(0.92 g, 63%) as colorless crystals, mp 150 °C (explodes); IR (KBr) 2280 cm^{-1}. Anal. Calcd for $C_6H_7N_5O$·HCl: C, 35.74; H, 4.00; N, 34.74. Found: C, 35.3; H, 3.79; N, 34.3.

A suspension of the hydrochloride of diazopyrazole **16b** (0.92 g, 4.56 mmol) in methylene chloride (50 mL) was treated consecutively with 2-chloroethyl isocyanate (2.5 mL) and 1,8-diazabicyclo[5.4.0]undec-7-ene (0.7 g, 4.60 mmol), and the mixture was stirred at room temperature (18 h). The mixture was concentrated and the residue triturated with petroleum ether. The insoluble residue was purified by flash chromatography (eluent ethyl acetate) and the obtained product crystallized from ethyl acetate, affording the pyrazolo[5,1-*d*]tetrazinone **17b** (0.38 g, 31%) as colorless crystals, mp 116–118 °C dec. Anal. Calcd for $C_9H_{11}ClN_6O_2$: C, 39.93; H, 4.1; Cl, 13.1; N, 31.05. Found: C, 39.7; H, 3.96; Cl, 13.1; N, 30.9.

Method O. Preparation of 17c by the Sequence 15c → 16c → 17c. A stirred saturated solution of hydrogen chloride in methanol (56 mL) was treated with **15c**[18] (2.0 g, 0.016 mol) and cooled to 0 °C. Amyl nitrite (7.2 g, 0.062 mol) was added dropwise. The mixture was stirred at 2 °C (1 h) and the resulting suspension was poured into ether (200 mL), giving **16c**·HCl (2.5 g, 92%) as a white solid, mp 113 °C dec (lit.[18] mp 104–105 °C). Anal. Calcd for $C_3HN_5O_2$·HCl: C, 20.53; H, 1.15; Cl, 20.2; N, 39.9. Found: C, 20.5; H, 0.91; Cl, 19.0; N, 40.8.

A suspension of 3-diazo-4-nitropyrazole (**16c**) hydrochloride salt (2.45 g, 0.014 mol) in methylene chloride (50 mL) was treated with 2-chloroethyl isocyanate (8.9 g) and then with 1,8-diazabicyclo[5.4.0]undec-7-ene (2.13 g, 0.014 mol) dropwise. The resulting solution was stirred at room temperature in the dark (1.5 h), concentrated, and triturated with petroleum ether (bp 60–80 °C). The residue was purified by flash chromatography (silica, eluent ethyl acetate/petroleum ether (bp 40–60 °C) 1:2), giving **17c** (0.384 g, 11%) as a pale yellow solid, mp 168–172 °C dec. Anal. Calcd for $C_6H_5ClN_6O_3$: C, 29.46; H, 2.06; Cl, 14.5; N, 34.4. Found: C, 30.4; H, 2.31; Cl, 15.6; N, 32.4.

Method P. Preparation of 17d by the Sequence 19d → 20d → 15d → 16d → 17d. A solution of (methylsulfonyl)acetonitrile (10.5 g, 0.089 mol) in triethyl orthoformate (21 mL, 0.14 mol) and acetic anhydride (21 mL, 0.2 mol) was heated at 160 °C with removal of evolved ethyl acetate (35 mL). The dark oily residue was dissolved in methanol (60 mL) and the solution was concentrated and distilled, giving **20d** (11.98 g, 78%) as a pale yellow oil, bp 165–168 °C (1.0 mm).

A solution of the 3-ethoxy-2-(methylsulfonyl)propenenitrile (**20d**) (3.49 g, 0.02 mol) in ethanol (20 mL) was treated with hydrazine hydrate (1 g, 0.02 mol) and heated under reflux (6 h). Concentration and purification of the residue by flash chromatography (silica, eluent $CHCl_3$/MeOH, 85:15) gave **15d** as a pink oil (1.37 g). This oil was dissolved in ethyl acetate (10 mL) and treated with ethereal HCl, yielding **15d**·HCl (0.97 g, 24%) as a colorless solid, mp 201–203 °C dec. Anal. Calcd for $C_4H_7N_3O_2S$·HCl: C, 24.3; H, 4.08; Cl, 17.9; N, 21.3; S, 16.2. Found: C, 24.4; H, 4.09; Cl, 17.9; N, 21.5; S, 16.6.

A stirred solution of sodium nitrite (0.46 g, 6.67 mmol) in water (3.5 mL) was treated dropwise with a solution of 5-amino-4-(methylsulfonyl)pyrazole hydrochloride (**15d**·HCl) (1.0 g, 5.06 mmol) in hydrochloric acid (1 M, 12.2 mL) at 0 °C. The solution was adjusted to pH 7 (sodium hydrogen carbonate) and extracted with ethyl acetate (3 × 50 mL). The extracts were dried ($MgSO_4$) and evaporated (30 °C (10 mm), then 30 °C (0.1 mm)), giving **16d** (0.68 g, 78%) as a yellow solid, mp 117–119 °C dec. Anal. Calcd for $C_4H_4N_4O_2S$: C, 27.9; H, 2.34; N, 32.5. Found: C, 27.8; H, 2.32; N, 31.9.

A solution of 5-diazo-4-(methylsulfonyl)pyrazole (1.3 g, 7.55 mmol) in 2-chloroethyl isocyanate (16 mL) was kept at room temperature (24 h). The solution was concentrated and the residue purified by flash chromatography (silica, eluent ethyl acetate/toluene, 4:6). The product was triturated with petroleum ether, giving **17d** (1 g, 48%) as a colorless solid, mp 184–185 °C dec. Anal. Calcd for $C_7H_8ClN_5O_3S$: C, 30.3; H, 2.90; N, 25.2. Found: C, 29.9; H, 2.81; N, 25.1.

Acknowledgment. We thank J. A. Stoneley and T. J. Lucas for technical assistance.

NMR Spectroscopic Studies of Intermediary Metabolites of Cyclophosphamide. 2. Direct Observation, Characterization, and Reactivity Studies of Iminocyclophosphamide and Related Species

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4-Hydroxy-5,5-dimethylcyclophosphamide (**6**) was synthesized as a stable (to fragmentation) analogue of 4-hydroxycyclophosphamide (**1**). In anhydrous Me_2SO-d_6 (≤0.03 mol % water), *cis*- and *trans*-**6** were observed by multinuclear NMR spectroscopy to equilibrate with α,α-dimethylaldophosphamide (**7**) and 5,5-dimethyliminocyclophosphamide (**8**). Identification of **8** was based on ^1H, ^{13}C, and ^{31}P chemical shifts, selective INEPT and two-dimensional NMR correlation experiments, and temperature-dependent equilibria data. The interconversion of *cis*-/*trans*-**6** and -**7** was also observed in lutidine buffer; **8** was not detected under the aqueous conditions. In Me_2SO-d_6, hydroxy metabolite **1** underwent dehydration to give iminocyclophosphamide (**5**), as evidenced by chemical shift data and a selective INEPT experiment. Concentrations of *cis*-/*trans*-**1**, aldophosphamide (**2**), and **5** were found to be temperature-dependent with higher temperatures favoring **2** and **5** in a reversible manner, thus indicating that **1**/**2**/**5** were interconverting. The addition of small amounts of water to Me_2SO-d_6 solutions of imine **5** resulted in the immediate disappearance of its NMR signals. The role of imine **5** in the conversion of **1** to C-4 substituted analogues of **1** was elucidated for the formation of 4-cyanocyclophosphamide (**3a**) from **1** and sodium cyanide in lutidine buffer.

There is considerable evidence that the unique therapeutic efficacy of the anticancer drug cyclophosphamide is related to its intermediary metabolites, 4-hydroxycyclophosphamide (**1**) and aldophosphamide (**2**) (Scheme I).[1-6] Mechanistic bases for the oncostatic selectivity of

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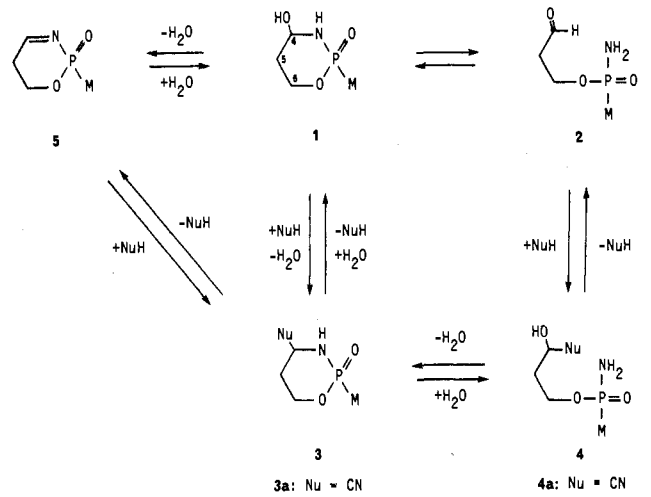
Scheme I[a]

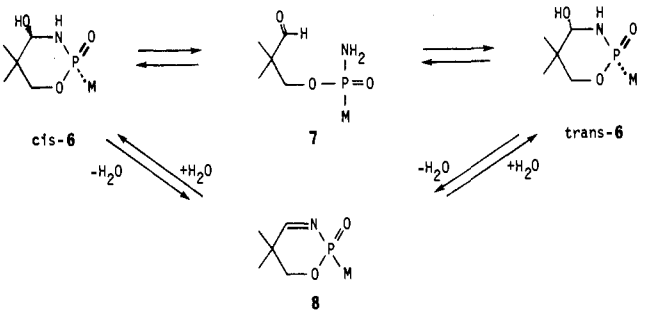


[a] M = $N(CH_2CH_2Cl)_2$.

cyclophosphamide have been reviewed, and among these is the theory of latentiation and/or transport of **1**/**2** via the reversible formation of general adducts **3**/**4** with nucleophilic biomolecules (NuH, Scheme I).[1,7,8] Although this theory has received some criticism,[9] interest in it continues and numerous models of the hypothetical transport forms **3**/**4** have been studied.[9-15]

Particular attention has focused on the conversion of **1** to **3** and the possible intermediacy of iminocyclophosphamide (**5**) in this reaction, as first proposed by Fenselau et al.[12] The use of trapping reagents has provided product, kinetic, and stereochemical data consistent with the formation of an imine from **1** or its analogues;[15-17] however, the proposed imines have not been isolated nor have they been characterized spectroscopically as a component of a reaction mixture. While the inability to detect **5** spectroscopically (and in particular by NMR spectroscopy) does not preclude its existence, it does suggest that under the conditions of the reported studies the concentration of **5**, if formed, is low.[9,15,18] Efforts to isolate and characterize **5** are further complicated by the known instability of its precursor, **1**.[9,18] Investigations of alternative pathways directed toward the synthesis of **5** have been unsuccessful.[19,20]

Scheme II[a]



[a] M = $N(CH_2CH_2Cl)_2$.

It became apparent to us that in order to optimize the conditions under which an imino oxazaphosphorine might form and be spectroscopically detected, possible addition and fragmentation pathways would have to be controlled. This led to the synthesis of 4-hydroxy-5,5-dimethylcyclophosphamide (**6**) for studies of its equilibria reactions in a nonnucleophilic solvent. Like **1**, hemiaminal **6** can undergo ring opening and dehydration reactions, thus affording α,α-dimethylaldophosphamide (**7**) and 5,5-dimethyliminocyclophosphamide (**8**), respectively (Scheme II); however, the absence of a C-5 proton in **6** blocks the formation of phosphoramide mustard by an α,β-elimination mechanism. Characterization of imine **8** would then provide spectral benchmarks applicable to imine **5**.

We now report NMR spectroscopic evidence for the formation of imino oxazaphosphorines **5** and **8** via the "spontaneous" dehydration of 4-hydroxyoxazaphosphorines **1** and **6**, respectively. Investigations of mechanisms for the conversion of **1** to **3** that do not require the intermediacy of imine **5** are also discussed (cf. Scheme I).

Synthesis of 4-Hydroxy-5,5-dimethylcyclophosphamide (6)

Metabolite **1** is conveniently obtained by the deoxygenation of 4-hydroperoxycyclophosphamide;[9] by analogy, 4-hydroperoxy-5,5-dimethylcyclophosphamide (**9**) was viewed as a logical precursor to 4-hydroxy-5,5-dimethylcyclophosphamide (**6**). An adaptation of Takamizawa's method for synthesizing C-4 oxidized oxazaphosphorines was used to produce **9** via the oxidative cyclization of butenyl phosphorodiamidate **10** (eq 1).[21,22] Hydroperoxide **9** was judged to be diastereomerically pure (with presumed cis stereochemistry[23,24]) on the basis of ^1H, ^{13}C, and ^{31}P

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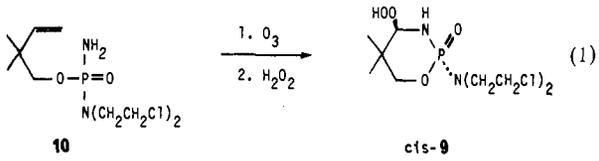
NMR spectroscopy (see Experimental Section).



10 cis-9 (1)

An aqueous solution of hydroperoxide **9** was reacted overnight with sodium thiosulfate ($Na_2S_2O_3$), and extraction of this reaction mixture with CH_2Cl_2 gave an oil, which crystallized from ether. ^{31}P and ^1H NMR spectra of these crystals were accumulated immediately upon dissolution in Me_2SO-d_6. Two, equally intense ^{31}P NMR signals were observed with chemical shifts characteristic of oxazaphosphorines in organic solvents (δ 8.77 and 7.11).[9,17,25] The ^1H NMR spectrum also featured two sets of signals, due to the presence of cis and trans isomers of **6** (vide infra). Most notable among these ^1H NMR signals were doublets at δ 5.92 and 5.70 (amino or hydroxyl protons), triplets at δ 5.19 and 5.06 (C-4 protons), and two pairs of diastereotopic methyl protons in the range δ 1.0–0.8. Further identification of this material as hydroxy **6** was made by electron-impact mass spectrometry (see Experimental Section).

In the ^1H NMR spectrum of **6**, no signals were *initially* observed for the CH=N moiety in imine **8**, expected in the range δ 8.5–6.5; however, after ca. 1 h, a doublet of doublets was observed at δ 8.25 ($^3J_{HP}$ = 53 Hz; $^4J_{HH}$ = 2.5 Hz). The chemical shift and coupling constants of this signal provided prima facie evidence for the presence of 5,5-dimethyliminocyclophosphamide (**8**).

Identification of Imine 8 in Me_2SO-d_6

Crystalline 4-hydroxy-5,5-dimethylcyclophosphamide (**6**, 0.05 mmol) was dissolved in Me_2SO-d_6 (0.6 mL, freshly opened ampule, molar percentage of water $\leq \sim 0.03$), and the solution was transferred to a 5-mm NMR tube containing several molecular sieves (type 4A, 10–16 mesh). The sample was allowed to equilibrate at room temperature for approximately 1 h prior to spectral analyses.

The ^1H NMR spectrum of this equilibrated sample is shown in Figure 1. The multiplet at δ 8.25 was in the chemical shift range anticipated for the C-4 imino proton in **8**. The splittings (53 and 2.5 Hz) of the doublet of doublets were independent of magnetic field strength (90 and 400 MHz) and, therefore, derived from scalar couplings. The coupling constant of 53 Hz was consistent with large $^3J_{HP}$ values found for model compounds containing an HC=NP moiety,[26] and this coupling was also observed in the ^1H-coupled ^{31}P NMR spectrum of the sample. The remaining resolved splitting (2.5 Hz) was attributed to coupling between the C-4 imino proton in **8** and one of the C-6 protons, which was confirmed by a homonuclear decoupling experiment (data not shown).

A ^{13}C NMR spectrum of the equilibrated sample of **6** was acquired overnight at 60 °C; a partial display is shown in Figure 2. The signal at δ 203.7 was attributed to the

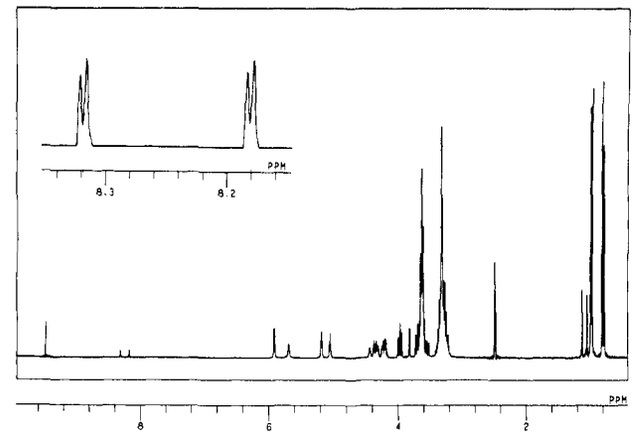


Figure 1. ^1H NMR (400-MHz) spectrum of an equilibrated sample of **6** in Me_2SO-d_6 at 20 °C (Me_2SO-d_5 at δ 2.49). (Inset) Expanded chemical shift region showing the signals for the C-4 proton in imine **8** (δ 8.25, $^3J_{HP}$ = 53 Hz, $^4J_{HH}$ = 2.5 Hz).

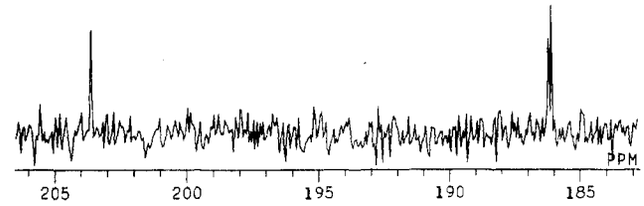


Figure 2. Partial display of the 100.5-MHz ^{13}C NMR spectrum of an equilibrated sample of **6** in Me_2SO-d_6 at 60 °C. The shown signals arise from **7** (CHO, δ 203.7) and **8** (C_4, δ 186.1, $^2J_{CP}$ = 12 Hz).

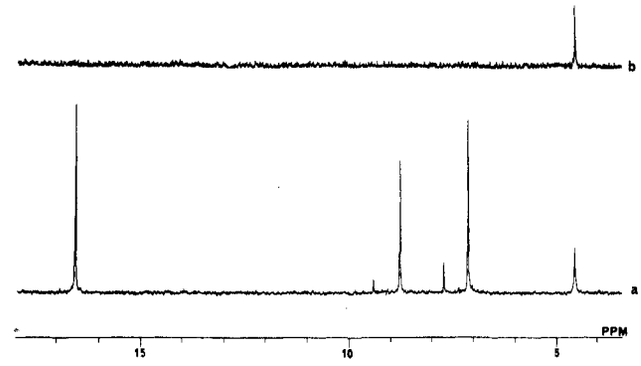


Figure 3. (a) ^{31}P NMR (162-MHz) spectrum of an equilibrated sample of **6** in Me_2SO-d_6 at 60 °C. Signal assignments include those for *cis*-**6** (δ 8.77), *trans*-**6** (δ 7.11), **7** and **7**·H_2O (δ 16.54), and **8** (δ 4.54). (b) ^{31}P NMR (162-MHz) spectrum derived from a ^1H–^{31}P selective INEPT experiment using soft-pulse irradiation (δ_H 8.25) on the same sample from which the spectrum shown in (a) was derived.

(24) For cyclophosphamide analogues that are monosubstituted at the C-4 position, cis and trans designations follow IUPAC nomenclature and refer to the relative orientation of the C-4 substituent and the P=O functionality [Farmer, P. B.; Jarman, M.; Facchinetti, T.; Pankiewicz, K.; Stec, W. J. *Chem.-Biol. Interact.* **1977**, *18*, 47]. For cyclophosphamide analogues that bear an oxygenated moiety as well as a second substituent at the C-4 position, cis and trans designations refer to the relative stereochemistry of the oxygenated moiety at C-4 and the P=O functionality. Throughout the present report, only the *R* configuration at phosphorus is shown in figures that illustrate stereochemistry.

(25) In previous papers[9,17] we referenced all ^{31}P NMR chemical shifts to external 25% H_3PO_4 in D_2O regardless of the solvent used in sample preparation. In the present report, ^{31}P chemical shifts have been referenced to external 25% H_3PO_4 in D_2O or to external 25% H_3PO_4 with an insert of Me_2SO-d_6 or $CDCl_3$, depending on the deuterium lock used in the sample. To compare chemical shifts with those cited in this report, subtract 3.30 ppm from previously reported ^{31}P chemical shifts for Me_2SO-d_6 solutions or add 1.64 ppm to previously reported ^{31}P chemical shifts for $CDCl_3$ solutions.

(26) Two acyclic phosphinylimines [PhCH=NP(O)H_2 and $(CH_3)_2CHCH_2CH$=NP(O)Ph_2] have shown $^3J_{HP}$ values of 32 Hz with the ^1H NMR chemical shift of the C*H*=N proton in each compound occurring at δ 8.8 in $CDCl_3$ [Stec, W. J.; Krzyzanowska, B., unpublished data].

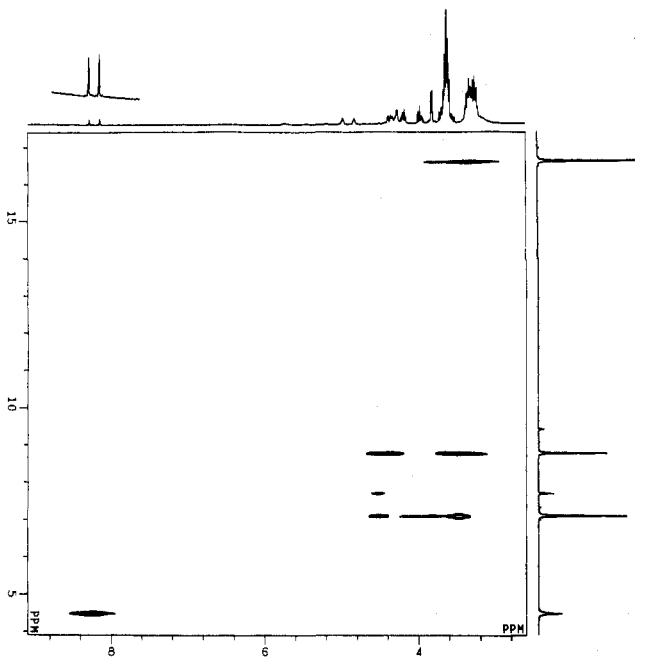


Figure 4. Partial display of a ^1H–^{31}P 2D correlation NMR spectrum (400-MHz ^1H) of an equilibrated sample of **6** in Me_2SO-d_6 at 60 °C. The parameters were chosen so as to optimize correlation peaks due to large couplings.

aldehydic carbon of **7** on the basis of the chemical shift (δ 207.5) of the analogous carbon of aldehyde **2** in polar solvent.[9] The chemical shift of the doublet at δ 186.1 ($^2J_{CP}$ = 12 Hz) was appropriate for the C-4 carbon in imine **8**. This was substantiated by a ^1H–^{13}C selective INEPT (Insensitive Nucleus Enhancement via Polarization Transfer)[27] experiment using "soft" ^1H pulses at 8.25 ppm (δ_H of the imino proton in **8**). In this experiment, only the ^{13}C signal for the carbon attached to the irradiated proton will be observed. The resulting spectrum displayed one ^{13}C signal at δ 186.1 (data not shown).

A ^{31}P NMR spectrum of the equilibrated sample of **6** at 60 °C is shown in Figure 3a. This display features signals attributable (vide infra) to *cis*- and *trans*-**6** (δ 8.77 and 7.11, respectively), aldehyde **7** and its hydrate (isochronous signals at δ 16.54), and an additional signal at δ 4.54. A selective ^1H–^{31}P INEPT experiment was performed with soft-pulse irradiation at the imine proton frequency. Under the conditions employed, only the signal for the ^{31}P nucleus that is coupled to the irradiated proton via multiple-bond, long-range coupling will be observed. The resulting spectrum (Figure 3b) contained only one ^{31}P signal at δ 4.54. A two-dimensional heteronuclear correlation spectrum (Figure 4) provided further evidence that the proton signal at δ_H 8.25 was coupled to the phosphorus signal at δ_P 4.54 and that these signals were due to imino species **8**. In the two-dimensional experiment, the parameters were chosen to optimize correlation peaks due to large (ca. 50 Hz) coupling at the expense of smaller couplings, thus enhancing the correlation between the ^1H and ^{31}P nuclei of the imino species even though it was in low concentration. In the ^1H-coupled ^{31}P NMR spectrum of this sample, the ^{31}P signal at δ 4.54 appeared as a doublet ($^3J_{HP}$ = 53 Hz) and, as expected, the ^{31}P signal at δ 4.54, which was obtained with selective ^1H irradiation at 8.25 ppm, displayed a collapse of the 53-Hz coupling (data not shown).

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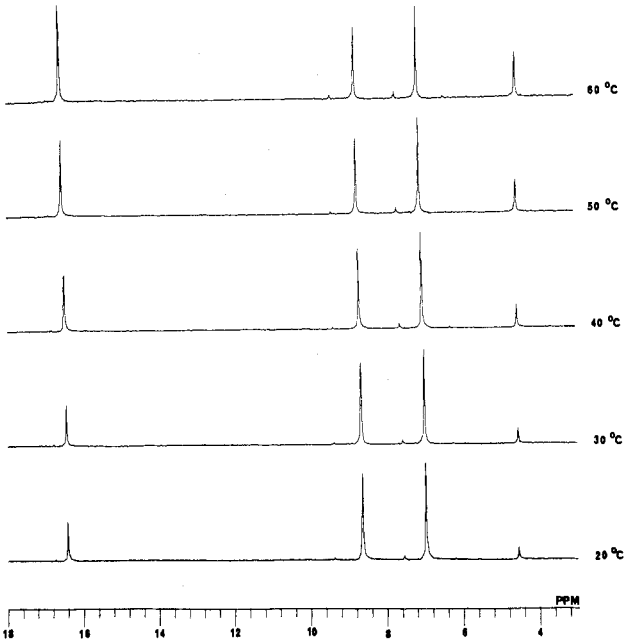


Figure 5. ^{31}P NMR (162-MHz) spectra of **6** in Me_2SO-d_6 following equilibration at various temperatures (as noted). Signal assignments include those for *cis*-**6** (δ 8.77), *trans*-**6** (δ 7.11), **7** and **7**·H_2O (δ 16.54), and **8** (δ 4.54).

The relative intensities of the signals assigned to *cis*- and *trans*-**6**, **7**/**7**·H_2O, and **8** were temperature-dependent and were readily quantified by ^{31}P NMR spectroscopy (Figure 5; relative intensities = 36:41:18:5, respectively, at 20 °C). Phosphorus peak heights were judged to be reliable measures of compound concentration on the basis of the following: (1) possible differential nuclear Overhauser effects were suppressed by gated decoupling and (2) the pulse delay time (10 s) used to acquire the spectra shown in Figure 5 was long enough so as to compensate for differences in relaxation times (T_1) among **6**/**7**/**8** [varying pulse delay times from 2 or 5 s to 20 s at 20 and 60 °C gave identical spectra (±1% in relative peak heights) at a given temperature]. The relative amounts of acyclic and imino species increased with increasing temperature while the relative ratio of *cis*- to *trans*-**6** was essentially temperature-independent. These changes in concentration were reversible, thus indicating that the species were in equilibrium. At higher temperatures or over a longer period of time, new species were seen (^{31}P NMR signals at δ 9.4 and 7.7); however, their relative concentrations did not decrease with lowered temperature, which indicated that they were not in equilibrium with **6**/**7**/**8**.

Aldehyde **7** was estimated to be approximately 30% hydrated at 20 °C by the following measurements. In the ^{31}P NMR spectrum at 20 °C (Figure 5), the relative signal intensities of acyclic to imino species (δ 16.54 vs. 4.54) was 3.4:1; however, under identical conditions, the ^1H NMR spectrum (Figure 1) indicated that the relative ratio of aldehydic to imino species (δ 9.5 vs. 8.25) was 2.3:1. These data implied that ca. 30% of the intensity of the ^{31}P signal at δ 16.54 was not due to aldehyde **7**; the additional signal intensity was attributed to **7**·H_2O, which could have arisen from the small amount of water in the Me_2SO-d_6 solvent and/or the water generated by the conversion of **6** to **8**. Due to the complexity of the ^1H NMR spectrum, it was not possible to unambiguously identify a signal for the $HC(OH)_2$ proton in **7**·H_2O; however, a control experiment with propanal supported the formation of an aldehyde hydrate under similar conditions. Propanal (0.08 mmol) in Me_2SO-d_6 (1.0 mL) with added molecular sieves was

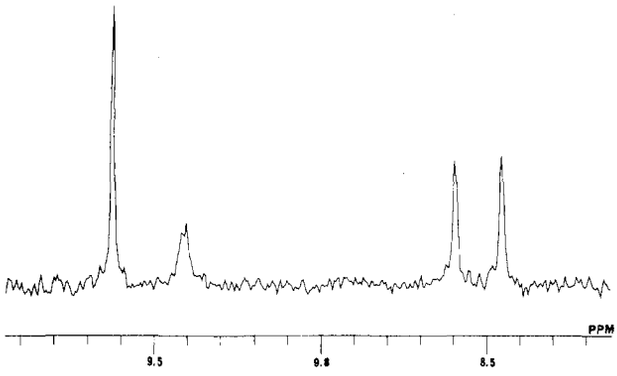


Figure 6. Partial display of a 400-MHz ^1H NMR spectrum recorded for the decomposition of *cis*-**1** in Me_2SO-d_6 at 20 °C. The shown signals arise from **2** (CHO, δ 9.63), acrolein (CHO, δ 9.41, $^3J_{HH}$ = 7 Hz), and **5** (HC=N, δ 8.53, $^3J_{HP}$ = 57 Hz).

shown by ^1H NMR spectroscopy to be ca. 30% hydrated at 26 °C by comparison of the relative integrations of the aldehydic proton (δ 9.63) and the methyl protons (δ 0.90).

In the course of these studies it was further established that the equilibrium component ascribed to **8** was highly susceptible to reaction with water. In either "wet" Me_2SO-d_6 or lutidine buffer (vide infra) or starting with noncrystalline **6** as an oil contaminated with water, ^1H and ^{31}P NMR signals for **8** were not observed.

The above described spectroscopic data (chemical shifts, selective INEPT spectra, two-dimensional correlation experiment, temperature-dependent equilibria data), which established the presence of 5,5-dimethyliminocyclophosphamide (**8**) in anhydrous Me_2SO-d_6, provided spectral guidelines for the characterization of iminocyclophosphamide (**5**) under similar conditions.

Identification of Imine 5 in Me_2SO-d_6

A sample of *cis*-4-hydroperoxycyclophosphamide (0.03 mmol) was deoxygenated with $(MeO)_3P$ (2 equiv) in Me_2SO-d_6 (1.0 mL). The solution was transferred to a 5-mm NMR tube containing molecular sieves, and this was allowed to sit at room temperature ca. 1.5 h prior to spectral analyses. The region of interest in the ^1H NMR spectrum of this sample is shown in Figure 6. On the basis of values of chemical shift and coupling constants found for imine **8**, the doublet at δ 8.53 ($^3J_{HP}$ = 57 Hz) was assigned to the C-4 proton in iminocyclophosphamide (**5**). Also apparent in this figure are signals attributable to the aldehydic protons in aldophosphamide (**2**, δ 9.63) and its fragmentation product, acrolein (doublet at δ 9.41, $^3J_{HH}$ = 7 Hz; verified with authentic material). The aldehydic proton in **2** appeared as a singlet, as did the analogous proton (δ 9.63) in model compound propanal under similar conditions.

The ^{31}P NMR spectrum of this sample is shown in Figure 7a. Signals that could be clearly identified included those derived from *cis*-**1** (δ 7.95; initial signal detected upon deoxygenation of the cis hydroperoxide precursor), *trans*-**1** (δ 8.08), aldehyde **2** (δ 17.1), and $(MeO)_3P/(MeO)_3PO$ (δ 139.2 and 2.16, respectively). Based on the ^{31}P NMR chemical shift of **8** (δ 4.54), it was anticipated that the ^{31}P signal at either δ 4.85 or 3.97 derived from imine **5**. A selective INEPT experiment similar to the one described above for **8** established that the ^{31}P signal at δ 3.97 arose from **5** (Figure 7b). This assignment was further supported by two additional experiments. First, changes in temperature resulted in reversible changes in the concentration of only four species, *cis*-/*trans*-**1**, **2**, and **5**. And second, the addition of 2 equiv water (relative to hydroperoxide starting material) followed by the immediate acquisition

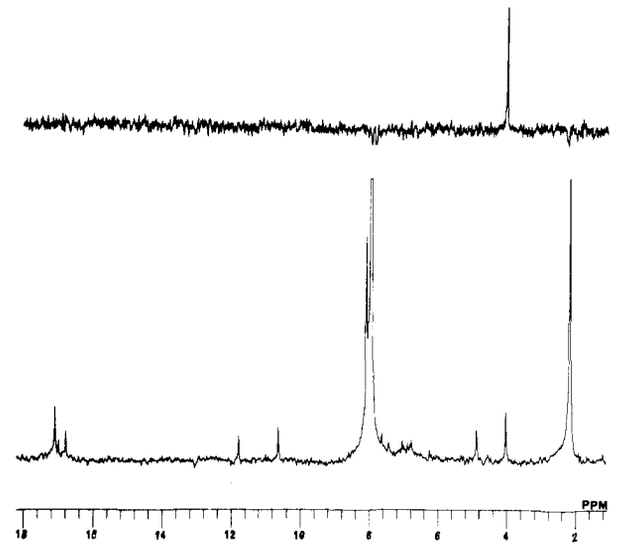


Figure 7. (a) Partial display of a 162-MHz ^{31}P NMR spectrum recorded for the decomposition of *cis*-**1** in Me_2SO-d_6 at 20 °C. Signal assignments include those for *cis*-**1** (δ 7.95), *trans*-**1** (δ 8.08), **2** (δ 17.1), **5** (δ 3.97), phosphoramide mustard (δ 7.5), and $(MeO)_3PO$ (δ 2.16). (b) Partial display of a 162-MHz ^{31}P NMR spectrum derived from a ^1H–^{31}P selective INEPT experiment using soft-pulse irradiation (δ_H 8.53) on the same sample from which the spectrum in (a) was derived.

of a ^{31}P NMR spectrum revealed the absence of the ^{31}P signal for **5** at δ 3.97, and no significant changes in the remaining ^{31}P signals over a 15-min observation period.

After the initial 1.5-h equilibration period at 20 °C, the relative amounts of *cis*-**1**, *trans*-**1**, **2**, and **5** were approximately 59:27:7:7, respectively. As with **6**–**8**, higher temperatures favored a shift in metabolite concentrations toward greater quantities of the aldehydic and imino species: e.g., at 40 °C, the ratios were 51:24:15:10, respectively. Fragmentation of aldehyde **2** to acrolein and phosphoramide mustard was slow at 20 °C (ca. 5% decomposition after 1.5 h), which was in accord with the fact that this reaction is pH-dependent.[9,15,18] On the other hand, phosphoramide mustard (δ_P 7.5) was found to be very unstable (half-life ca. 10 min at 26 °C), and it gave rise to products with ^{31}P NMR chemical shifts of δ 26.3, 11.8, and −0.4.[28] One of the ^{31}P NMR signals at δ 17.0 and 16.7 can be attributed to the hydrate of aldehyde **2**; the intensities of these signals indicate that **2** was 30–35% hydrated under the conditions of the experiment. Other ^{31}P signals that are seen in Figure 7a were attributed to other as yet unidentified decomposition pathways available to **1**/**2**/**5**.

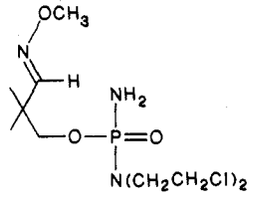
Chemistry of 6 in Lutidine Buffer

Hydroxy compound **6** was synthesized on the basis of the assumption that it would undergo reversible ring opening and dehydration reactions analogous to those proposed for cyclophosphamide metabolite **1**, but that **6** would be inert toward phosphoramide mustard production. To investigate these points and to establish chemical shift assignments, **6** was studied under conditions identical with those used previously for **1**/**2**. The use of ^{31}P NMR spectroscopy to characterize the reversible and irreversible reactions of **1**/**2** in 2,6-dimethylpyridine ("lutidine") buffer has been described in detail elsewhere.[9]

Hydroperoxide *cis*-**9** (0.03 mmol) was dissolved in 1 M lutidine buffer (1.5 mL, 10% D_2O, pH 7.4) and to this was

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

added $Na_2S_2O_3$ (4 equiv). The pH of the solution was readjusted to 7.4 (2 M HCl) and the sample was then filtered into a 10-mm NMR tube. The ^{31}P NMR spectrum at 37 °C at "time zero" (15 min after dissolution of the sample) showed only one product signal at δ 12.93; residual *cis*-**9** (δ 12.15) was not observed. Subsequent spectra, taken at 5-min intervals, revealed that the intensity of the peak at δ 12.93 decreased while signals at δ 20.26, 19.96, and 12.39 appeared. The absolute intensities of these four signals remained constant after 25 min, and no other signals were detected over a 3-week period at 37 °C. The addition of *O*-methylhydroxylamine hydrochloride[29] (2 equiv relative to *cis*-**9**) resulted in the conversion of all four species to one product (δ 20.09). This product was isolated and identified as the *O*-methyloxime derivative of aldehyde **7**, i.e., compound **11**.



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On the basis of the chemical shifts of *cis*-**1** (δ 12.22), *trans*-**1** (δ 12.42), and **2**/**2**·H_2O (δ 20.40, isochronous signals) under identical conditions, these data were consistent with the rapid, stereospecific reduction of hydroperoxide *cis*-**9** to give hydroxy compound *cis*-**6** (δ 12.93) followed by the equilibration of *cis*- and *trans*-**6** (δ 12.39) through aldehyde **7** (δ 20.26) and **7**·H_2O (δ 19.96).[30] The equilibrium ratios of *cis*-**6**/*trans*-**6**/**7**/**7**·H_2O were 45:36:7:12, which were close to the relative ratios found previously for the metabolites of cyclophosphamide after 20 min of reaction time under identical conditions (*cis*-**1**/*trans*-**1**/**2**/**2**·H_2O = 57:30:4:9);[9] imines **5** and **8** were not detected under these aqueous conditions. It was thus apparent that the methyl groups in hemiaminal **6** did not significantly perturb the equilibria relative to the interconversions of *cis*-/*trans*-**1** and **2**.

Mechanisms for 1 → 3

Several mechanistic rationale (Scheme I) have been proposed for the reaction **1** → **3**: (a) conversion involving the intermediacy of iminocyclophosphamide (**5**),[12] (b) direct nucleophilic displacement of the hydroxyl group in **1**,[10] and (c) the reaction sequence **1** → **2** → **4** → **3**.[31] Since the reaction of **1** with sodium cyanide leads to trapping products **3a**/**4a**, which have been synthesized and characterized,[12,16,17,32] we decided to study the proposed mechanisms using cyanide as the nucleophile and ^{31}P NMR spectroscopy as the analytical tool.

Sodium thiosulfate (3.7 equiv) and sodium cyanide (4 equiv) were added to a solution of *cis*-4-hydroperoxycyclophosphamide (0.03 mmol) in 1 M lutidine buffer (1.5 mL, 10% D_2O). The pH of the solution was adjusted to 7.2, and ^{31}P NMR spectra were acquired at 4-min intervals (37 °C). In the "time-zero" spectrum (ca. 15 min after sample preparation), ^{31}P signals were observed for *cis*-**1** [δ 12.22 (48%)], *cis*-/*trans*-4-cyanocyclophosphamide[24] [**3a**; δ 11.68 (7%) and 10.91 (8%)], and the cyanohydrin adduct of aldophosphamide, **4a** [δ 20.37 (37%)]. After 45 min of reaction time, *cis*-**1** was no longer observed and the signal for **4a** represented 83% of the phosphorus content. The remaining 17% of the phosphorus intensity was nearly equally distributed between the two diastereomers of **3a**; *trans*-**1** was not detected.

The formation of nearly equal quantities of *cis*-/*trans*-**3a** at nearly equal rates from *cis*-**1** was most readily accounted for by one of the following reaction sequences: (1) *cis*-**1** → **5** → *cis*-/*trans*-**3a** or (2) *cis*-**1** → **2** → **4a** → *cis*-/*trans*-**3a**. A direct substitution mechanism was least probable unless *cis*-**1** reacted with cyanide in a stereospecific displacement reaction to produce *trans*-**3a**, and then this species either (a) reacted with excess cyanide in solution to effect stereomutation at C-4 or (b) cyanide was eliminated to form imine **5**, which subsequently led to the formation of *cis*-/*trans*-**3a**. These latter mechanisms could not be disregarded since it has been reported that one isomer of **3a** "readily" converts to a more stable diastereomer of **3a**,[16] and it has also been predicted that **3a** would more readily undergo an elimination reaction to form **5** than would hemiaminal **1**.[15] Authentic **3a** and **4a** were synthesized to investigate these various mechanisms.

4-Hydroperoxycyclophosphamide (75 mg) was reduced with thiosulfate (3.5 equiv) in water (15 mL) and the resultant solution was treated with sodium cyanide (12 equiv). After 1 h, the reaction mixture (pH ca. 11) was extracted (CH_2Cl_2) and the concentrated extract was fractionated by preparative TLC (silica gel, $CHCl_3$–CH_3OH, 9:1) to give three products: fast-eluted **3a** [R_f 0.57; ^{31}P δ 8.5 ($CDCl_3$)], slow-eluted **3a** [R_f 0.51; ^{31}P δ 8.4 ($CDCl_3$)], and cyanohydrin **4a** [R_f 0.43; ^{31}P δ 19.3 ($CDCl_3$)].[25]

A mixture of fast- and slow-eluted **3a** (4 mg, 3:7, respectively) was dissolved in 1 M lutidine (0.62 mL, 10% D_2O) and to this was added sodium cyanide (3 equiv) and thiosulfate (2.7 equiv). The pH was adjusted to 7.2 and the sample was monitored by ^{31}P NMR spectroscopy at 37 °C. The signals for fast- and slow-eluted **3a** were observed at δ 11.7 and 10.9 in a ratio of 3:7, respectively, and this ratio was invariant (±1%) over 19 h; no other signals were detected.

A mixture of slow-eluted **3a** and **4a** (4 mg, 7:3, respectively) was treated exactly as described above. The signals arising from slow-eluted **3a** (δ 10.9) and cyanohydrin **4a** (δ 20.37) maintained the respective 7:3 ratio (±1%) over an 18-h period. No other signals were observed.

A diastereomerically pure sample of fast-eluted **3a** (4 mg) was dissolved in 1 M lutidine (pH 7.4) and to this was added thiosulfate, but no cyanide. The sample, which displayed one ^{31}P signal at δ 11.7 was kept at 37 °C for 21 h; no spectral changes could be observed.

From these data it was concluded that, under the conditions used in the studies, the two diastereomers of **3a**

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

(30) It can be noted that the chemical shift of *trans*-**1** (δ 12.42) is downfield relative to that of *cis*-**1** (δ 12.22); however, *trans*-**6** (δ 12.39) is shifted upfield relative to *cis*-**6** (δ 12.93). Since the assignment of cis stereochemistry to hydroperoxide **9** is tentative (see Experimental Section), it is possible that the chemical shift assignments for *cis*-/*trans*-**6** should be reversed. On the other hand, phosphorus chemical shifts are dependent on the axial/equatorial disposition of the P=O moiety and other analogues of **1** have shown this "reversal" in chemical shifts. For example, *cis*-4-hydroxyifosfamide has a ^{31}P NMR chemical shift of δ 13.42 while its trans isomer appears at δ 13.09 in lutidine buffer.[19] Likewise, the ^{31}P signal for *cis*-4-hydroperoxyifosfamide (δ 9.75) is downfield relative to that of its trans diastereomer (δ 9.46) in CD_3OD [Takamizawa, A.; Matsumoto, S.; Iwata, T.; Makino, I. *Chem. Pharm. Bull.* **1977**, *25*, 1877].

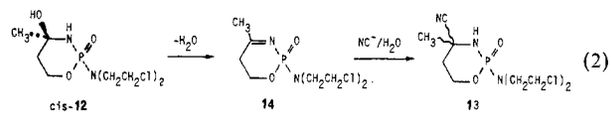
(31) Peter, G.; Wagner, T.; Hohorst, H.-J. *Cancer Treat. Rep.* **1976**, *60*, 429.

(32) Myles, M.; Fenselau, C.; Friedman, O. M. *Tetrahedron Lett.* **1977**, 2475 (1977).

were not subject to rapid interconversion either in the presence or absence of excess cyanide. In addition, *cis-/trans*-**3a** and **4a** were not in equilibrium, and ring closure of **4a** to **3a** did not occur at an appreciable rate. *These conclusions, together with the data described above for the reaction of cis-1 with cyanide, support the formation of 3a via the intermediacy of imine 5.* Caution must be used in generalizing this mechanistic conclusion to other reactions of the type **1** → **3**. In particular, the use of thiols as nucleophiles results in sulfhydryl analogues of **3**/**4** (Scheme I, Nu = SR), which are much more labile under aqueous conditions than are cyano adducts **3a**/**4a**. As a result, the sulfhydryl compounds are involved in a complex kinetic manifold including reversible reactions with **1**/**2**.[9] Further studies would be required to determine the precise role of imine **5** in these reactions.

It must also be emphasized that in the reaction described above for *cis*-**1** and cyanide, the time-zero ^{31}P NMR spectrum revealed that approximately half of the *cis*-**1** had already reacted and that 15% of the ^{31}P intensity arose from *cis-/trans*-**3a**. However, during the next 45 min, as the remainder of the *cis*-**1** disappeared, the total concentration of **3a** increased by only 2%, which was not significant within the experimental error limits. Thus, at pH 7.2, the rate of dehydration of *cis*-**1** to form imine **5** was negligible relative to the rate of ring opening to produce aldehyde **2**, which subsequently reacted rapidly and irreversibly to give **4a**. The presence of **3a** in the time-zero spectrum could be attributed to its formation at elevated pH, prior to the adjustment of the solution pH, as mixing 4-hydroperoxycyclophosphamide, thiosulfate, and cyanide gave an ambient pH of ca. 9. Under these basic conditions, hydroxide and/or cyanide could have catalyzed the dehydration of *cis*-**1**; evidence for the formation of **5** via general-base catalysis has been reported.[15] This was supported by repeating the experiment with *cis*-**1** and cyanide (4 equiv) at pH 8 whereupon the yield of **3a** increased to 21%. At pH 10, with 12 equiv of cyanide, *cis-/trans*-**3a** represented 50% of the product mixture. To summarize, two competing and irreversible pathways contributed to the disappearance of *cis*-**1**: (1) *cis*-**1** → **5** → **3a** and (2) *cis*-**1** → **2** → **4a**. However, the first reaction was significant only under conditions (elevated pH and cyanide concentration) that favored the catalytic formation of **5**.

Additional evidence for cyanide-catalyzed dehydration of a hemiaminal has been reported[17] for *cis*-4-hydroxy-4-methylcyclophosphamide (*cis*-**12**). In 1 M lutidine with added Me_2SO-d_6 (8:2), *cis*-**12** was reacted with cyanide (4 equiv) at pH 7.2, 37 °C, to provide two diastereomers of 4-cyano-4-methylcyclophosphamide (**13**). The concentration of **13** increased from 56% (time-zero ^{31}P NMR spectrum) to 64% upon the disappearance of starting material *cis*-**12** (half-life ca. 4 min). Formation of **13** via ring closure of a cyanohydrin adduct was ruled out in a separate experiment. Assuming, then, that **13** was produced via the intermediacy of 4-methyliminocyclophosphamide (**14**, eq 2), the greater yield of product **13** relative to **3a** reflected



(2)

the greater stability (increased substitution) of imine **14** relative to **5**. However, *in the absence of cyanide, cis*-**12** (half-life 15 min) underwent irreversible ring opening to form an acyclic ketone without coformation of *trans*-**12**. Since addition of water to imine **14**, by analogy to nonselective addition of hydrogen peroxide to imine **5**,[15] would give both *cis*- and *trans*-**12**, the formation of **14** under the latter conditions is not reasonable.

We conclude that the conversion of **1** to **3** does, in some cases, involve the intermediacy of imine **5**, but that in the absence of agents that catalyze the formation of **5**, the "spontaneous" rate of dehydration of **1** is slow relative to other reactions of *cis-/trans*-**1** and **2**.

Conclusion

4-Hydroxy-5,5-dimethylcyclophosphamide (**6**) was synthesized as a cyclophosphamide metabolite-analogue that could be expected to undergo reversible ring opening and dehydration reactions akin to those proposed for 4-hydroxycyclophosphamide (**1**), with one important difference. The inability of **6** to decompose to phosphoramide mustard by an α,β-elimination pathway allowed for extensive NMR spectroscopic studies of solutions of **6** and obviated the time constraints associated with studies of **1**. It was found that the methyl groups in **6** did not significantly perturb the equilibria reactions of **6** relative to those of **1** in lutidine buffer.

Under anhydrous conditions, it was possible to spectroscopically detect and characterize 5,5-dimethyliminocyclophosphamide (***8***) by using a variety of homonuclear and heteronuclear coupling experiments. Spectral characterization of this imino oxazaphosphorine provided useful guidelines for the identification of the elusive cyclophosphamide metabolite, iminocyclophosphamide (**5**). *In addition to achieving the first spectral identification of 5, we have shown that 5 is highly susceptible to addition reactions and that its concentration in aqueous solutions must be extremely low.*

The mechanistic studies of the conversion of **1** to **3** provide compelling evidence for the intermediacy of imine **5** with cyanide as the nucleophile. It was also established that ring opening of *cis*-**1** to aldehyde **2** is kinetically favored over dehydration of *cis*-**1** to imine **5** at pH 7.2. Since the rate constant for the reaction *cis*-**1** → **2** is smaller than that for the reaction *trans*-**1** → **2** (0.06 vs. 0.21 min^{-1}, respectively),[9] it is suggested that dehydration of *trans*-**1** might also be slower than ring opening. It must be noted that the cited rate constants were determined assuming no contribution from imine **5** in the interconversions of **1** and **2**; however, the data derived from the cyanide trapping experiments with **1** and its analogue 4-hydroxy-4-methylcyclophosphamide (**12**) support the conclusion that the kinetic contribution from **5** in these reactions is small.

Iminocyclophosphamide has been shown to be a viable metabolite of cyclophosphamide; however, even under the conditions that were selected to favor its formation, the concentration of **5** was low relative to that of **1**/**2**. On the other hand, the low concentration and/or high reactivity of a species do/does not preclude its participation in biochemical reactions. While the intermediacy of iminocyclophosphamide in the metabolism of cyclophosphamide cannot be disregarded, the role and significance of this metabolite in the oncostatic selectivity of cyclophosphamide have yet to be defined.

Experimental Section

Tetrahydrofuran (THF) and benzene were dried and distilled prior to use. Reaction mixtures that did not contain water were carried out under nitrogen. Ozone (ca. 2 g/h) was produced by a Model 03V10-0 ozone generator (Ozone Research and Equipment Corp.). Melting points were obtained with a Thomas-Hoover capillary apparatus and are uncorrected. Analytical TLC employed 2.5 × 10 cm plates coated with a 250-μm layer of silica gel GF and preparative TLC used 20 × 20 cm plates with a 1-mm layer of silica gel GF (Analtech); I_2 was used for component visualization. Silica gel from either J. T. Baker Chemicals (60–200 mesh) or EM Reagents (<250 mesh for columns run at 5 °C) was

used for column chromatography. Me_2SO-d_6 was obtained from Merck Sharp and Dohme (minimum isotopic purity 99.96 atom % D; 0.5 mL ampules) and Aldrich Chemical Co. (minimum isotopic purity 99.9 atom % D; 1.0 mL ampules). Phosphramide mustard, as the cyclohexylammonium salt, was a gift from The National Cancer Institute.

^1H NMR spectra (60-MHz) were recorded on a Varian EM360-A spectrometer. ^1H (89.55-MHz), ^{13}C (22.49-MHz)and ^{31}P NMR (36.23-MHz) spectra were obtained with a JEOL FX-90Q broad-banded spectrometer. ^1H (399.97-MHz), ^{13}C (100.5-MHz), and ^{31}P NMR (161.6-MHz) spectra were recorded on a JEOL GX-400 spectrometer. ^1H and ^{13}C NMR chemical shifts were referenced to Me_4Si or Me_2SO-d_5; ^{31}P NMR chemical shifts were referenced to external 25% H_3PO_4 in D_2O or external 25% H_3PO_4 with an insert of Me_2SO-d_6 or $CDCl_3$, depending on the deuterium lock in the sample.[25]

Ethyl 2,2-Dimethylacetoacetate. The title compound was synthesized by an adaptation of the method reported by Falkers and Adkins.[33] A solution of ethyl acetoacetate (6.4 mL, 0.05 mol) in benzene (25 mL) was added dropwise to an ice-cooled suspension of sodium hydride (0.22 mol) in benzene (200 mL). This was followed by the addition (via syringe) of iodomethane (9.3 mL, 0.15 mol). After the mixture was stirred for several minutes, more ethyl acetoacetate (6.4 mL, 0.05 mol) in benzene (25 mL) and then iodomethane (9.3 mL, 0.15 mol) were added, and the reaction was refluxed for 6 h. The reaction mixture was filtered, and the benzene and excess iodomethane were removed by distillation. The residual material was identified as ethyl 2,2-dimethylacetoacetate (9.4 g, 0.06 mol, 60% yield) and was used without further purification: ^1H NMR (60 MHz, $CDCl_3$) δ 4.29 (q, J = 7 Hz, 2 H, OCH_2), 2.11 (s, 3 H, $CH_3C{=}O$), 1.32 [s, 6 H, $C(CH_3)_2$], and 1.22 (t, J = 7 Hz, 3 H, CH_2CH_3).

Ethyl 2,2-Dimethyl-3-hydroxybutanoate. Ethyl 2,2-dimethylacetoacetate (10.3 g, 65 mmol) in absolute ethanol (50 mL) was added dropwise to a suspension of sodium borohydride (0.95 g, 20 mmol, 23% excess) in absolute ethanol (50 mL). After being stirred for 2 days at room temperature, the reaction mixture was made slightly acidic (litmus paper) by the gradual addition of HCl (3–4 M). After filtration, ethanol was removed on a rotary evaporator with minimal heating (40–50 °C). The residual oil was dissolved in $CHCl_3$ (50 mL) and washed with water (20 mL). The organic layer was dried ($MgSO_4$) and the solvent was removed at reduced pressure with minimal heating. The remaining oil was identified as ethyl 2,2-dimethyl-3-hydroxybutanoate (8.5 g, 53 mmol, 82% yield): ^1H NMR (89.55 MHz, $CDCl_3$) δ 4.17 (q, J = 7 Hz, 2 H, OCH_2), 3.88 (q, 1 H, J = 7 Hz, $CHOH$), 2.70 (s, 1 H, OH), 1.27 (t, J = 7 Hz, 3 H, CH_2CH_3), 1.17 [s, 6 H, $C(CH_3)_2$], and 1.14 (d, 3 H, J = 7 Hz, $CHCH_3$).

Ethyl 2,2-Dimethyl-3-butenoate. Phosphorus pentoxide [5 g, ca. 35 mmol (hygroscopic)] was added to a solution of ethyl 2,2-dimethyl-3-hydroxybutanoate (10.3 g, 64 mmol) in benzene (10 mL); this addition induced reflux. The reaction was refluxed for 4 h and the benzene was then removed by pipet from the dark solids. Fresh benzene (50 mL) was added to the reaction flask and this was refluxed for an additional 4 h. The two benzene layers were combined, and the solvent was removed by distillation. The residual material was identified as ethyl 2,2-dimethyl-3-butenoate (4.3 g, 30 mmol, 47% yield) and was used without further purification: ^1H NMR (89.55 MHz, $CDCl_3$) δ 6.22–5.76 (m, 1 H, vinylic), 5.20–4.92 (m, 2 H, vinylic), 4.12 (q, J = 7 Hz, 2 H, OCH_2), 1.30 [s, 6 H, $C(CH_3)_2$], 1.23 (t, J = 7 Hz, CH_2CH_3).

2,2-Dimethyl-3-buten-1-ol. The title compound was prepared in 39% yield according to the procedure of Bly and Swindell.[34]

2,2-Dimethyl-3-butenyl *N*,*N*-Bis(2-chloroethyl)-phosphorodiamidate (10). A hexane solution of *n*-butyllithium (11.1 mL of 1.60 M, 17.8 mmol) was added dropwise to a stirred solution of 2,2-dimethyl-3-buten-1-ol (1.78 g, 17.8 mmol) in THF (20 mL) at −23 °C (CCl_4–CO_2 bath). Stirring at −23 °C continued for 2 h, and the resultant suspension was removed with a syringe and then added dropwise to a stirred solution of *N*,*N*-bis(2-chloroethyl)phosphoramidic dichloride (4.61 g, 17.8 mmol) in THF (25 mL) at −23 °C. Stirring at −23 °C was continued for 3 h, and then NH_3 was bubbled through the reaction mixture for 15 min at ca. 5 °C. The stoppered reaction flask stood at room temperature overnight prior to suction filtration and concentration of the filtrate at reduced pressure. The residual material was chromatographed on silica gel (3 × 30 cm column) with ether (ca. 500 mL) to remove fast-eluting components. Elution with $CHCl_3$–CH_3OH (9:1) gave **10** as a white solid [mp 55–58 °C; R_f 0.67 ($CHCl_3$–CH_3OH, 9:1), 2.06 g, 6.8 mmol, 38% yield]: ^1H NMR (89.55 MHz, $CDCl_3$) δ 6.02–5.65 (m, 1 H, vinylic), 5.20–4.90 (m, 2 H, vinylic), 3.79–3.10 (m, 12 H, CH_2O, 2 NCH_2CH_2Cl, and NH_2), and 1.05 (s, 6 H, 2 CH_3); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 144.2 ($CH_2{=}CH$), 112.7 ($CH_2{=}CH$), 72.79 (d, $^2J_{CP}$ = 5.9 Hz, CH_2O), 49.33 (d, $^2J_{CP}$ = 4.4 Hz, 2 NCH_2), 42.34 (2 CH_2Cl), 37.69 [d, $^3J_{CP}$ = 7.3 Hz, $C(CH_3)_2$], 23.42 (2 CH_3); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 15.95.

***cis*-4-Hydroperoxy-5,5-dimethylcyclophosphamide [(2*R*,4*R*/2*S*,4*S*)-*N*,*N*-Bis(2-chloroethyl)tetrahydro-2*H*-4-hydroperoxy-5,5-dimethyl-1,3,2-oxazaphosphorin-2-amine 2-Oxide, *cis*-9].** Ozone was bubbled through a solution of **10** (0.43 g, 1.4 mmol) in acetone–water (2:1, 15 mL) at ca. 5 °C for 15 min. The volume of the solution was adjusted to 15 mL with more acetone, aqueous H_2O_2 (0.6 mL of a 30% solution) was added, and the reaction flask was then stoppered and kept at room temperature overnight. Acetone was removed with 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 concentrated at reduced pressure and ambient temperature. The residual oil was chromatographed at 5 °C on a column (2.5 × 25 cm) of silica gel (<250 mesh) using $CHCl_3$–acetone (1:1) eluent (ca. 3 mL/h flow rate; ca. 1.5-mL fractions). The product (R_f 0.65) was obtained as an oil that crystallized from ether (mp 57 °C, 77 mg, 0.24 mmol, 17% yield): ^1H NMR (89.55 MHz, $CDCl_3$) δ 4.65 (apparent d, J = 12 Hz, 1 H, C_4-H), 4.47 (d, $^3J_{HP}$ = 4 Hz, 2 H, CH_2O), 3.90–3.20 (m, 10 H, 2 NCH_2CH_2Cl, OH, and NH), 1.25 (s, 3 H, CH_3), and 1.00 (s, 3 H, CH_3); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 93.87 (d, $^2J_{CP}$ = 4.3 Hz, C_4), 72.45 (d, $^2J_{CP}$ = 5.9 Hz, CH_2O), 48.78 (d, $^2J_{CP}$ = 5.9 Hz, 2 NCH_2), 41.95 (2 CH_2Cl), 34.11 (d, $^3J_{CP}$ = 4.4 Hz, C_5), 24.20 (CH_3), and 20.49 (CH_3); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 10.66. Anal. ($C_9H_{19}Cl_2N_2O_4P\cdot{}^1/_2H_2O$) C, H, N.

The assignment of cis stereochemistry to hydroperoxide **9** is tentative but precedented.[23] The value of the $^3J_{HP}$ coupling constant (12 Hz) for the doublet assigned to the C-4 proton in **9** is less than that found for the corresponding proton in *cis*-4-hydroperoxycyclophosphamide ($^3J_{HP}$ = 26 Hz).[9] However, if the solution structure of **9** includes some contribution from a chair conformation wherein the P=O and OOH moieties are both equatorial, then a $^3J_{HP}$ value of 12 Hz would be reasonable for the C-4 proton in an axial position.[35,36]

4-Hydroxy-5,5-dimethylcyclophosphamide [*N*,*N*-Bis(2-chloroethyl)tetrahydro-2*H*-4-hydroxy-5,5-dimethyl-1,3,2-oxazaphosphorin-2-amine 2-Oxide, 6]. A solution of hydroperoxide **9** (20 mg, 0.06 mmol) and $Na_2S_2O_3\cdot5H_2O$ (31 mg, 0.12 mmol, 2 equiv) in water (10 mL) was stirred overnight at room temperature. The reaction mixture was extracted with CH_2Cl_2 (2 × 15 mL), and the combined extracts were dried ($MgSO_4$) and concentrated on a rotary evaporator. The residual oil was crystallized from ether at −20 °C to give **6** as an equimolar mixture of cis (2*R*,4*R*/2*S*,4*S*) and trans (2*S*,4*R*/2*R*,4*S*) diastereomers (5 mg, 0.02 mmol, 33% yield, mp 96–99.5 °C): ^1H NMR (400 MHz, Me_2SO-d_6) δ 5.92 and 5.70 (d's, J = 5 Hz, diastereomeric OH or NH), 5.19 and 5.06 (apparent t's, J = 5.2 Hz, diastereomeric C_4-H), 4.40–4.18 (2 m's, diastereomeric CH_2O), 3.7–3.2 [m, diastereomeric $N(CH_2CH_2Cl)_2$ and NH or OH], and 1.00, 0.98, 0.82, and 0.79 (s's, two pairs of diastereotopic CH_3 groups); ^{31}P NMR (36.23 MHz, Me_2SO-d_6) δ 8.77 and 7.11; electron-impact mass spectrometry, m/z 237 ($M^{\bullet+} - H_2O - {}^\bullet CH_2Cl$), 164 [$M^{\bullet+} - {}^\bullet N(CH_2CH_2Cl)_2$], 84 [$(HN{=}CHC(CH_3)_2CH_2)^{\bullet+}$], 56 [$CH_2{=}C(CH_3)_2^{\bullet+}$], and ions arising from the $N(CH_2CH_2Cl)_2$ moiety (106 and 63). The expected isotope pattern for ions containing chlorine was also apparent.[37]

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Synthesis of α,α-Dimethylaldophosphamide *O*-Methyloxime (11). A solution of hydroperoxide **9** (20 mg, 0.06 mmol) and *O*-methylhydroxylamine hydrochloride (5 mg, 0.06 mmol) in water (1 mL) was stirred overnight at room temperature. The solution was saturated with NaCl and then extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated at reduced pressure. Spectroscopic analysis of the residual material indicated that predominantly (>86%) one diastereomer of **11** (presumably the *E* isomer[38,39]) had been isolated: ^1H NMR (89.55 MHz, $CDCl_3$) δ 7.3 (s, 1 H, N=CH), 4.1-3.2 (m, 10 H, CH_2O and 2 NCH_2CH_2Cl), 3.8 (s, 3 H, OCH_3), 3.1-2.7 (br s, 2 H, NH_2), and 1.13 and 1.11 [2 s, 3 H each, $C(CH_3)_2$].

NMR Measurements in Me_2SO-d_6. A JEOL GX-400 NMR spectrometer was used to record ^1H, ^{13}C, and ^{31}P NMR spectra in Me_2SO-d_6. The instrument was equipped with a variable-temperature unit, which maintained the sample temperature to ±0.1 °C. One-dimensional ^{13}C measurements were made by using bilevel ^1H decoupling (4-W decoupling power during the relaxation delay interval and 9 W during acquisition), and ^{31}P measurements were made with decoupling (9 W) during the acquisition period only so as to suppress possible nuclear Overhauser effects. Measurements for the equilibrium studies were made with a pulse delay of 10 s; in all other cases the pulse delay was 3 s.

^1H-^{31}P selective INEPT data were obtained by using the following pulse sequence: $90°_x(^1H)$-$\Delta_1/2$-$180°_y(^1H)$, $180°_x$-(^{31}P)-$\Delta_1/2$-$90°_{\pm y}(^1H)$, $90°_x(^{31}P)$-$\Delta_2/2$-$180°_x(^1H)$, $180°_x$-(^{31}P)-$\Delta_2/2$-acquire with broad-band decoupling.

Acquisition parameters were as follows: $90°(^1H)$ = 10 ms; $90°(^{31}P)$ = 19 μs; Δ_1 = 20 ms; Δ_2 = 20 ms; pulse delay = 3 s; and total scans = 40.

^{31}P-detected two-dimensional ^1H-^{31}P correlation NMR data were obtained with the pulse sequence: $90°_x(^1H)$-$\Delta_1/2$-$180°_x$-(^{31}P)-$\Delta_1/2$-Δ_2-$90°_x(^1H)$, $90°_x(^{31}P)$-Δ_3-acquire with broad-band decoupling.

Values for Δ_2 and Δ_3 of 10 and 5 ms, respectively, were used for optimum polarization transfer without severe signal loss due to T_2 relaxation. Values for Δ_1 ranged from 0.00 to 6.93 ms in steps of 110 μs for a total of 64 experimental data points in t_1. The block size in t_2 was 1K. The data were collected in ca. 50 min with 16 scans per t_2 slice and a repetition delay of 3 s. The final power spectrum was obtained by using a 3-Hz exponential filter in t_2 and no filtering in t_1.

Acknowledgment. This investigation was supported in part by PHS Grant No. CA21345 [to G.Z. (1-4 years) and S.M.L. (4-6 years)] and CA37323 (to S.M.L.) awarded by the National Cancer Institute, DHHS. We are grateful to Lawrence R. Phillips of the Food and Drug Administration for the electron-impact mass spectrum and we thank Dr. Wojciech J. Stec of the Polish Academy of Sciences for helpful discussions.

(37) A molecular ion was not observed in the EI-MS of a number of C-4 oxidized cyclophosphamide analogues. For example, like **6**, 4-hydroxy-5-methoxycyclophosphamide gave a base peak (*m/z* 239) corresponding to the combined loss of H_2O and $^•CH_2Cl$ from the molecular ion;[19] an analogous species (*m/z* 209) was detected in the EI-MS of 4-hydroperoxycyclophosphamide [Przybylski, M.; Ringsdorf, H.; Lenssen, U.; Peter, G.; Voelcker, G.; Wagner, T.; Hohorst, H. J. *Biomed. Mass Spectrom.* **1977**, *4*, 209]. The presence of ionic species observed at *m/z* 84 and 56 in the EI-MS of **6** was supportive of the expected structural similarities among **6** and other 5,5-dimethyloxazaphosphorines that had been previously investigated [Edmundson, R. S. *Org. Mass Spectrom.* **1982**, *17*, 558].

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Synthetic and Enzyme Inhibition Studies of Pepstatin Analogues Containing Hydroxyethylene and Ketomethylene Dipeptide Isosteres

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Synthetic details for the preparation of a series of hydroxyethylene and ketomethylene dipeptide isosteres with control of stereochemistry at C(2) are described. Incorporation of the isosteres into peptide sequences derived from pepstatin afforded potent inhibitors of the aspartic protease porcine pepsin. When Leu$\overset{OH}{—}$Ala or Leu$\overset{OH}{—}$Phe was substituted for statine ((3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid), inhibitors equipotent to the parent compound were obtained, whereas Leu$\overset{OH}{—}$Gly was a much less effective replacement for statine. A similar trend was evident in the corresponding ketones. The finding that structural features for good substrates do not closely parallel those for good inhibitors is discussed.

The study of natural inhibitors of proteases as a means for understanding enzyme mechanisms and as an aid to the development of potential therapeutic agents has been the subject of recent discussions.[2] Also, particular attention has lately been directed toward isosteric peptide bond replacements in efforts to prepare modified peptides with improved properties as drugs.[3,4] Previous studies from this laboratory[2,5] on the inhibition of the aspartic protease porcine pepsin by analogues of the natural peptide pepstatin have led us to address in some detail the question of how the structure of pepstatin is related to that of a normal substrate for the enzyme.

The pepstatin analogue Iva-Val-Sta-Ala-Iaa (**1**) is a potent (3 nM) inhibitor of pepsin that demonstrates a

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(1) Abbreviations used follow the IUPAC-IUB commission on Biochemical Nomenclature recommendations. Additional abbreviations are as follows: Sta (statine), 3-hydroxy-4-amino-6 methylheptanoic acid; Sto (Statine), 3-oxo-4-amino-6-methylheptanoic acid; Iva, isovaleryl; Iaa, isoamylamide; Iba, isobutylamide; Leu$\overset{OH}{—}$Ala, hydroxyethylene dipeptide isostere; Leu$\overset{K}{—}$Ala, ketomethylene dipeptide isostere; DMF, *N,N*-dimethylformamide; PDC, pyridinium dichromate.

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0022-2623/87/1830-0374$01.50/0