Synthesis and Evaluation of Nitroheterocyclic Phosphoramidates as Hypoxia-Selective Alkylating Agents

Richard F. Borch,*,[†,‡] Jiwen Liu,[†] James P. Schmidt,[†] Joseph T. Marakovits,[†] Carolyn Joswig,[†] Jerry J. Gipp,[§] and R. Timothy Mulcahy[§]

Departments of Chemistry and Pharmacology and the Cancer Center, University of Rochester, Rochester, New York 14642, Department of Medicinal Chemistry and Molecular Pharmacology and the Cancer Center, Purdue University, West Lafayette, Indiana 47907, and Department of Human Oncology, University of Wisconsin, Madison, Wisconsin 53792

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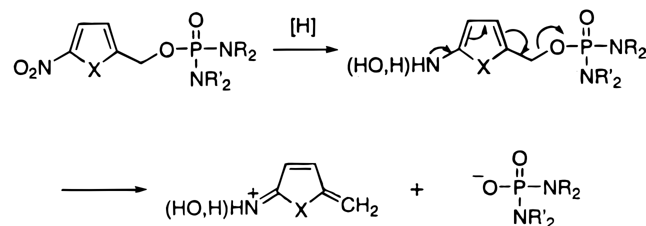
A series of novel nitroheterocyclic phosphoramidates has been prepared, and the cytotoxicity of these compounds has been evaluated in clonogenic assays against B16, wild-type and cyclophosphamide-resistant MCF-7, and HT-29 cells under aerobic conditions and HT-29 cells under hypoxic conditions. All compounds were comparable in toxicity to wild-type and resistant MCF-7 cells and were also selectively toxic to HT-29 cells under hypoxic conditions (selectivity ratios 1.7 to >20). Analogues lacking the nitro group were not cytotoxic. Electron-withdrawing substituents increased cytotoxicity under aerobic conditions and thereby decreased hypoxic selectivity. In contrast, an electron-donating substituent markedly decreased both aerobic and hypoxic cytotoxicity but enhanced hypoxic selectivity. Chemical reduction of the nitro group resulted in rapid expulsion of the cytotoxic phosphoramide mustard. The most potent of these compounds show significant cytotoxicity under both aerobic and hypoxic conditions.

Introduction

The presence of hypoxia and hypoxic cells in many solid tumors is well-established.[1] Hypoxic cells represent a therapeutic challenge in that these cells are refractory to radiation therapy and resistant to many of the cytotoxic drugs used in chemotherapy. However, hypoxia can also be exploited via the design of hypoxia-selective agents (HSA) that undergo bioreductive activation to generate cytotoxic species under hypoxic conditions. These HSAs should exhibit significantly greater toxicity to hypoxic compared to aerobic cells. Many classes of HSAs have been synthesized and evaluated preclinically for antitumor activity, and several compounds have entered clinical trials.[2] Nitroaromatic compounds have been widely used in the development of HSAs because of their favorable redox potentials and the marked reversal of electron affinity that results from reduction to the hydroxylamine or amine.[3] Many of these nitroaromatic compounds are metabolized directly to cytotoxic intermediates under hypoxic conditions. Other nitroheterocyclic compounds have been developed with latent alkylating moieties that are activated by reduction of the nitro group.[4] We have a continuing interest in the exploitation of phosphoramidate activation mechanisms to deliver cytotoxins to tumor cells,[5] including the bioreductive activation of nitroaromatic compounds to deliver cytotoxic phosphoramide mustards to the hypoxic cell. We report herein the synthesis and evaluation of a series of nitroheterocyclic phosphoramidates that are highly cytotoxic to both aerobic and hypoxic tumor cells.

Scheme 1



Results and Discussion

Chemistry. The mechanistic rationale for the proposed activation of these compounds is shown in Scheme 1. The nitroheterocyclic phosphoramidates should be stable toward intramolecular cyclization and aziridinium ion formation. However, enzymatic reduction of the nitro group is expected to lead to the arylhydroxylamine or the arylamine intermediate; expulsion of the phosphoramidate anion from this intermediate will activate the alkylating moiety.

The phosphoramidates listed in Tables 1 and 2 were prepared from the corresponding hydroxymethyl nitroheterocycles by the reaction sequences shown in Schemes 2 and 3. For those compounds containing the bis(haloethyl)phosphoramidate, the hydroxymethyl compound was deprotonated with lithium hexamethyldisilazide at −78 °C (Scheme 2) and the resulting alkoxide reacted with the appropriate bis(haloethyl)phosphoryl dichloride. The intermediate was then reacted with anhydrous ammonia at −20 °C to give the desired product. For those compounds containing the tetrakis(haloethyl)phosphoramidate group, the greater reactivity of phosphorus(III) chlorides was exploited (Scheme

* To whom correspondence should be addressed. Address: Dept. of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907-1333. Tel: (765)494-1403. Fax: (765)494-1414. E-mail: rickb@pharmacy.purdue.edu.
[†] University of Rochester.
[‡] Purdue University.
[§] University of Wisconsin.

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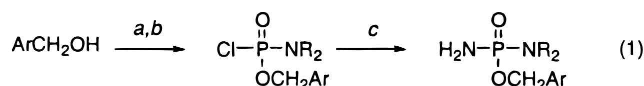
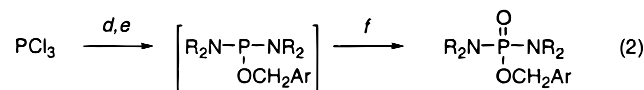
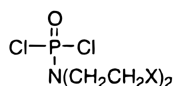
Scheme 2[a]



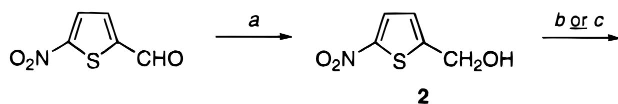


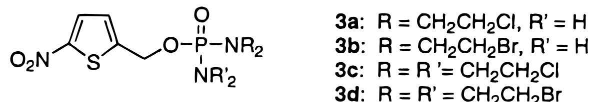


1a, X = Cl
1b, X = Br

[a]Reagents: a) LiHMDS, -78 °C; b) **1a** or **1b**, -78°C; c) NH_3, -20 °C; d) HX·HN(CH_2CH_2X)$_2$ (2 equiv),Et_3N, -40 °C; e) $ArCH_2OH$, Et_3N, -40 °C; f) t-BuOOH, -40 °C

Scheme 3[a]





[a]Reagents: a) $NaBH_4$, THF/H_2O; b) Cl_2PONR_2, then NH_3; c) ClP(NR_2)$_2$, then t-BuOOH

3).[5c] Phosphorus trichloride was reacted with 2 equiv of bis(haloethyl)amine hydrohalide in the presence of triethylamine, and the hydroxymethyl compound was then added. The resulting phosphoramidite was then oxidized to the desired product with *tert*-butyl hydroperoxide or ozone.

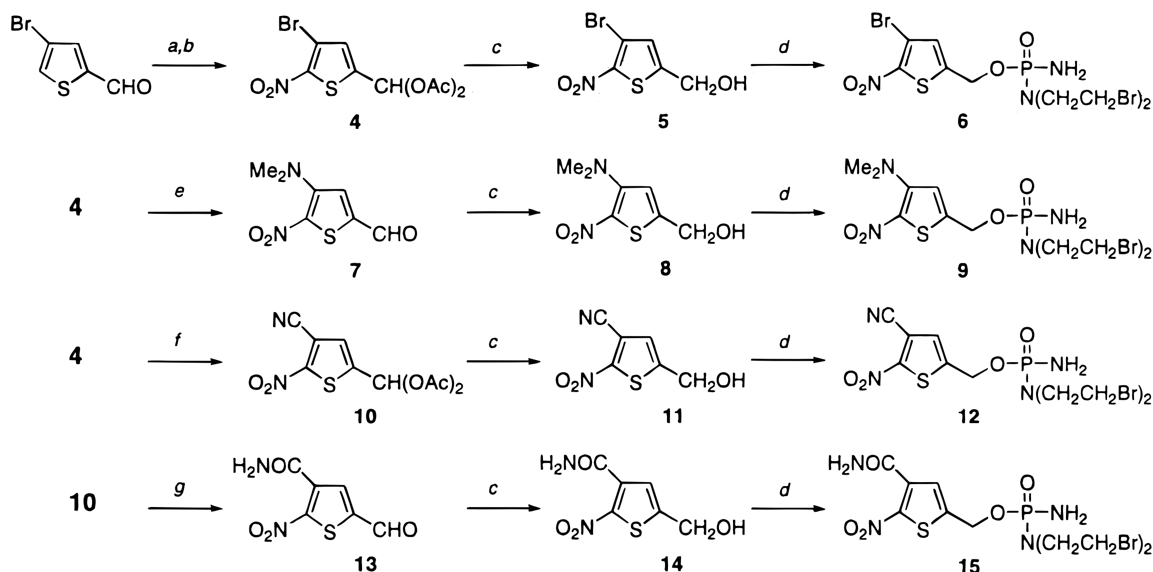
To explore the substituent effects on the cytotoxicity of these compounds, the series of nitrothiophenes **6, 9, 12,** and **15** was prepared according to the routes outlined in Scheme 4. The α,α-diacetoxy bromide **4** proved to be a key intermediate in the synthesis of all four compounds. Compound **4** was prepared from 4-bromothiophene-1-carboxaldehyde by reaction with acetic anhydride followed by nitration of the diacetoxy intermediate.[6] Treatment of **4** with aqueous sodium borohydride cleaved the ester and reduced the aldehyde to the corresponding alcohol **5**. Reaction of **4** with excess dimethylamine in refluxing chloroform[7] led to displacement of the bromide and cleavage of the diacetoxy groups to give the amino aldehyde **7**, which was reduced with sodium borohydride to the alcohol **8**. Reaction of **4** with CuCN in refluxing DMF[8] afforded the diacetoxy nitrile **10** which was reduced to the cyano alcohol **11**. Finally, the nitrile **10** was converted to the amido aldehyde **13** by reaction with aqueous sulfuric acid,[9] and this intermediate was subsequently reduced to the alcohol **14**.

The pyrrole **19** (Scheme 5) was prepared by reduction of the corresponding aldehyde **18** which in turn was obtained as the minor isomer from nitration of 1-methylpyrrole-2-carboxaldehyde.[10] The aldehyde precursor to imidazole **22** was prepared by reaction of the 2-methyl compound with DMF diethyl acetal followed by osmium tetroxide/periodate cleavage as described.[11] Phosphorylation of the respective alcohols was carried out using either P^{III} or P^{V} chemistry as outlined above.

Cytotoxicity. The cytotoxicity of the target compounds was assessed using clonogenic assays against B16 murine melanoma cells, wild-type (/WT), and 4-hydroperoxycyclophosphamide-resistant (/CP) MCF-7 human breast cancer cells. Selected compounds were also tested against HT-29 human colon carcinoma cells under aerobic and hypoxic conditions as described previously.[5c] The results for the nitrothiophenes are presented in Table 1. The presence of the nitro group significantly enhances cytotoxicity (see compounds **24** and **25**, Scheme 5). Most of the bis(bromoethyl) compounds are 1−2 orders of magnitude more potent than 4-hydroperoxycyclophosphamide (4-HC), and they are comparable in toxicity against wild-type and cyclophosphamide-resistant MCF-7 cells. Although the potency of the bis- and tetrakis(haloethyl)phosphoramidates is similar, cytotoxicity decreases in the order: tetrakis-(bromoethyl) > bis(bromoethyl) > tetrakis(chloroethyl) > bis(chloroethyl) as observed for the aldophosphamide analogues.[5a] The phosphoramidates also reduced HT-29 cell survival under both aerobic and hypoxic conditions; the unsubstituted nitrothiophene **3b** showed a hypoxic/aerobic differential of approximately 8-fold (determined from the ratio of LC_{99} values calculated from the log-linear portion of the aerobic and hypoxic survival curves). The effect of substituents on toxicity and hypoxic selectivity to HT-29 cells is apparent from a comparison of compounds **3b**, **6**, **9**, **12**, and **15** (Table 1 and Figure 1). The introduction of a substituent at position 4 of the thiophene ring decreases hypoxic toxicity, perhaps because of steric interference with the coplanarity of the nitro group. The effect is small for the linear cyano group but is dramatic (50-fold) for the dimethylamino group, where conjugation of the nitrogen lone pair places the methyl group in the plane of the ring. The introduction of electron-withdrawing groups increases aerobic toxicity 1.5−2-fold; in contrast, introduction of the electron-donating dimethylamino group decreases aerobic toxicity 130-fold. Presumably the dimethylamino compound has a significantly more negative reduction potential, and its one-electron reduction product is more easily reoxidized by oxygen. The results in Table 2 demonstrate that the nitrofurans are comparable to the nitrothiophenes in their cytotoxicities to these cell lines. In contrast, the nitropyrroles are ca. 1 order of magnitude less potent than the corresponding nitrofurans and nitrothiophenes. The 5-nitroimidazole **23** is essentially nontoxic to all cell lines under aerobic conditions; however, it shows modest toxicity to HT-29 cell lines under hypoxic conditions and appears to be the most hypoxia-selective compound in this series.

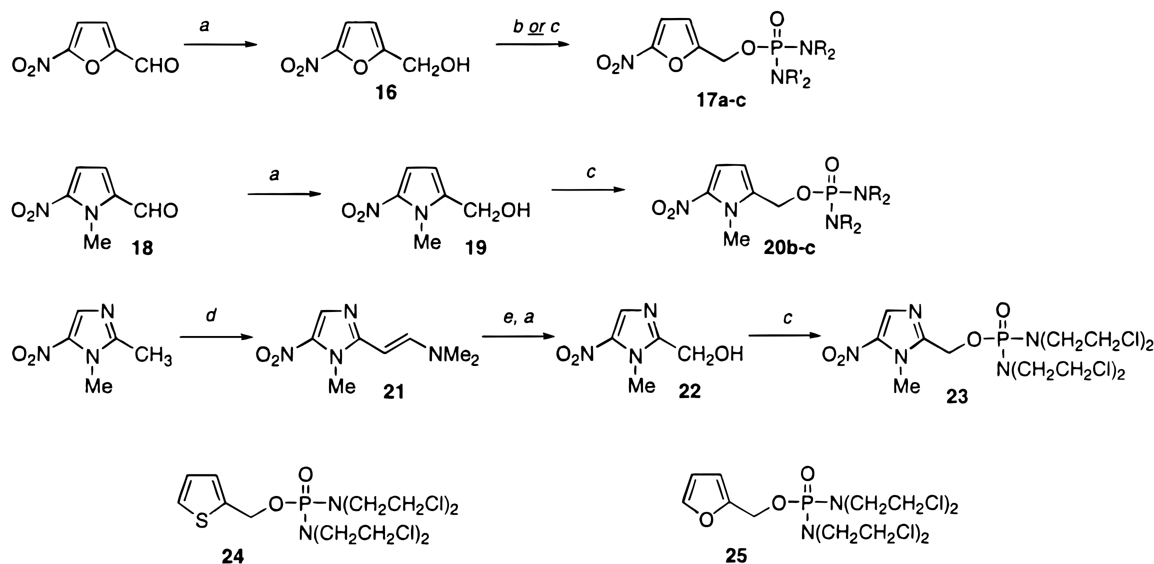
Compounds **3b** and **17a** were evaluated in the NCI human tumor cell line in vitro screen. Both compounds were broadly cytotoxic in this assay, with mean LC_{50} values of 20 and 25 μM, respectively. However, there was only modest cytotoxic selectivity among the nine cell line panels. The cytotoxicity results were also examined to determine whether there was a correlation with expression levels of the reducing enzymes DT diaphorase, cytochrome b_5 reductase, or cytochrome P450 reductase in these cell lines. The Pearson correla-

Scheme 4[a]



[a]Reagents: a) Ac_2O, H_2SO_4; b) HNO_3, Ac_2O; c) $NaBH_4$, THF/H_2O; d) $Cl_2PON(CH_2CH_2Br)_2$, then NH_3; e) Me_2NH, Et_3N; f) CuCN, DMF; g) H_2SO_4

Scheme 5[a]



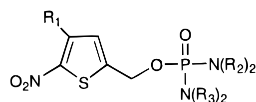
[a]Reagents: a) $NaBH_4$, THF/H_2O; b) Cl_2PONR_2, then NH_3; c) $ClP(NR_2)_2$, then t-BuOOH; d) $HC(OEt)_2NMe_2$; e) OsO_4, $NaIO_4$

tion coefficients for **3b** were 0.18, 0.02, and 0.17, respectively, and the coefficients for **17a** were 0.22, 0.09, and 0.18, respectively, suggesting that none of these enzymes is primarily responsible for the aerobic toxicity of the compounds.

Reductive Activation. To confirm that reduction of the nitroheterocycle leads to expulsion and activation of the phosphoramidate, nitrothienyl phosphoramidate **3a** was reduced with sodium dithionite (acetonitrile/cacodylate buffer, pH 7.4, 37 °C) and the reaction monitored by ^{31}P NMR. In the absence of reducing agent, **3a** is stable under these conditions for >12 h. Addition of sodium dithionite to the solution resulted in rapid disappearance of the resonance corresponding to **3a** at −5.8 ppm and the appearance of the resonance of the bis(chloroethyl)phosphoramidate anion (−12.2 ppm); this conversion was complete within 4 min. The phosphoramidate anion subsequently disappeared with $t_{1/2} \sim 23$ min as previously reported.[12]

Other mechanisms for the activation of these compounds might also be considered. For example, nucleophilic attack by glutathione at the methylene carbon with or without catalysis by glutathione S-transferases would also lead to expulsion of the phosphoramidate anion. Two pieces of evidence argue against this hypothesis. First, a possible correlation was explored

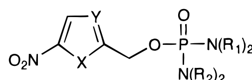
Table 1. Cytotoxicity of Nitrothienyl Phosphoramidates in Vitro[a]



compd	R_1	R_2	R_3	LC_{99} (μM)[b] B16[c]	MCF-7/WT[c]	MCF-7/CP[c]	HT-29/O_2[d]	HT-29/N_2[d]	ratio
3a	H	CH_2CH_2Cl	H	5.0	10.7	9.9	nd	nd	
3b	H	CH_2CH_2Br	H	1.8	6.1	4.1	1.4	0.18	7.8
3c	H	CH_2CH_2Cl	CH_2CH_2Cl	5.1	7.9	6.5	10.8	6.2	1.7
3d	H	CH_2CH_2Br	CH_2CH_2Br	1.1	2.4	1.9	nd	nd	
6	Br	CH_2CH_2Br	H	0.44	0.62	1.3	0.91	0.25	3.6
9	NMe_2	CH_2CH_2Br	H	52	88	110	180	8.9	20
12	CN	CH_2CH_2Br	H	0.45	0.11	0.24	0.62	0.22	2.8
15	$CONH_2$	CH_2CH_2Br	H	1.4	1.6	1.5	0.73	0.33	2.2
24[e]				120	211	205	nd	nd	
4-HC[f]				11	14	145	nd	nd	

[a] See Experimental Section for details of the cytotoxicity assays. nd = not determined. [b] Concentration that reduces clonogenic survival by 2 log units (99%). [c] 2-h drug treatment. [d] 4-h drug treatment. [e] See Scheme 5 for structure. [f] 4-Hydroperoxycyclophosphamide.

Table 2. Cytotoxicity of Nitroheterocyclic Phosphoramidates in Vitro[a]



compd	X	Y	R_1	R_2	LC_{99} (μM)[b] B16[c]	MCF-7/WT[c]	MCF-7/CP[c]	HT-29/O_2[d]	HT-29/N_2[d]	ratio
17a	O	CH	CH_2CH_2Br	H	2.0	4.5	2.9	1.8	0.22	8.2
17b	O	CH	CH_2CH_2Cl	CH_2CH_2Cl	9.3	20	22	12	3.4	3.5
17c	O	CH	CH_2CH_2Br	CH_2CH_2Br	1.7	1.9	2.3	nd	nd	
20b	NMe	CH	CH_2CH_2Cl	CH_2CH_2Cl	70	205	245	nd	nd	
20c	NMe	CH	CH_2CH_2Br	CH_2CH_2Br	14	57	51	nd	nd	
23	NMe	N	CH_2CH_2Cl	CH_2CH_2Cl	150	>600	>600	>600	28	>20
25[e]					53	300	230	nd	nd	

[a] See Experimental Section for details of the cytotoxicity assays. nd = not determined. [b] Concentration that reduces clonogenic survival by 2 log units (99%). [c] 2-h drug treatment. [d] 4-h drug treatment. [e] See Scheme 5 for structure.

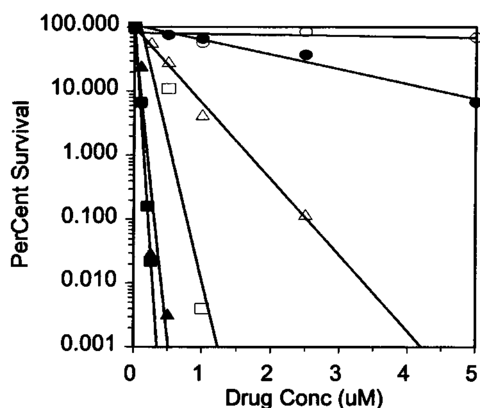


Figure 1. Clonogenic survival of HT-29 cells under aerobic (open symbols) or hypoxic (closed symbols) conditions following 4-h drug treatment: (○,●) **9**; (△,▲) **3b**; (□,■) **12**. See Experimental Section for details of the cytotoxicity assays.

between the NCI cytotoxicity data and GST-P or GST-M mRNA expression or glutathione levels in the cell lines. The Pearson correlation coefficients for **3b** were −0.19, −0.14, and 0.02, respectively; similar values were obtained for **17a**. These results suggest that cytotoxicity is independent of GST expression and GSH levels. Second, the reaction of **17a** and glutathione (40 mM, 3-fold excess, pH 7.4, 37 °C) was monitored by ^{31}P NMR as described above. Under these conditions **17a** was stable for >2 h, demonstrating that glutathione does not catalyze expulsion of the phosphoramidate under model physiological conditions.

Conclusions

A novel series of reductively activated cytotoxic phosphoramidates with moderate hypoxic selectivity has been prepared. We have demonstrated that the nitro group enhances cytotoxicity and that chemical reduction leads to expulsion of the cytotoxic phosphoramide mustards. Electron-withdrawing substituents on the nitroheterocycle increase cytotoxicity under aerobic conditions but decrease hypoxic selectivity as a result. The proposed mechanism of aerobic toxicity is based on reductive activation, but the activating enzymes have not been identified. No correlation was observed between growth inhibition or toxicity of **3b** or **17a** and DT diaphorase, cytochrome P450 reductase, or cytochrome b_5 reductase enzyme activities in the NCI cell line panel. Although an alternate activation mechanism involving nucleophilic substitution at the methylene carbon by glutathione might be considered, cytotoxicity did not correlate with GST expression or with cellular GSH levels. A bioreductive drug with exceptional hypoxic selectivity must be evaluated in combination with other drugs that will kill the rapidly proliferating tumor cell population. However, highly potent and moderately selective drugs such as **3b** or **17a** offer promise as single agents because of their ability to kill both aerobic and hypoxic cell populations. Compounds **3b** and **17a** have been selected for further preclinical antitumor and

toxicologic evaluation; the results of these studies will be reported in due course.

Experimental Section

NMR spectra were recorded on a Bruker WP-270SY spectrometer. ^1H chemical shifts are reported relative to internal TMS in the following format: chemical shift (number of protons, multiplicity). ^{31}P NMR spectra were recorded at 109.368 MHz with broadband ^1H decoupling using a 10-mm probe. Chemical shifts are reported relative to 1% triphenylphosphine oxide in toluene-d_8 as a coaxial reference. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN, or Midwest Microlab, Indianapolis, IN. Mass spectral data were obtained from the Mass Spectrometry Facility in the School of Pharmacy, University of California, San Francisco, CA. Melting points were measured on a Meltemp apparatus and are uncorrected. Chromatographic separations were carried out using flash chromatography on silica gel grade 60. Thin-layer chromatography was performed on Analtech silica gel plates (250 μm). All reactions were carried out in flame-dried flasks under nitrogen atmosphere unless specified otherwise or reagents containing water were used.

***N,N*-Bis(2-bromoethyl)phosphoramidic Dichloride (1b).** Bis(2-bromoethyl)amine hydrobromide (1.0 g, 3.2 mmol) was suspended in CH_2Cl_2 (22 mL) and cooled to −40 °C. Phosphorus oxychloride (0.3 mL, 3.2 mmol) was added dropwise, and a solution of triethylamine (1.2 mL, 8.6 mmol) in CH_2Cl_2 (3 mL) was added dropwise over 5 min. The mixture was warmed to 0 °C over 2 h and the reaction was quenched by addition of saturated NH_4Cl (20 mL). The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were dried (Na_2SO_4), the filtrate was concentrated under reduced pressure and the residue was purified by chromatography (3:1 hexanes:ethyl acetate) to give the product **1b** as a white solid (761 mg, 68%): mp 43−44 °C; R_f = 0.57 (3:1 hexanes:ethyl acetate); ^1H NMR ($CDCl_3$) δ 3.70 (4H, dt), 3.55 (4H,t); ^{31}P NMR ($CDCl_3$) δ −8.09.

5-Nitro-2-hydroxymethylthiophene (2). 5-Nitrothiophene-2-carboxaldehyde (2.21 g, 14 mmol) was dissolved in 20 mL of THF. Sodium borohydride (2.66 g, 70 mmol) in 5 mL of water was added slowly to the THF solution at 0 °C. After the addition, the ice bath was removed, and the reaction mixture was allowed to warm to room temperature and stirred for 1 h. Water (10 mL) was added and the reaction mixture was extracted with ethyl acetate (2 × 30 mL). The combined organic extracts were washed with saturated aqueous NaCl (2 × 40 mL) and dried ($MgSO_4$). The filtrate was evaporated to give the product (1.61 g, 73%) as a viscous oil: R_f = 0.55 (CH_2Cl_2); ^1H NMR ($CDCl_3$) δ 7.79 (1H, d), 6.92 (1H, d), 4.86 (2H, s), 3.08 (1H, bs).

(5-Nitro-2-thienyl)methyl *N,N*-Bis(2-bromoethyl)phosphorodiamidate (3b). Lithium bis(trimethylsilyl)amide (17.25 mL, 17.25 mmol, 1.0 M in THF) was added slowly to **2** (2.5 g, 15.7 mmol) in THF (50 mL) at −78 °C. After stirring for 2−3 min, a solution of **1b** (6.0 g, 17.2 mmol) in THF (4 mL) was added in one portion at −78 °C. The reaction mixture was stirred at −78 °C for 50 min and then warmed rapidly to −20 °C. Anhydrous gaseous ammonia was passed through the reaction mixture for 8−10 min. The reaction mixture was stirred for 10 min at −20 °C, water (40 mL) was added, and the reaction mixture was warmed to room temperature and extracted with $CHCl_3$ (20 mL). The aqueous layer was extracted with ethyl acetate (3 × 40 mL), and the combined organic extracts were washed with saturated aqueous NaCl, dried (Na_2SO_4) and evaporated. The crude product was purified by column chromatography (24:1 $CHCl_3$:methanol) to give **3b** (5.60 g, 72%) as a tan solid: mp 103−104 °C; R_f = 0.35 (24:1 $CHCl_3$:methanol); ^1H NMR ($CDCl_3$) δ 7.83 (1H, d), 7.03 (1H, d), 5.21 (2H, m), 3.52 (8H, m), 2.92 (2H, bs); ^{31}P NMR (methanol) δ −5.97. Anal. ($C_9H_{14}Br_2N_3O_4PS$) C, H, N.

(5-Nitro-2-thienyl)methyl *N,N*-bis(2-chloroethyl)phosphorodiamidate (3a) was prepared as described above from **2** and **1a** on a 1.0-mmol scale to give **3a** as an oil (0.31 g, 72%): R_f = 0.42 (ethyl acetate); ^1H NMR ($CDCl_3$) δ 7.91(1H, d, J = 3.9 Hz), 7.03 (1H, d, J = 3.9 Hz), 5.17 (2H, m), 3.71 (4H, m), 3.45 (4H, m), 2.96 (2H, bs); ^{31}P NMR ($CDCl_3$) δ −8.52. Anal. ($C_9H_{14}Cl_2N_3O_4PS$) C, H, N.

(5-Nitro-2-thienyl)methyl *N,N,N',N'*-Tetrakis(2-chloroethyl)phosphorodiamidate (3c). Phosphorus trichloride (10 mL, 2.0 M in CH_2Cl_2, 20 mmol) was dissolved in CH_2Cl_2 (200 mL). Bis(2-chloroethyl)amine hydrochloride (8.0 g, 45 mmol) was added with stirring at −10 °C. Triethylamine (17 mL, 122 mmol) was added over 5 min with vigorous agitation at −10 °C and the reaction mixture was stirred for an additional 10 min. A solution of **2** (3.0 g, 19 mmol) in a minimum of dry THF was added, and the mixture was stirred at −10 °C for 30 min. The reaction mixture was cooled to −78 °C and a stream of ozone was bubbled through the reaction mixture for 30 min. The reaction mixture was allowed to warm to room temperature and concentrated. The residue was chromatographed (1:1 hexanes:ethyl acetate) to give **3c** (2.19 g, 42%) as a viscous oil: R_f = 0.28 (1:1 hexanes:ethyl acetate); ^1H NMR ($CDCl_3$) δ 7.83 (1H, d, J = 4.1 Hz), 7.05 (1H, d, J = 4.1 Hz), 5.24 (2H, d, J = 7.7 Hz), 3.66 (8H, m), 3.46 (8H, m); ^{31}P NMR ($CDCl_3$) δ −8.08. Anal. ($C_{13}H_{20}Cl_4N_3O_4PS$) C, H, N.

(5-Nitro-2-thienyl)methyl *N,N,N',N'*-tetrakis(2-bromoethyl)phosphorodiamidate (3d) was prepared on a 3.0-mmol scale as described for **3c** above except that bis(2-bromoethyl)amine hydrobromide was used. The product was chromatographed to give **3d** (1.05 g, 53%) as a brown oil: R_f = 0.30 (1:1 hexanes:ethyl acetate); ^1H NMR ($CDCl_3$) δ 7.84 (1H, d), 7.06 (1H, d), 5.23 (2H, d), 3.50 (16H, m); ^{31}P NMR ($CDCl_3$) δ −8.73. Anal. ($C_{13}H_{20}Br_4N_3O_4PS$) C, H, N.

5-Nitro-4-bromo-2-thiophenecarboxaldehyde Diacetate (4). 4-Bromo-2-thiophenecarboxaldehyde[6] (5.6 g, 29.3 mmol) was dissolved in acetic anhydride (40 mL) and concentrated H_2SO_4 (3 drops) was added. The solution was stirred at room temperature for 1 h and then poured into ice water. The aqueous mixture was extracted with ether (3 × 30 mL). The organic extracts were washed with 10% K_2CO_3 and water, dried ($MgSO_4$) and evaporated to afford 4-bromo-2-thiophenecarboxaldehyde diacetate (8.07 g, 94%) as a colorless oil: R_f = 0.55 (7:1 hexanes:ethyl acetate); ^1H NMR ($CDCl_3$) δ 7.83 (1H, s), 7.27 (1H, s), 7.17 (1H, s), 2.14 (6H, s). A portion of the crude product (4.92 g, 16.8 mmol) in 6.7 mL of acetic anhydride (6.7 mL) was added dropwise to a solution of fuming nitric acid (1.0 mL, d 1.49−1.50) in glacial acetic acid (8.3 mL) at 0 °C. The mixture was stirred at room temperature for 2.5 h and then poured over crushed ice. The yellow precipitate was filtered, washed with water and dried to give **4** (5.45 g, 96%) as a yellow powder: mp 69−70 °C; R_f = 0.63 (3:1 hexanes: ethyl acetate); ^1H NMR ($CDCl_3$) δ 7.78 (1H, s), 7.23 (1H, s), 2.18 (6H, s).

5-Nitro-4-bromo-2-hydroxymethylthiophene (5). Sodium borohydride (0.3 g, 7.93 mmol) in water (5 mL) was added slowly to a solution of **4** (1.01 g, 2.99 mmol) in THF (50 mL) at 0 °C. The mixture was stirred at room temperature for 30 min. Water (40 mL) was added and the mixture was extracted with ethyl acetate (3 × 30 mL). The organic extracts were washed with saturated aqueous NaCl, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography (1:1 hexanes:ethyl acetate) to give **5** (0.66 g, 93%) as a yellow solid: mp 84−86 °C; R_f = 0.55 (1:1 hexanes:ethyl acetate); ^1H NMR ($CDCl_3$) δ 7.00 (1H, s), 4.88 (2H, s), 2.00 (1H, bs).

(5-Nitro-4-bromo-2-thienyl)methyl *N,N*-Bis(2-bromoethyl)phosphorodiamidate (6). Lithium bis(trimethylsilyl)amide (2.9 mL, 2.9 mmol, 1.0 M in THF) was added slowly to a solution of **5** (0.63 g, 2.65 mmol) in THF (10 mL) at −78 °C. After stirring for 2 min, the alkoxide solution was added to a solution of **1b** (1.08 g, 3.1 mmol) in THF (10 mL) at −78 °C. The reaction mixture was stirred at −78 °C for 50 min and then warmed to −20 °C. Anhydrous gaseous ammonia was passed through the reaction mixture for 10 min; the mixture was stirred for an additional 10 min and then allowed to warm to room temperature. Water (10 mL) was added and the reaction mixture was extracted with ethyl acetate (3 × 15 mL). The combined organic extracts were washed with saturated

aqueous NaCl (3 × 30 mL) and dried ($MgSO_4$). The filtrate was evaporated and the crude product was purified by column chromatography (20:1 ethyl acetate:methanol) to give **6** as an oil (0.91 g, 65%): R_f = 0.55 (ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.08 (1H, s), 5.16 (2H, s), 3.52 (8H, m), 2.50 (2H, bs); ^{31}P NMR (methanol) δ −5.77; HRMS ($C_9H_{14}Br_3N_3O_4PS$) (M + H$^+$) calcd 527.7993, found 527.7974.

5-Nitro-4-dimethylamino-2-thiophenecarboxaldehyde (7). Triethylamine (8.7 mL, 62.4 mmol) was added to a mixture of **4** (0.70 g, 2.07 mmol) and dimethylamine hydrochloride (1.70 g, 20.8 mmol) in $CHCl_3$ (30 mL).[7] The mixture was refluxed for 2 h. Water (20 mL) was added and the layers were separated. The aqueous layer was extracted with CH_2Cl_2. The combined organic extracts were washed with saturated aqueous NaCl solution, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography (1:1 hexanes:ethyl acetate) to give **7** (0.30 g, 73%) as a yellow solid: mp 150−153 °C; R_f = 0.44 (1:1 hexanes:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 9.87 (1H, s), 7.37 (1H, s), 3.15 (6H, s).

5-Nitro-4-dimethylamino-2-hydroxymethylthiophene (8). Sodium borohydride (0.10 g, 2.64 mmol) in water (5 mL) was added slowly to a solution of **7** (0.51 g, 2.55 mmol) in THF (40 mL) at 0 °C. The mixture was stirred at room temperature for 50 min. Water (20 mL) was added, and the mixture was extracted with ethyl acetate (3 × 20 mL). The organic extracts were washed with saturated aqueous NaCl, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography (ethyl acetate) to give **48** (0.42 g, 82%) as a yellow solid: mp 100−103 °C; R_f = 0.60 (ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 6.66 (1H, s), 4.75 (2H, s), 3.13 (6H, s), 2.18 (1H, bs).

(5-Nitro-4-dimethylamino-2-thienyl)methyl *N,N*-bis(2-bromoethyl)phosphorodiamidate (9) was prepared and purified on a 2.0-mmol scale as described for **6** to give 660 mg (67%) of **9** as an amorphous solid: R_f = 0.53 (20:1 ethyl acetate: methanol); ^{1}H NMR ($CDCl_3$) δ 6.68 (1H, s), 5.08 (2H, m), 3.50 (8H, m), 3.13 (6H, s), 2.75 (2H, bs); ^{31}P NMR (methanol) δ −5.88; HRMS ($C_{11}H_{20}Br_2N_4O_4PS$) (M + H$^+$) calcd 492.9311, found 492.9290.

5-Nitro-4-cyano-2-thiophenecarboxaldehyde Diacetate (10). CuCN (0.56 g, 6.21 mmol) was added to a solution of **4** (1.40 g, 4.14 mmol) in DMF (25 mL).[8] The mixture was refluxed for 1.5 h and then poured into a solution of ferric chloride (4.2 g) in cold 15% HCl (20 mL). The aqueous mixture was extracted with ethyl acetate (3 × 20 mL). The organic extracts were washed with saturated aqueous NaCl, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography (3:1 hexanes:ethyl acetate) to afford **10** (0.95 g, 81%) as an amorphous solid: R_f = 0.45 (3:1 hexanes:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.79 (1H, s), 7.47 (1H, s), 2.19 (6H, s).

5-Nitro-4-cyano-2-hydroxymethylthiophene (11). Sodium borohydride (0.4 g, 10.6 mmol) in water (1.5 mL) was added slowly to a solution of **10** (1.34 g, 4.72 mmol) in THF (50 mL) at 0 °C. The mixture was stirred at room temperature for 1 h. Water (40 mL) was added and the mixture was extracted with ethyl acetate (3 × 30 mL). The organic extracts were washed with saturated aqueous NaCl, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography (1:1 hexanes:ethyl acetate) to give **11** (0.76 g, 88%) as a yellow solid: mp 50−52 °C; R_f = 0.45 (1:1 hexanes:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.20 (1H, s), 4.94 (2H, s), 2.33 (1H, bs).

(5-Nitro-4-cyano-2-thienyl)methyl *N,N*-bis(2-bromoethyl)phosphorodiamidate (12) was prepared on a 2.0-mmol scale as described for **6** and purified by chromatography (ethyl acetate) to give 600 mg (64%) of **12** as an oil: R_f = 0.48 (ethyl acetate); ^{1}H NMR (5% DMSO-d_6 in $CDCl_3$) δ 7.37 (1H, s), 5.21 (2H, m), 4.13 (2H, bs), 3.51 (8H, m); ^{31}P NMR (methanol) δ −5.70; HRMS ($C_{10}H_{14}Br_2N_4O_4PS$) (M + H)$^+$ calcd 474.8841, found 474.8825.

5-Nitro-4-carbamoyl-2-thiophenecarboxaldehyde (13). Compound **10** (1.44 g, 5.07 mmol) was added to 97% sulfuric acid (20 mL).[9] The mixture was stirred at 75−80 °C for 1 h, cooled, and poured slowly into crushed ice. The aqueous mixture was extracted with ethyl acetate (3 × 30 mL). The combined organic extracts were washed with saturated aqueous NaCl, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography (ethyl acetate) to give **13** (0.66 g, 65%) as a yellow solid: mp 151−154 °C; R_f = 0.57 (ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 10.07 (1H, s), 8.06 (1H, s), 7.65 (1H, bs), 7.31 (1H, bs).

5-Nitro-4-carbamoyl-2-hydroxymethylthiophene (14). Sodium borohydride (0.5 g, 13.2 mmol) in water (2 mL) was added slowly to a solution of **13** (0.40 g, 2.0 mmol) in THF (20 mL) at 0 °C. The mixture was stirred at room temperature for 30 min. Water (10 mL) was added and the mixture was extracted with ethyl acetate (3 × 20 mL). The organic extracts were washed with saturated aqueous NaCl, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography (ethyl acetate) to give **14** as a yellow oil (0.33 g, 82%): R_f = 0.35 (ethyl acetate); ^{1}H NMR (acetone-d_6) δ 7.52 (1H, s), 7.14 (1H, s), 7.07 (1H, s), 5.21 (1H, t), 4.86 (2H, d).

(5-Nitro-4-carbamoyl-2-thienyl)methyl *N,N*-bis(2-bromoethyl)phosphorodiamidate (15) was prepared and purified on a 2-mmol scale as described for **6** to give 600 mg (61%) of **15** as an amorphous solid: R_f = 0.40 (20:1 ethyl acetate: methanol); ^{1}H NMR (10% DMSO-d_6 in $CDCl_3$) δ 7.72 (1H, bs), 7.46 (1H, s), 6.98 (1H, bs), 5.15 (2H, m), 4.30 (2H, bs), 3.50 (8H, m); ^{31}P NMR (methanol) δ −5.75. Anal. ($C_{10}H_{15}Br_2N_4O_5PS$) C, H, N.

5-Nitro-2-hydroxymethylfuran (16). 5-Nitro-2-furaldehyde (8.04 g, 57 mmol) was dissolved in THF (100 mL). Sodium borohydride (1.80 g, 47.6 mmol) in water (8 mL) was added slowly to the THF solution at 0 °C. After the addition, the ice bath was removed and the reaction mixture was allowed to warm to room temperature and stir for 1 h. Water (50 mL) was added and the reaction mixture was extracted with ethyl acetate (3 × 30 mL). The combined organic extracts were washed with saturated aqueous NaCl, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography (1:1 hexanes:ethyl acetate) to give **16** (7.55 g, 93%) as a tan solid: mp 30−32 °C; R_f = 0.59 (1:1 hexanes:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.29 (1H, d), 6.56 (1H, d), 4.73 (2H, s), 2.40 (1H, bs).

(5-Nitro-2-furyl)methyl *N,N*-bis(2-bromoethyl)phosphorodiamidate (17a) was prepared from **16** on a 14.3-mmol scale as described for **3b**. The crude product was purified by column chromatography (24:1 $CHCl_3$:methanol) to give **17a** (5.1 g, 83%) as a tan solid: mp 91−92 °C; R_f = 0.32 (24:1 $CHCl_3$:methanol); ^{1}H NMR ($CDCl_3$) δ 7.29 (1H, d), 6.68 (1H, d), 5.05 (2H, d), 3.50 (8H, m), 3.00 (2H, bs); ^{31}P NMR (methanol) δ −5.66. Anal. ($C_9H_{14}Br_2N_3O_5P$) C, H, N.

(5-Nitro-2-furyl)methyl *N,N,N',N'*-Tetrakis(2-chloroethyl)phosphorodiamidate (17b). Phosphorus trichloride (10.0 mL, 2.0 M in CH_2Cl_2, 20 mmol) was dissolved in CH_2Cl_2 (100 mL), and bis(2-chloroethyl)amine hydrochloride (8.0 g, 45 mmol) was added at −10 °C and stirred for 10 min. Alcohol **16** (2.60 g, 18.2 mmol) was dissolved in CH_2Cl_2 (50 mL), added at −10 °C and stirred for 30 min. The reaction mixture was cooled to −20 °C, and *tert*-butyl hydroperoxide (7.0 mL, 3.0 M in 2,2,4-trimethylpentane, 21 mmol) was added. The reaction mixture was allowed to warm to room temperature. Ethyl acetate (150 mL) was added, and the mixture was filtered through a short column of silica gel. The filtrate was evaporated and chromatographed (5% acetone:CH_2Cl_2) to give **17b** (4.22 g, 49%) as an oil: R_f = 0.55 (ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.29 (1H, d, J = 3.7 Hz), 6.68 (1H, d, J = 3.7 Hz), 5.10 (2H, d, J = 9.0 Hz), 3.65 (8H, m), 3.45 (8H, m); ^{31}P NMR ($CDCl_3$) δ −7.57. Anal. ($C_{13}H_{20}Cl_4N_3O_5P$) C, H, N.

(5-Nitro-2-furyl)methyl *N,N,N',N'*-tetrakis(2-bromoethyl)phosphorodiamidate (17c) was prepared on a 3-mmol scale and purified as described for **17b** to give 0.96 g (48%) of product as an oil: R_f = 0.6 (ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.33 (1H, d, J = 3.7 Hz), 6.68 (1H, d, J = 3.7 Hz), 5.11 (2H, d, J = 9.0 Hz), 3.49 (16H, m); ^{31}P NMR ($CDCl_3$) δ −8.40. Anal. ($C_{13}H_{20}Br_4N_3O_5P$) C, H, N.

2-Hydroxymethyl-1-methyl-5-nitropyrrole (19). A solution of 1-methyl-5-nitropyrrole-2-carboxaldehyde[10] (2.23 g, 14.4 mmol) was dissolved in methanol (50 mL) and THF (50 mL)

and cooled to 0 °C. Sodium borohydride (0.60 g, 15.9 mmol) was added in portions over 15 min, and the reaction was stirred for an additional 15 min while warming to room temperature. The reaction mixture was poured into water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The extract was dried ($MgSO_4$) filtered and evaporated and the crude product chromatographed (1:2 hexanes:ethyl acetate) to give **19** (1.67 g, 74%) as a yellow solid: mp 83−86 °C; R_f = 0.22 (1:2 hexanes:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.14 (1H, d), 6.17 (1H, d), 4.66 (2H, d), 4.01 (3H, s), 1.72 (1H, t).

(1-Methyl-5-nitro-2-pyrrolyl)methyl *N,N,N',N'*-tetrakis(2-chloroethyl)phosphoramidate (20b) was prepared on a 3-mmol scale from alcohol **19** as described for the preparation of **17b** to give the product (0.26 g, 18%) as an oil: R_f = 0.5 (1:1 hexanes:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.18 (1H, d), 6.32 (1H, d), 5.09 (2H, d), 4.02 (3H, s), 3.62 (8H, m), 3.4 (8H, m); ^{31}P NMR ($CDCl_3$) δ −8.74. Anal. ($C_{14}H_{23}Cl_4N_4O_4P$) C, H, N.

(1-Methyl-5-nitro-2-pyrrolyl)methyl *N,N,N',N'*-tetrakis(2-bromoethyl)phosphoramidate (20c) was prepared on a 2.7-mmol scale from alcohol **19** as described for the preparation of **17b** to give the product (0.23 g, 18%) as an oil: R_f = 0.42 (1:2 hexanes:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.18 (1H, d), 6.34 (1H, d), 5.11 (2H, d), 4.03 (3H, s), 3.45 (16H, m); ^{31}P NMR ($CDCl_3$) δ −8.89. Anal. ($C_{14}H_{23}Br_4N_4O_4P$) C, H; N: calcd, 8.46; found, 7.97.

2-(2-*N,N*-Dimethylaminovinyl)-1-methyl-5-nitroimidazole (21). 1,2-Dimethyl-5-nitroimidazole (20.0 g, 142 mmol) was dissolved in a mixture of DMF (50 mL) and dimethylformamide dimethyl acetal (50 mL).[11] Trifluoroacetic acid (1.0 mL) was added and the reaction mixture was heated at 100 °C for 12 h. The reaction mixture was cooled to room temperature and then maintained at −20 °C for 12 h. The product was collected by filtration, washed with ether (2 × 50 mL) and dried under vacuum to give **21** (25.5 g, 91%) as red needles: mp 91 °C; ^{1}H NMR ($CDCl_3$) δ 8.01 (1H, s), 7.88 (1H, d), 4.71 (1H, d), 3.83 (3H, s), 3.01 (6H, s).

2-Hydroxymethyl-1-methyl-5-nitroimidazole (22). Compound **21** (7.0 g, 35.7 mmol) was dissolved in a mixture of CH_2Cl_2 (180 mL) and methanol (20 mL) and cooled to −78 °C. Ozone was bubbled through the solution until the deep red color faded to pale yellow (ca. 2 h). Dimethyl sulfide (5.30 mL, 72.2 mmol) was added at −78 °C and the reaction mixture was allowed to warm to room temperature overnight. The mixture was evaporated to a dark red oil which was dissolved in CH_2Cl_2 (200 mL) and filtered through a short column of silica gel. The column was washed with CH_2Cl_2 (200 mL), and the combined solutions were evaporated to give the aldehyde (2.84 g, 51%) as a solid: mp 91 °C; R_f = 0.65 (3:1 hexanes: ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 9.96 (1H, s), 8.13 (1H, s), 4.39 (3H, s). A portion of the crude aldehyde (0.50 g, 3.22 mmol) was dissolved in absolute ethanol (10 mL). Sodium borohydride (0.061 g, 1.6 mmol) was added followed by CH_2Cl_2 (40 mL). The mixture was stirred for 1 h at room temperature, water (2 × 50 mL) was added, and the layers were separated. The organic solution was dried ($MgSO_4$), filtered and evaporated to give **22** (0.31 g, 61%) as a tan solid: mp 112 °C; R_f = 0.31 (ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.92 (1H, s), 4.77 (2H, s), 4.05 (3H, s).

(1-Methyl-5-nitro-2-imidazoyl)methyl *N,N,N',N'*-Tetrakis(2-chloroethyl)phosphoramidate (23). Phosphorus trichloride (2.5 mL, 2.0 M in CH_2Cl_2, 5 mmol) was added to CH_2Cl_2 (25 mL). Bis(2-chloroethyl)amine hydrochloride (1.8 g, 10 mmol) was added with stirring at room temperature. Triethylamine (4.2 mL, 30 mmol) was added dropwise over 10 min and stirred 10 min at room temperature. Compound **22** (0.64 g, 4.1 mmol) in CH_2Cl_2 (5 mL) was added and stirred for 30 min. The reaction mixture was cooled to −20 °C. *tert*-Butyl hydroperoxide (1.7 mL, 3.0 M in 2,2,4-trimethylpentane, 5.1 mmol) was added, and the reaction mixture was maintained overnight at −20 °C. The reaction mixture was poured into 10% hydrochloric acid, the organic layer separated, dried ($MgSO_4$), filtered through a short column of silica gel and evaporated. The residue was chromatographed (1:1 hexanes: ethyl acetate) to give **23** (0.89 g, 37%) as a brown oil: R_f = 0.31 (1:1 hexanes:ethyl acetate); ^{1}H NMR (acetone-d_6) δ 7.29 (1H, s), 5.29 (2H, d), 4.13 (3H, s), 3.76 (8H, m), 3.47 (8H, m); ^{31}P NMR ($CDCl_3$) δ −7.69. Anal. ($C_{13}H_{22}Cl_4N_5O_4P$) C, H; N: calcd, 14.44; found, 13.99.

2-Thienylmethyl *N,N,N',N'*-tetrakis(2-chloroethyl)phosphorodiamidate (24) was prepared on a 5-mmol scale from 2-hydroxymethylthiophene as described for the preparation of **17b** to give the product (0.94 g, 43%) as an oil: R_f = 0.3 (2:1 hexanes:ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.39 (1H, d), 7.12 (1H, d), 7.01 (1H, m), 5.23 (2H, d), 3.65 (8H, m), 3.38 (8H, m); ^{31}P NMR ($CDCl_3$) δ −9.21. Anal. ($C_{13}H_{21}Cl_4N_2O_2PS$) C, H, N.

2-Furylmethyl *N,N,N',N'*-tetrakis(2-chloroethyl)phosphorodiamidate (25) was prepared on a 5-mmol scale from furfuryl alcohol as described for the preparation of **17b** to give the product (1.36 g, 64%) as an oil: R_f = 0.36 (4:1 hexanes: ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 7.47 (1H, d), 6.44 (1H, d), 6.38 (1H, m), 5.02 (2H, d), 3.60 (8H, m), 3.35 (8H, m); ^{31}P NMR ($CDCl_3$) δ −8.91. Anal. ($C_{13}H_{21}Cl_4N_2O_3P$) C, H, N.

Cytotoxicity Studies in Vitro. 1. B16 and MCF-7 Cell Lines. The B16 murine melanoma cell line was obtained from National Cancer Institute, Bethesda, MD. Wild-type and 4-hydroxycyclophosphamide-resistant MCF-7 human breast carcinoma cell lines were kindly provided by Beverly Teicher, Dana-Farber Cancer Institute, Boston, MA. B16 cells were grown in Eagle's minimum essential medium supplemented with L-glutamine, antibiotics, and 10% fetal bovine serum (FBS). MCF-7 cells were grown in Dulbeccos's modified Eagle's medium supplemented with L-glutamine, penicillin−streptomycin, and 10% FBS. Clonogenic assays of cytotoxicity were initiated by suspension of the cells in appropriate unsupplemented medium at a final density of 2−30 000 cells/mL. Drug stock solutions were prepared in unsupplemented medium or ethanol depending on the solubility of the drug. Appropriate volumes of drug stock solutions were added to the cell suspensions to give final drug concentrations of 1−400 μM; the maximum drug concentration for each compound was selected to provide >99% cell kill. The drug-treated cell suspensions were incubated for 2 h at 37 °C and 5% CO_2. The cells were washed with supplemented medium (3 × 3 mL) and then resuspended in 5 mL of supplemented medium. Cells were counted, and the number of cells required for plating was determined with 2 or 3 different dilutions per drug concentration. Cells were plated at densities between 50 and 4 × 10^5 cells/mL (5 mL of medium/60-mm culture dish). B16 and MCF-7 colonies were stained with 0.5% crystal violet after 8 and 14 days, respectively. Colonies with 50 or more cells were counted. The ratio of surviving colonies relative to untreated controls were plotted as log(surviving fraction) vs drug concentration. The LC_{99} (the drug concentration required to reduce colony formation to 1% of controls) was determined by linear regression from these data.

2. Hypoxia-Selectivity Assay in HT-29 Cells. HT-29 human colon carcinoma cells were cultured in α-MEM supplemented medium with 10% FBS and antibiotics. Cytotoxicity was assayed in duplicate under both aerobic and hypoxic conditions as previously described; the maximum drug concentration for each compound was selected to provide >99% cell kill.[5c] The ratio of surviving colonies relative to untreated controls were plotted as log(surviving fraction) vs drug concentration. The LC_{99} (the drug concentration required to reduce colony formation to 1% of controls) was determined by linear regression from these data.

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