opened

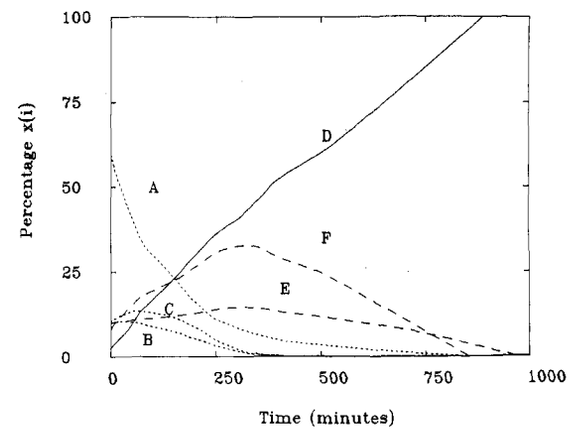
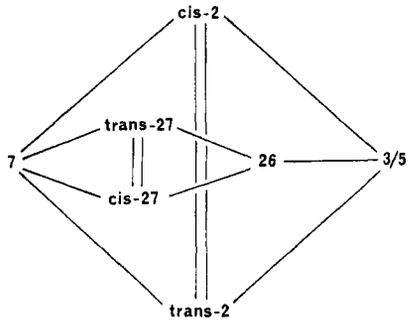



**Figure 7.** $^{31}P$ NMR derived time courses (~20 °C) for the reactants referred to in the legend to Figure 6. The "zero" time corresponds to ~15 min after the mixing of the initial reactants. The ordinate values refer to relative concentrations (peak heights) of the *i*th reaction component (A–F), expressed as a percentage of the total signal intensity, and the indicated times are relative to the start of data acquisition. Each curve was generated by mathematically smoothing (3 times a 5-point smooth) and interpolating the data points, which were obtained from 20 spectra and are not shown for the sake of clarity: A = *cis*-**2**, B = **3**/**5**, C = *trans*-**2**, D = **4**/**16**, E = **26**, and F = *cis*- and *trans*-**27**. See Figure 8 for the experimental data points for *cis*-**2**.

congeners of "**3**", while the signal assignments for the diastereomers of **27** were based on their independent synthesis from racemic *cis*-**12**, as well as the reaction of *N*-acetyl-L-cysteine with an enantiomerically pure sample of (2*S*,4*S*)-*cis*-**12**, which halved the number of signals observed for **27**, and the reaction of racemic *cis*-**12** with an achiral sulfhydryl model compound, viz., 2-mercaptoethanol (see Experimental Section for details).

The $^{31}P$ NMR determined time courses for the observed reactants are presented in Figure 7. Each curve was generated by mathematically smoothing (3-times a 5-point smooth) and interpolating the data points, which were

(102) If one allows for possible tautomerization, pseudorotation (at phosphorus), $S_N2$ (at C-4), E2, and addition processes, then all of these reversible prefragmentation reactions may be conveniently displayed by the following graph in which any




pair of adjacent vertices (reaction components) and their connection line (or lines) represent a reaction pathway (or pathways). The two lines of connection for *cis*- and *trans*-**2**, as well as *cis*- and *trans*-**27**, represent possible interconversions by either direct displacement or pseudorotation. Also worthwhile to note here is that the principle of microscopic reversibility states that the pathways of forward and reverse reactions at equilibrium are described by the same energy surface; it does *not* state that the profile of such a surface must be symmetrical with respect to the reaction path [cf. Mislow, K. *Acc. Chem. Res.* **1970**, *3* 321].

# Error

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Table III

| time, min | $A_{540}$ | residual alkylating act., % |
|---|---|---|
| "zero" | 0.568 | 90 |
| 18 | | 79 |
| 20 | 0.546 | |
| 33 | | 72 |
| 35 | 0.530 | |
| 60 | 0.458 | 57 |
| 120 | 0.376 | 35 |
| 140 | | 29 |
| 150 | 0.347 | |
| 180 | 0.378 | |
| 14 × 60 | | 0 |
| 18 × 60 | 0.048 | |

Draeger et al.[47b] were due primarily to the acid-induced stability of **2**/**3** and the mustard-bearing reaction fragments, rather than the inertness of **26**/**27**. This explanation was supported by control studies of the reaction of *N*-acetyl-L-cysteine with **2**/"**3**" in 70 mM phosphate "buffer" at 37 °C with manual titration to maintain a pH value of ~7 (see Experimental Section for details).

## Conclusions

Multinuclear Fourier-transform NMR spectroscopy proved to be a very useful analytical tool for studies of the transient metabolites of **1**. We believe that the present results have provided the first persuasive evidence for the existence and the time scale of the interconversion of *cis*- and *trans*-**2** via aldehyde **3** and hydrate **5**. Our spectroscopic observation of the complete epimerization of *cis*- and *trans*-**2** within minutes after the formation of this cis isomer at pH 7.4 and 37 °C indicates that the theoretically interesting question of the *stereoselectivity* of liver microsomal oxidation of the C-4 position in a given enantiomer of **1** will be very difficult to assess. By the same token, it will be difficult to establish whether any one of these metabolites serves as a type of "chemoselective" agent for differential cellular uptake. Nevertheless, the general and time-dependent features of the $^{31}P$ NMR spectra found for the reactions of **2** and **3** also obtain for the corresponding metabolite analogues derived from 4-methyl-, 4-phenyl-, and 5,5-dimethylcyclophosphamide and isophosphamide,[71] which thereby provide a new approach to metabolite structure–chemistry–activity correlations in studies of the mechanism of action of cyclophosphamide and its congeners.[6,7]

In lutidine buffer at pH 7.4 and 37 °C, the reversible reactions of **2**, **3**, and **5** compete with the pH-sensitive, rate-limiting abstraction of a proton from **3** to give acrolein and **4**. The ~38 min apparent half-life measured for *cis*-**2**, *trans*-**2**, and **3**/**5** in 1 M lutidine at pH 7.4 and 37 °C was remarkably close to the 37 ± 6 min apparent plasma half-life reported[52] for **2** in rats injected with **1**. It has yet to be determined whether this is a coincidental equivalence of unrelated kinetic phenomena or if it is due to a common kinetic element, viz., rate-limiting metabolite fragmentation to give acrolein and **4**. The rate-limiting nature of the fragmentation of **3** *in lutidine buffer* was best supported by $^{31}P$ NMR measurements of the primary kinetic isotope effect ($k_H/k_D$ = 5.6 ± 0.4) that resulted from perdeuteration of the methylene group adjacent to the carbonyl moiety in **3**. An aggregate kinetic isotope effect of similar magnitude (5.3) has been found[70] by mass spectrometric determination of the ratio of acrolein/acrolein-$\alpha$-*d* collected during the initial incubation of an equimolar ratio of **1** and **1**-5,5-$d_2$ with rat-liver microsomes *in phosphate buffer* (0.15 M) at 37 °C. On the other hand, $^{31}P$ NMR studies in conjunction with UV spectroscopic measurements of the acrolein produced *in phosphate buffer* have led to the conclusion that ring opening of **2** to **3** is rate limiting,[35] which does not account for the aforementioned, independently determined, isotope effects unless one evokes a rather unusual participation mechanism.[105] It is possible that differences in experimental reaction conditions could lead to "crossover" with regard to the nature of the rate-determining step in the reaction manifold for **2**/"**3**"; however, further studies are needed to resolve this issue, especially in view of the suggestion[35] that the fragmentation kinetics may be related to the oncostatic specificity of **1**. For this reason it is also important to clarify how amino-bearing compounds influence the chemistry and kinetics of **2**/"**3**"; hydrolytically reversible Schiff base or hemiaminal adducts of **3** are chemically reasonable metabolite conjugates, and synthetic versions of these materials may provide new "preactivated" cyclophosphamide analogues[7] with improved therapeutic properties.

The $^{31}P$ NMR data for the chemistry and kinetics of **2**/"**3**" in the presence of *N*-acetyl-L-cysteine support the earlier report by Draeger et al.[48] that this prototypal sulfhydryl compound "leads to an equilibrium between the reaction product [i.e., **26**/**27**] and the starting substrates [i.e., **2**/"**3**"]". While the formation of **26**/**27** would be expected from the fact that sulfhydryl addition to an aldehyde is *thermodynamically* more favorable than the corresponding hydrate by at least 4 kcal/mol,[106] the major new insight provided by our results is that the reaction mixture with ≤4 equiv of sulfhydryl compound does *not* provide a constant level of "alkylating capacity" at pH ~7,[48] since the total concentration of the alkylating species of interest, viz., **2**, "**3**", **26**, and **27**, was observed spectroscopically to gradually decrease. The sometimes misleading nature of colorimetric methods, such as the NBP assay, for assessing "alkylating capacity" has also been demonstrated by our NMR studies of **4**[32] and the anticancer drug "thiotepa".[107] Since the lifetimes ($\tau_{1/2}$ < 2–7 min) for the diastereomers of **27** in water under irreversible reaction conditions were shown to be substantially less than the apparent lifetimes ($\tau^*_{1/2} \approx$ 38 min) for *cis*-**2**, "**3**", and *trans*-**2**, the pharmacokinetic significance of analogous conjugates in vivo should be critically dependent on the availability of reactive SH groups, the rate of diffusion of **2**/**3** from the ejected nucleofuge, local pH, and thermodynamics. In this connection, it is interesting to note that the presence of sodium 4-[(2-sulfonatoethyl)thio]cyclophosphamide (**7**, R = $CH_2CH_2SO_3^-Na^+$) has been reported[48] as a component in the urine of patients who received a combination of **1** and $HSCH_2CH_2SO_3^-Na^+$ ("mesnum"); however, no chemical details were given.

The previously cited[6] hydrolytic stability of PhCH=NP(O)$Ph_2$ and our inability to detect **8** by either $^{31}P$, $^{13}C$,

(105) By analogy to solvolytic reactions,[92] it could be argued that the proposed[35] ring opening of **2** to **3** was accompanied by substantial positive charge development at C-4 and concurrent neighboring group participation by *both* of the C-5 hydrogens/deuteriums in the activated complex (the isotope effects for a *single* "participating" hydrogen are in the range of the primary effects reported for intramolecular 1,2-hydride shifts; i.e., $k_H/k_D$ = 1.5–3.0 per deuterium). Another factor to consider is the claim [Kwart, H. *Acc. Chem. Res.* **1982**, *15*, 401] that the precise transition-state geometry for a rate-determining H-transfer reaction cannot be deduced in the absence of information about the temperature dependence of the isotope effect.

(106) Kanchunger, M. S.; Byers, L. D. *J. Am. Chem. Soc.* **1979**, *101*, 3005.

(107) Zon, G.; Egan, W.; Stokes, J. B. *Biochem. Pharmacol.* **1976**, *25*, 989.

$^2H$, or $^1H$ NMR spectroscopy under a wide variety of reaction conditions for **2**/"**3**" strongly suggest that this potential metabolite of **1** is not readily observable by NMR methods that employ chemical reactions of **2**/"**3**" in aqueous solution at pH 5.5–7.8 and 37 °C. Since these results cannot, of course, be used to address the question of possible enzyme-mediated processes that involve **8** as a transient species in vivo, other experimental tests regarding **8** are needed. In our opinion, the possible intermediacy of **8** is especially interesting when one considers the recent report[108] of oncostatic phosphorylated imines and the strikingly similar in vivo chemistry that might exist for **2**/**8** as compared to active metabolites of the anticancer agents maytansine,[109] saframycin,[110] and related antibiotics that have a reactive hemiaminal moiety like that in **2**.

The present applications of NMR spectroscopy to monitor simultaneously the individual metabolites of **1** are now being extended to cyclophosphamide analogues[7] and other anticancer drugs in an effort to compare and contrast reported binding,[111–114] cell uptake,[115] and intracellular pH factors.[32,35, 116,117]

## Experimental Section

THF and $Et_2O$ refer to anhydrous solvents, "hexanes" were a commercially available mixture, and petroleum ether refers to the hydrocarbon mixture having a 30–60 °C boiling point range. Reaction mixtures that do not include $H_2O$ were carried out with exclusion of atmospheric moisture. Ozone (~5 g/h) was produced by a Model 03V5-0 ozone generator (Ozone Research & Equipment Corp.) Elemental analyses were performed by the National Institutes of Health Microanalytical Laboratory. Melting points were obtained with a Thomas-Hoover capillary apparatus and were not corrected. Cyclophosphamide monohydrate and the cyclohexylammonium salt of phosphoramide mustard were obtained from the National Cancer Institute; the monohydrate was also purchased from Aldrich Chemical Co. Analytical and preparative TLC employed 2.5 × 10 cm and 20 × 20 cm plates coated with a 250-μm layer of silica gel GF; a 250-nm UV lamp and $I_2$ vapor were used for component visualization. 60-MHz $^1H$ NMR spectra were recorded with a Varian EM360-A instrument. Details regarding Fourier-transform $^1H$ NMR spectroscopy at 89.55 and 300 MHz, $^{31}P$ NMR spectroscopy at 36.23 and 121.51 MHz, and $^{13}C$ NMR spectroscopy at 22.49 MHz have been previously reported.[20,60h] $^2H$ NMR spectra at 46 MHz were recorded with a Bruker WM-300 spectrometer using 10-mm sample tubes, a 1000-Hz spectral window, a $\pi/2$ pulse of 35 μs, and a 1-s pulse recycle time; the $^2H$-depleted water solvent had a deuterium content that was 1.02% of natural abundance. Unless specified otherwise, $^1H$ NMR chemical shifts ($\delta$) refer to $Me_4Si$ as an internal reference. $^{31}P$ NMR $\delta$ values refer to external 25% $H_3PO_4$ in $D_2O$; the phosphorus chemical shifts in aqueous media are pH dependent. $^{13}C$ NMR $\delta$ values refer to external TSP in water or $Me_4Si$ in $CDCl_3$. NMR sample temperatures were measured by immersion of a precalibrated copper–constantan thermocouple attached to a digital-readout meter. Values of solution pH were measured with a precalibrated standard glass electrode; the "pH" values for $D_2O$-containing solutions correspond to the observed reading and were not corrected for deuterium isotope effects.[118] Lutidine buffers were prepared with commercially available 2,6-dimethylpyridine and used 2–3 M HCl for adjustment of the solution pH.

***cis*-4-Hydroperoxycyclophosphamide [2-[Bis(2-chloroethyl)amino]-4-hydroperoxytetrahydro-2*H*-1,3,2-oxazaphosphorin 2-Oxide, *cis*-12].** A hexane solution of *n*-BuLi (6.54 mL of 1.53 M, 10 mmol) was added dropwise to a stirred solution of 3-buten-1-ol (0.86 mL, 10 mmol) in THF (10 mL) at −23 °C ($CCl_4$–$CO_2$ bath). Stirring at −23 °C was continued for 2 h, and the resultant suspension was removed with a syringe and then added to a stirred solution of *N*,*N*-bis(2-chloroethyl)phosphoramidic dichloride (2.59 g, 10 mmol) in THF (10 mL) at −23 °C. Stirring at −23 °C was continued for 3 h, and $NH_3$ was then bubbled through the reaction mixture for 15 min at ~5 °C. The stoppered reaction flask stood at room temperature overnight prior to suction filtration, concentration of the filtrate on a rotary evaporator, and then chromatography of the residual material on silica gel (2 × 25 cm column) with $Et_2O$ (200–300 mL) to remove fast-eluting components. Elution with $CHCl_3$–MeOH (9:1) gave 3-butenyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate as an oil [$R_f$ 0.50 ($CHCl_3$-MeOH, 9:1), $R_f$ 0.15 ($Et_2O$); 1.10 g, 4 mmol; 40% yield] that was spectroscopically identified: $^1H$ NMR (60 MHz, $CDCl_3$) $\delta$ 6.15–5.43 (m, 1 H, vinylic), 5.28–4.87 (m, 2 H, vinylic), 4.00 (apparent q, $J$ = 6.5 Hz, 2 H, $CH_2O$), 3.77–3.03 (m, 10 H, $CH_2CH_2Cl$, $NH_2$), 2.40 (apparent q, $J$ = 6.5 Hz, 2 H, $CH_2CH_2O$); $^{13}C$ NMR (22.49 MHz, $CDCl_3$) $\delta$ 133.65 and 117.40 (vinylic), 64.49 (d, $J_{CP}$ = 4.9 Hz, $CH_2O$), 49.36 (d, $J_{CP}$ = 4.9 Hz, $CH_2N$), 42.55 ($CH_2Cl$), 34.82 (d, $J_{CP}$ = 7.3 Hz, $CH_2CH_2O$).

Ozone was bubbled through a solution of the aforementioned 3-butenyl ester (612 mg, 2.22 mmol) in acetone–$H_2O$ (2:1, 13.5 mL) at ~5 °C for 15 min. The volume of the solution was adjusted to 13.5 mL with more acetone, aqueous $H_2O_2$ (0.69 mL of a 30% solution) was added, and the reaction flask was then stoppered and kept at room temperature overnight. The reaction mixture was concentrated on a rotary evaporator at ambient temperature, and the residual aqueous solution was extracted with $CH_2Cl_2$ (6 × 25 mL). The combined extracts were dried ($MgSO_4$) and rotary evaporated at ambient temperature, and the residual material was then dissolved in a minimal volume of $Et_2O$ for storage at −20 °C for 3 days. The *cis*-**12** product was obtained as a white microcrystalline solid (180 mg, 0.61 mmol; 27% yield), mp 103 °C dec (lit.[63] mp 107–108 °C dec), which was >95% pure by $^{31}P$ NMR spectroscopy (36.23 MHz, $CDCl_3$, $\delta$ 8.35) and gave rise to a $^1H$ NMR spectrum (300 MHz, $CDCl_3$) that supported the assigned[64,69] stereochemistry: $\delta$ 5.15 (doubled q, $^3J_{HP}$ = 26 Hz, $J_{HH} \cong$ 2 Hz, 1 H, $C_4$ H), 4.75–4.64 (m, 1 H, $C_6$ H) 4.68 (s, 1 H, OH), 4.16 (m, 1 H, $C_6$ H), 3.62 (t, $J$ = 6.8 Hz, 4 H, 2 $CH_2Cl$), 3.57–3.32 (m, 4 H, 2 $NCH_2$), 2.30–2.02 (m, 2 H, NH and $C_5$ H), 1.95 (broadened d, $J$ = 14 Hz, $C_5$ H).

***cis*-4-Hydroperoxycyclophosphamide-5,5-$d_2$ (*cis*-12-5,5-$d_2$).** The 2,2-dideuterio-3-deuteroxypropionitrile starting material was prepared (72% yield) from 3-hydroxypropionitrile and deuterium oxide (>99.8 atom % $^2H$) according to the procedure reported by Jarman and Taylor:[70b] $^1H$ NMR (60 MHz, $CDCl_3$) $\delta$ 3.80 (s, $CH_2O$); $^{13}C$ NMR (22.49 MHz, $CDCl_3$) $\delta$ 118.3 (CN) and 57.01 ($CH_2O$) (the $CD_2$ resonance was not detected, as expected[60d]). A solution of the labeled nitrile (4.05 g, 0.055 mol) in THF (75 mL) was added dropwise to a stirred suspension of $LiAlH_4$ (6.23 g, 0.164 mol) in THF (200 mL), and the mixture was then refluxed overnight. The reaction mixture was hydrolyzed at room temperature by the slow, sequential addition of water (6 mL), 15% aqueous NaOH (6 mL), and then more water (18 mL). White solids were removed from the hydrolysate by suction filtration, and the filtrate was dried ($MgSO_4$) and then concentrated at reduced pressure to give an oil (1.6 g). Soxhlet extraction (THF, overnight) of the aforementioned white solids afforded additional material (1.04 g) that was combined with the initially obtained

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oil to give the 1-amino-2,2-dideuteriopropan-3-ol product (2.64 g, 0.034 mol, 62% yield): $^1$H NMR (60 MHz, $CDCl_3$) $\delta$ 3.70 (broadened s, 2 H, $CH_2O$), 2.85 (broadened s, 2 H, $CH_2N$), 2.40 (broadened s, 3 H, OH and $NH_2$); $^{13}$C NMR (22.49 MHz, $CDCl_3$) $\delta$ 59.90 ($CH_2O$), 38.96 ($CH_2N$) [the unlabeled 1-aminopropan-3-ol has an additional absorption at $\delta$ 34.31 ($CH_2CH_2CH_2$)]. A portion of the labeled amino alcohol was used to synthesize cyclophosphamide-*5,5-d*$_2$ (1-*5,5-d*$_2$) according to the procedure of Jarman and Taylor[70b] on a 0.014-mol scale based on the amount of *N,N*-bis(2-chloroethyl)phosphoramidic dichloride starting material. The resultant crude product was chromatographed on silica gel (2 × 25 cm column) with $CHCl_3$–MeOH (9:1) to give anhydrous 1-*5,5-d*$_2$ as a pale yellow oil [$R_f$ 0.63 ($CHCl_3$–MeOH, 9:1); 86% yield]: $^1$H NMR (60 MHz, $CDCl_3$) $\delta$ 4.55–3.95 (m, 2 H, $C_4$ methylene), 3.90–2.85 (m, 11 H, remaining protons) (the $C_5$ methylene moiety was not detectable at $\delta$ 2.0–1.8[60b]); $^{13}$C NMR (22.49 MHz, $CDCl_3$) $\delta$ 67.59 (d, $J_{CP}$ = 7.2 Hz, $C_6$), 48.77 (d, $J_{CP}$ = 5.8 Hz, 2 $NCH_2CH_2Cl$), 42.33 (s, 2 $NCH_2CH_2Cl$), 41.35 (d, $J_{CP}$ = 2.9 Hz, $C_4$) ($C_5$ was not detectable at $\delta$ 25.85[60b]); $^{31}$P NMR (36.23 MHz, $CDCl_3$) $\delta$ 10.61. Ozone was bubbled through a solution of 1-*5,5-d*$_2$ (2.14 g, 7.6 mmol) in a mixture of water (22.5 mL), acetone (11.5 mL), and 30% aqueous $H_2O_2$ (1.9 mL) for 3 h at ~5 °C. The volume of the solution was kept at ~36 mL by the addition of acetone as needed, and more of the 30% aqueous $H_2O_2$ (1.9 mL) was added after the 1st and 2nd h of reaction. The reaction mixture was concentrated on a rotary evaporator at ambient temperature, and the residual aqueous solution was extracted with $CH_2Cl_2$ (6 × 50 mL). The combined extracts were dried ($MgSO_4$) and rotary evaporated at ambient temperature, and the residual oil (4.12 g) was divided into two equal portions for separate chromatography on silica gel (EM Reagents, <230 mesh, 2 × 26 cm column) at ~5 °C with acetone–$CHCl_3$ (1:1) at a flow rate of ~8 mL/h. The fractions (3–4 mL each) that were collected at 8–12 h of elution contained the *cis*-**12**-*5,5-d*$_2$[121] product [$R_f$ 0.6–0.7 (acetone–$CHCl_3$, 1:1)], which was followed closely by 4-ketocyclophosphamide (**10**). Solvent from the pooled product fractions was removed on a rotary evaporator at ambient temperature to give *cis*-**12**-*5,5-d*$_2$ as a colorless oil (84 mg, 0.28 mmol, 4% yield) that was crystallized from $CHCl_3$ at −20 °C: $^1$H NMR (89.55 MHz, $CDCl_3$) $\delta$ 5.35–3.90 (m, 4 H, OOH, $CH_2O$ and $C_4$ H), 3.75–3.20 (m, 9 H, 2 $NCH_2CH_2Cl$ and NH); $^{13}$C NMR (22.49-MHz, $CDCl_3$) $\delta$ 86.36 (d, $J_{CP}$ = 4.9 Hz, $C_4$), 63.21 (d, $J_{CP}$ = 6.1 Hz, $C_6$), 48.77 (d, $J_{CP}$ = 4.9 Hz, 2 $NCH_2$), 42.06 (s, 2 $CH_2Cl$); $^{31}$P NMR (36.23 MHz, $CDCl_3$) $\delta$ 8.18.

**Dimethoxymethane-$^{13}C$.**[121] The following procedure was an adaptation of a literature report.[119] Formaldehyde-$^{13}C$ (90 atom % $^{13}$C; 6 g of a 20% aqueous solution, 38.7 mmol) was added dropwise to a mixture of MeOH (5.74 mL, 141 mmol), concentrated HCl (82 $\mu$L, 0.98 mmol), and anhydrous $CaCl_2$ (3.25 g, 29.5 mmol) at ~5 °C. The mixture was heated to its boiling point, and it was then allowed to stand at room temperature for 6 h. Distillation afforded the product (bp 41.5 °C) in 54% yield (1.61 g, 20.8 mmol): $^1$H NMR (89.55 MHz, $CDCl_3$) $\delta$ 4.57 (d, $^1J_{CH}$ = 162.1 Hz, 2 H, $^{13}CH_2$), 3.36 (d, $^3J_{CH}$ = 4.5 Hz, 6 H, 2 $CH_3$) [the 10 mol % of $CH_2(OCH_3)_2$ that was present gave rise to relatively low intensity singlets at $\delta$ 4.57 and 3.36]; $^{13}$C NMR (22.49 MHz, $CDCl_3$) $\delta$ 97.34.

**1,3-Dithiane-2-$^{13}C$.** The following procedure was adapted from the method of Corey and Seebach.[120] A solution of dimethoxymethane-$^{13}C$ (1.87 mL, 21 mmol) and 1,3-propanedithiol (1.95 mL, 19.4 mmol) in $CHCl_3$ (30 mL) was added dropwise over a 5-h period to a refluxing mixture of $BF_3$·$Et_2O$ (2.34 mL, 19 mmol) and acetic acid (4.68 mL, 82 mmol) in $CHCl_3$ (8 mL). The reaction mixture was then cooled to room temperature and washed sequentially with water (4 × 15 mL), 10% aqueous KOH (2 × 20 mL), and more water (2 × 15 mL). The organic layer was dried ($K_2CO_3$) and then concentrated carefully at reduced pressure. The resultant solid was boiled with MeOH (4 mL), and the hot solution was filtered. The filtrate was kept at −20 °C overnight, and the resultant crystals, which were removed by suction filtration of the cold mixture, were washed with cold MeOH. Concentration of the filtrate and low-temperature crystallization afforded a second crop of crystals, which were combined with the initial material to give the labeled dithiane product (1.26 g, 10.4 mmol, 54% yield): $^1$H NMR (89.55 MHz, $CDCl_3$) $\delta$ 3.78 (d, $^1J_{CH}$ = 152 Hz, 2 H, $^{13}CH_2$), 2.82 (apparent q, $J$ = 6 Hz, 4 H $CH_2S$), 2.21–1.91 (m, 2 H, $CH_2CH_2CH_2$); $^{13}$C NMR (22.49 MHz, $CDCl_3$) $\delta$ 31.96 (s, $C_2$) [$C_2$ in the nondecoupled spectrum appeared as a triplet of quintets ($^1J_{CH}$ = 149 Hz and $^3J_{CH}$ = 5 Hz)].

**2-(2-Hydroxyethyl)-1,3-dithiane-2-$^{13}C$.**[121] A hexane solution of *n*-BuLi (3.5 mL of 1.7 M, 6.0 mmol) was added dropwise to a stirred solution of 1,3-dithiane-2-$^{13}C$ (664 mg, 5.5 mmol) in THF (50 mL) that was cooled with a $CH_3CN$–$CO_2$ bath. The coolant was then changed to a $CCl_4$–$CO_2$ bath, and the reaction mixture was stirred for 2.5 h. The $CH_3CN$–$CO_2$ bath was then used during the addition of an excess of chilled ethylene oxide (1.0 mL) in one portion (**caution**: avoid inhalation of vapors and skin contact when handling ethylene oxide). The coolant was removed after 3 h, water (1.8 mL) was added at room temperature, and the resultant mixture was then filtered. Concentration of the filtrate at reduced pressure gave an aqueous solution, which was diluted with water (3 mL) and extracted with $CH_2Cl_2$ (6 × 20 mL). The combined extracts were dried ($MgSO_4$) and concentrated on a rotary evaporator, and the resultant oil was subjected to flash chromatography[122] on silica gel (2.9 × 21.5 cm column) with hexanes–EtOAc (3:2), which gave the desired product as an oil (537 mg, 3.25 mmol, 59% yield): $R_f$ 0.47 (hexanes–EtOAc, 3:2); $^1$H NMR (89.55 MHz, $CDCl_3$) $\delta$ 4.25 (d of t, $^1J_{CH}$ = 154 Hz, $^3J_{HH}$ = 7 Hz, 1 H, $C_2$ H), 3.84 (apparent q, $J$ = 6 Hz, 2 H, $CH_2O$), 3.15–2.75 (m, 4 H, 2 $CH_2S$), 2.57 (s, 1 H, OH), 2.30–1.80 (m, 4 H, $C_4$ methylene and $CH_2CH_2O$); $^{13}$C NMR (22.49 MHz, $CDCl_3$) $\delta$ 43.99 (s, $C_2$) [$C_2$ in the nondecoupled spectrum appeared as a doublet of septuplets ($^1J_{CH}$ = 152 Hz and $J_{CH}$ = 4.4 Hz)].

**2-(1,3-Dithian-2-$^{13}C$-2-yl)ethyl *N,N*-Bis(2-chloroethyl)-phosphorodiamidate (13).** A solution of 2-(2-hydroxyethyl)-1,3-dithiane-2-$^{13}C$ (638 mg, 3.9 mmol) in $CH_2Cl_2$ (6 mL) was added dropwise to a stirred solution of *N,N*-bis(2-chloroethyl)-phosphoramidic dichloride (1.0 g, 3.9 mmol) and $Et_3N$ (0.54 mL, 3.9 mmol) in $CH_2Cl_2$ (5 mL). The reaction mixture was refluxed overnight, and $NH_3$ was then bubbled through the reaction mixture at ~5 °C. The resultant solids were removed by suction filtration, and the concentrated filtrate was then subjected to flash chromatography[122] on silica gel (2.9 × 15.5 cm column) with $CHCl_3$–EtOAc (1:1), which gave **13** as a white solid (572 mg, 1.7 mmol, 44% yield): mp 69–71 °C; $R_f$ 0.29 ($CHCl_3$–EtOAc, 1:1); $^1$H NMR (89.55 MHz, $CDCl_3$) $\delta$ 4.20 (d of t, $^1J_{CH}$ = 153 Hz, $^3J_{HH}$ = 7 Hz, 1 H, $C_2$ H), 4.30–4.00 (m, 2 H, $CH_2O$), 3.80–3.20 (m, 8 H, 2 $NCH_2CH_2Cl$), 3.00–2.65 (m, 6 H, 2 $CH_2S$ and $NH_2$), 2.30 (m, 4 H, $C_4$ methylene and $CH_2CH_2O$); $^{13}$C NMR (22.49 MHz, $CDCl_3$) $\delta$ 43.62 (s, $C_2$) [$C_2$ in the nondecoupled spectrum appeared as a doublet of septuplets ($^1J_{CH}$ = 153 Hz and $J_{CH}$ = 4.9 Hz)]; $^{31}$P NMR (36.23 MHz, $CDCl_3$) $\delta$ 13.87. Anal. ($C_{10}H_{21}N_2O_2Cl_2PS_2$, unlabeled) C, H, N, P.

***cis*-4-Hydroperoxycyclophosphamide-4-$^{13}C$ (*cis*-12-4-$^{13}C$).**[121] A solution of **13** (246 mg, 0.7 mmol) in a minimal amount of THF (0.9 mL) was added in one portion to a stirred suspension of red HgO powder (290 mg, 1.34 mmol) and 35% aqueous $HBF_4$ (0.66 mL) in THF (3.3 mL) at room temperature. The reaction mixture was cooled to ~5 °C after 10 min, and it was then neutralized by the addition of solid $NaHCO_3$ (440 mg) in one portion. The resultant mixture was diluted immediately with THF (5 mL), dried ($MgSO_4$), and filtered, and the filtrate was then concentrated on a rotary evaporator at ambient temperature to afford an oil (120 mg), which was judged by $^1$H, $^{13}$C, and $^{31}$P NMR spectral analyses to be a mixture of **14** (major) and **12**-*4*-$^{13}C$ (minor). A portion of this oil (70 mg) was dissolved in $D_2O$–acetone (2:1, 5 mL) that contained $H_2O_2$ (0.5 mL of a 30% aqueous solution). $^{31}$P NMR spectroscopic comparisons with authentic samples of unlabeled *cis*-**12** and **10** indicated that, after 5 days at ~25 °C, the reaction products were an 87:13 mixture of *cis*-**12**-*4*-$^{13}C$ (vide infra) and **10**-*4*-$^{13}C$ [$\delta_P$ 7.86 ($CDCl_3$)]. The combined

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(121) Trial syntheses of this poduct using unlabeled starting material indicated that the yield was sometimes substantially lower than that presently reported; consequently, batchwise preparation of the labeled product is recommended.

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extracts ($CH_2Cl_2$, 6 × 15 mL) from this reaction mixture were dried ($MgSO_4$) and concentrated on a rotary evaporator at ambient temperature, and the resultant oil was dissolved in a minimal amount of $Et_2O$, which afforded crystalline *cis*-**12**-4-$^{13}C$ [10 mg, 0.34 mmol, 8% yield (corrected)] after 3 days at −20 °C: $^{13}C$ NMR (22.49 MHz, $CDCl_3$) δ 79.11 (d, $^2J_{CP}$ = 4.2 Hz, $C_4$); $^{31}P$ NMR (36.23 MHz, $CDCl_3$) δ 8.72 (d, $^2J_{PC}$ = 4.2 Hz).

**Synthesis of Deuterium-Labeled Aldophosphamide *O*-Methyloxime (21).** $MeONH_2$·HCl (8.3 mg, 0.1 mmol) was added to a solution of *cis*-**12**-5,5-$d_2$ (14.4 mg, 0.05 mmol) in 0.05 M lutidine buffer (1.5 mL, pH 7.4). A $^{31}P$ NMR spectrum that was recorded after 2 days at ~25 °C showed the absence of the labeled starting material. The reaction mixture was saturated with NaCl and then extracted with $CH_2Cl_2$ (4 × 20 mL). The combined extracts were dried ($MgSO_4$) and concentrated at reduced pressure, and the resultant oil was subjected to preparative TLC with $CHCl_3$–acetone (2:1). A wide band that excluded the fast-eluting lutidine and noneluting material was desorbed with $CH_2Cl_2$–MeOH (1:1) and afforded a sample of **21** (11 mg, 0.036 mmol, 73% yield), which was recrystallized from $CHCl_3$–petroleum ether (mp 70–72 °C, unlabeled **21**[42] mp 72–75 °C) and spectroscopically identified: $^1H$ NMR (89.55 MHz, $CDCl_3$) δ 7.40 (s , N=CH, *E* isomer), 6.70 (s, N=CH, *Z* isomer) (the N=CH proton resonances in unlabeled *E*- and *Z*-**21**[42] appear as triplets), 4.30–4.00 (m, 2 H, $CH_2O$), 3.89 (s, $OCH_3$, *Z* isomer), 3.83 (s, $OCH_3$, *E* isomer), 3.80–3.25 (m, 8 H, 2 $NCH_2CH_2Cl$), 2.85 (broadened s, 2 H, $NH_2$) (absorptions at δ 2.50[42] and 2.62[42] for *CH*CH=N were not detectable); $^{13}C$ NMR (22.49 MHz, $CDCl_3$) δ 147.1 and 146.8 (*E*/*Z* C=N), 62.37, 62.10, 61.78, 61.45 (*E*/*Z* $OCH_2$/$OCH_3$), 49.37 and 49.26 (2 d, $J_{CP}$ = 4.9 Hz, *E*/*Z* $NCH_2$), 42.52 (isochronous *E*/*Z* $CH_2Cl$) (absorptions for $CD_2CH$=N were not detected as expected[60d]; $^{31}P$ (36.23 MHz, $CDCl_3$) δ 14.01 (isochronous signals for the *E*/*Z* isomers).

**$^{31}P$ NMR Kinetic Studies. General Procedure.** NMR sample solutions of *cis*-**12** (~20 mM) were prepared immediately prior to their use by the addition of lutidine buffer (1.35 mL of 1 M) and $D_2O$ (0.15 mL) to a glass vial that contained the crystalline hydroperoxide (~10 mg). Dissolution was facilitated by the use of a high-speed vortex mixer, and after 1–2 min the solution was transferred to a vial that contained $Na_2S_2O_3$·$5H_2O$ (31 mg, 0.125 mmol, ~4 molar equiv). Use of the high-speed vortex mixer led to dissolution after 1–2 min, and the pH of the reaction medium was then adjusted rapidly by titration with 2–3 M HCl. The solution was placed in a 10-mm NMR tube, a Teflon vortex plug was inserted, and the sample was then allowed to thermally equilibrate for 2–3 min in the spectrometer probe (37 ± 2 °C) before optimization of the magnetic field homogeneity. At time "zero", which was ~15 min after the dissolution of *cis*-**12**, 36.23-MHz $^{31}P$ NMR data accumulation was initiated using a 5-kHz spectral window, 8192 data points, a π/2 pulse of 20 μs, low-power $^1H$ decoupling, and a pulse recycle time of 2 s. The free-induction decay (FID) signal that was obtained after 100 pulses was stored on a diskette, and the next spectral acquisition was initiated at time *t*, relative to the "zero" time. The stored FID signals were exponentially multiplied so as to result in an additional 0.97-Hz of line broadening in the frequency-domain spectra. Possible nuclear Overhauser effects (NOE) were not suppressed by gated decoupling. In kinetic runs that assessed the effects of various compounds on the metabolite equilibria/decomposition, the material was added to the solution obtained from *cis*-**12** and $Na_2S_2O_3$, and the resultant mixture was processed as described above. Signal intensities (peak heights) of the individual reaction components were compared as a measure of relative concentrations, and reactions were generally followed for at least 2 half-lives.

Following eq 4, we can write a set of coupled differential equations (the kinetic model), which describe the concentrations of the various species as a function of time:

$$\dot{A} = -k_1[A] + k_{-1}[B]$$

$$\dot{B} = k_1[A] + k_{-2}[C] - [B](k_2 + k_{-2} + k_3)$$

$$\dot{C} = k_2[B] - k_{-2}[C]$$

$$\dot{D} = k_3[B]$$

[*A*] = [*cis*-**2**]; [B] = [**3** + **5**] = ["**3**"]; [*C*] = [*trans*-**2**]; [*D*] = [**4** + byproducts]

The NMR data were analyzed in terms of the above model to provide the least-squares estimates for $k_1$, $k_{-1}$, $k_2$, $k_{-2}$, and $k_3$ that are found in Table I; the curve fitting was accomplished with the MLAB facilities at NIH.[86] After obtaining the best values of the rate constants, we then allowed the initial values of *A*–*D* to also be varied; this had virtually no effect on the estimated rate constants. The data points were not weighted. Having obtained the estimated rate constants, we integrated the above equations to obtain curves for *A*(*t*) through *D*(*t*), and these curves were then compared to the input data, as shown in Figure 2; visual comparison was good, and the fit shown in Figure 2 was typical.

**Kinetic Studies of 2/3 in Phosphate Buffer. NBP Assay.** The sample was prepared by the dissolution of *cis*-**12** (~10 mg, ~0.034 mmol) in potassium phosphate buffer (70 mM, 1.35 mL, pH 7.0) and $D_2O$ (0.15 mL) with the aid of a vortex mixer (3 min). $Na_2S_2O_3$·$5H_2O$ (31 mg, 0.125 mmol) was added, and after complete dissolution (vortex mixer, 1–2 min) *N*-acetyl-L-cysteine (11 mg, 0.068 mmol) was added. The pH of the reaction medium was adjusted to 7.0 by titration with 1 N NaOH, and at this point (ca. 10 min after the dissolution of *cis*-**12**) a timer was started (*t* = "zero"). The reaction mixture was placed in a constant-temperature bath (37 °C), and aliquots (0.1 mL) were taken at *t* = 0, 20, 35, 60, 120, 150, and 180 min and at 18 h. Each aliquot was placed in a screw-cap test tube, and to this was added 1 N HCl (1.0 mL). The acidified solution was heated at 100 °C for 10 min, and it was then neutralized with 1 N NaOH (1 mL). An aliquot (0.2 mL) of the resultant hydrolysate was added to a screw-cap test tube and was diluted with $H_2O$ (2.8 mL). The test tube was inserted into crushed ice, sodium acetate buffer (1.0 mL, pH 4.6) was then added and this was followed by the addition of a solution of 4-(*p*-nitrobenzyl)pyridine (NBP) in acetone (5%, w/v, 0.4 mL). The resultant mixture was heated at 100 °C for 20 min and then cooled to room temperature, and acetone (2.0 mL), EtOAc (5 mL), and 0.25 N NaOH (1.5 mL) were then added successively. The capped test tube was shaken immediately (20 times), and the contents were then centrifuged for 30 s. An aliquot (1 mL) of the upper layer was added to a 1-cm cuvette and the absorbance (*A*) at 540 nm was measured 90 s after the previously mentioned addition of 0.25 N NaOH. The results are listed in Table III.

**$^{31}P$ NMR Spectroscopic Analysis.** Corresponding NMR samples were prepared exactly as described for the NBP assay. Spectra at 37 °C were recorded at *t* = 0, 18, 33, 60, 120, and 140 min and at 14 h. The percentage of residual alkylating activity was defined as the total peak height for 2 plus "3" divided by the total peak height for all phosphorus signals. The results are listed in Table III.

**Control Studies Related to $^{13}C$ NMR Data for the Acrolein Fragment.** (A) $^{13}C$ NMR spectrum for acrolein in 1 M lutidine, pH 7.4: δ 201.5, 144.2, 140.1; for acrolein plus 1 equiv of $Na_2S_2O_3$ in 1 M lutidine, pH 7.4: δ 201.4, 144.3, 140.1.

(B) $^{13}C$ NMR spectrum for acrolein plus 1 equiv of HCl in 1 M lutidine, pH 7.4: δ 201.5, 91.70, and 90.98, with 12 relatively minor resonance absorptions.

(C) $^{13}C$ NMR spectrum for acrolein plus 1 equiv of **4** (cyclohexylammonium salt) in 1 M lutidine, pH 7.4: eight signals at δ 25–55; no resonance absorption for C=O; a similar spectrum was obtained for acrolein plus 1 equiv of **4** after passage of the latter compound through a Dowex ion-exchange column in the $Na^+$ form.

(D) $MeONH_2$·HCl (5.7 mg, 68 μmol) was added to a solution of acrolein (2.3 μL, 34 μmol) in 1 M lutidine (1.35 mL, pH 7.3) and $D_2O$ (0.15 mL): $^{13}C$ NMR δ 154.3 and 154.1 (ca. 2:1, respectively) for C=N.

(E) $MeONH_2$·HCl (5.7 mg, 68 μmol) was added to a solution of *cis*-**12** (10 mg, 34 μmol) and $Na_2S_2O_3$·$5H_2O$ (31 mg, 3.7 equiv) in 1 M lutidine (1.35 mL, pH 7.4) and $D_2O$ (0.15 mL), and the spectrum was recorded after 3 h at ~20 °C: $^{13}C$ NMR δ 153.1, 152.8 (ca. 1:1) for C=N.

**Control Studies Related to Adducts 22–24.** A sample of *cis*-**12** (10 mg) was deoxygenated with $Na_2S_2O_3$ (4 equiv) in water and then treated with Tris (6 equiv) at ambient pH (~9). $^{31}P$ NMR spectroscopic analysis indicated the presence of ~50% **22**–**24**, and an overnight $^{13}C$ NMR spectral acquisition was begun at ~27 °C, after which time the relative proportion of **22**–**24** had decreased to ~15%; the $^{13}C$ spectrum featured signals at δ 92.80 and 91.76 (~2:1), but no *C*H=N signals were evident. The

resonance absorption at δ 92.80 was subsequently shown to arise from the reaction of acrolein with Tris. The aforementioned experiment with *cis*-**12** and Tris was used to record the $^1H$ NMR spectrum for a reaction mixture that contained ~70% **22–24**; no signals were observed at δ >5 for *CH*=N.

**Reaction of 2/3 with Sulfur-Containing Compounds.** The following procedures were adapted from synthetic methods reported by Peter and Hohorst.[66] A solution of *cis*-**12** (100 mg, 0.34 mmol) and $Ph_3P$ (89 mg, 0.34 mmol) in $CH_2Cl_2$ (0.5 mL) was stirred for 15 min at ambient temperature and then concentrated on a rotary evaporator without heating. The resultant oil was dissolved in a minimal volume of $Et_2O$, a seed crystal of $Ph_3PO$ was then added, and the solution was stored at −20 °C overnight. The ether solution was removed and concentrated first on a rotary evaporator and then under high vacuum to afford crude **2/3** as a white semisolid.

A solution of crude **2/3** (~0.34 mmol) in $CH_2Cl_2$ (0.2 mL) was added to a mixture of **2-mercaptoethanol** (48 μL, 0.68 mmol) and trichloroacetic acid (0.5 mg, 3 μmol) in $CH_2Cl_2$ (0.1 mL) at ~5 °C, and the resultant solution was stirred for 1 h at ~5 °C before removal of volatile material in vacuo. The residual oil was dissolved in lutidine buffer (1.35 mL of 1 M, pH 7.4) and $D_2O$ (0.15 mL) for $^{31}P$ NMR analysis, which showed the presence of two major peaks (ca. 1:1 ratio) at δ 11.35 and 12.59 that were ascribed to the expected[66] products, viz., *cis*- and *trans*-**7** (R = $CH_2CH_2OH$); a trace amount of the acyclic hemithioacetal conjugates was seen at δ 20.36. Attempts to isolate these products by either low-temperature crystallization or chromatography on silica gel were unsuccessful.

A solution of crude **2/3** (~0.34 mmol) in $CH_2Cl_2$ (1 mL) was added to a mixture of ***N*-acetyl-L-cysteine** (55 mg, 0.34 mmol) and trichloroacetic acid (0.5 mg, 3 μmol) in acetone (2 mL) at ~5 °C, and the resultant solution was stirred for 1.5 h at ~5 °C before removal of volatile material in vacuo. $^{31}P$ NMR analysis of the residual solid, as described above, showed the presence of major peaks at δ 11.25 and 11.35 for *cis*-**27** and at δ 12.29 and 12.56 for *trans*-**27** in approximately equimolar amounts, as well as less intense signals at δ 20.36 and δ 13.8–13.9 for **26** and **4**/alkylation products, respectively. Repetition of this procedure with crude **2/3**, derived by ozonation (vide supra) of (*S*)-**1**, led to $^{31}P$ NMR signals for *cis*- and *trans*-**27** at δ 11.25 and 12.29, respectively.

To a solution of crude **2/3** (~0.34 mmol) in acetone (2 mL) at ~5 °C was added $Na_2S_2O_3 \cdot 5H_2O$ (84 mg, 0.34 mmol) and trichloroacetic acid (0.5 mg, 3 μmol). The resultant suspension was allowed to warm to room temperature, more acetone (1 mL) was added, and the reaction mixture was then stirred for 8 h at room temperature. Removal of solvent in vacuo afforded a semisolid, which was dissolved in lutidine buffer (1.35 mL of 1 M, pH 7.4) and $D_2O$ (0.15 mL) for $^{13}C$ and $^{31}P$ NMR analyses, which failed to show the presence of signals indicative of either a cyclic ($\delta_P$ 11–13) or an acyclic ($\delta_P$ 20–21) conjugate; signals ascribed to **17** ($\delta_C$ 69.78 and $\delta_P$ 10.01) were also not evident.

**Acknowledgment.** This investigation was supported in part by research Grant CA-21345 to G.Z. (01–04 years) and S.M.L. (04–present) from the National Institutes of Health. We are grateful to The Catholic University of America Chemical Instrumentation Center for the use of its JEOL FX-90Q NMR spectrometer. We thank Dr. Edward M. Sweet for helpful suggestions during the course of this work and Ellen I. Kirshbaum and Crystal B. Honemond in the preparation of the manuscript.

**Registry No.** **1**-5,5-$d_2$, 59720-09-1; *cis*-**2**, 88852-77-1; *trans*-**2**, 88852-78-2; **3**, 35144-64-0; **4**, 10159-53-2; **5**, 88685-78-3; *cis*-**7**, 61903-94-4; *trans*-**7**, 61903-98-8; **10**-*14*-$^{13}C$, 27046-19-1; (±)-*cis*-**12**, 62435-42-1; *cis*-**12**-5,5-$d_2$, 88803-00-3; *cis*-**12**-*4*-$^{13}C$, 88803-05-8; **13**, 88803-04-7; **13** (hydroxy derivative), 88803-03-6; **16**, 88802-97-5; **17**, 88825-35-8; (*E*)-**21**, 88803-06-9; (*Z*)-**21**, 88803-07-0; L-(*S*)-**26**, 88802-98-6; L-(*R*)-**26**, 88852-79-3; *cis*-**27** (isomer 1), 88802-99-7; *cis*-**27** (isomer 2), 88852-80-6; *trans*-**27** (isomer 1), 88852-81-7; *trans*-**27** (isomer 2), 88852-82-8; Tris, 77-86-1; L-R*SH, 616-91-1; $Cl_2P(O)M$, 127-88-8; $MeO^{13}CH_2OMe$, 88803-02-5; $^{13}CH_2O$, 3228-27-1; MeOH, 67-56-1; $HS(CH_2)_3SH$, 109-80-8; $MeONH_2 \cdot HCl$, 593-56-6; acrolein, 107-02-8; 3-buten-1-ol, 627-27-0; 3-butenyl *N*,*N*-bis-(2-chloroethyl)phosphorodiamidate, 39800-29-8; 2,2-dideuterio-3-deuteroxypropionitrile, 88803-01-4; 1-amino-2,2-dideuteriopropan-3-ol, 59720-08-0; 1,3-dithiane-2-$^{13}C$, 88157-07-7; 2-mercaptoethanol, 60-24-2.

# Base-Catalyzed Hydrolysis of 4-Hydroperoxycyclophosphamide: Evidence for Iminocyclophosphamide as an Intermediate

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*cis*-4-Hydroperoxycyclophosphamide (**5**) undergoes facile reaction with aqueous phosphate or Tris buffers at pH 7–8 and 30 °C. The kinetics of **5** are complex, and the *trans*-4-hydroperoxy isomer **6** is produced and subsequently disappears over the course of the reaction. Addition of hydrogen peroxide to the reaction mixture retards the disappearance rate of **5** and increases the amount of **6** generated. Rate constants for the reversible disappearance of **5** and appearance of **6** and 4-hydroxycyclophosphamide (**2**) have been determined by nonlinear least-squares methods. The reaction is catalyzed by hydroxide ion, Tris free base, and $HPO_4^{2-}$, with catalytic constants of 0.032 $min^{-1}$ (pH 8.0), 0.052, and 0.115 $M^{-1}$ $min^{-1}$, respectively. The major product in the presence of Tris is the oxazolidine **8b** arising from the addition of Tris to aldophosphamide, not **2** as assumed previously. These results are consistent with a mechanism involving general-base-catalyzed elimination to produce iminocyclophosphamide **7** as a transient intermediate; the imine can react with the hydrogen peroxide evolved in the reaction to give **5** and **6**, with water to give **2**, or, in general, by addition of a nucleophile to C-4. The significance of these findings with respect to other 4-substituted cyclophosphamides is discussed.

Cyclophosphamide (**1**) continues to be one of the most widely used and intensively studied agents available for the treatment of malignancy. The chemistry, pharmacology, and metabolism of **1** have been reviewed;[1–3] the activation process appears to involve initial hydroxylation by the hepatic cytochrome P450 system to produce 4-hydroxycyclophosphamide (**2**) of unknown stereochemistry. Intermediate **2** undergoes ring opening to aldophosphamide **3**, which in turn suffers β-elimination to produce acrolein and phosphoramide mustard **4**, generally

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