^{31}P NMR and Chloride Ion Kinetics of Alkylating Monoester Phosphoramidates

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^{31}P NMR spectroscopy was used to study the solvolysis kinetics of a novel series of alkylating monoester phosphoramidates (**4a–d**) under model physiologic conditions. Halide ion kinetics were used to determine the rate of aziridinium ion formation. The solvolysis rates showed the expected dependence upon substitution at the reactive nitrogen; comparison of **4a** with phosphoramide mustard (**1a**) indicated that replacement of the amido group by alkoxy decreased the solvolysis rate by approximately 10-fold. The rate of conversion of starting compound (**4a–d**) to solvolysis product was essentially equal to the rate of halide ion release, suggesting that the aziridinium ion is a short-lived intermediate. ^{1}H NMR and ^{31}P NMR kinetics experiments performed in the absence and presence of trapping agent (dimethyldithiocarbamate) confirmed that the aziridinium ion was too short-lived to be observed via NMR. These compounds were also tested for cytotoxicity against L1210 leukemia and B16 melanoma cells in vitro; the monoalkylators **4c** and **4d** showed no activity, **4a** was weakly cytotoxic, and **4b** was comparable in activity to phosphoramide mustard.

Introduction

Cyclophosphamide is a widely used and highly effective antitumor agent.[1] Phosphoramide mustard **1a**, produced metabolically from cyclophosphamide, is the bisalkylating agent responsible for alkylating the N-7 position of guanine and cross-linking DNA.[2-7] It has been proposed that this alkylation proceeds via an aziridinium ion (**1b**),[4,8,9] which undergoes nucleophilic attack to form the monosubstituted product **1c** as shown in Scheme I. The disubstituted product is then formed by nucleophilic attack of a second aziridinium ion (**1d**). Upon the basis of our interest in the analogous phosphoramidate esters (e.g., **4**) as potential antitumor agents, we have investigated the effects of oxygen substitution on the rates of phosphoramidate solvolysis and cytotoxic activity.

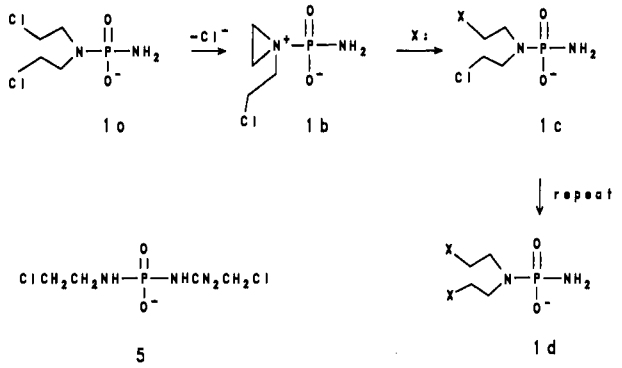
Results and Discussion

The series of monoester phosphoramidates **4a–d** was prepared as shown in Scheme II. Phenyl phosphorodichloridate was reacted with the appropriate amine hydrochloride in the presence of triethylamine to form **2a–d**. These compounds were then treated with the anion of (hydroxymethyl)cyclopentane to afford **3a–d**. The (hydroxymethyl)cyclopentane substituent was selected in the synthesis of these compounds to serve as a potential model for a nucleoside substituent. The protecting group was removed by hydrogenation using Adam's catalyst in ethanol. Once hydrogenation was complete, cyclohexylamine was immediately added to generate the cyclohexylamine salts **4a–d**.

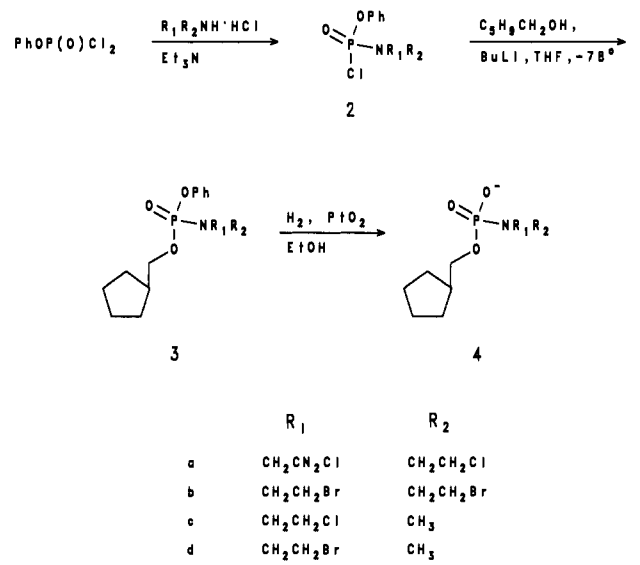
Chloride Ion and ^{31}P NMR Kinetics. ^{31}P NMR solvolysis experiments were performed with **4a** in cacodylate buffer (0.4 M, pH 7.4, 37 °C). Figure 1 shows the sequential conversion of **4a** into two solvolysis products. Peaks B and C represent the mono- and bis(hydroxyethyl) phosphoramidates, analogous to those reported for phosphoramide mustard **1a**.[4] Similar reaction of **4b** (−16.49 ppm) gave two new peaks at −15.39 and −14.28 ppm, respectively. Under the same conditions, **4c** generated the series of spectra shown in Figure 2, in which only one new resonance appeared upon depletion of the starting material. Similarly, reaction of **4d** (−15.06 ppm) led to the formation of a single resonance at −13.88 ppm.

Halide ion release was measured with a chloride ion selective electrode in cacodylate buffer (0.3 M, pH 7.4, 37 °C). Halide ion release after four half-lives corresponded to 2 equiv when experiments were performed with **4a** and **4b** and 1 equiv with **4c** and **4d**. The rate constants were obtained from nonlinear least-squares minimization for a two-step sequential reaction using the Simplex algorithm;

Scheme I



Scheme II



	R_1	R_2
a	CH_2CN_2Cl	CH_2CH_2Cl
b	CH_2CH_2Br	CH_2CH_2Br
c	CH_2CH_2Cl	CH_3
d	CH_2CH_2Br	CH_3

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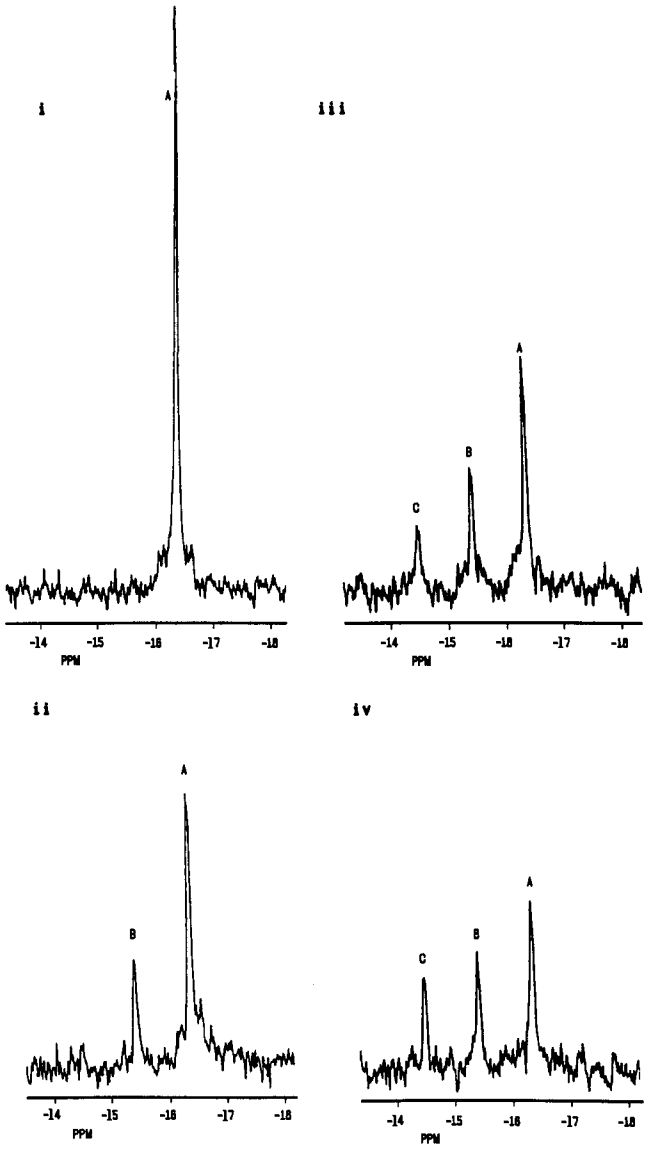

Figure 1. Solvolysis of **4a** (peak A) to form peaks B and C (0.4 M cacodylate, pH 7.4, 37 °C): (i) 12 min, (ii) 212 min, (iii) 292 min, (iv) 372 min.

Table I. Solvolysis Rates Obtained from ^{31}P NMR Spectroscopy and Chloride Ion Electrode Experiments

	disappearance of peak A (^{31}P NMR)		loss of first equiv halide ion (chloride ion electrode)	
no.	$k \times 10^3$, min^{-1}	$t_{1/2}$,[a] min	$k \times 10^3$, min^{-1}	$t_{1/2}$,[a] min
4a	2.8	250 ± 17	3.0[b]	230 ± 8
4b	53	13 ± 1	58[b]	12 ± 1
4c	9.6	72 ± 2	13	54 ± 12
4d	126	5.5 ± 1.0	131	5.3 ± 1.5
1a	29	24 ± 3	41[b]	17 ± 2
5	8.9	78 ± 12	10[c]	69 ± 3

[a] Results expressed as mean ± standard deviation for at least three experiments. [b] Rate constant for loss of second equivalent of halide ion was essentially equal to that of the first equivalent. [c] Rate constant for loss of second equivalent of chloride ion; $k = 3.9 \times 10^{-3}$ min^{-1} ($t_{1/2}$ = 179 min).

the results are presented in Table I. It is apparent that halide ion is released at the same rate for which the ^{31}P resonance of the starting material is disappearing. The data also indicate that the bisalkylating compounds **4a** and **4b** lose the first and second equivalent of halide ion at essentially the same rate, in contrast to ifosfamide mustard (**5**), which loses the first chloride ion twice as fast as the second.[10] Rate constants for **1a** and **5** were also obtained for comparison and are in accord with the ^{31}P NMR rate constants previously reported.[4,10] The rate constant observed for the monoamide monoester analogue (**4a**) of phosphoramide mustard is approximately 10-fold slower than that for phosphoramide mustard (**1a**), presumably resulting from the increased electronegativity of oxygen and its effect on the electron density of the nitrogen involved in aziridinium ion formation. This trend is also apparent from the 8-fold slower solvolysis rate for the phenoxy analogue of **1a** ($t_{1/2}$ = 1800 min)[2] compared to that of the alkoxy compound.

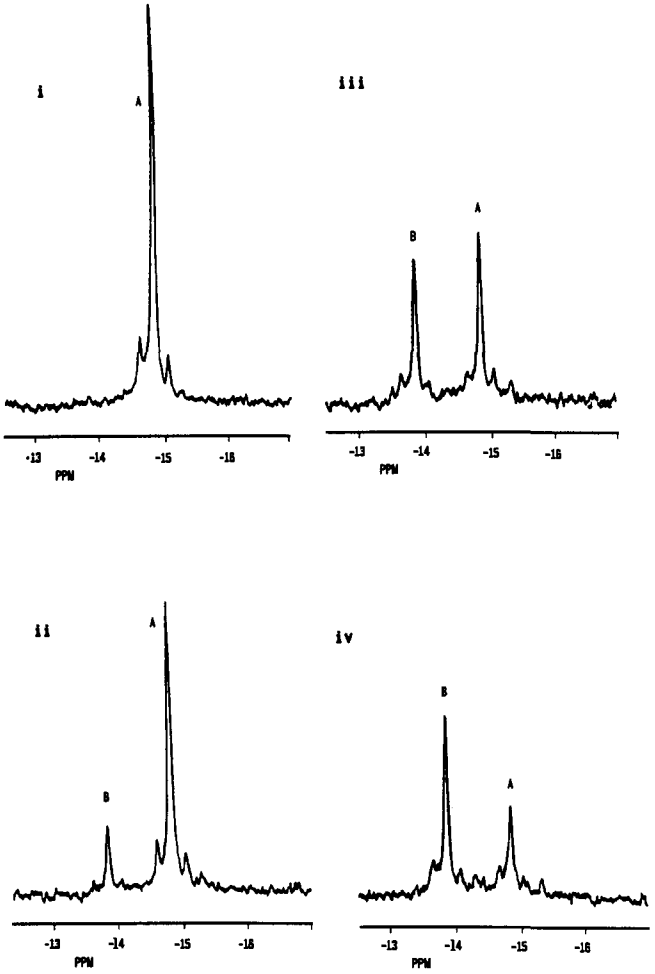

Figure 2. Solvolysis of **4c** (peak A) to form peak B (0.4 M cacodylate, pH 7.4, 37 °C): (i) 10 min, (ii) 30 min, (iii) 70 min, (iv) 110 min.

Trapping Agent Kinetics. It was desirable to acquire more details regarding the lifetime of the aziridinium ion and whether this intermediate could be observed by NMR.[4,7,9] In order to exclude the possibility that the aziridinium ion intermediate occurs at the same ^{31}P chemical shift as the starting material or one of the solvolysis products, the solvolysis reaction was carried out in the presence of dimethyldithiocarbamate (DDC) in order to trap the aziridinium ion intermediate as it was formed. DDC (20% excess) was added to a buffered solution of **4c** (pH 7.4, 37 °C), and the reaction was monitored by ^{31}P NMR (Figure 3). A new peak appeared at higher field (−14.7 ppm, peak X) compared to the solvolysis peak generated in Figure 2. The product corresponding to peak X was isolated and characterized by ^1H NMR as the di-

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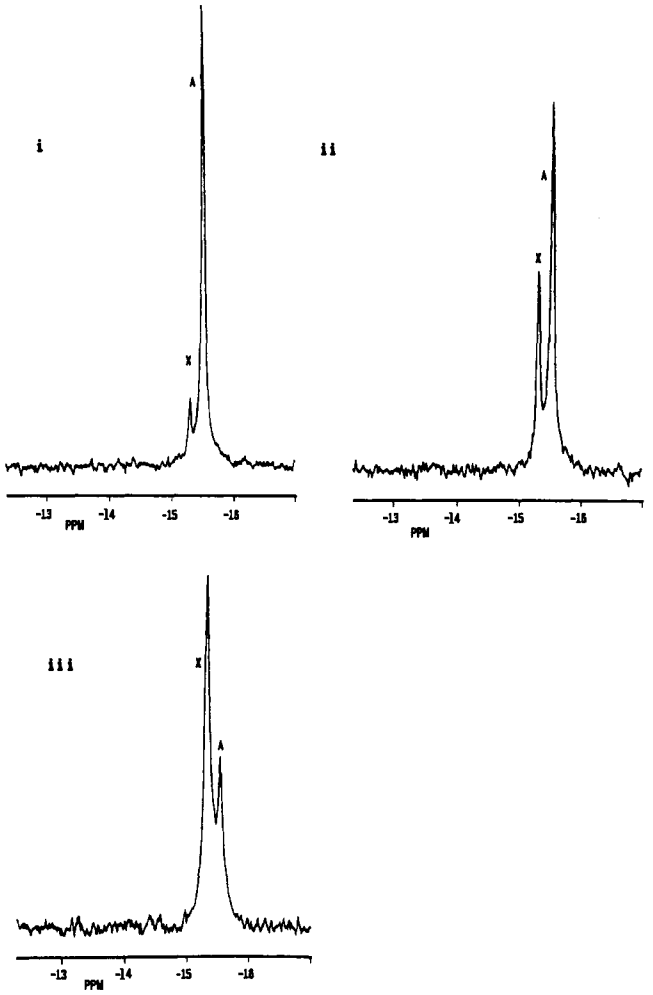

Figure 3. Reaction of **4c** (peak A) in the presence of dimethyldithiocarbamate (1.2 equiv) as a trapping agent (0.4 M cacodylate, pH 7.4, 37 °C): (i) 11 min, (ii) 31 min, (iii) 91 min.

thiocarbamate substituted product. This reaction proceeded with a rate constant = 10.4×10^{-3} min^{-1}, which is comparable to that found for solvolysis of **4c** in the absence of DDC (Table I). These observations indicate that the failure to observe the aziridinium ion does not result from coincidence of chemical shift with the starting compound, because in that case DDC would have reacted rapidly with the aziridinium ion intermediate and increased the apparent rate of disappearance of peak A.

In order to rule out the possibility that the aziridinium ion intermediate had the same chemical shift as the solvolysis product, **4c** was reacted in aqueous buffer until the peaks for **4c** and solvolysis product were approximately equal (^{31}P NMR). DDC (20% excess) was then added; the residual peak for **4c** was subsequently converted to the dithiocarbamate substitution product peak, but the intensity of the solvolysis peak remained unchanged. If a signal corresponding to the aziridinium ion had been present at the same chemical shift as the solvolysis product, the reaction with DDC would have led to the conversion of this resonance to the DDC-product resonance.

^1H NMR Experiments. The reactions of **4a–d** in buffer were allowed to go to completion (monitored by ^{31}P NMR), and the products were isolated and characterized by ^1H NMR (D_2O). The same product (peak C, Figure 1) was produced from both **4a** and **4b** as expected and showed a change in chemical shift of the terminal methylene protons to 3.76 ppm, which were assigned as CH_2OH. To explore further the lifetime of the aziridinium ion intermediate, a ^1H NMR experiment was performed by dissolving **4c** in phosphate buffer in D_2O (0.3 M, pD 7.4, 37 °C). This solvolysis reaction showed no evidence of aziridinium ion accumulation in the ^1H NMR spectrum (1–2 ppm); **4c** was converted directly to solvolysis product. Similar results were observed in the reaction of **4d** and the products formed from the reaction of **4c** and **4d** had identical ^1H NMR spectra (CH_2OH, 3.67 ppm; CH_3, 2.51 ppm).

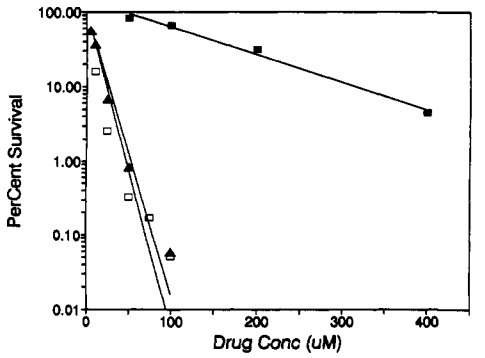

Figure 4. Clonogenic survival of L1210 leukemia cells following 2-h exposure to drug: ■, **4a**; ▲, **4b**; □, **1a**.

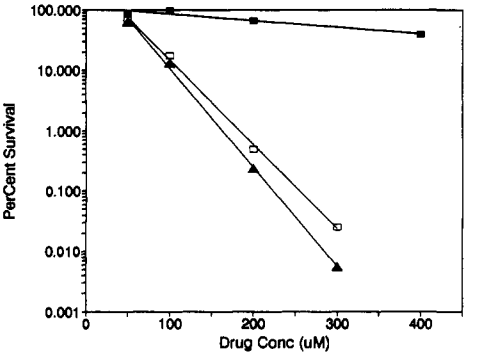

Figure 5. Clonogenic survival of B16 melanoma cells following 2-h exposure to drug: ■, **4a**; ▲, **4b**; □, **1a**.

In Vitro Cytotoxicity. Compounds **4a–d** were tested for cytotoxicity against L1210 and B16 cell lines in vitro. As expected, the monoalkylating compounds **4c** and **4d** were not cytotoxic (data not shown), since they cannot cross-link DNA. Although **4a** was minimally cytotoxic, the cytotoxicity of **4b** was comparable to that of phosphoramide mustard (**1a**) in this assay (Figures 4 and 5), suggesting that the decrease in cytotoxicity resulting from substitution of ester for amide is compensated by the increased reactivity of the bromo compared to the chloro substituent.

Summary

Several conclusions can be drawn from the data presented. First, the aziridinium ion formed by cyclization of the mixed ester–amide phosphoramidates is a very short-lived species and cannot be detected by NMR experiments. Aziridinium ion formation is presumably the rate-limiting step for alkylation of solvent or other nucleophiles. Second, the reactivity of the phosphoramidates is highly dependent upon the electronic effects of the substituents. Replacement of the amide moiety of **1a** by an ester group retards aziridinium ion formation approximately 10-fold. However, this decrease in reactivity can be compensated for by replacing the chlorine with a better leaving group. Substitution of a methyl group for the electron-withdrawing haloethyl group increases aziridinium ion formation approximately 3-fold (or a factor of 5–6 if the statistical factor for the bis(haloethyl) compounds is

considered). Third, cytotoxicity parallels the solvolysis rate for those compounds capable of cross-linking DNA. Finally, phosphoramidate esters with a broad range of alkylating activity can be prepared. The development of analogues targeted to sites other than DNA is currently under investigation.

Experimental Section

NMR Spectra. ^{31}P NMR spectra were recorded on a Bruker WP-270 SY instrument equipped with a VSP multinuclear probe tuned for 109.368 MHz using 10-mm sample tubes, 5000-Hz spectral width, and 128 acquisitions. The temperature was maintained at 37 °C with a VT1000 variable-temperature unit. Broadband gated decoupling was used. Chemical shifts are reported in parts per million from 5% triphenylphosphine oxide in toluene-d_8 as a coaxial reference. ^1H NMR spectra were recorded on the same instrument using 5-mm sample tubes and 32 acquisitions. Chemical shifts are reported in parts per million from tetramethylsilane.

^{31}P NMR Kinetics Experiments. Cacodylate buffer (1.6 mL, 0.4 M) and D_2O (0.4 mL) were placed in a 10-mm NMR tube in the thermostated probe for 1 h. The phosphoramidate (20–30 mM) was added to the buffer solution, and it was allowed to equilibrate at 37 °C in the probe for about 7 min before acquisition. Experiments using a trapping agent were performed with a 20% excess of sodium dimethyldithiocarbamate, which was added immediately after buffer addition to the sample. Spectra were taken every 5 min for compounds **1a**, **4b**, and **4d** and every 15 min for compounds **4a**, **4c**, and **5** and time points for each spectrum were assigned at the midpoint of the data acquisition. The FID spectra were processed by exponential multiplication with a line broadening of 2 Hz. The relative concentrations of the intermediates were determined from integration of the peak areas.

Chloride Ion Electrode Experiments. Potentials were measured in millivolts using an Orion combination chloride electrode. Five standard sodium chloride solutions were prepared from NaCl (0.1 M) stock solution. Bromide ion standards were prepared from NaBr (0.1 M) stock solution. Standards were diluted with cacodylate buffer (25 mL, 0.3 M) after addition of $NaNO_3$ (0.25 mL, 5 M) to maintain a constant ionic strength. The standards were maintained at 37 °C in a temperature-controlled water bath.

The sample solution was prepared immediately prior to use by addition of $NaNO_3$ (0.25 mL, 5 M) to a volumetric flask (25 mL). NaCl (0.1 M) or NaBr (0.1 M) stock solution (2.5 μL) was then added to provide a baseline concentration of chloride or bromide ion, respectively. The solution was diluted with cacodylate buffer (25 mL, 0.3 M) and equilibrated at 37 °C for 1 h. Phosphoramidate (1.0 mmol) was added to the warmed solution and allowed to equilibrate for 7 min. Potential measurements were taken at selected time intervals.

Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. IR spectra were recorded on a Perkin-Elmer 1310 infrared spectrometer. Melting points were determined on a Mel-Temp apparatus and are uncorrected. Purification by flash chromatography was carried out with silica gel and elution with 1:4 ethyl acetate/hexane.

Determination of Rate Constants. The rate constants were determined by nonlinear least-squares minimization using the Simplex algorithm.[11,12] The data were fit to the equation representing the sequential reaction $A \rightarrow Cl^- + B$; $B \rightarrow Cl^- + C$.

Phosphoramide mustard (1a) was prepared as described previously and isolated as the cyclohexylamine salt:[13] ^1H NMR (DMSO-d_6) 3.64 (dt, 4 H, CH_2Cl), 3.17 (dt, $-CH_2N-$) ppm; ^{31}P NMR (D_2O) −11.30 ppm.

Ifosfamide mustard (5) was prepared as described previously and isolated as the cyclohexylamine salt:[14,15] ^1H NMR (DMSO-d_6) 3.53 (dt, 4 H, CH_2Cl), 2.96 (m, 5 H, overlapping $-CH_2N-$, cyclohexylamine) ppm; ^{31}P NMR (D_2O) −11.56 ppm.

Phenyl *N*,*N*-Bis(2-chloroethyl)phosphoramidate Chloride (2a). Triethylamine (8.58 mL, 61 mmol) was added dropwise at 0 °C to a stirred solution of bis(2-chloroethyl)amine hydrochloride (5 g, 28 mmol) and phenyl phosphorodichloridate (4.18 mL, 28 mmol) in methylene chloride (50 mL) under nitrogen. The mixture was allowed to stir for 14 h at room temperature and was then poured over ice. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The product was isolated as an oil after purification by flash chromatography (7.09 g, 80%): ^1H NMR ($CDCl_3$) 7.33 (m, 5 H, phenyl), 3.73 (dt, 4 H, CH_2Cl), 3.61 (dt, 4 H, $-CH_2N-$) ppm; ^{31}P NMR ($CDCl_3$) −13.46 ppm.

Cyclopentylmethyl Phenyl *N*,*N*-Bis(2-chloroethyl)phosphoramidate (3a). Butyllithium (4.74 mL, 7.6 mmol, 1.6 M) was added at 0 °C to a stirred solution of (hydroxymethyl)cyclopentane (0.75 mL, 7.0 mmol) and a crystal of 4-(phenylazo)diphenylamine indicator in THF (3 mL) under nitrogen. This alkoxide was added dropwise at −78 °C to a stirred solution of **2a** (2.5 g, 0.0062 mol) in THF (6 mL) under nitrogen. The mixture was allowed to stir under nitrogen at −78 °C for 3 h, at which time the purple solution turned yellow. The mixture was warmed to room temperature and the solvent was removed under reduced pressure. The residue was purified by flash chromatography and the product was isolated as an oil (2.67 g, 89%): ^1H NMR ($CDCl_3$) 7.26 (m, 5 H, phenyl), 3.99 (dd, 2 H, $-CH_2O-$), 3.57 (dt, 4 H, CH_2Cl), 3.47 (dt, 4 H, $-CH_2N-$) ppm; ^{31}P NMR ($CDCl_3$) −20.73 ppm.

Cyclopentylmethyl *N*,*N*-Bis(chloroethyl)phosphoramidate Cyclohexylamine Salt (4a). Compound **3a** (0.5 g, 1.3 mmol) was dissolved in ethanol (25 mL). $PtO_2 \cdot H_2O$ (0.15 g, 0.6 mmol) was added and the solution was hydrogenated at room temperature and 1 atm for 1 h. Cyclohexylamine (0.15 mL, 1.3 mmol) was then added to the stirred solution. After filtration of the catalyst, the solvent was removed under reduced pressure and the residue was triturated with ether. The product was isolated as a white solid (0.37 g, 70%): mp 140–141 °C; ^1H NMR (DMSO-d_6) 3.57 (dd, 2 H, $-CH_2O-$), 3.35 (dt, 4 H, CH_2Cl), 3.15 (dt, 4 H, $-CH_2N-$) ppm; ^{31}P NMR (D_2O) −16.38 ppm; IR (KBr) 3460–3640, 2930, 2780, 2650, 2570, 2190, 1210, 1040 cm^{-1}. Anal. ($C_{16}H_{33}N_2O_3PCl_2 \cdot {}^1/_2H_2O$) C, H, N.

Phenyl *N*,*N*-bis(bromoethyl)phosphoramidate chloride (2b) was prepared from bis(2-bromoethyl)amine hydrobromide (3.0 g, 10 mmol) as described above. The crude product was purified by flash chromatography and was isolated as an oil (3.1 g, 79%): ^1H NMR ($CDCl_3$) 7.27 (m, 5 H, phenyl), 3.65 (dt, 4 H, CH_2Br), 3.27 (dt, 4 H, $-CH_2N-$) ppm; ^{31}P NMR ($CDCl_3$) −13.13 ppm.

Cyclopentylmethyl phenyl *N*,*N*-bis(2-bromoethyl)phosphoramidate (3b) was prepared from **2b** (2.0 g, 5.0 mmol) as described above. The residue was purified by flash chromatography and the product was isolated as an oil (2.0 g, 85%): ^1H NMR 7.12 (m, 5 H, phenyl), 3.86 (dd, 2 H, $-CH_2O-$), 3.38 (dt, 4 H, CH_2Br), 3.24 (dt, 4 H, $-CH_2N-$) ppm; ^{31}P NMR ($CDCl_3$) −21.55 ppm.

Cyclopentylmethyl *N*,*N*-Bis(bromoethyl)phosphoramidate Cyclohexylamine Salt (4b). Compound **3b** (0.5 g, 1.1 mmol) was hydrogenated as described above. The product was isolated as a white solid (0.36 g, 70%): mp 120–122 °C; ^1H NMR (DMSO-d_6) 3.78 (dd, 2 H, $-CH_2O-$), 3.57 (dt, 4 H, CH_2Br), 3.37 (dt, 4 H, $-CH_2N-$) ppm; ^{31}P NMR (D_2O) −16.49 ppm. IR (KBr) 3300–3660, 2930, 2880, 2650, 2560, 2190, 1780, 1210, 1070 cm^{-1}. Anal. ($C_{16}H_{33}N_2O_3PBr_2 \cdot {}^1/_2H_2O$) C, H, N.

Phenyl *N*-methyl-*N*-(2-chloroethyl)phosphoramidate chloride (2c) was prepared from *N*-methyl-(2-chloroethyl)amine hydrochloride (5 g, 38 mmol) as described above. The residue was purified by flash chromatography and the product was isolated as an oil (8.14 g, 70%): ^1H NMR ($CDCl_3$) 7.35 (m, 5 H, phenyl), 3.68 (dt, 2 H, CH_2Cl), 3.53 (dt, 2 H, $-CH_2N-$), 2.75 (d, 3 H, CH_3) ppm; ^{31}P NMR ($CDCl_3$) −12.95 ppm.

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Cyclopentylmethyl phenyl *N*-methyl-*N*-(2-chloroethyl)-phosphoramidate (3c) was prepared from **2c** (2.0 g, 7.4 mmol) as described above. The residue was purified by flash chromatography and was isolated as an oil (2.21 g, 85%): ^{1}H NMR ($CDCl_3$) 7.27 (m 5 H, phenyl), 3.98 (dd, 2 H, $-CH_2O-$), 3.54 (dt, 2 H, $ClCH_2$), 3.40 (dt, 2 H, $-CH_2N-$), 2.81 (d, 3 H, CH_3) ppm; ^{31}P NMR ($CDCl_3$) -19.53 ppm.

Cyclopentylmethyl *N*-methyl-*N*-(2-chloroethyl)-phosphoramidate cyclohexylamine salt (4c) was prepared from **3c** (0.5 g, 1.5 mmol) as described above. The product was isolated as a white solid (0.4 g, 75%): mp 149-152 °C; ^{1}H NMR (DMSO-d_6) 3.74 (dd, 2 H, CH_2O), 3.55 (dt, 2 H, $ClCH_2$), 3.27 (dt, 2 H, $-CH_2N-$), 2.55 (d, 3 H, CH_3) ppm; ^{31}P NMR (D_2O) -14.86 ppm; IR (KBr) 3200-3700, 2940, 2860, 2650, 2560, 2210, 1190, 1040 cm^{-1}. Anal. ($C_{15}H_{32}N_2O_3PCl \cdot {}^1/_2H_2O$) C, H, N.

Phenyl *N*-methyl-*N*-(2-bromoethyl)phosphoramidate chloride (2d) was prepared as described above from *N*-methyl-2-bromoethylamine hydrobromide (3.0 g, 14 mmol). The residue was purified by flash chromatography and the product was isolated as an oil (3.21 g, 75%): ^{1}H NMR ($CDCl_3$) 7.25 (m, 5 H, phenyl), 3.52 (dt, 2 H, $BrCH_2$), 3.35 (dt, 2 H, $-CH_2N-$), 2.81 ppm (d, 3 H, CH_3). ^{31}P NMR ($CDCl_3$) -12.67 ppm.

Cyclopentylmethyl phenyl *N*-methyl-*N*-(2-bromoethyl)-phosphoramidate (3d) was prepared from **2d** (3.0 g, 9.6 mmol) as described above. The residue was purified by flash chromatography and was isolated as an oil (3.25 g, 87%): ^{1}H NMR ($CDCl_3$) 7.25 (m, 5 H, phenyl), 3.97 (dd, 2 H, CH_2O), 3.52 (dt, 2 H, $BrCH_2$), 3.38 (dt, 2 H, $-CH_2N-$), 2.80 (d, 3 H, CH_3) ppm; ^{31}P NMR ($CDCl_3$) -19.57 ppm.

Cyclopentylmethyl *N*-methyl-*N*-(2-bromoethyl)-phosphoramidate cyclohexylamine salt (4d) was prepared from **3d** (0.5 g, 1.3 mmol) as described above. The product was isolated as a white solid (0.37 g, 72%): mp 113-116 °C; ^{1}H NMR (DMSO-d_6) 3.74 (dd, 2 H, $-CH_2O-$), 3.59 (dt, 2 H, $BrCH_2$), 3.26 (dt, 2 H, $-CH_2N-$), 2.56 (d, 3 H, CH_3) ppm; ^{31}P NMR (D_2O) -15.06 ppm; IR (KBr) 3250-3680, 2940, 2860, 2650, 2570, 2200, 1740, 1200, 1040 cm^{-1}. Anal. ($C_{15}H_{32}N_2O_3PBr \cdot {}^1/_2H_2O$) C, H, N.

Characterization of the Dimethyldithiocarbamate Trapped Product of 4c. When the reaction had gone to completion (^{31}P NMR), the solution was acidified and extracted with $CHCl_3$. The extracts were evaporated, and the crude product was characterized by NMR: ^{1}H NMR (DMSO-d_6) 3.70 (dd, 2 H, $-CH_2O-$), 3.55 (d, 6 H, $(CH_3)_2N$), 3.48 (dt, 2 H, $-CH_2S-$), 3.25 (dt, 2 H, $-CH_2N-$), 2.80 (d, 3 H, CH_3N) ppm; ^{31}P NMR (D_2O) -15.31 ppm.

In Vitro Cytotoxic Activity. Cultured mouse L1210 murine leukemia and B16 melanoma cells were obtained from the National Cancer Institute, Bethesda, MD. A soft agar clonogenic assay was used with L1210 cells as described previously.[16] Clonogenic survival of B16 cells was determined according to the method of Mirabelli et al.[17] L1210 and B16 cells, in exponential growth, were suspended in the appropriate unsupplemented medium (10 mL) at a final density of 2-3 × 10^5 cells/mL. Drug stock solutions (4 mM) were prepared with medium without supplement and sterilized by passage through a 0.22-μm filter. Appropriate volumes (0.125-1 mL) of drug stock solution were added to the cell suspension to give a final drug concentration of 5-400 μM. The drug-cell suspensions were then incubated for 2 h at 37 °C. The cells were washed (3×) with supplemented medium (3 mL) and then resuspended in medium (5 mL). Cells were counted (Coulter counter), and the number of cells required for plating was determined with 2 or 3 different dilutions per drug concentration.

L1210 cells were plated in soft agar (0.2%) at densities between 25 000 and 100 000 cells/mL, and colonies were counted 10 days later. B16 cells were plated by using the monolayer clonogenic assay at densities between 5000 and 20 000 cells/mL (5 mL of medium per 60-mm culture dish). Colonies were stained with crystal violet and counted 7 days later.

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Antimalarial Polyamine Analogues

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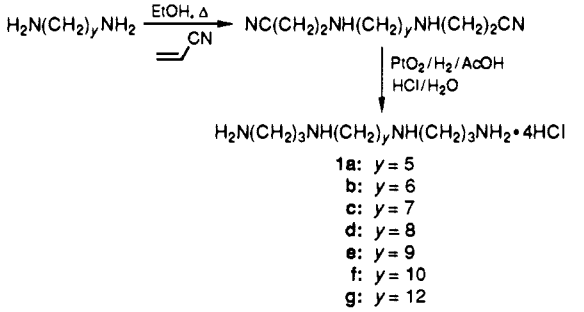
A series of novel tetraamines of the general formula $RNH(CH_2)_xNH(CH_2)_yNH(CH_2)_xNHR$ was synthesized and examined for activity against growth of *Plasmodium falciparum* in vitro. Within the series, dibenzyl analogues (R = benzyl) were found to be the most effective growth inhibitors, with IC_{50} values of about 10^{-6} M. Further modifications of the tetraamine provided the optimum chain length for antimalarial activity of $y = 7$, $x = 3$. Compound **8** (MDL 27,695) with the structure $y = 7$, $x = 3$, R = benzyl, in combination with the ornithine decarboxylase inhibitor α-(difluoromethyl)ornithine, resulted in radical cures when tested against experimental *Plasmodium berghei* infections in mice. The structure-activity relationships of the series are discussed.

Introduction

In spite of past progress in the prevention and treatment of malaria, half the world's population is still threatened by the disease. Its incidence is presently increasing due to the development of resistance of the malaria parasite to current drugs.[1,2] We have shown that parasitic protozoa are sensitive to polyamine biosynthesis inhibitors. Most notably, we found that α-(difluoromethyl)ornithine (DFMO, eflornithine),[3] an irreversible inhibitor of ornithine decarboxylase, is effective against African trypanosomiasis both in the laboratory[4] and in the clinic.[5]

Scheme I



Additionally, DFMO inhibits the growth of blood stages of the human parasite *Plasmodium falciparum* in vitro[6]

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