Synthesis and Antitumor Activity of Cyclophosphamide Analogues. 4. Preparation, Kinetic Studies, and Anticancer Screening of "Phenylketophosphamide" and Similar Compounds Related to the Cyclophosphamide Metabolite Aldophosphamide

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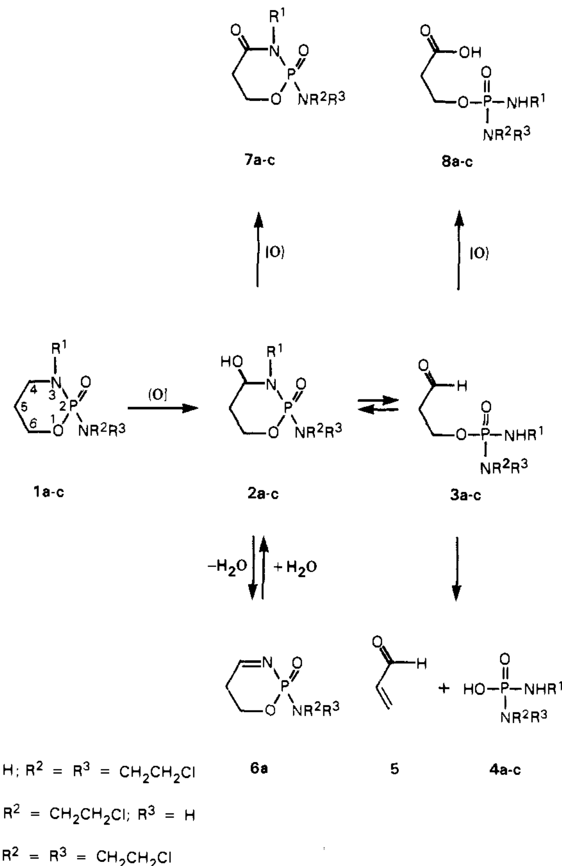
Phenyl ketone phosphorodiamidates [$C_6H_5C(O)CH_2CH_2OP(O)NHR^1NR^2R^3$] were synthesized in conjunction with an ongoing investigation into the effects of substituents on the dynamical solution chemistry of the metabolites of cyclophosphamide (**1a**). In contrast to aldophosphamide (**3a**), which readily interconverts with its cyclic isomer 4-hydroxycyclophosphamide (**2a**), phenylketophosphamide (**14a**: R^1 = H, R^2 = R^3 = CH_2CH_2Cl) exhibited an apparent "resistance" toward an intramolecular addition reaction such that 4-hydroxy-4-phenylcyclophosphamide (**13a**) could not be detected either spectroscopically (^{31}P or ^{13}C NMR) or chemically (NaCN trapping experiment). Control studies that compared the relative reactivities of **14a** and methylketophosphamide [**20**: $CH_3C(O)CH_2CH_2OP(O)NH_2N(CH_2CH_2Cl)_2$] revealed that the factors that modulate the ring closure/opening reactions were not peculiar to the phenyl group; however, differences between phenyl and methyl profoundly influenced the rates of fragmentation of **14a** and **20**. ^{31}P NMR spectroscopy was used to determine the rates at which each compound generated a cytotoxic alkylating agent. Under a standard set of reaction conditions [1 M lutidine buffer with added Me_2SO (8:2), pH 7.4, 37 °C], the half-lives of **2a**/**3a**, **14a**, phenylketoifosfamide (**14b**: R^1 = R^2 = CH_2CH_2Cl, R^3 = H), phenylketotrofosfamide (**14c**: R^1 = R^2 = R^3 = CH_2CH_2Cl), and **20** were 72, 66, 63, 56, and 173 min, respectively. Analogues **14a** and **14b** exhibited good anticancer activity against a variety of test systems.

Cyclophosphamide (**1a**) is one of the most useful drugs in the treatment of human cancers and, as such, it has engendered a multitude of analogues, perhaps the most notably effective of which are ifosfamide (**1b**) and trofosfamide (**1c**).[1–3] It is generally accepted that the metabolism of **1a** includes the key transformations depicted in Scheme I; similar reactions hold for **1b** and **1c**.[2,3] In short, the enzyme-catalyzed oxidation of **1a** produces 4-hydroxycyclophosphamide (**2a**), and the subsequent formation of aldophosphamide (**3a**) leads to phosphoramide mustard (**4a**), which is the ultimate alkylating agent, and acrolein (**5**). Evidence has been provided for the transient existence of iminophosphamide (**6a**) in vitro and this species must also be considered part of the metabolic scheme.[4,5] Detoxification products 4-ketocyclophosphamide (**7a**) and carboxyphosphamide (**8a**) result from enzymatic oxidations of **2a** and **3a**, respectively.

While the metabolism of **1a** is well established, mechanisms for the oncostatic selectivity of **1a** are less clear.[3,6] It has been suggested that the aforementioned detoxification reactions, in particular the aldehyde dehydrogenase mediated conversion of aldehyde **3a** into acid **8a**, occur to a greater extent in normal cells as compared with cancer cells.[7,8] Tests of this hypothesis have produced data subject to conflicting interpretations;[9–12] however, this premise has been used as a basis to predict that lower therapeutic activities will be found for analogues of **1a** that cannot be converted into 4-keto or 4-carboxy derivatives akin to **7a** or **8a**.[13] Representative of these analogues are oxazaphosphorine ring systems bearing substituents at the C-4 position, such as 4-phenylcyclophosphamide (**9**)[14] and 4-methylcyclophosphamide (**10**).[15] On the other hand, substituents at the C-4 position must influence the reactivities of such analogues throughout their metabolism, not just at the detoxification stage. In an effort to probe the overall steric and electronic consequences attending C-4 substitution in cyclophosphamide, we have synthesized

Scheme I



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(1) Zon, G. *Progr. Med. Chem.* **1982**, *19*, 205.
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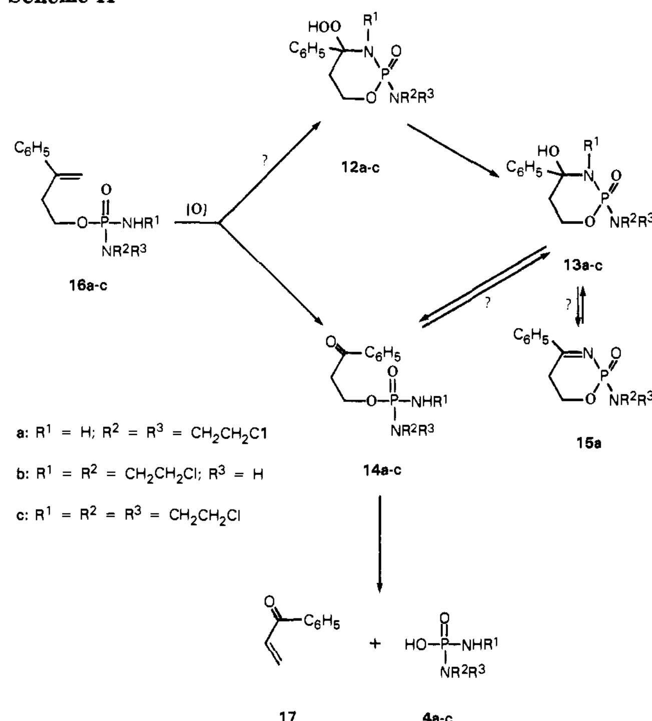
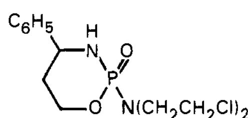
Scheme II



an oxidized derivative of 4-phenylcyclophosphamide for studies of its solution chemistry and anticancer activity. For comparative purposes, similar oxidized aryl analogues of ifosfamide and trofosfamide have been prepared, as well as oxidized derivatives of 4-methylcyclophopshamide.



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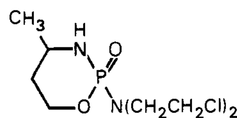

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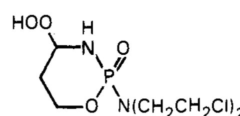
Synthesis of Oxidized Analogues of 4-Phenylcyclophosphamide. Access to the active metabolites of cyclophosphamide (**1a**) is routinely provided by the reduction of 4-hydroperoxycyclophosphamide (**11**), the synthesis of which is given by the reaction of **1a** with ozone and hydrogen peroxide.[16] A similar ozonation of 4-



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phenylcyclophosphamide (**9**) was envisioned as a synthetic route to 4-hydroperoxy-4-phenylcyclophosphamide (**12a**), which would then serve as a precursor to the compounds of interest, namely, 4-hydroxy-4-phenylcyclophosphamide (**13a**), its acyclic isomer **14a**, and 4-phenyliminophosphamide (**15a**, Scheme II). However, the oxidation of either the cis or the trans diastereomer of **9** led to poor yields, difficult purifications, and variable results. An adaptation of Takamizawa's method of synthesizing C-4-oxidized analogues of **1a** by the oxidative cyclization of butenyl phosphorodiamidates was pursued as an alternative pathway.[17,18]

Reaction of the lithium salt of 3-phenyl-3-buten-1-ol[19] with *N,N*-bis(2-chloroethyl)phosphoramidic dichloride [$Cl_2P(O)N(CH_2CH_2Cl)_2$] followed by ammonolysis afforded 3-phenyl-3-butenyl *N,N*-bis(2-chloroethyl)phosphorodiamidate (**16a**, Scheme II). Treatment of **16a** with ozone and hydrogen peroxide led to the isolation of a crystalline product. The identification of this product as the acyclic ketone **14a** ("phenylketophosphamide"[20]) was based on spectral data that included absorption signals characteristic of an aromatic ketone in the ^{13}C nuclear magnetic resonance (NMR) spectrum (δ 197.4) and infrared (IR) spectrum (1670 cm^{-1}). The expected hydroperoxide **12a** was not isolated as a reaction product.

Oxidation of butenyl phosphorodiamidate **16b** (Scheme II) prepared from the reaction of 3-phenyl-3-buten-1-ol, phosphorus oxychloride [$P(O)Cl_3$], and 2 equiv of 2-chloroethylamine hydrochloride, led to an analogous phenyl ketone, **14b** ("phenylketoifosfamide",[20] Scheme II; ^{13}C NMR δ 197.4, C=O). Substitution of ammonia in the synthesis of butenyl **16a** with 2-chloroethylamine hydrochloride gave butenyl phosphorodiamidate **16c**; ozonation of **16c** yielded **14c** ("phenylketotrofosfamide",[20] Scheme II; ^{13}C NMR δ 197.2, C=O). 4-Hydroperoxy-4-phenylifosfamide (**12b**) and 4-hydroperoxy-4-phenyltrofosfamide (**12c**) were not isolated from the reactions that produced ketones **14b** and **14c**, respectively (Scheme II).

Solution Chemistry of Phenyl Ketones 14a–c. Our previous studies of the metabolites of cyclophosphamide have utilized ^{31}P NMR spectroscopy to monitor the interconversion of *cis*- and *trans*-4-hydroxycyclophosphamide (*cis*- and *trans*-**2a**) with aldophosphamide (**3a**) and the fragmentation of **3a** to phosphoramide mustard (**4a**).[16]

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(17) Takamizawa, A.; Matsumoto, S.; Iwata, T.; Katagiri, K.; Tochino, Y.; Yamaguchi, K. *J. Am. Chem. Soc.* **1973**, *95*, 985.
(18) Takamizawa, A.; Matsumoto, S.; Iwata, T.; Tochino, Y.; Katagiri, K.; Yamaguchi, K.; Shiratori, O. *J. Med. Chem.* **1975**, *18*, 376.
(19) Hawkins, E. G. E.; Thompson, R. D. *J. Chem. Soc.* **1961**, 370.
(20) As the phenyl ketone analogue of aldehyde aldophosphamide (**3a**), **14a** has been given the trivial name "phenylketophosphamide". Similarly, "phenylketoifosfamide" and "phenylketotrofosfamide" have been adopted to described **14b** and **14c**, the phenyl ketone analogues of aldehydic metabolites aldoifosfamide (**3b**) and aldotrofosfamide (**3c**), respectively. It follows that the methyl ketone analogue of aldophosphamide is "methylketophosphamide" (compound **20**).

Table I. ^{31}P NMR Chemical Shifts in 1 M Lutidine-Me_2SO (8:2) at 37 ± 2 °C

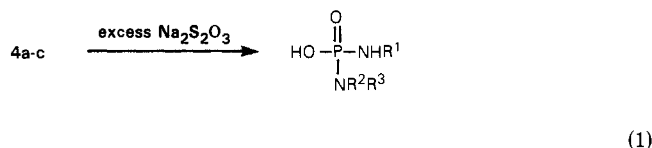
compd	pH[a]	δ[b]	compd	pH[a]	δ[b]
cis-**2a**	6.3	14.01	4c/18c	6.3	14.9–15.2
	7.4	14.01		7.4	15.0–15.4
trans-**2a**	6.3	14.17	**14a**	6.3	22.08
	7.4	14.17		7.4	22.03
3a	6.3	22.25	**14b**	6.3	20.18
	7.4	22.22		7.4	20.09
4a/18a	6.3	15.1–15.2	14c	6.3	20.50
	7.4	15.5–15.7		7.4	20.37
4b/18b	6.3	15.1	*cis*-**19**	7.4	14.25
	7.4	15.1–15.2	**20**	7.4	22.08

[a] Observed pH (uncorrected for the influence of Me_2SO) ± 0.2 pH unit. [b] Relative to external 25% H_3PO_4.

This spectroscopic approach was extended to the investigation of the dynamical solution chemistry of phenyl ketones **14a–c** (Scheme II).

NMR samples were prepared by adding 1 M 2,6-dimethylpyridine ("lutidine") buffer to deuterated dimethyl sulfoxide (Me_2SO) solutions of ketones **14a**, **14b**, or **14c** (final volume ratio of lutidine buffer to Me_2SO = 8:2). Sodium thiosulfate ($Na_2S_2O_3$, 3–5 molar equiv) was added to each sample to provide a nucleophilic trapping agent for the expected fragmentation products, phosphoramide mustard (**4a**), isophosphoramide mustard (**4b**), or trophosphoramide mustard (**4c**). The pH of each solution was then checked, and the values reported herein are those that were observed (i.e., uncorrected for the influence of Me_2SO).

The initial ("time zero") ^{31}P NMR (36.23 MHz) spectrum of phenylketophosphamide (**14a**) at pH 7.4, 37 °C, displayed one resonance absorption signal for starting material at δ 22.03. In each subsequent spectrum (acquired at 4-min intervals), the signal intensity of this peak decreased, and an array of signals appeared at δ 15.5–15.7. Control experiments using authentic phosphoramide mustard (**4a**)[21] and $Na_2S_2O_3$ demonstrated that these product signals could be ascribed to **4a** and bis-adduct **18a** (eq 1), which results from two sequential reactions of **4a**



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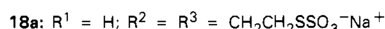
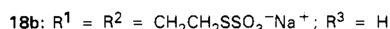
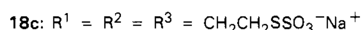






with thiosulfate.[22]

By analogy to the formation of 4-hydroxycyclophosphamide (**2a**) from aldophosphamide (**3a**) in the **2a/3a** equilibrium, phenylketophosphamide (**14a**) could undergo reversible intramolecular cyclization to afford 4-hydroxy-4-phenylcyclophosphamide (**13a**, Scheme II). Since ketone **14a** and **3a** had similar ^{31}P NMR chemical shifts (δ 22.03 and 22.22, respectively, Table I), it was expected that the chemical shift of the resonance absorption signal for **13a** would roughly correspond to that for **2a** (δ 14.0–14.2, Table I). The absence of any observable signals other than those arising from fragmentation products **4a/18a** was evidence against the presence of **13a** in significant concentrations ($\geq$5% of the reaction components). Considering that the

(21) Phosphoramide mustard, as the cyclohexylammonium salt, was a gift from the National Cancer Institute.

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

^{31}P NMR signal for hydroxy compound **13a** could have been obscured by signals for **4a/18a**, another test for the formation of **13a** was desirable. A ^{13}C NMR (75.47 MHz) spectrum of a sample of phenylketophosphamide (**14a**, lutidine–Me_2SO, pH 7.4) was accumulated over several hours at 20 °C; no signals were observed between δ 60 and 120, a range of values that would certainly include the chemical shift of the C_4 nucleus in **13a**.[16]

In addition to providing further evidence against the presence of hydroxy compound **13a**, the ^{13}C NMR spectrum acquired during the decomposition of phenylketophosphamide (**14a**) gave other information regarding the nature of the species in solution. With use of the hydrated carbon in aldophosphamide hydrate (**3a**·H_2O) as a chemical shift model [^{13}C NMR δ ~91 for $HC(OH)_2R$ in lutidine buffer],[16] it was concluded that the ketone moiety in **14a** was not detectably hydrated ($\leq$20%, no signals between δ 60 and 120). A ^{13}C NMR signal attributable to a carbonyl carbon was observed at δ 202, but it was unclear whether this signal arose from **14a** and/or its fragmentation product phenyl vinyl ketone (**17**, Scheme II). Control spectra of authentic phenyl vinyl ketone[23,24] did not resolve this ambiguity; however, phenyl vinyl ketone was unstable under the aqueous reaction conditions, and the same signals corresponding to its presumed polymerization product(s)[25] were observed in the ^{13}C NMR spectrum of phenylketophosphamide (**14a**) in lutidine–Me_2SO (most notably aromatic signals and one fairly intense peak at δ ~30).

4-Phenyliminophosphamide (**15a**, Scheme II) was not detected in either the ^{31}P or ^{13}C NMR spectra of samples of phenylketophosphamide. An intense ^{13}C signal (δ 158.1) due to lutidine did, however, partially obscure the region of interest for an imino carbon (δ 150–160).

^{31}P NMR spectral analyses of phenylketoifosfamide (**14b**) and phenylketotrofosfamide (**14c**) gave results that paralleled those obtained for **14a**. The initial spectrum of **14b** displayed one resonance absorption signal for starting material at δ 20.09, the intensity of which gradually decreased concomitant with the appearance of signals at δ 15.1–15.2. As confirmed by control experiments using authentic materials, the product signals were assigned to isophosphoramide mustard (**4b**) and its adducts with thiosulfate (**18b**, eq 1). Likewise, the initial signal at δ 20.37 arising from **14c** disappeared with time, and this was linked to the growth of an array of signals at δ 15.0–15.4. Use of authentic materials allowed for the assignment of the latter signals to trophosphoramide mustard (**4c**) and its various thiosulfate alkylation products (**18c**, eq 1). In each ^{31}P NMR spectrum, there was no evidence for an intramolecular cyclization reaction of **14b** or **14c** to give 4-hydroxy-4-phenylifosfamide (**13b**) or 4-hydroxy-4-phenyltrofosfamide (**13c**), respectively.

Measurement of Half-Lives. Linear least-squares fits of pseudo-first-order plots of the disappearance of individual ^{31}P NMR resonance absorption signals provided the half-life ($\tau_{1/2}$) values given in Table II. ^{31}P NMR signal intensities (peak heights) were used to measure component–concentration as a function of time. The addition of methylphosphonate to selected NMR kinetic samples

(23) Gras, J.-L. *Tetrahedron Lett.* **1978**, 2955.

(24) Vaidya, N. A.; Nixon, W. J., Jr.; Fatmi, A. A.; Blanton, C. D., Jr. *J. Org. Chem.* **1982**, *47*, 2483 (see footnote 8 in this reference).

(25) For the reactions of acrolein and related systems, see: (1) Nielsen, A. T.; Moore, D. W.; Schuetze, B. D., Jr. *Polish J. Chem.* **1981**, *55*, 1393. (2) Keiko, N. A.; Stepanova, L. G.; Vainberg, N. N.; Bannikova, O. B.; Voronkov, M. G. *Zh. Org. Khim.* **1983**, *19*, 480.

Table II. ^{31}P NMR Derived Half-Lives for Compounds in 1 M Lutidine-Me_2SO (8:2) at 37 ± 2 °C

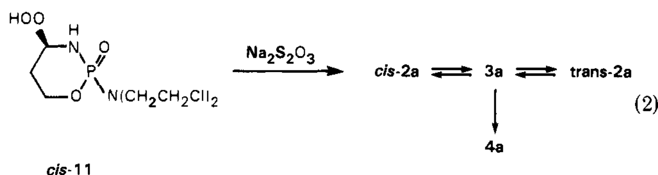
compd	concn, mM	pH[a]	molar equiv of $Na_2S_2O_3$	$\tau_{1/2}$,[b] min
cis-**2a**	20[c]	6.3	4	115 ± 3 (0.996 ± 0.002)[d,e]
	20[c]	7.4	4	73 ± 2 (0.986 ± 0.002)[d,f]
trans-**2a**	20[c]	6.3	4	124 ± 8 (0.968 ± 0.019)[d,e]
	20[c]	7.4	4	65 ± 5 (0.998 ± 0.002)[d,f]
3a	20[c]	6.3	4	123 (0.944)[d]
	20[c]	7.4	4	77 ± 6 (0.913 ± 0.040)[d,f]
14a	20	6.3	3	92 ± 10 (0.996 ± 0.004)[g]
	7	6.3	3	83 ± 1 (0.991 ± 0.001)[e]
	20	7.4	3	66 ± 2 (0.998 ± 0.001)[g]
	10	7.4	3	66 (0.994)
14b	20	6.3	3	92 ± 1 (0.999 ± 0.001)[e]
	20	7.4	3	63 ± 1 (0.998 ± 0.001)[e]
14c	10	6.3	5	78 (0.976)
	7	6.3	5	80 ± 1 (0.992 ± 0.002)[e]
	10	7.4	5	56 ± 4 (0.995 ± 0.003)[g]
cis-**19**	20[h]	7.4	4	15 ± 1 (0.998 ± 0.001)[f]
20	20[h]	7.4	4	173 ± 1 (0.990 ± 0.005)[e]

[a] Observed pH, uncorrected for the influence of Me_2SO; $\Delta pH \leq 0.2$ after complete reaction. [b] The values in parentheses refer to the linear least-squares correlation coefficients; the estimated error limits are ±5–10%. [c] The concentration refers to that of precursor *cis*-11 prior to deoxygenation with thiosulfate. [d] Apparent half-life (see ref 28). [e] Average of two runs. [f] Average of three runs. [g] Average of four runs. [h] The concentration refers to that of precursor *cis*-24 prior to deoxygenation with thiosulfate.

gave a concentration-invariant standard against which other peaks could be normalized. In each case studied, the half-life values calculated with and without normalizing agreed to within a precision error of ±5%. Thus, phenyl ketones **14a–c** were found to have half-lives of 66, 63, and 56 min, respectively, in lutidine–Me_2SO at pH 7.4, 37 °C (Table II).

Due to the increased lipophilicity of phenylketotrofosfamide (**14c**), kinetic runs using this compound required lower sample concentrations relative to those generally used for solutions of **14a** and **14b** (i.e., 7–10 mM vs. 20 mM). However, as would be expected for reactions following first-order kinetics, control experiments with phenylketophosphamide (**14a**) and phenylketotrofosfamide (**14c**) demonstrated that, within the experimental error limits (±~10%), variations in compound–concentration had no influence on the half-life of the compound (see Table II).

Comparisons between the Chemistry of Ketone 14a and Aldehyde 3a. A kinetic analysis of aldophosphamide (**3a**) in lutidine–Me_2SO was done so as to compare the chemistry of **3a** with that of the more lipophilic phenylketophosphamide (**14a**) under identical reaction conditions. The generation of **3a** and other active metabolites of cyclophosphamide as well as detailed NMR spectroscopic analyses of their reactions in 1 M lutidine has been reported;[16] the presence of Me_2SO in the buffer did not significantly influence the chemistry of these metabolites (eq 2). The rapid, stereospecific reduction of *cis*-4-



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hydroperoxycyclophosphamide (*cis*-**11**) with $Na_2S_2O_3$ in lutidine–Me_2SO, pH 7.4, 37 °C, was observed by ^{31}P NMR spectroscopy to give *cis*-4-hydroxycyclophosphamide (*cis*-**2a**, δ 14.01). The reversible stereomutation of *cis*-**2a** with its trans isomer (δ 14.17) through the intermediacy of aldophosphamide (**3a**, δ 22.22)[26] resulted in the development of a pseudoequilibrium[27] mixture of these metabolites. After 45 min of reaction time, the relative ratio of the three interconverting isomers remained constant (*cis*-**2a**/*trans*-**2a**/**3a**[26] = 54:32:14); however, the absolute signal intensity for each species continually decreased as **3a** fragmented irreversibily to mustard **4a**. With use of spectral data accumulated after the pseudoequilibrium state was established, the individual, apparent half-lives[28] of *cis*-**2a**, *trans*-**2a**, and **3a** were calculated, and within the experimental error limits (±10%), these values were identical, as would be expected for rapidly interconverting species (average apparent half-life for **2a**/**3a** = 72 min, Table II).

The rate of production of phosphoramide mustard (**4a**) from phenylketophosphamide (**14a**, half-life = 66 min) was, therefore, comparable to its rate of production from **2a**/**3a** (average half-life = 72 min). Conversely, **14a** was not observed to cyclize to its hydroxy isomer **13a** while hydroxy compounds *cis*- and *trans*-**2a** were clearly more favored thermodynamically than acyclic aldehyde **3a** (pseudoequilibrium ratio of **2a**/**3a**[26] = 86:14). We have observed a similar preponderance of cyclic metabolites over their aldehydic isomers for several analogues of cyclophosphamide (**1a**), including the metabolites of ifosfamide (**1b**) and trofosfamide (**1c**).[29] Since the reasons for the apparent "resistance" to ring closure exhibited by phenylketophosphamide (**14a**) and its analogues **14b** and **14c** were unclear to us, companion studies of a related C-4 substituted system were desirable. This led to the synthesis of the oxidized metabolites of 4-methylcyclophosphamide (**10**).

Synthesis of Oxidized Analogues of 4-Methylcyclophosphamide. As in the metabolism of cyclophosphamide, it is believed that 4-methylcyclophosphamide (**10**) undergoes in vivo oxidation to 4-hydroxycyclophosphamide (**19**), which leads to the acyclic "methylketophosphamide" (**20**)[20] structure and, possibly, 4-methyliminophosphamide (**21**, Scheme III).[3] Fragmentation of methyl ketone **20** affords phosphoramide mustard (**4a**) and methyl vinyl ketone (**22**, Scheme III). It has been reported that the synthesis of 4-hydroxy-4-methylcyclophosphamide (**19**) resulted from the potassium permanganate oxidation of 4-methylcyclophosphamide (**10**); however, the authors did note that the IR and ^{1}H NMR spectra of the isolated material were more consistent with methylketophosphamide (**20**) than with hydroxy compound **19**.[13] In view of the ambiguities associated with the composition of the product given by the potassium permanganate reaction, we chose to pursue an alternative

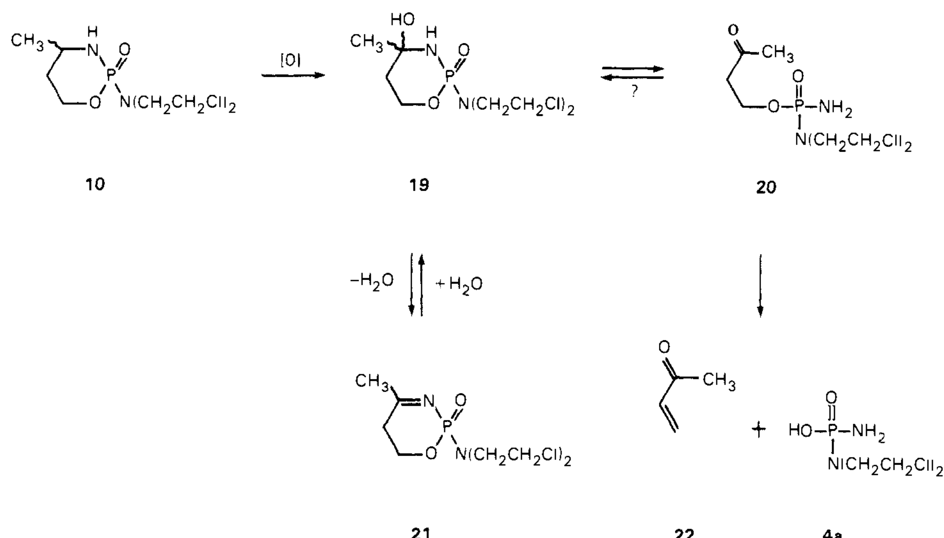
(26) The ^{31}P NMR resonance absorption signal ascribed to **3a** actually arises from isochronous signals for **3a** and its hydrate.[5,16] The ^{31}P NMR derived calculation of the relative abundance of **3a** in aqueous solution is, therefore, an expression of the combined quantities of free aldehyde and its hydrate, **3a**·H_2O.

(27) The *absolute* concentrations of *cis*- and *trans*-**2a** and **3a** (and **3a**·H_2O[26]) do not maintain a steady-state and, therefore, the species do not reach a true equilibrium. However, these species are said to be in "pseudoequilibrium" since the ratio of their *relative* concentrations reaches a constant value.

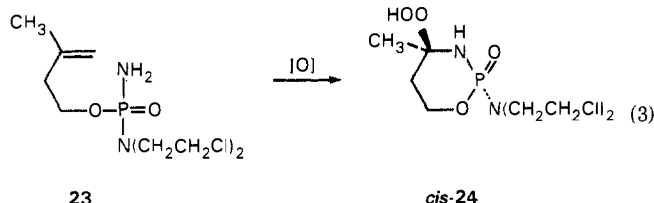
(28) Under conditions that allow for a rapid interconversion of *cis*-**2a**, *trans*-**2a**, and **3a** (and **3a**·H_2O[26]), each species is characterized by an apparent lifetime, which is longer than that which would be observed if each compound reacted in a unidirectional manner. It has been determined that **3a** and **3a**·H_2O can be treated as a single kinetic species.[16]

(29) In 1 M lutidine buffer, pH 7.4, 37 °C, the ratio of *cis*/*trans*-4-hydroxyifosfamide (*cis*/*trans*-**2b**) to aldehyde **3b** (plus **3b**·H_2O) is 89:11 at pseudoequilibrium. Under identical conditions, the ratio of *cis*/*trans*-4-hydroxytrofosfamide (*cis*/*trans*-**2c**) to aldehyde **3c** (plus **3c**·H_2O) is 80:20.

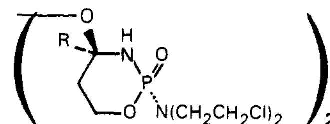
Scheme III



synthetic route to the metabolites of **10**. Butenyl phosphorodiamidate **23** was viewed as a possible precursor to *cis*-4-hydroperoxy-4-methylcyclophosphamide (*cis*-**24**, eq 3);[30] reduction of *cis*-**24** was expected to then afford hydroxy compound **19** and ketone **20**.



Reaction of the lithium salt of 3-methyl-3-buten-1-ol with $Cl_2P(O)N(CH_2CH_2Cl)_2$ followed by ammonolysis yielded 3-methyl-3-butenyl *N*,*N*-bis(2-chloroethyl)-phosphorodiamidate (**23**). Treatment of **23** with ozone and hydrogen peroxide yielded two major products by thin-layer chromatography (TLC). Silica gel chromatography at low temperature (5 °C) allowed the separation of these two products, which had virtually identical ^1H NMR (89.55 MHz) spectra. On the basis of TLC (acetone–$CHCl_3$, 1:1) and ^{31}P NMR ($CDCl_3$) spectral comparisons with *cis*-4-hydroperoxycyclophosphamide (*cis*-**11**: R_f 0.65; δ 8.45), the slower eluting material was tentatively identified as *cis*-4-hydroperoxy-4-methylcyclophosphamide (*cis*-**24**: R_f 0.54; δ 8.86). The same comparisons between *cis*-4-peroxycyclophosphamide (*cis*-**25**: R_f 0.82; δ 10.76)[16] and the faster eluting material provided the bases for the tentative structural assignment of this product as *cis*-4-methyl-4-peroxycyclophosphamide (*cis*-**26**: R_f 0.76; δ 10.67). Additional comparisons of the ^{13}C NMR spectra of these ozonolysis products with those reported for hydroperoxide **11** and peroxy dimer **25** (unspecified stereochemistry) provided further support for the assigned structures.[31] On the basis of ^{31}P NMR chemical shifts, both the faster and slower eluting materials gave the same cyclic reduction product upon treatment with $Na_2S_2O_3$. Since this reduction was stereospecific,[16] it followed that if the assignment of cis stereochemistry to hydroperoxide **24** was correct, then it was also correct for peroxy dimer **26**.



cis-**25**: R = H

cis-**26**: R = CH_3

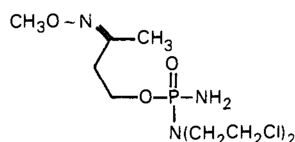
Solution Chemistry of the Oxidized Analogues of 4-Methylcyclophosphamide. An NMR sample of *cis*-4-hydroperoxy-4-methylcyclophosphamide (*cis*-**24**) was deoxygenated with $Na_2S_2O_3$ in lutidine–Me_2SO, pH 7.4, and ^{31}P NMR spectra were accumulated at 37 °C at 4-min intervals. The initial spectrum displayed two resonances at δ 14.25 and 22.08 in a 64:36 ratio, respectively; no residual hydroperoxide *cis*-**24** (δ 13.43) was observed. After 1 h, the signal at δ 14.25 had disappeared and the intensity of the peak at δ 22.08 represented 68% of the total phosphorus content; the other 32% was attributed to fragmentation products **4a**/**18a** (δ 15.5–15.7, eq 1). As time progressed, the concentration of **4a**/**18a** increased at the expense of the component giving rise to the signal at δ 22.08.

These data were consistent with the stereospecific reduction of hydroperoxide *cis*-**24** to give *cis*-4-hydroxy-4-

(30) 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 substituent at the C-4 position 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 alkyl/aryl group as well as an oxygenated moiety at the C-4 position, cis and trans designations refer to the relative stereochemistry of the oxygenated moiety at the C-4 position and the P=O functionality. For example, the two enantiomers of *cis*-4-phenylcyclophosphamide have absolute configurations of 2*R*,4*S* and 2*S*,4*R*; the two enantiomers of *cis*-4-hydroperoxy-4-methylcyclophosphamide have absolute configurations of 2*R*,4*R* and 2*S*,4*S*. Throughout the present report, only the *R* configuration at phosphorus is shown in figures that illustrate stereochemistry.

(31) The ^{13}C NMR chemical shifts (ppm, $CDCl_3$) for 4-hydroperoxycyclophosphamide (**11**) as compared with those for the slower eluting compound (values in parentheses) are as follows: C-4, 86.60 (91.15); C-5, 27.78 (33.77 or 26.43); C-6, 63.44 (63.76); C-α,α′, 48.97 (48.63); C-β,β′, 42.15 (42.10). The ^{13}C NMR chemical shifts (ppm, $CDCl_3$) for 4-peroxycyclophosphamide (**25**) as compared with those for the faster eluting compound (values in parentheses) are as follows: C-4, 86.85 (91.73); C-5, 28.33 (35.67 or 26.68); C-6, 62.76 (63.13); C-α,α′, 49.19 (49.29); C-β,β′, 41.97 (41.98). NMR data for **11** and **25** were taken from Struck, R. F.; Thorpe, M. C.; Coburn, W. C., Jr.; Laster, W. R., Jr. *J. Am. Chem. Soc.* **1974**, *96*, 313.

methylcyclophosphamide (*cis*-**19**, δ 14.25), followed by the apparent undirectional ring opening of *cis*-**19** to afford methylketophosphamide (**20**, δ 22.08). Fragmentation of **20** then yielded phosphoramide mustard (**4a**). A signal for 4-methyliminophosphamide (**21**) was not observed. The signal assignments for hydroxy compound **19** and ketone **20** were supported by the following: (1) the chemical shifts of the signals at δ 14.25 and 22.08 were consistent with those found for related cyclic and acyclic compounds, respectively (e.g., see Table I, *cis*-**2a** vs. **3a**), and (2) the transient species **19**/**20** could be trapped with *O*-methylhydroxylamine[32] to give an adduct identified as **27**, viz., the *E* and *Z* diastereomers of the *O*-methyloxime of **20** (see Experimental Section Section for details).



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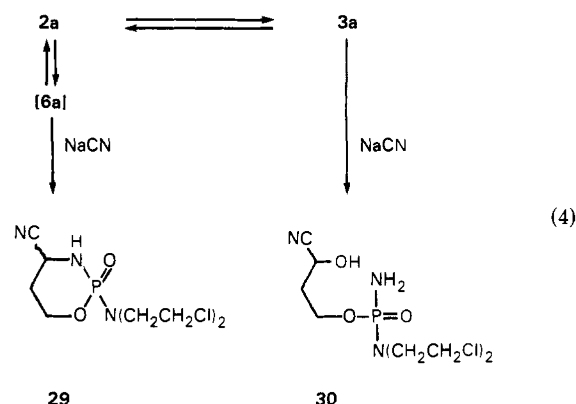
Linear least-squares fits of pseudo-first-order plots of the disappearance of *cis*-4-hydroxy-4-methylcyclophosphamide (*cis*-**19**) over the first hour of reaction time gave a half-life of 15 min (Table II). With use of data from spectra accumulated after the disappearance of *cis*-**19**, the half-life of methylketophosphamide (**20**) was calculated to be 173 min (Table II).

Comparisons between the Reactivities of Phenyl Ketone 14a and Methyl Ketone 20. The thermodynamic favorability of methylketophosphamide (**20**) over its cyclic isomer **19** paralleled the stability of phenylketophosphamide (**14a**) over its cyclic isomer **13a**. It could be concluded, therefore, that the apparent "resistance" of **14a** toward ring closure was not due to an effect peculiar to the phenyl group but rather might be attributed to the lower reactivity of ketones, relative to aldehydes, in this general type of addition reaction. On the other hand, the rates at which phenylketophosphamide (**14a**) and methylketophosphamide (**20**) produced phosphoramide mustard (**4a**) were quite different: the fragmentation of **14a** was almost 3 times faster than that of **20** (half-lives = 66 and 173 min, respectively). This rate difference was rationalized by considering the opposite inductive effects exerted by phenyl vs. methyl on the methylene protons adjacent (α) to the carbonyl group in **14a** and **20**, in relation to the mechanism of fragmentation. Previously reported kinetic analyses of aldophosphamide that was perdeuterated at the carbon α to the carbonyl group provided a value of 5.6 ± 0.4 for the kinetic isotope effect on the rate of fragmentation;[16] this was consistent with rate-determining removal of a proton in an α,β-elimination reaction. The transient intermediacy of an enol (**28**) could not be ruled out, but the studies indicated that if fragmentation proceeded by **3a** → **28** → **4a**, then this reaction would most likely be unidirectional and rate limiting at the first step. In either case, the acidity of the methylene protons adjacent to the carbonyl group would have a direct bearing on the reaction rate. By virtue of its negative inductive effect, the phenyl group would increase the acidity of these protons in **14a** while the acidity of the corresponding protons in **20** would be decreased due to the positive inductive effect of the methyl moiety. Since electron-withdrawing capabilities follow the trend phenyl > H > methyl, the predicted order of the rates of fragmentation would be **14a** > **3a** > **20**. While this was the reactivity order determined experimentally (see Table II), it must be emphasized that aldophopshamide (**3a**) participates in a considerably more complex reaction manifold than either **14a** or **20**.[28]

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

Cyanide Trapping Experiment. In ^{31}P NMR spectra of phenylketophosphamide (**14a**), the absence of discrete signals for the two diastereomers of 4-hydroxy-4-phenylcyclophosphamide (**13a**) did not preclude the formation of these stereoisomers via cyclization of **14a** in a "weighted" equilibrium process. An alternative method for examining the possible formation of hemiaminal **13a** was desirable. If a trapping agent were to be used to detect the transient existence of **13a**, it was necessary to establish that the expected product could be traced back to a reaction between the trapping reagent and **13a** rather than ketone **14a**. Fenselau, Colvin, and co-workers reported that 4-hydroxycyclophosphamide (**2a**) and aldophosphamide (**3a**) were individually trapped with cyanide to give 4-cyanocyclophosphamide (**29**) and cyanohydrin adduct **30**, respectively (eq 4).[4] While these investigators postulated

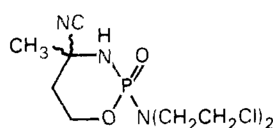


(4)

that **29** was formed from **2a** via the intermediacy of iminophosphamide (**6a**), we believed that further studies were needed prior to using cyanide as a test for the presence of either phenyl hydroxy compound **13a** or 4-phenyliminophosphamide (**15a**, Scheme II). Two control experiments were thus conducted with the preactivated analogues of 4-methylcyclophosphamide. Use of these analogues allowed us to monitor the effects of adding cyanide to reaction mixtures that contained both 4-hydroxy-4-methylcyclophosphamide (**19**) and methylketophosphamide (**20**) and of adding cyanide to solutions that contained **20** but not **19** (see the section that discusses the solution chemistry of **19** and **20**).

To an NMR sample of *cis*-4-hydroperoxy-4-methylcyclophosphamide (*cis*-**24**) in lutidine–Me_2SO was added $Na_2S_2O_3$ followed immediately by sodium cyanide (NaCN, 4 equiv). The solution pH was adjusted to 7.2, and ^{31}P NMR spectra were accumulated at 37 °C. The time-zero spectrum featured signals at δ 22.15 (13%), 22.06 (15%), 14.27 [*cis*-4-hydroxy-4-methylcyclophosphamide (*cis*-**19**), 16%], 13.50 (26%), and 11.28 (30%). Within 12 min, the signal arising from *cis*-**19** was no longer observed and the other four signals had increased in intensity. Over the next 4 h, the two downfield signals diminished with the attendant growth of peaks attributable to fragmentation products **4a**/**18a** (eq 1); the absolute intensities of the two upfield signals were essentially invariant (±5%). The sample was kept at 37 °C for 6 days prior to the accumulation of a final spectrum: the combined intensities of the two upfield signals at δ 13.50 and 11.28 (ca. 1:1 ratio) still accounted for ca. 50% of the total phosphorus content (the other half arising mainly from trapping product **18a**).

On the basis of chemical shift comparisons with authentic *cis/trans*-4-cyanocyclophosphamide (**29**, δ 13.37 and 12.76 in lutidine-Me_2SO),[33] the signals at δ 13.50 and 11.28 were assigned to *cis/trans*-4-cyano-4-methylcyclophosphamide (**31**). To support these assignments, the compounds giving rise to these signals were isolated by lyophilizing the NMR sample and extracting the residue with methylene chloride. Concentration of the organic layer gave a material that exhibited two ^{31}P NMR signals ($CDCl_3$, δ 6.70 and 4.18, ca. 1:1 ratio); in comparison, authentic *cis/trans*-4-cyanocyclophosphamide (**29**) gave overlapped signals ($CDCl_3$, δ 6.84). The ^1H NMR spectrum of the extracted material was almost identical with that of **29** with the exception that the extract gave a pair of equally intense doublets (δ 1.6–1.8) characteristic of diastereomeric methyl groups coupled to phosphorus.[34] All of these spectral data provided evidence for the isolation of the two diastereomers of 4-cyano-4-methylcyclophosphamide (**31**).



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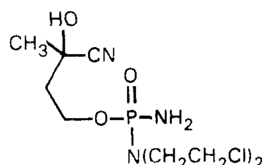

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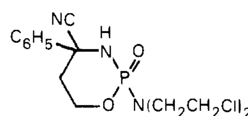
The trapping experiment was repeated with a single variation: NaCN (4 equiv) was added to the NMR sample *after* the signal arising from *cis*-4-hydroxy-4-methylcyclophosphamide (*cis*-**19**) could no longer be observed; at this point the signal for methylketophosphamide (**20**, δ 22.08) had reached maximum intensity. The sample was monitored over a 28-h period and during this time signals at δ 22.15 and 22.06 were evident, as were those given by **4a**/**18a**; however, the presence of 4-cyano-4-methylcyclophosphamide (**31**) was never detected. The signals at δ 22.15 and 22.06 were consistent with the formation of diastereomeric cyanohydrin adducts (**32**) of methylketophosphamide (**20**).

Conclusions to be drawn from these two experiments included (1) 4-cyano-4-methylcyclophosphamide (**31**) was a relatively stable trapping product formed in the presence of 4-hydroxy-4-methylcyclophosphamide (**19**), (2) methylketophosphamide (**20**) and cyanohydrin **32** were not precursors to 4-cyano-4-methylcyclophosphamide (**31**), (3) the formation of two diastereomers of **31** in nearly equal amounts from *cis*-**19** suggested that this reaction proceeded via the intermediacy of a compound that was functionally achiral at the C-4 center, e.g., 4-methyliminophosphamide (**21**, Scheme III), and (4) cyclization of ketone **20** to hydroxy compound **19** did not proceed at an appreciable rate. In light of these data, it was determined that the NaCN trapping experiment could be used to probe the formation of 4-hydroxy-4-phenylcyclophosphamide (**13a**) and 4-phenyliminophosphamide (**15a**).

NaCN (8 equiv) was added to a lutidine-Me_2SO solution of phenylketophosphamide (**14a**); new ^{31}P NMR signals attributable to 4-cyano-4-phenylcyclophosphamide (**33**) were not observed. It was unclear whether the signal that was observed at δ ~22 was due to phenylketophosphamide (**14a**) and/or cyanohydrin adduct **34**. The intensity of this signal decreased 50% in 62 min; this was coupled to the growth of signals for **4a**/**18a** (eq 1). In the absence of cyanide, the half-life of **14a** was 66 min. In a separate experiment, the addition of trapping agent *O*-methylhydroxylamine (4 equiv) to an NMR sample of **14a** also gave one downfield signal, the intensity of which decreased 50% in 59 min. On the basis of previous trapping experiments with *O*-methylhydroxylamine, it was anticipated that the formation of oxime **35** would have resulted in a stabilizing effect.[32] The apparent insensitivity of the ketone moiety in phenylketophosphamide (**14a**) toward addition reactions with cyanide or *O*-methylhydroxylamine, as well as the absence of trapping products arising from the reaction of cyanide with 4-hydroxy-4-phenylcyclophosphamide (**13a**) and/or 4-phenyliminophosphamide (**15a**) suggested that an intramolecular addition reaction of **14a** to form **13a** was unlikely.



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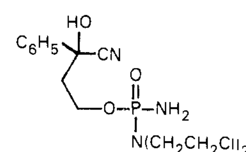

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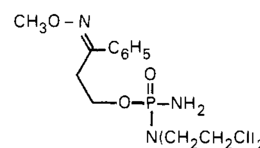

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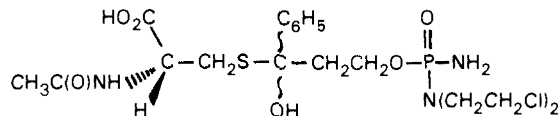
pH and Sulfhydryl Effects. A brief examination of some chemical factors that might influence the reactivities of phenyl ketones **14a–c** revealed the following. Paralleling the behavior of the cyclophosphamide metabolites under acidic conditions, an approximate 40% increase in the lifetime of phenylketophosphamide (**14a**) was observed at pH 6.3 ($\tau_{1/2}$ = ca. 92 min); phenylketoifosfamide (**14b**, $\tau_{1/2}$ = 92 min) and phenylketotrofosfamide (**14c**, $\tau_{1/2}$ = ca. 80 min) responded in similar fashion (Table II). These results were not unexpected in view of the aforementioned kinetic isotope data.

The addition of *N*-acetyl-L-cysteine (4 equiv) to an NMR sample of phenylketophosphamide (**14a**) did not result in the appearance of new resonance absorption signals; however, it was possible that signals arising from **14a** and its hemithioketal addition product **36** were isochronous.



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More telling, perhaps, was the fact that the 59-min half-life for the one observable signal at δ ~22 in the presence of *N*-acetyl-L-cysteine was essentially equal to the 66-min half-life of **14a** in the absence of the thiol. This suggested that, under the conditions of this experiment (lutidine-Me_2SO, pH 7.4, 37 °C), the formation of **36** was insignificant and/or the reverse reaction (**36** → **14a**) proceeded

(33) 4-Cyanocyclophosphamide (**29**) was a gift from Dr. Michael Colvin, The Johns Hopkins Oncology Center, Baltimore, MD. Its synthesis has been reported.[4]

(34) For 4-cyanocyclophosphamide (**29**), ^1H NMR (89.55 MHz, $CDCl_3$) chemical shifts (ppm) are 4.73–4.15 (CH_2O), 3.84–3.26 (NH, C_4-H, and 2 NCH_2CH_2Cl), and 2.46–2.05 (CH_2CH_2O). For 4-cyano-4-methylcyclophosphamide (**31**), chemical shifts (ppm) under the same conditions are 4.65–4.02 (CH_2O), 3.80–3.31 (NH and 2 NCH_2CH_2Cl), 2.32–1.92 (CH_2CH_2O), and 1.83–1.65 (2 doublets, nonequivalent CH_3).

Table III. Screening Data against L1210 Lymphoid Leukemia in Mice[a]

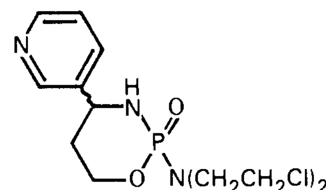
compd	dose, mg/kg	% ILS[b]	survivors on day 60[b]
14a	7.5	10.7	0/6
	15	16.3	0/6
	30	35.1	0/6
1a	100	59.6	0/6
14a	25	22.9 (23.6)	0/7 (0/7)
	50	43.9 (32.8)	0/7 (0/7)
	100	−34.0 (43.5)	0/7 (0/7)
1a	100	52.9 (66.3)	0/7 (0/7)
14a	5 × 7[c]	20.6	0/7
	10 × 7[c]	29.7	0/7
	20 × 7[c]	58.8	0/7
1a	100 × 1	66.3	0/7
14a	50[d]	8.0	0/7
	100[d]	14.0	0/7
	200[d]	−19.1	0/7
1a	100	52.9	0/7
14b	50	20.2	0/7
	100	51.7 (79.4)	0/7 (0/7)
	200	>206.9 (>234.9)	1/7 (2/7)
	400	>443.1	5/7
1b	50[e]	21.4	0/7
	100[e]	62.2	0/7
	200[e]	78.5	0/7
	100	60.3	0/7
	200	99.8	0/7
	400	>617.7	7/7
1a	200	>422.7	4/7
14c	100	29.9	0/7
	200	60.6	0/7
	400	-19.7	0/7
1a	200	>422.7	4/7

[a] Male BDF_1 mice, 6 weeks old, were implanted ip with 1×10^5 cells of L1210 lymphoid leukemia on day 0. Unless specified otherwise, each sample was given ip on day 2, and the vehicle was 10% Me_2SO. Positive controls done simultaneously are grouped with the test compound. Screening data provided by Otsuka Pharmaceutical Co. [b] Numbers in parentheses are duplicate runs. [c] Administered ip day 2 through day 8. [d] Administered po. [e] The vehicle was saline.

at a rate faster than that of the fragmentation of **14a**. In either case, it could be concluded that in vivo deactivation of **14a** by sulfhydryl compounds would be dependent on a fortuitous set of reaction conditions and/or special effects such as binding to proteins. Similar conclusions have been drawn for the deactivation of 4-hydroxycyclophosphamide (**2a**) and aldophosphamide (**3a**) by sulfhydryl compounds.[16,35]

Anticancer Screening Data. Phenyl ketones **14a–c** were tested for their in vivo anticancer activity against L1210 lymphoid leukemia in mice by using standard protocols established by Otsuka Pharmaceutical Co. (Japan) (Table III). In addition, **14a** and **14b** were evaluated against a variety of other test systems, as reported in Table IV.

When phenylketophosphamide (**14a**) was administered in multiple doses (7 × 20 mg/kg), its activity against L1210 lymphoid leukemia was comparable to that given by a single injection (100 mg/kg) of cyclophosphamide [% increased life span (ILS) = 58.8 and 66.3, respectively]. The activity of **14a** was clearly superior to that of its unactivated precursor, 4-phenylcyclophosphamide (**9**), in the same test system [% ILS = −2.9 (cis) and −11.9 (trans), 120 mg/kg].[30] The improved efficacy of **14a** relative to **9** could be a result of the ketone's circumvention of the inital oxidation step that is required for **9**. That the phenyl group introduces some steric factor in **9** that is incompatible with efficient enzymatic oxidation was suggested by previously reported screening data against L1210 lymphoid leukemia [% test/control (T/C) = 105 (cis) and 149 (trans), 250 mg/kg];[14] similar results have been found for the 4-pyridyl analogue **37** [% ILS = −0.7 (cis) and 34.3 (trans), 120 mg/kg].[30,36]



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Phenylketoifosfamide (**14b**) showed very promising activity and also produced some cures (Table IV); however, approximately twice as much compound was required to achieve the same response given by cyclophosphamide (**1a**) or ifosfamide (**1b**, Tables III and IV). Relative to all other test compounds, phenylketotrofosfamide (**14c**) was therapeutically uninteresting. The lower activity of **14c**, relative to **14a,b**, was presumably due to a combination of factors, including increased lipophilicity, shorter solution lifetime, and the difference in alkylating agent produced (i.e., **4c** rather than **4a** or **4b**).

While phenylketophosphamide (**14a**) and phenylketoifosfamide (**14b**) exhibited good activity against L1210 lymphoid leukemia, the lipophilicity of these compounds must have affected their transport properties relative to the metabolites of cyclophosphamide. It was determined spectroscopically that the partition coefficients for **14a** and **14b** were greater than 49; in comparison, the value obtained for cyclophosphamide by the same technique was 5.4 (see Experimental Section for details).[37] By virtue of their lipophilicity, these compounds may have a greater impact as anticancer agents for cancers of the central nervous system. Compounds **14a** and **14b** are currently being tested for their activity against brain cancers, and preliminary data have been reported.[38]

Conclusion. Phenyl ketones **14a–c** were synthesized in conjunction with an ongoing investigation into the effects of substituents on the dynamical solution chemistry of the metabolites of cyclophosphamide (**1a**). It was demonstrated that replacement of the aldehydic moiety in aldophosphamide (**3a**) with a phenyl ketone group in phenylketophosphamide (**14a**) resulted in a dramatic shift from cyclic to acyclic metabolites such that 4-hydroxy-4-phenylcyclophosphamide (**13a**) could not be detected either spectroscopically (^{31}P or ^{13}C NMR) or chemically (NaCN trapping experiment). The insensitivity of **14a** toward carbonyl addition reactions was further evidenced by the apparent absence of a reaction between **14a** and cyanide, *O*-methylhydroxylamine, or *N*-acetyl-L-cysteine. On the other hand, phenyl ketones **14a–c** were functionally

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(36) Ludeman, S. M.; Zon, G.; Secor, H. V., unpublished results.

(37) An unpublished partition coefficient value of 4.27 has been cited for **1a** in octanol/water: Hansch, C.; Leo, A. In "Substituent Constants for Correlation Analysis in Chemistry and Biology"; Wiley: New York, 1979; p 223.

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Table IV. Anticancer Screening Data against Various Test Systems

test system (test facility)	compd	dose, mg/kg	% T/C[a]	toxicity day survivors[a]	cures[b]
male CD_2F_1 mice, P388 lymphocytic leukemia (1 × 10^6 cells), ip injection on days 1 and 5, toxicity day = day 5, saline–Tween 80 vehicle (National Cancer Institute)	**14a**	400 × 2	*c*	5/5	0
		200 × 2	89	6/6	0
		100 × 2	221	6/6	0
		200 × 2[d]	*c*	5/6	0
		100 × 2[d]	92	6/6	0
		50 × 2[d]	223	6/6	1
		25 × 2[d]	163	5/6	0
male C57BL mice, Lewis lung carcinoma (5 × 10^5 cells), ip injection from day 2 through day 7 or on day 2 only, toxicity day = day 15, 10% Me_2SO vehicle (Otsuka Pharmaceutical)	**14a**	5 × 6	106	4/7	
		10 × 6	96	7/7	
		20 × 6	84	5/7	
	1a	20 × 6	43	7/7	
		100 × 1	2	7/7	
male BALB/c mice, Meth A sarcoma (5 × 10^5 cells), ip injection on day 2, toxicity day = day 21, 10% Me_2SO vehicle (Otsuka Pharmaceutical)	**14a**	100 × 1	63	5/7	
	1a	100 × 1	76	7/7	
female CD_2F_1 mice, L120 lymphoid leukemia (1 × 10^6 cells), ip injection on day 1, toxicity day = day 5, saline–Tween 80 vehicle (National Cancer Institute)	**14b**	500 × 1	291 (*c*)	5/6 (4/6)	3 (1)
		250 × 1	165 (229)	6/6 (6/6)	2 (2)
		125 × 1	138 (166)	6/6 (6/6)	2 (1)
		62.5 × 1	117 (142)	6/6 (6/6)	0 (0)

[a] Values in parentheses are duplicate tests. [b] Cures were determined by the National Cancer Institute Test Facility but not by Otsuka Pharmaceutical. Values in parentheses are duplicate tests. [c] Toxic dose. [d] Female mice.

analogous to aldophosphamide in one significant respect: each produced a cytotoxic alkylating agent (**4a–c**) through an elimination reaction. Control studies that compared the relative reactivities of phenylketophosphamide (**14a**) and methylketophosphamide (**20**) revealed that the factors that modulate the ring closure/opening reactions were not peculiar to the phenyl group; however, differences (in inductive effects?) between phenyl and methyl profoundly influenced the rates of fragmentation of **14a** and **20**.

In light of the anticancer activity exhibited by phenylketophosphamide (**14a**) and phenylketoifosfamide (**14b**), it can be inferred that the 4-hydroxycyclophosphamide/aldophosphamide (**2a**/**3a**) equilibrium can be markedly perturbed in the direction of the acyclic metabolite without a concomitant loss of activity. From the screening data reported herein, it is more difficult to ascertain the consequence of **14a** and **14b** being unable to detoxify to metabolites akin to 4-ketocyclophosphamide (**7a**) and carboxyphosphamide (**8a**). If anticancer activity, increased host survival times, and the occurrence of cures are associated with the oncostatic selectivity of a drug, then the screening data do indicate that oxidative detoxification may not be a necessary criterion for the therapeutic efficacy of cyclophosphamide analogues. It is possible that alternative detoxification routes, such as the reduction of the ketone moiety to an alcohol, may intervene in the metabolism of **14a** and **14b**.[39] The toxicity of phenyl vinyl ketone, a product generated by the fragmentation of **14a** and **14b**, is not yet known but is under investigation. Despite these uncertainties, the studies do reveal that the inactivity of 4-methylcyclophosphamide (**10**) as an anticancer drug need not be solely ascribed to the inability of its metabolites to undergo oxidative detoxification; instead, the inactivity could result from the slow rate of phosphoramide mustard (**4a**) production relative to that of cyclophosphamide or phenylketophosphamide (**14a**). That an empirical correlation may exist between therapeutic efficacy and the rate of fragmentation has already been suggested through studies of 5,5-dideuteriocyclophosphamide (**1a**-*5,5-d*$_2$). Relative to **1a**, **1a**-*5,5-d*$_2$ exhibits a 7- to 13-fold decrease in tumor toxicity.[40] It has been demonstrated that deuterium substitution has no therapeutic consequence on the oxidative activation and detoxification reactions in the metabolism of **1a**.[40] Furthermore, we have shown that the distribution of hydroxy and aldehydic metabolites of **1a**-*5,5-d*$_2$ is nearly identical with that of the metabolites of unlabeled **1a**; only the rate of production of **4a** is significantly influenced by the incorporation of deuterium at the C-5 position (k_H/k_D = 5.6 for fragmentation).[16] Investigations of the relationship between anticancer activity and the rate of fragmentation are being pursued through the study of other metabolite–analogues.[41]

Compounds **14a–c** represent additional prototypes of nonaldehydic, preactivated, analogues of aldophosphamide that need only undergo an elimination reaction to produce a cytotoxic alkylating agent. Lipophilic **14a** and **14b**, which exhibited good activity against L1210 lymphoid leukemia when administered systemically, may have an even greater impact delivered locally against cancers of the central nervous system or other tumors resistant to treatment with **1a**. Derivatives of **14a–c** may have future applications in the field of drug targeting, since appropriate modifications to the aromatic ring would allow for coupling reactions between these compounds and synthetic polymers or monoclonal antibodies.[42,43] In any case, the success of **14a** and **14b** in anticancer screening studies suggests that there is more flexibility in designing analogues of aldophosph-

(39) For a review article on ketone reductases in mammalian cells, see: Felsted, R. L.; Bachur, N. R. *Drug Metab. Rev.* **1980**, *11*, 1.

(40) Cox, P. J.; Farmer, P. B.; Foster, A. B.; Gilby, E. D.; Jarman, M. *Cancer Treat. Rep.* **1976**, *60*, 483.

(41) For a preliminary communication, see: Ludeman, S. M.; Boyd, V. L.; Regan, J. B.; Gallo, K. A.; Zon, G.; Ishii, K. *Drugs Exp. Clin. Res.* in press.

(42) Gros, L.; Ringsdorf, H.; Schup, H. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 305.

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amide than previous data might have indicated.[13,44]

Experimental Section

Tetrahydrofuran (THF) and Et_2O refer to anhydrous solvents. Reaction mixtures that do not include water were carried out under nitrogen. Ozone (~5–10 g/h) was produced by either a Model 03V5-0 or 03V10-0 ozone generator (Ozone Research and Equipment Corp.). Elemental analyses were performed by Galbraith Laboratories, Inc. 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 (Analtech); a 250-nm UV lamp and I_2 were used for component visualization. Column chromatography employed silica gel from either J. T. Baker Chemicals (60–200 mesh) or EM reagents (<250 mesh for columns run at 5 °C, and 230–400 mesh for "flash" chromatography). 60-MHz ^1H NMR spectra were recorded on a Varian EM360-A spectrometer. 89.55-MHz ^1H, 36.23-MHz ^{31}P, and 22.49-MHz ^{13}C NMR spectra were obtained with a JEOL FX-90Q broad-banded spectrometer. 75.47-MHz ^{13}C spectra were recorded on a Bruker WM300 spectrometer. Unless specified otherwise, ^1H NMR chemical shifts (δ) refer to Me_4Si as an internal reference. ^{31}P δ values refer to external 25% H_3PO_4 in D_2O; the phosphorus shifts in aqueous media are pH dependent. ^{13}C NMR δ 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 read-out meter. Values of solution pH were measured with a precalibrated standard glass microprobe; the pH values for D_2O or Me_2SO-d_6 solutions correspond to the observed readings and were not corrected. IR spectra were recorded with a Perkin-Elmer Model 337 grating spectrophotometer.

3-Phenyl-3-buten-1-ol. 3-Phenyl-3-butenyl acetate was prepared (50%) from the reaction of paraformaldehyde and α-methylstyrene in acetic acid/acetic anhydride according to the procedure of Hawkins and Thompson:[19] ^1H NMR (60 MHz, $CDCl_3$) δ 7.48–7.08 (m, 5 H, aromatic), 5.31 (d, $^2J_{HH}$ = ~1 Hz, 1 H, vinylic), 5.07 (d, $^2J_{HH}$ = ~1 Hz, 1 H, vinylic), 4.14 (t, J = 7 Hz, 2 H, CH_2O), 2.79 (apparent t, J = 7 Hz, 2 H, CH_2CH_2O), and 1.91 (s, 3 H, CH_3).

A solution of 3-phenyl-3-butenyl acetate (20.4 g, 107 mmol) and KOH (12.0 g, 214 mmol) in aqueous ethanol (50%, 214 mL) was refluxed (45 min). Upon cooling, the reaction mixture was neutralized with HCl (1 M, 107 mL). Ethanol was removed on a rotary evaporator and the residual water layer was extracted with Et_2O (3 × 100 mL). The combined Et_2O extracts were washed with 5% $NaHCO_3$ (1 × 150 mL), dried ($MgSO_4$), and concentrated at reduced pressure. Vacuum distillation of the crude material afforded 3-phenyl-3-buten-1-ol as a colorless liquid (bp 80 °C (0.025 mm) (lit.[19] bp 130–131 °C (13 mm)) in 69% yield (10.9 g, 74 mmol): ^1H NMR (60 MHz, $CDCl_3$) δ 7.53–7.10 (m, 5 H, aromatic), 5.34 (d, $^2J_{HH}$ = ~1 Hz, 1 H, vinylic), 5.09 (d, $^2J_{HH}$ = ~1 Hz, 1 H, vinylic), 3.66 (t, J = 7 Hz, 2 H, CH_2O), 2.73 (apparent t, J = 7 Hz, 2 H, CH_2CH_2O), and 2.32 (s, 1 H, OH); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 140.5, 128.3, 127.5, and 126.0 (aromatic), 144.8 and 114.2 (vinylic), 60.94 (CH_2O), and 38.47 (CH_2CH_2O).

3-Phenyl-3-butenyl *N,N*-Bis(2-chloroethyl)phosphorodiamidate (16a). A hexane solution of *n*-BuLi (8.8 mL of 1.53 M, 13.5 mmol) was added dropwise to a stirring solution of 3-phenyl-3-buten-1-ol (2.0 g, 13.5 mmol) in THF (15 mL) at −23 °C (CCl_4/CO_2 bath). Stirring at −23 °C was continued for 2 h, and then the resultant dark red mixture was removed with a syringe and added to a stirring solution of *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (3.5 g, 13.5 mmol) in THF (15 mL) at −23 °C. After the solution was stirred for 3 h at −23 °C, NH_3 was bubbled through the pale yellow reaction mixture for 15 min at 5 °C. The stoppered reaction flask stood overnight at room temperature prior to suction filtration and concentration of the filtrate on a rotary evaporator. The residual material was chromatographed on silica gel (4 × 33 cm column) with Et_2O eluent (~1000 mL) to remove fast-eluting impurities. Subsequent elution with $CHCl_3$–CH_3OH (9:1) gave **16a** as a pale yellow oil (2.5 g, 7.1 mmol, 53% yield): R_f 0.69 ($CHCl_3$–CH_3OH, 9:1); R_f 0.11 (Et_2O); ^1H NMR (89.55 MHz, $CDCl_3$) δ 7.49–7.18 (m, 5 H, aromatic), 5.37 (d, $^2J_{HH}$ = ~1 Hz, 1 H, vinylic), 5.16 (d, $^2J_{HH}$ = ~1 H, 1 H, vinylic), 4.04 (apparent q, J = 7 Hz, 2 H, CH_2O), 3.74–3.12 (m, 8 H, 2 NCH_2CH_2Cl), 3.02 (br d, $^2J_{HP}$ = 4 Hz, 2 H, NH_2), and 2.86 (t, J = 7 Hz, 2 H, CH_2CH_2O); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 140.1, 128.4, 127.6, and 125.9 (aromatic), 144.1 and 114.7 (vinylic), 63.86 (d, J_{CP} = 5.9 Hz, CH_2O), 49.20 (d, J_{CP} = 4.40 Hz, 2 NCH_2), 42.34 (2 CH_2Cl), and 36.10 (d, J_{CP} = ~8 Hz, CH_2CH_2O); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 14.11.

3-Phenyl-3-butenyl *N,N'*-Bis(2-chloroethyl)phosphorodiamidate (16b). A solution of 3-phenyl-3-buten-1-ol (2.0 g, 13.5 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a stirring solution of $POCl_3$ (1.26 mL, 13.5 mmol) in CH_2Cl_2 (15 mL) at −10 °C (ice/salt bath). After 3 h at −10 °C, more solvent (20 mL) was added, followed by the sequential addition of 2-chloroethylamine hydrochloride (3.16 g, 27 mmol) and Et_3N (7.51 mL, 67.5 mmol). The reaction stirred at room temperature overnight and was then filtered and concentrated on a rotary evaporator. The residue was chromatographed on silica gel (2.9 × 25 cm column) with $CHCl_3$–CH_3OH (9:1) as the eluent. The collected material (R_f 0.67) was further purified by flash chromatography (2.9 × 15 cm column) using ethyl acetate as the eluent. Product **16b** (R_f 0.22, ethyl acetate) was isolated in 24% yield as an oil (1.12 g, 3.2 mmol): ^1H NMR (89.55 MHz, $CDCl_3$) δ 7.54–7.18 (m, 5 H, aromatic), 5.39 (d, $^2J_{HH}$ = ~1 Hz, 1 H, vinylic), 5.16 (d, $^2J_{HH}$ = ~1 Hz, 1 H, vinylic), 4.07 (apparent q, J = 7 Hz, 2 H, CH_2O), 3.69–2.98 (m, 10 H, 2 $HNCH_2CH_2Cl$), and 2.87 (t, J = 7 Hz, 2 H, CH_2CH_2O); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 140.2, 128.4, 127.6, and 125.9 (aromatic), 144.1 and 114.7 (vinylic), 63.93 (d, J_{CP} = 5.9 Hz, CH_2O), 45.59 (d, J_{CP} = 5.9 Hz, 2 NCH_2), 42.79 (2 CH_2Cl), and 36.13 (d, J_{CP} = 7.3 Hz, CH_2CH_2O); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 12.79.

3-Phenyl-3-butenyl *N,N,N'*-Tris(2-chloroethyl)phosphorodiamidate (16c). A hexane solution of *n*-BuLi (8.8 mL of 1.53 M, 13.5 mmol) was added dropwise to a stirring solution of 3-phenyl-3-buten-1-ol (2.0 g, 13.5 mmol) in THF (10 mL) at −23 °C (CCl_4/CO_2 bath). Stirring at −23 °C was continued for 2 h, and then the resultant mixture was removed by syringe and added to a solution of *N,N*-bis(2-chloroethyl)phosphoramidic dichloride (3.5 g, 13.5 mmol) in THF (10 mL) at −23 °C. After the solution was stirred for 3 h at −23 °C, the mixture was added, through an addition funnel, to a suspension of 2-chloroethylamine hydrochloride (recrystallized from acetone, 1.56 g, 13.5 mmol) and Et_3N (3.75 mL, 27 mmol) in THF (15 mL) at 5 °C. After stirring overnight at room temperature, the reaction mixture was filtered, concentrated at reduced pressure, and chromatographed on silica gel (2.9 × 25 cm column) with $CHCl_3$–CH_3OH (9:1) as the eluent. The collected material (R_f 0.83) was further purified by flash chromatography using ethyl acetate as eluent. Product **16c** (R_f 0.57, ethyl acetate) was isolated in 28% yield as an oil (1.54 g, 3.7 mmol): ^1H NMR (89.55 MHz, $CDCl_3$) δ 7.53–7.24 (m, 5 H, aromatic), 5.42 (d, $^2J_{HH}$ = ~1 Hz, 1 H, vinylic), 5.18 (d, $^2J_{HH}$ = ~1 Hz, 1 H, vinylic), 4.10 (apparent q, J = 7 Hz, 2 H, CH_2O), 3.75–2.99 (m, 13 H, all NH and NCH_2CH_2Cl), and 2.89 (t, J = 7 Hz, 2 H, CH_2CH_2O); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 140.0, 128.4, 127.7, and 125.9 (aromatic), 144.1 and 114.9 (vinylic), 64.06 (d, J_{CP} = 5.9 Hz, CH_2O), 49.14 [d, J_{CP} = 4.4 Hz, $N(CH_2CH_2Cl)_2$], 45.53 (d, J_{CP} = 5.9 Hz, $HNCH_2$), 42.66 ($HNCH_2CH_2Cl$), 42.34 [$N(CH_2CH_2Cl)_2$], and 36.07 (d, J_{CP} = 7.3 Hz, CH_2CH_2O); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 13.40.

3-Phenyl-3-oxopropyl *N,N*-Bis(2-chloroethyl)phosphorodiamidate ("Phenylketophosphamide", 14a). Ozone was bubbled through a solution of **16a** (1.0 g, 2.8 mmol in acetone–water (2:1, 21 mL) at 5 °C for 30 min. The volume of the solution was adjusted to 21 mL with more acetone, aqueous H_2O_2 (0.87 mL of a 30% solution) was added, and the stoppered reaction flask stood overnight at room temperature. Acetone was removed at ambient temperature on a rotary evaporator, and the residual aqueous solution was extracted with CH_2Cl_2 (6 × 40 mL). The combined extracts were dried ($MgSO_4$) and concentrated at room temperature, and the residual material was dissolved in minimal Et_2O and stored at −20 °C. After several days, **14a** was obtained as a white microcrystalline solid in 30% yield (0.3 g, 0.85 mmol): mp 82–84 °C; ^1H NMR (89.55 MHz, $CDCl_3$) δ 8.08–7.93 (m, 2 H, aromatic), 7.71–7.39 (m, 3 H, aromatic), 4.62–4.27 (m, 2 H, CH_2O), 3.77–3.24 (m, 10 H, $CH_2C{=}O$ and 2 NCH_2CH_2Cl), and 3.10–2.88

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(br m, 2 H, NH_2); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 197.4 (C=O), 136.5, 133.5, 128.7, and 128.1 (aromatic), 60.58 (d, J_{CP} = 4.4 Hz, CH_2O), 49.33 (d, J_{CP} = 4.4 Hz, 2 NCH_2), 42.44 (2 CH_2Cl), and 38.56 (d, J_{CP} = ~8 Hz, CH_2C=O); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 14.38; IR (Nujol) ~3390, 3300, 1670, 1210, 1085, 1030, 970, 745, and 690 cm^{-1}. Anal. ($C_{13}H_{19}Cl_2N_2O_3P$) C, H, N.

3-Phenyl-3-oxopropyl *N*,*N*'-Bis(2-chloroethyl)phosphorodiamidate ("Phenylketoifosfamide", 14b). Ozone was bubbled through a solution of **16b** (450 mg, 1.3 mmol) in acetone–water (2:1, 21 mL) at 5 °C for 30 min. Workup as described above for **14a** (using 0.6 mL of 30% H_2O_2) afforded **14b** as an oil. Dissolution of **14b** in minimal Et_2O and the addition of a tiny seed crystal of **14a** induced crystallization. The product was then obtained as a white, microcrystalline solid in 33% yield (150 mg, 0.4 mmol): mp 78–80 °C; ^1H NMR (89.55 MHz, $CDCl_3$) δ 8.13–7.91 (m, 2 H, aromatic), 7.77–7.35 (m, 3 H, aromatic), 4.46 (apparent q, J = 7 Hz, 2 H, CH_2O), 3.77–3.51 (m, 4 H, 2 CH_2Cl), and 3.51–3.04 (m, 8 H, 2 $HNCH_2$ and CH_2C=O); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 197.4 (C=O), 136.4, 133.5, 128.7, and 128.1 (aromatic), 60.71 (d, J_{CP} = 4.4 Hz, CH_2O), 45.82 (d, J_{CP} = 4.4 Hz, 2 NCH_2), 42.92 (two CH_2Cl), and 38.80 (d, J_{CP} = 7.3 Hz, CH_2C=O); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 13.33. Anal. ($C_{13}H_{19}Cl_2N_2O_3P$), C, H, N.

3-Phenyl-3-oxopropyl *N*,*N*,*N*'-Tris(2-chloroethyl)phosphorodiamidate ("Phenylketotrofosfamide", 14c). Ozone was bubbled through a solution of **16c** (500 mg, 1.2 mmol) in acetone–water (2:1, 21 mL) for 90 min at 5 °C. The volume of the solution was adjusted to 21 mL with more acetone after 30, 60, and 90 min of reaction time. Aqueous H_2O_2 (0.6 mL of a 30% solution) was then added, and the stoppered reaction flask sat overnight at room temperature. Acetone was removed at ambient temperature on a rotary evaporator, 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 (room temperature), and the product was flash chromatographed on silica gel (2.9 × 15 cm column) with ethyl acetate as the eluent. Product **14c** (R_f 0.44) crystallized from minimal Et_2O at −20 °C in 11% yield (130 mg, 0.3 mmol): mp 48–50 °C; ^1H NMR (89.55 MHz, $CDCl_3$) δ 8.11–7.90 (m, 2 H, aromatic), 7.76–7.37 (m, 3 H, aromatic), 4.72–4.17 (m, 2 H, CH_2O), and 3.79–3.03 (m, 15 H); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 197.2 (C=O), 136.4, 133.6, 128.7, and 128.1 (aromatic), 60.78 (d, J_{CP} = 4.4 Hz, CH_2O), 49.27 [d, J_{CP} = 4.4 Hz, $N(CH_2CH_2Cl)_2$], 45.82 (d, J_{CP} = 4.4 Hz, $HNCH_2$), 42.66 ($HNCH_2CH_2Cl$), 42.53 [$N(CH_2CH_2Cl)_2$], and 38.80 (d, J_{CP} = 7.3 Hz, CH_2C=O); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 13.84. Anal. ($C_{15}H_{22}Cl_3N_2O_3P$) C, H, N.

***cis*-4-Hydroperoxy-4-methylcyclophosphamide [(2*R*,4*R*/2*S*,4*S*)-2-[Bis(2-chloroethyl)amino]-4-hydroperoxy-4-methyltetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide, *cis*-24] and Peroxy Dimer *cis*-26.** Incorporation of 3-methyl-3-buten-1-ol (Aldrich Chemical Co.) into the synthesis described above for **16a** gave 3-methyl-3-butenyl *N*,*N*-bis(2-chloroethyl)phosphorodiamidate (**23**) in 61% yield: R_f 0.3 (Et_2O); R_f 0.61 ($CHCl_3$–CH_3OH, 9:1); ^1H NMR (60 MHz, $CDCl_3$) δ 4.99–4.69 (m, 2 H, vinylic), 4.13 (apparent q, J = 7 Hz, 2 H, CH_2O), 3.89–3.16 (m, 8 H, 2 NCH_2CH_2Cl), 3.16–2.76 (br m, 2 H, NH_2), 2.41 (t, J = 7 Hz, 2 H, CH_2CH_2O), and 1.79 (s, 3 H, CH_3); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 141.1 (C=CH_2), 112.2 (C=CH_2), 63.15 (d, J_{CP} = 5.9 Hz, CH_2O), 49.14 (d, J_{CP} = 4.4 Hz, 2 NCH_2), 42.21 (2 CH_2Cl), 38.15 (d, J_{CP} = 7.3 Hz, CH_2CH_2O), and 22.05 (CH_3); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 14.68.

Ozone was bubbled through an acetone–water (2:1, 21 mL) solution of **23** (1.0 g, 3.5 mmol) at 5 °C for 30 min. The reaction mixture was transferred to a round-bottom flask with use of acetone (14 mL), H_2O_2 (1.08 mL of a 30% solution) was added, and the stoppered flask stood overnight at room temperature. Acetone was removed on a rotary evaporator at room temperature, and the residual water solution was extracted with CH_2Cl_2 (6 × 40 mL). The organic layers were combined, dried ($MgSO_4$), and concentrated at ambient temperature on a rotary evaporator. The residual oil was chromatographed at 5 °C on a column (2.5 × 25 cm) of silica gel (<250 mesh) with $CHCl_3$–acetone (1:1) as the eluent. The flow rate was ~3 mL/h, and fractions were collected in ~1.5-mL volumes.

Fractions 24–30 (component R_f 0.76) were combined and concentrated at reduced pressure (ambient temperature) to give an oil (150 mg) which was assigned structure *cis*-**26**: ^1H NMR (89.55 MHz, $CDCl_3$) δ 4.94–3.86 (m, CH_2O), 3.77–3.24 (m, NH and 2 NCH_2CH_2Cl), 2.12–1.90 (m, CH_2CH_2O), and 1.55 (d, J_{HP} = 4 Hz, CH_3); ^{13}C NMR (25 MHz, $CDCl_3$) δ 91.73 (d, J_{CP} = 3.7 Hz, C_4), 63.13 (d, J_{CP} = 6.1 Hz, C_6), 49.29 (d, J_{CP} = 3.7 Hz, 2 NCH_2), 41.98 (2 CH_2Cl), and 35.67 (d, J_{CP} = 4.3 Hz) and 26.68 (d, J_{CP} = 11 Hz), C_5 and CH_3; ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 10.67.

Fractions 42–46 (component R_f 0.54) were combined and concentrated on a rotary evaporator at ambient temperature to give a white solid (48 mg) which was assigned structure *cis*-**24**: mp 71–77 °C; ^1H NMR (89.55 MHz, $CDCl_3$) δ 4.90–3.74 (m, CH_2O and OOH), 3.74–3.16 (m, NH and 2 NCH_2CH_2Cl), 2.09–1.86 (m, CH_2CH_2O), and 1.54 (d, J_{HP} = 4 Hz, CH_3); ^{13}C NMR (25 MHz, $CDCl_3$) δ 91.15 (d, J_{CP} = 3.7 Hz, C_4), 63.76 (d, J_{CP} = 6.1 Hz, C_6), 48.63 (d, J_{CP} = 4.3 Hz, 2 NCH_2), 42.10 (2 CH_2Cl), and 33.77 (d, J_{CP} = 3.7 Hz) and 26.43 (d, J_{CP} = 10 Hz), C_5 and CH_3; ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 8.86.

Synthesis of Methylketophosphamide *O*-Methyloxime (27). A solution of *O*-methylhydroxylamine hydrochloride (33 mg, 0.4 mmol) in water (1 mL) was adjusted to pH 7.4 with 1 M NaOH and was then added to a solution of hydroperoxide *cis*-**24** (31 mg, 0.1 mmol) in 0.05 M lutidine (1 mL, pH 7.4). After being stirred for 2 days at room temperature, the reaction mixture was saturated with NaCl and extracted with CH_2Cl_2 (4 × 10 mL). The organic layers were combined, dried ($MgSO_4$), and concentrated at reduced pressure. The residual oil was subjected to TLC (two 10 × 20 cm plates, 250-μm silica gel) with acetone–$CHCl_3$ (1:2) as the eluent. The product (R_f 0.44) was desorbed with CH_2Cl_2–CH_3OH (1:1) and was recovered in 55% yield (17 mg) as a 71:29 mixture of *E*/*Z* isomers of **27**: ^1H NMR (60 MHz, $CDCl_3$) δ 4.38–3.92 (m, 2 H, CH_2O), 3.81 (apparent, s, 3 H, OCH_3), 3.75–3.15 (m, 8 H, 2 NCH_2CH_2Cl), 3.08–2.75 (m, 2 H, NH_2), 2.68 and 2.53 (2 t, J = ~7 Hz, 2 H total in 29:71 ratio, respectively, nonequivalent CH_2CH_2O), and 1.93 and 1.85 (2 s, 3 H total in 29:71 ratio, respectively, nonequivalent N=CCH_3); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 14.1 (major) and 14.0 (minor).

Isophosphoramide Mustard [*N*,*N*'-Bis(2-chloroethyl)phosphorodiamidic Acid, 4b]. Phenyl *N*,*N*'-bis(2-chloroethyl)phosphorodiamidate [$PhOP(O)(NHCH_2CH_2Cl)_2$] was prepared (28%) according to the procedure of Struck et al.[45] and was purified on silica gel [$CHCl_3$–CH_3OH (9:1); R_f 0.63]: ^1H NMR (89.55 MHz, $CDCl_3$) δ 7.49–7.05 (m, 5 H, aromatic), and 3.74–3.07 (m, 10 H); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 129.71, 124.70, 120.25 (d, J_{CP} = 4.27 Hz, COP), 45.68 (d, J_{CP} = 5.49 Hz, NCH_2), and 43.06 (CH_2Cl); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 8.96.

Phenyl *N*,*N*'-bis(2-chloroethyl)phosphorodiamidate was hydrogenated according to the method of Struck et al.,[45] and **4b** was obtained as a white, crystalline solid in 5% yield: ^{31}P NMR (36.23 MHz, Me_2SO-d_6) δ 15.15 (verified with authentic material[46]).

Trophosphoramide Mustard–Cyclohexylamine [Cyclohexylammonium *N*,*N*,*N*'-Tris(2-chloroethyl)phosphorodiamidate, 4c·CHA]. A solution of phenol (1.88 g, 20 mmol) in benzene (5 mL) was added dropwise to an ice-cooled suspension of NaH (0.53 g, 22 mmol) in benzene (14 mL). After being stirred overnight at room temperature, the reaction mixture was transferred by pipet to a solution of bis(2-chloroethyl)phosphoramidic dichloride (5.18 g, 20 mmol) in benzene (20 mL) at 5 °C. The reaction was allowed to stir overnight at ambient temperature prior to filtration under N_2. Concentration of the filtrate at reduced pressure gave an oil [$PhOP(O)(Cl)N(CH_2CH_2Cl)_2$] which was 85% pure by ^{31}P NMR (δ 9.09, $CDCl_3$). This oil was dissolved in benzene (20 mL) and was added dropwise to a stirring suspension of 2-chloroethylamine hydrochloride (2.32 g, 20 mmol, recrystallized from acetone) and Et_3N (2.78 mL, 40 mmol) in benzene (15 mL) at 5 °C. After being stirred overnight at ambient temperature, the mixture was filtered and the filtrate was concentrated at reduced pressure. Phenyl *N*,*N*,*N*'-tris(2-chloroethyl)phosphorodiamidate [$PhOP(O)(NHCH_2CH_2Cl)N(CH_2CH_2Cl)_2$] was crystallized from ether in 36% yield (2.59 g, 7 mmol): mp 59–62 °C; ^1H NMR (89.55 MHz, $CDCl_3$) δ 7.26–7.04

(45) Struck, R. F.; Dykes, D. J.; Corbett, T. H.; Suling, W. J.; Trader, M. W. *Br. J. Cancer.* **1983**, *47*, 15.

(46) Authentic isophosphoramide mustard (**4b**) was a gift from Dr. W. J. Stec, The Polish Academy of Sciences, Lodz, Boczna, Poland.

(m, 5 H, aromatic), and 3.84–3.06 (m, 13 H); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 9.83.

A suspension of phenyl *N,N,N'*-tris(2-chloroethyl)phosphorodiamidate (0.5 g, 1.4 mmol) and PtO_2 (50 mg) in absolute ethanol (5 mL) was hydrogenated at 50 psi for 2 h with use of a Paar medium-pressure shaker–hydrogenator. The reaction mixture was then diluted with ethanol (100 mL) and was stirred 10 min prior to filtration. Cyclohexylamine (0.64 mL, 5.6 mmol) was added and the solution was stirred 10 min and was then concentrated at reduced pressure. The residue was taken up in minimal $CHCl_3$ and Et_2O was added to cloudiness. Storage of this mixture at –20 °C gave **4c**·CHA as a white solid (140 mg, 0.37 mmol, 26% yield): mp 152–162 °C; ^1H NMR (89.55 MHz, $CDCl_3$) δ 5.65 (br s), 3.72–2.62 (m), and 2.18–0.71 (m); ^{13}C NMR (22.49 MHz, $CDCl_3$) δ 50.5 [d, J_{CP} = 4.4 Hz, $N(CH_2CH_2Cl)_2$], 46.08 (d, J_{CP} = 7.3 Hz, $HNCH_2$), 43.71 (CH_2Cl), 43.12 [$N(CH_2CH_2Cl)_2$], and 50.21, 31.93, 24.91, and 24.46 (cyclohexylamine); ^{31}P NMR (36.23 MHz, $CDCl_3$) δ 8.86 [impurity (14%) at δ 4.45].

^{31}P NMR Kinetic Studies. General Procedure. NMR sample solutions were prepared immediately prior to use by first dissolving the compound (0.012–0.034 mmol) in Me_2SO-d_6 (0.34 mL) and then adding 1 M lutidine buffer (1.35 mL). The solution was transferred to a vial containing $Na_2S_2O_3·5H_2O$ (3–5 molar equiv) and use of a high-speed vortex mixer effected dissolution in 1–2 min. The solution pH was checked and adjusted, if necessary, with 2–4 M HCl. The sample was then placed in a 10-mm NMR tube and a vortex plug was inserted. The sample was allowed to thermally equilibrate for 2–3 min in the spectrometer probe (37 ± 2 °C) prior to optimization of the magnetic field homogeneity. At time "zero", which was 10–15 min after dissolution of compound, 36.23-MHz ^{31}P NMR data accumulation was initiated, using a 5-kHz spectral window, 8192 data points, a $\pi/2$ 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. Signal intensities (peak heights) were used to measure relative concentrations of components as a function of time. Select kinetic runs used samples with added methylphosphonate as a standard. Normalizing the peak heights of the reaction components relative to that of methylphosphonate gave half-lives which were within ±5% of those calculated without normalizing.

Partition Coefficients. A 14 mM solution of cyclophosphamide (**1a**) monohydrate in water (8 mg/2 mL) was vortexed (5 min) with an equal volume (2 mL) of octanol. After the solution was allowed to stand (5–10 min), the layers were separated and each was analyzed by ^{31}P NMR spectroscopy, using an identical set of acquisition and display parameters (0.15 mL of D_2O or Me_2SO-d_6 was added to each layer as an NMR lock signal). The signal intensities [(peak height) × (width at half-height)] given after 1000 pulses were used to determine the relative concentration of **1a** in the octanol and water layers. The partition coefficient ($P = [\mathbf{1a}]_{octanol}/[\mathbf{1a}]_{water}$) was 5.42.[37]

Phenylketophosphamide (**14a**) and phenylketoifosfamide (**14b**) were treated as above, except that each was first dissolved in octanol (10 mg/2 mL; 14 mM solutions) and then extracted with water. Under the conditions of the NMR experiment, **14a** or **14b** could not be detected in the water layers. Control experiments with **14a** indicated that if 2 mg of **14a** had been extracted into water, its signal would have been observed under the NMR conditions used, and the partition coefficient would have been 49. Therefore, the partition coefficients for **14a**, and by analogy **14b**, must be greater than 49.

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Registry No. *cis*-**2a**, 100993-78-0; *trans*-**2a**, 100993-79-1; **3a**, 35144-64-0; **4a**, 10159-53-2; **4b**, 31645-39-3; **4c**·CHA, 100993-71-3; **14a**, 100993-68-8; **14b**, 100993-69-9; **14c**, 100993-70-2; **16a**, 100993-65-5; **16b**, 100993-66-6; **16c**, 100993-67-7; **18a**, 88802-97-5; **18b**, 100993-80-4; **18c**, 100993-81-5; *cis*-**19**, 100993-82-6; **20**, 100993-83-7; **23**, 100993-75-7; *cis*-**24**, 100993-73-5; *cis*-**26**, 100993-74-6; (*E*)-**27**, 100993-76-8; (*Z*)-**27**, 100993-77-9; $P(O)(Cl)_2N(CH_2CH_2Cl)_2$, 127-88-8; $PhOP(O)(NHCH_2CH_2Cl)_2$, 70772-68-8; $PhOP(O)(Cl)N(CH_2CH_2Cl)_2$, 4798-75-8; $PhOP(O)(NHCH_2CH_2Cl)N(CH_2CH_2Cl)_2$, 100993-72-4; 3-phenyl-3-butenyl acetate, 7306-12-9; paraformaldehyde, 30525-89-4; α-methylstyrene, 98-83-9; 3-phenyl-3-buten-1-ol, 3174-83-2; 2-chloroethylamine hydrochloride, 870-24-6; phenol, 108-95-2; 3-methyl-3-buten-1-ol, 763-32-6; *O*-methylhydroxylamine hydrochloride, 593-56-6.

Isohelical Analysis of DNA Groove-Binding Drugs

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Many antitumor drugs, and many carcinogens, act by binding within the minor groove of double-helical DNA, interfering with both replication and transcription. Several of these, including netropsin and distamycin, are quite base specific, recognizing and binding only to certain base sequences. The repeating pyrrole–amide unit of netropsin, and the repeated benzimidazole unit of the DNA stain and carcinogen Hoechst 33258, both are approximately 20% too long for synchronous meshing with base pairs along the floor of the minor groove in B DNA. We have carried out a systematic computer search for possible repeating drug backbones that are isohelical with DNA and that also provide chemical groups capable of reading and differentiating between A·T and G·C base pairs. These isohelical sequence-reading drug polymers or "isolexins" should offer the possibility of targeting synthetic drug analogues specifically against one region of a genome rather than another, or against neoplastic cells in preference to normal cells.

Some DNA-binding antitumor antibiotics such as actinomycin D or daunomycin bind by intercalation; that is, the DNA helix is expanded so that a flat polycyclic group can be inserted between two adjacent base pairs as though it was itself another base pair.[1,2] Other drugs such as netropsin and distamycin bind within the minor groove of the double helix without gross helical distortion.[3-6]

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