Synthesis and Evaluation of Pteroic Acid-Conjugated Nitroheterocyclic Phosphoramidates as Folate Receptor-Targeted Alkylating Agents

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A novel nitroheterocyclic bis(haloethyl)phosphoramidate prodrug linked through lysine to a pteroic acid has been prepared and evaluated as a potential alkylating agent to target tumor cells that overexpress the folate receptor. The prodrug exhibited IC_{50} values in the micromolar range and was 10−400-fold less cytotoxic in vitro than the phosphoramidate that lacks the lysine-pteroyl moiety. The data does not support a contribution of the folate receptor to cytotoxicity. In an attempt to determine the basis for the decreased cytotoxicity in the pteroyl-lysyl analogue, compounds were prepared in which the lysine-pteroyl moiety was replaced with lysine alone or with an *n*-propyl group. The *n*-propyl and the lysyl analogues were on average 3.8- and 21-fold less potent than the unsubstituted bis(haloethyl)phosphoramidate, respectively. Chemical reduction of the prodrugs followed by ^{31}P NMR kinetics demonstrated that all of the phosphoramidate anions cyclized to the aziridinium ion at similar rates and gave comparable product distributions, suggesting that changes in chemical activation did not account for the differences in cytotoxicity. It is likely that folate receptor-mediated transport is not sufficient to deliver adequate intracellular concentrations of the cytotoxic phosphoramide mustard.

Introduction

A recent approach to cancer treatment involves enhancement of the differential specificity of anticancer agents by selective targeting mechanisms. The vitamin folic acid has attracted considerable attention as a potential means of delivering covalently bound drug conjugates.[1] Folate uptake is mediated by a family of membrane receptors termed the folate-binding proteins (FBP). FBPs have K_D values for folate that vary from 10^{-9} to 10^{-11} M and exhibit a strong preference for folic acid over its reduced counterparts. Many human cancer cell lines have highly overexpressed levels of the protein that binds folic acid,[2] a finding which is being exploited with the preparation of folate−drug conjugates.[3]

Bioreductive activation is a well-established concept for achieving selective toxicity of antitumor agents to hypoxic cells.[4] Recently we reported that nitroheterocyclic phosphoramidates can be activated under both aerobic and hypoxic conditions.[5] These prodrugs were highly potent but only moderately hypoxia-selective. The activation of these prodrugs involves bioreduction of the nitro group and subsequent release of the cytotoxic phosphoramide mustard (PDA).[5,6] We have attempted to exploit the selective targeting afforded by folate−drug conjugates by combining a pteroic acid moiety with the high potency of the nitroheterocyclic phosphoramidate prodrugs to design a folate receptor-selective cytotoxic agent.

The prodrug includes several components: a nitroheterocyclic delivery group, an alkylating group, and a ligand for the folate receptor. When the nitroheterocyclic delivery group is bioreduced a phosphoramide mustard is released intracellularly; this process serves to activate the alkylating moiety. The pteroic acid ligand for the folate receptor is introduced to achieve folate receptor selectivity. It has been established that folate receptors have a structural requirement of a pteroyl moiety appended to the α-amino group of an amino acid containing a free α-carboxyl group that is essential for ligand binding.[7] With respect to the amino acid substituent, the receptor will tolerate extensive substitution. Lysine has been selected to connect the phosphoramidate to the pteroyl moiety. Lysine fulfills the α-amino acid requirement of the receptor when it is coupled to the pteroyl moiety, and at the same time the ϵ-amino group can be exploited to attach the phosphoramidate. Here we report the synthesis, evaluation in vitro, and activation mechanisms of substituted nitrofuryl phosphorodiamidates.

Results and Discussion

Chemistry. The mechanistic hypothesis underlying the proposed activation of these compounds is shown in Scheme 1. Enzymatic reduction is expected to proceed to the corresponding hydroxylamine derivative. Lone pair-assisted expulsion of the phosphorodiamidate anion from this intermediate will activate the alkylating moiety.

The nitrofuryl phosphorodiamidates **1a** and **1b** were prepared as outlined in Scheme 2. Lysine methyl ester (**7**) was reacted with the phosphoramidic chlorides **2a** and **2b** in the presence of diisopropylethylamine to give the lysine phosphoramidates **3a** and **3b**. The methyl esters were then hydrolyzed using pig liver esterase in acetone/phosphate buffer to give the free acids **4a** and **4b**. To confirm that phosphorylation took place on the ϵ-amino group of the lysine, an NMR decoupling experiment was carried out on **3b**. If phosphorylation occurred on the ϵ-amino group, irradiation of the protons on the

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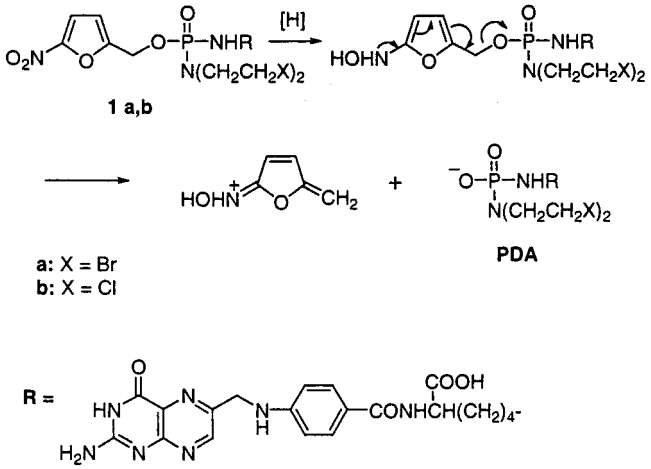
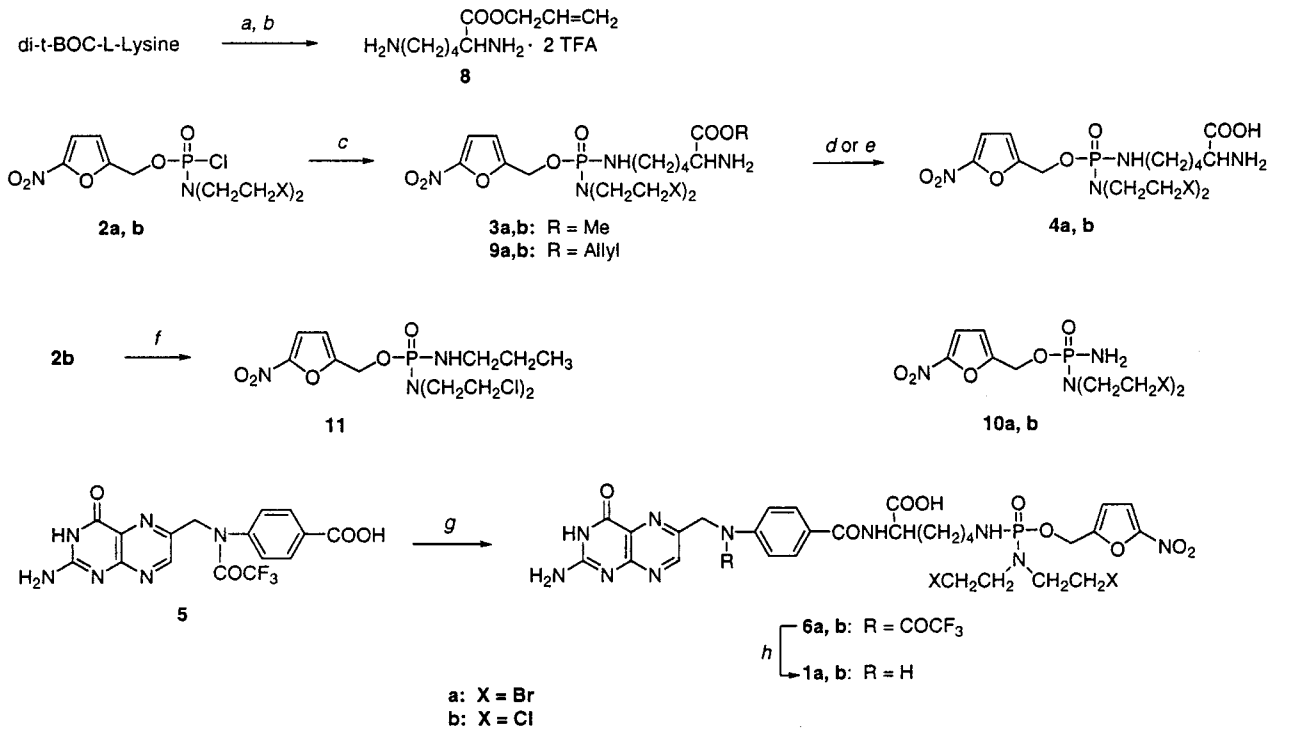
Scheme 1



δ-carbon should give a doublet for the ϵ-protons arising from three-bond proton−phosphorus coupling. If phosphorylation occurred on the α-amino group, however, the ϵ-protons should appear as a singlet following irradiation. In fact, irradiation of the δ-methylene at 1.6 ppm caused collapse of the ϵ-proton peak at 2.9 ppm to a doublet with $J = 9.7$ Hz, consistent with phosphorylation of the ϵ-amino group. Unfortunately, the yields for the enzymatic hydrolysis were not reproducible and ranged between 5% and 65%. An alternative route was then adopted (Scheme 2). The dicyclohexylammonium salt of di-*t*-Boc-lysine was converted to the allyl ester with allyl bromide in the presence of diisopropylethylamine, and the amine groups were deprotected with trifluoroacetic acid. The resulting lysine allyl ester **8** was then phosphorylated under the same conditions as described for the methyl ester. The allyl esters **9a** and **9b** were cleaved in a palladium-catalyzed reaction in the presence of sodium toluenesulfinate to give **4a** and **4b**, which were identical to the products obtained from the enzymatic cleavage of the methyl esters **3a** and **3b**. Coupling of the nitrofuryl lysylphosphoramidates **4a** and **4b** to 10-trifluoroacetylpteroic acid **5** was carried out using isobutyl chloroformate and triethylamine to give the pteroyllysyl intermediates **6a** and **6b**. Finally, the trifluoroacetyl group was removed using 3 equiv of potassium carbonate to give the prodrugs **1a** and **1b**. Compounds **10a** and **10b** were synthesized as previously described.[5] They are known to be highly cytotoxic and were used as positive controls in the cytotoxicity studies.

Biological Activity. The cytotoxicities of **1a** and **10a** were assessed against cell lines with overexpressed folate receptors (IGROV human ovarian carcinoma[8] and MDA231 human breast adenocarcinoma[9]) and against cell lines with low levels of folate receptors (A549 human lung carcinoma cells[3c] and A498 human kidney carcinoma cells[9]). To assess the contribution of folate transport to drug activity, cytotoxicity was compared using overexpressed receptor cell lines in folate-containing and folate-deficient media. Folate competes for the receptor and therefore inhibits receptor-mediated drug transport, so differences between folate-deficient and folate-containing media reflect the contribution of the folate transport system to drug activity. Cell viability was measured after 72-h drug incubation using the MTT assay;[10] the results are presented in Table 1. The cytotoxicity of **1a** is modest, with IC_{50} values in the low-micromolar range, and is 1−2 orders of magnitude less toxic than the unsubstituted prodrug **10a**. Although **1a** is somewhat more toxic to IGROV and MDA231 cells in folate-deficient compared to folate-containing media, the differences are much smaller than one would expect

Scheme 2[a]



[a] Reagents: (a) allyl bromide, *i*-Pr_2NEt, 70 °C; (b) TFA−CH_2Cl_2; (c) lysine methyl ester **7** or allyl ester **8**, *i*-Pr_2NEt; (d) pig liver esterase, 0.1 M KH_2PO_4, pH 7, 10% acetone; (e) $Pd(PPh_3)_4$, $ArSO_2Na$, THF/H_2O; (f) *n*-$PrNH_2$, *i*-Pr_2NEt; (g) *i*-BuOCOCl, Et_3N, then **4a** and **4b**; (h) K_2CO_3, MeOH/THF.

Table 1. Growth Inhibition of Human Tumor Cell Lines in Vitro[a]

	IC_{50} (μM)				
cell line	**1a**	**10a**	**4b**	**10b**	**11**
IGROV[b,c]	0.83	0.041			
IGROVfd[b,c]	0.57	0.049			
MDA231[c]	8.0	0.032	16	0.52	1.9
MDA231fd[b,c]	3.6	0.022			
A549[d]	11	0.27	41	0.33	1.9
A549fd[b,d]	23	0.26			
A498[d]	11	0.045	26	1.1	1.4
A498fd[b,d]	19	0.029			
UMUC3	5.2	0.013	0.62	0.055	0.72
HT29	0.30	0.060	20	0.38	17
PC3	11	0.25	21	1.0	2.3
PACA2	16	0.16	2.4	0.13	0.45

[a] See Experimental Section for details of the in vitro assays. [b] All cell lines designated "fd" were grown in folate-deficient medium. [c] IGROV and MDA231 cell lines have overexpressed folate receptors. [d] A549 and A498 cell lines have low levels of folate receptors.

if selective transport were essential for activity. We do not understand why folate receptor overexpression does not enhance the toxicity of these compounds. A diverse array of small and large molecules can be linked to folic acid to give conjugates with excellent receptor binding affinity, but the γ-carboxyl group is the only linkage site that provides high-affinity conjugates. Folate conjugates penetrate cells exclusively by receptor-mediated endocytosis;[1] although sequestration in vesicular structures is known to be a problem with folate−protein conjugates, free folic acid and low-molecular-weight conjugates are not sequestered.[3] Folate-mediated tumor targeting has been exploited for other low-molecular-weight antitumor agents, but these compounds must be linked via the γ-carboxyl group of pteroylglutamic acid.[11] Conceivably the γ-carboxyl group contributes to receptor binding, and the lysine conjugates are not transported because they lack the ester carbonyl group. The compounds were then examined in seven cell lines in folate-containing medium where folate-specific transport should not occur (see Table 1). The pteroate conjugate **1a** is significantly less cytotoxic than the unsubstituted nitrofuryl phosphoramidate **10a** in all of the cell lines tested. To determine the possible effects of phosphoramidate substitution on cytotoxicity, the unsubstituted, the lysyl, and the *n*-propyl bis(chloroethyl)phosphoramidates **10b**, **4b**, and **11**, respectively, were prepared and evaluated in vitro. Introduction of the *n*-propyl, the lysine, and the pteroyllysine groups increases the relative IC_{50} values by approximately 4-, 20-, and 64-fold, respectively (Table 1). The cytotoxicity of the nitrofuryl phosphoramidate prodrugs requires that the compounds undergo intracellular conversion to the phosphoramidate anion followed by cyclization to the reactive aziridinium ion.[5] To assess whether substitution influences the activation of these compounds, the chemical reduction and subsequent phosphoramidate anion chemistry were assessed using ^{31}P NMR.

^{31}P NMR Kinetics. Compound **4b** was reduced with sodium dithionite (cacodylate buffer, pH 7.4, 37 °C), and the reaction was monitored by ^{31}P NMR. Addition of sodium dithionite to the solution resulted in rapid disappearance of the resonance corresponding to **4b** (−5.7 ppm) and the appearance of the resonance for the substituted bis(chloroethyl)phosphoramidate anion (−11.5 ppm); the phosphoramidate anion subsequently disappeared with a half-life of 10.9 min. Similarly, reduction of **11** with sodium dithionite led to rapid disappearance of the resonance corresponding to **11** (−5.5 ppm) and the appearance of the analogous phosphoramidate anion (−11.9 ppm). This intermediate subsequently disappeared with a half-life of 13.0 min. Finally, dithionite reduction of **10b** (−5.5 ppm) led to formation of phosphoramide mustard (−12.3 ppm) which disappeared with a half-life of 18 min. These modest differences in rate of phosphoramidate activation are unlikely to account for the differences in cytotoxicity observed among the prodrugs. Furthermore, in each case the bis(chloroethyl)phosphoramidate reacts to give a mixture of products arising from initial formation of the aziridinium ion and subsequent reactions of water and other nucleophiles at both carbon and phosphorus.[12,13] Although there are minor changes in the product distribution of the phosphoramidate anions derived from **4b**, **10b**, and **11**, none of these changes would account for the observed differences in cytotoxicity. Therefore it is unlikely that differences in activation chemistry account for the variation in cytotoxicity among these prodrugs.

Conclusions

The pteroyllysine-conjugated nitroheterocyclic phosphoramidate **1a** showed modest cytotoxicity that was significantly diminished compared to that of the unsubstituted phosphoramidate **10a**; the basis for this diminished toxicity is not understood. Differences in cytotoxicity under folate-deficient conditions to cells that are high and low in folate receptor levels were modest, and addition of folate to the medium gave only a modest increase in the IC_{50} for the folate receptor-overexpressed cell lines. These results suggest that folate receptors are not contributing significantly to the intracellular delivery of the cytotoxin. The rates of phosphoramidate anion cyclization and product distribution were not significantly altered by the nature of the phosphoramidate substitution, indicating that the decreased cytotoxicity is not related to changes in prodrug activation. Finally, it is likely that successful development of a pteroate-conjugated drug that can exploit folate receptor overexpression will require delivery of a cytotoxin that is much more potent than a phosphoramide mustard.

Experimental Section

All ^{1}H NMR spectra were measured on a 250-MHz Bruker NMR system equipped with a Tecmag data acquisition system and 5-mm multinuclear probe. The NMR program MacNMR was used for acquisition and processing of data. ^{1}H chemical shifts are reported in parts per million from tetramethylsilane. All ^{31}P NMR spectra were obtained on the same instrument using broadband gated decoupling. Chemical shifts are reported in parts per million from a coaxial insert containing 5% phosphoric acid in H_2O (or in D_2O), except for ^{31}P kinetics experiments where a coaxial insert containing 1% triphenylphosphine oxide in benzene-d_6 was used. The chemical shift difference between phosphoric acid and triphenylphosphine oxide inserts is 25.2 ppm. ^{31}P NMR kinetics were carried out as described previously.[12]

Chromatographic separations were done on silica gel grade 60 or on 40-μm octadecyl (C_{18}) supports. High-performance liquid chromatography (HPLC) analyses were performed using a Beckman System Gold with a 126 solvent module, a 168 detector set to 313 nm, and an Econosphere C18 5-μm column (250 mm; Alltech Associates). Mass spectral data were ob-

tained from the Purdue University Mass Spectrometry Service, West Lafayette, IN, using electrospray (ESI) or fast atom bombardment (FAB) ionization. All mass spectral data for halogenated compounds show the appropriate isotope ratios; however only the lowest-molecular-weight peak is reported. All anhydrous reactions were carried out in oven-dried flasks under argon. Reagents and solvents were introduced via syringe where appropriate. All solvents were distilled prior to use over calcium hydride or sodium.

(5-Nitro-2-furyl)methyl N^{ϵ}-(*O*-Methyllysyl)-*N*,*N*-bis(2-bromoethyl)phosphorodiamidate (3a). Lysine methyl ester dihydrochloride was dried over P_2O_5 at 45 °C for 48 h and then dissolved (244 mg, 1.05 mmol) in dry acetonitrile (15 mL) and cooled to −40 °C under argon. Diisopropylethylamine (0.60 mL, 3.45 mmol) was added, followed by the addition of a small amount of 18-crown-6 to improve solubility. Phosphoramidic chloride **2a**[5] (476 mg, 1.05 mmol) was dissolved in dry acetonitrile (3 mL) and added to the reaction mixture. The mixture was warmed to −10 °C and allowed to stir for 3 h. The reaction was quenched with water at −10 °C and extracted with CH_2Cl_2 (3×). The combined organic layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. Reverse-phase chromatography using a gradient of acetonitrile/water:acetonitrile 20% to 80% afforded **3a** as a mixture of diastereomers (440 mg, 72%): ^{1}H NMR ($CDCl_3$) δ 7.31 (d, 1H), 6.75 (d, 1H), 5.08 (dd, 2H), 3.25−3.71 (bm, 12H), 2.95 (bs, 2H), 1.34−1.94 (bm, 6H); ^{31}P NMR ($CDCl_3$) δ 16.82; MS (ESI) *m*/*z* 577 $(M + H)^+$.

(5-Nitro-2-furyl)methyl N^{ϵ}-(*O*-methyllysyl)-*N*,*N*-bis(2-chloroethyl)phosphorodiamidate (3b) was prepared as described for **3a** (1.7 mmol scale) using bis(chloroethyl)-phosphoramidic chloride **2b**.[5] The crude product was purified by reverse-phase chromatography using a gradient of acetonitrile/water:acetonitrile 20% to 80% to give **3b** (582 mg, 70%) as a mixture of diastereomers: ^{1}H NMR ($CDCl_3$) δ δ 7.30 (d, 1H), 6.75 (d, 1H), 5.07 (dd, 2H), 3.26−3.70 (bm, 12H), 2.93 (bs, 2H), 1.34−1.93 (bp, 6H); ^{31}P NMR ($CDCl_3$) δ 17.26;. MS (ESI) *m*/*z* 489 $(M + H)^+$.

(5-Nitro-2-furyl)methyl N^{ϵ}-Lysyl-*N*,*N*-bis(2-bromoethyl)phosphorodiamidate (4a). Methyl ester **3a** (180 mg, 0.31 mmol) was dissolved in acetone (0.6 mL) and KH_2PO_4 (5.4 mL, 0.1 M, pH 7) was added with stirring. Pig liver esterase (800 units) was added to the reaction mixture, and it was stirred at room temperature for 36 h. During that time the pH was maintained by dropwise addition of 0.5 M NaOH (to a total of 1 equiv). The reaction progress was monitored using HPLC. When the reaction was complete, the solvent was removed under reduced pressure and the residue redissolved in methylene chloride. The suspension was filtered, evaporated and the residue was redissolved in acetonitrile and purified by reverse-phase chromatography using a gradient of acetonitrile/water:acetonitrile 20% to 80% to give **4a** as a mixture of diastereomers (116 mg, 66%): ^{1}H NMR ($CDCl_3$) δ 7.28 (d, 1H), 6.82 (d, 1H), 5.02 (dd, 2H), 3.18−3.79 (bm, 9H), 2.91 (bs, 2H), 1.30−1.90 (bm, 6H); ^{31}P NMR ($CDCl_3$) δ 17.07; MS (ESI) *m*/*z* 563 $(M + H)^+$.

(5-Nitro-2-furyl)methyl N^{ϵ}-lysyl-*N*,*N*-bis(2-chloroethyl)phosphorodiamidate (4b) was prepared as described for **4a** (0.37 mmol scale). The crude residue was purified by reverse-phase chromatography using a gradient of acetonitrile/water:acetonitrile 20% to 80% to give **4b** as a mixture of diastereomers (112 mg, 64%): ^{1}H NMR ($CDCl_3$) δ 7.25 (d, 1H), 6.69 (d, 1H), 4.96 (dd, 2H), 3.09−3.82 (bm, 9H), 2.93 (bs, 2H), 1.29−1.91 (bm, 6H); ^{31}P NMR ($CDCl_3$) δ 17.45; MS (ESI) ($C_{15}H_{25}N_4O_7PCl_2$) calcd 475.0916, obsd 475.0903.

Allyl Lysinate Bistrifluoroacetate (8). N^{α},N^{ϵ}-Di-*t*-Boc-lysine dicyclohexylammonium salt (2 g, 3.8 mmol) was dissolved in allyl bromide (10 mL), and diisopropylethylamine (1.32 mL, 7.58 mmol) was added to the solution. The mixture was heated for 2 h at 70−75 °C. It was diluted with ethyl acetate (200 mL) and washed with 0.1 N HCl (3 × 100 mL), saturated $NaHCO_3$ (3 × 100 mL), and saturated NaCl (3 × 100 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The resultant yellow oil (1.8 g) was dissolved in trifluoroacetic acid−methylene chloride (60 mL, 1:1 v/v), and the solution was stirred at room temperature for 50 min. The solution was concentrated under reduced pressure and the residue triturated repeatedly with diethyl ether. The product was isolated as a yellow oil (1.64 g, 86% overall yield): R_f = 0.28 ($CHCl_3$−MeOH−HOAc, 75:23:2); ^{1}H NMR (DMSO-d_6) δ 5.94 (m, 1H), 5.35 (dd, 2H), 4.70 (d, 2H), 4.06 (t, 1H), 2.75 (m, 2H), 1.17−1.92 (m, 6H); MS (ESI) *m*/*z* 187 $(M + H)^+$.

(5-Nitro-2-furyl)methyl N^{ϵ}-(*O*-Allyllysyl)-*N*,*N*-bis(2-bromoethyl)phosphorodiamidate (9a). Lysine ester **8** (445 mg, 1.08 mmol) was dissolved in acetonitrile (5 mL) and cooled to −30 °C, and diisopropylethylamine (0.62 mL, 3.55 mmol) was added to the solution. Phosphoramidic chloride **2a** (489 mg, 1.08 mmol) was dissolved in dry acetonitrile (3 mL) and added to the reaction mixture, which was then warmed to −10 °C and stirred for 2.5 h. The reaction was quenched with water at −10 °C and extracted with CH_2Cl_2 (3×). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to give **9a** as an oil (565 mg, 87%): ^{1}H NMR ($CDCl_3$) δ 7.30 (d, 1H), 6.74 (m, 1H), 5.90 (m, 1H), 5.35 (dd, 2H), 5.02 (m, 2H), 4.63 (d, 2H); 4.10 (t, 1H), 3.24−3.65 (bm, 8H), 2.92 (m, 2H), 1.28−1.99 (m, 6H); ^{31}P NMR ($CDCl_3$) δ 17.06 (diastereomers, broad peak); MS (ESI) *m*/*z* 603 $(M + H)^+$.

(5-Nitro-2-furyl)methyl N^{ϵ}-(*O*-allyllysyl)-*N*,*N*-bis(2-chloroethyl)phosphorodiamidate (9b) was prepared as described for **9a** (2.86 mmol scale) using phosphoramidic chloride **2b**. The crude product was used without further purification (1.33 mg, 90%): ^{1}H NMR ($CDCl_3$) δ 7.30 (d, 1H), 6.73 (m, 1H), 5.91 (m, 1H), 5.34 (dd, 2H), 5.02 (m, 2H), 4.61 (d, 2H), 3.89 (t, 1H), 3.24−3.75 (bm, 8H), 2.92 (m, 2H), 1.29−2.01 (m, 6H); ^{31}P NMR ($CDCl_3$) δ 17.11 (diastereomers, broad peak); MS (ESI) *m*/*z* 515 $(M + H)^+$.

(5-Nitro-2-furyl)methyl N^{ϵ}-Lysyl-*N*,*N*-bis(2-bromoethyl)phosphorodiamidate (4a). $Pd(PPh_3)_4$ (47 mg, 0.041 mmol) was added to a solution of phosphoramidate **9a** (490 mg, 0.81 mmol) in THF (10 mL). *p*-Toluenesulfinic acid (173 mg, 0.97 mmol) was dissolved in water (0.5 mL) and added to the THF solution. The reaction mixture was stirred for 30 min at room temperature, diluted with water, washed with diethyl ether (5×), and the water layer was then concentrated under reduced pressure. Reverse-phase chromatography using a gradient of acetonitrile/water:acetonitrile 20% to 80% afforded **4a** as a yellow oil (244 mg, 53%). This product was identical in all respects to that prepared by enzymatic hydrolysis of the methyl ester.

(5-Nitro-2-furyl)methyl N^{ϵ}-lysyl-*N*,*N*-bis(2-chloroethyl)phosphorodiamidate (4b) was prepared as described for **4a** (2.16 mmol scale) using phosphorodiamidate **9b**. The crude residue was purified by reverse-phase chromatography using a gradient of acetonitrile/water:acetonitrile 20% to 80%) to give **4b** as a yellow oil (0.564 g, 55%). This product was identical in all respects to that prepared by enzymatic hydrolysis of the methyl ester.

(5-Nitro-2-furyl)methyl N^{ϵ}-(N^{10}-(Trifluoroacetyl)pteroyllysyl)-*N*,*N*-bis(2-bromoethyl)phosphorodiamidate (6a). N^{10}-(Trifluoroacetyl)pteroic acid **5** was dried over P_2O_5 for 48 h at 45 °C. A solution of **5** (10 mg, 0.03 mmol) in anhydrous DMF (0.5 mL) under argon was cooled to 0 °C, and triethylamine (0.007 mL, 0.05 mmol) was added, followed by isobutyl chloroformate (0.005 mL, 0.04 mmol). The reaction mixture was protected from light and stirred at 0 °C for 1 h. Phosphorodiamidate **4a** (21 mg, 0.04 mmol) was dissolved in anhydrous DMF (0.5 mL), added to the reaction mixture, and stirred for 2 h at 0 °C. Water (3 mL) was added to the reaction mixture and the precipitated solid was collected by centrifugation to give **6a** as a yellow powder (18 mg, 76%): ^{1}H NMR (DMSO-d_6) δ 8.60 (s, 1H), 7.91 (d, 2H), 7.49−7.76 (bm, 3H), 6.90 (d, 1H), 5.10 (s, 2H), 4.92 (bd, 2H); 4.25 (bs, 1H), 2.88 (bd, 2H), 1.24−1.90 (m, 6H); ^{31}P NMR (DMSO-d_6) δ 17.67; MS (ESI) *m*/*z* 953 $(M + H)^+$.

(5-Nitro-2-furyl)methyl N^{ϵ}-(N^{10}-(trifluoroacetyl)pteroyllysyl)-*N*,*N*-bis(2-chloroethyl)phosphorodiamidate (6b) was prepared as described for **6a** (0.025 mmol scale) using phosphorodiamidate **4b**: ^{1}H NMR (DMSO-d_6) δ 8.50 (s, 1H), 7.77

(d, 2H), 7.30−7.64 (bm, 3H), 6.78 (d, 1H), 5.00 (s, 2H), 4.83 (bd, 2H), 4.17 (bs, 1H), 2.74 (bd, 2H), 1.38−1.82 (m, 6H); ^{31}P NMR (DMSO-d_6) δ 17.87; MS (ESI) *m/z* 865 $(M + H)^+$.

(5-Nitro-2-furyl)methyl N^{ϵ}-(Pteroyllysyl)-*N*,*N*-bis(2-bromoethyl)phosphorodiamidate (1a). (5-Nitro-2-furyl)-methyl N^{ϵ}-(N^{10}-(trifluoroacetyl)pteroyllysyl)-*N*,*N*-bis(2-bromoethyl)phosphorodiamidate **6a** (13 mg, 0.014 mmol) was suspended in THF/MeOH (1.6 mL, 1:1), and aqueous K_2CO_3 (5.8 mg, 0.04 mmol; 0.02 mL) was added. The resulting homogeneous solution was stirred for 6 days at room temperature. Aqueous trifluoroacetic acid (3 mL, 0.1%) was added to the reaction mixture, and the precipitated solid was collected by centrifugation to give **1a** as a yellow powder (10.4 mg, 89%): ^{1}H NMR (DMSO-d_6) δ 11.40 (s, 1H), 8.63 (s, 1H), 7.65 (bm, 3H), 6.88 (bm, 2H), 6.61 (d, 2H), 4.93 (bd, 2H), 4.46 (s, 2H), 4.26 (bs, 1H), 2.72 (bd, 2H), 1.38−1.82 (m, 6H); ^{31}P NMR (DMSO-d_6) δ 17.82; MS (FAB) ($C_{29}H_{35}N_{10}O_9PBr_2$) calcd 857.0771, obsd 857.0789.

(5-Nitro-2-furyl)methyl N^{ϵ}-(pteroyllysyl)-*N*,*N*-bis(2-chloroethyl)phosphorodiamidate (1b) was prepared as described for **1a** (0.012 mmol scale) using phosphorodiamidate **6b**: ^{1}H NMR (DMSO-d_6) δ 11.40 (s, 1H), 8.64 (s, 1H), 7.66 (bm, 3H), 6.89 (bm, 2H), 6.24 (d, 2H), 4.93 (bd, 2H), 4.47 (s, 2H), 4.26 (bs, 1H), 2.73 (bd, 2H), 1.38−1.79 (m, 6H); ^{31}P NMR (DMSO-d_6) δ 17.98; MS (negative ion FAB) ($C_{29}H_{35}N_{10}O_9PCl_2$) calcd 767.1625, obsd 767.1621.

(5-Nitro-2-furyl)methyl *N*,*N*-Bis(2-chloroethyl)-*N*-propylphosphorodiamidate (11). Propylamine (0.07 mL, 0.85 mmol) was dissolved in methylene chloride (10 mL) and cooled to −20 °C under argon. Diisopropylethylamine (0.18 mL, 1.02 mmol) was added, followed by addition of phosphoramidic chloride **2b** (310 mg, 0.85 mmol) in methylene chloride (1 mL). The mixture was warmed to −10 °C and allowed to stir for 3 h. The reaction was quenched by addition of water at −10 °C and extracted with CH_2Cl_2 (3×). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The product was isolated as a yellow oil after purification by flash chromatography (123 mg, 77%): ^{1}H NMR ($CDCl_3$) δ 7.30 (d, 1H), 6.67 (d, 1H), 5.06 (dd, 2H), 3.29−3.70 (bm, 8H), 2.88 (m, 2H), 1.54 (m, 2H), 0.93 (t, 3H); ^{31}P NMR ($CDCl_3$) δ 16.40; MS (FAB) ($C_{12}H_{20}N_3O_5PCl_2$) calcd 388.0596, obsd 388.0595.

^{31}P NMR Studies. Compounds **4b**, **10b** and **11** were dissolved in 100 μL of CH_3CN, and cacodylate buffer (400 μL, 0.4M, pH7.4) was added. The solution was placed in a 5-mm NMR tube, and sodium dithionite (3 equiv) in 100 μL of buffer was added to the NMR tube. The acquisition was started and the start time recorded. Spectra were taken every 2.5 min for 0.5 h, then every 5 min for 0.5 h, then every 10 min for 1 h, and time points for each spectrum were assigned from the beginning of the data acquisition. The temperature of the probe was maintained at 37 °C, using the Bruker variable temperature unit. Chemical shifts are reported in parts per million from a coaxial insert containing 1% triphenylphosphine oxide in benzene-d_6. The relative concentrations of the intermediates were determined from integration of the peak areas.

Growth Inhibition Studies in Vitro. Stock solutions of drugs were prepared in DMSO and added to cell suspensions so there was <1% DMSO in the final medium. The cells were inoculated into each well of a 96-well plate (0.2 mL/well) and cultured at 37 °C for 24 h in McCoys 5A media supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Supplemented McCoys 5A media (0.1 mL) containing the drug in DMSO was added, and culture of the cells was continued at 37 °C for 72 h. Five serial 10-fold dilutions were tested for each drug sample using a maximum final drug concentration of 100 μg/mL. Adriamycin and compound **10** were used as positive controls. The number of viable cells was estimated by the MTT method[10] to determine the IC_{50} for each drug.

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