Synthesis and Biological Activity of Novel 5-Fluoro-2′-deoxyuridine Phosphoramidate Prodrugs

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A series of novel haloethyl and piperidyl phosphoramidate FdUMP prodrug analogues has been synthesized, and the growth inhibitory activity of these compounds has been evaluated against L1210 mouse leukemia cells. All compounds exhibited potent inhibition of L1210 cell proliferation with IC_{50} values in the nanomolar range. Growth inhibition was reversed by the addition of 5 μM thymidine, suggesting a mechanism of action involving the intracellular release of FdUMP. ^{31}P NMR studies carried out on model haloethyl phosphoramidates confirm the release of nucleotide via cyclization of the phosphoramidate anion to the aziridinium ion intermediate followed by hydrolysis of the P−N bond. The data suggests that <50% of the prodrug is converted to FdUMP intracellularly by this pathway. Piperidyl phosphoramidate analogues are also converted to nucleotide intracellularly, presumably by the action of an endogenous phosphoramidase.

Introduction

Inhibition of DNA biosynthesis has become a key objective in the design of anticancer chemotherapeutic agents.[1,2] To this end, several modified nucleoside and pyrimidine antimetabolites requiring intracellular phosphorylation have been developed as inhibitors of the enzyme thymidylate synthase (TS), a key enzyme for the endogenous production of thymidine 5′-monophosphate (TMP).[3,4] The emergence of resistance to such analogues, likely as a result of reduced enzymatic activation by the tumor cell,[5] has prompted efforts to develop prodrugs that release nucleotides intracellularly and circumvent the requirement for intracellular phosphorylation.[6−8] For example, the amino acid phosphoramidate derivatives of antiviral and antitumor nucleosides[7] have demonstrated potential as nucleotide prodrugs. However, the initial step in the activation of these compounds can potentially occur extracellularly, because it relies on the function of nonspecific esterases. Furthermore, the final activation step requires cleavage of the P−N bond, presumably catalyzed by an endogenous phosphoramidase;[8] this process may be sufficiently slow that little intracellular accumulation of the nucleotide occurs.[9]

Our interest in the development of phosphoramidate nucleotide prodrugs[10] has led to the design and synthesis of a new series of haloethyl nucleoside phosphoramidates, **1a**,**b** and **2a**,**b** (Scheme 1), that undergo facile P−N bond hydrolysis following intracellular activation to liberate the known nucleotide TS inhibitor, 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP). The benzotriazolyl and nitrofuryl moieties were introduced as delivery groups for the corresponding 5-fluorodeoxyuridyl phosphoramidates. It was anticipated that the benzotriazolyl moiety would undergo direct hydrolysis while the nitrofuryl moiety would undergo bioreductive activation[11] to generate the same phosphoramidate anion (**B**) intracellularly as shown in Scheme 2. Three amide groups were investigated; the methyl bromoethyl and bis(bromoethyl) groups were selected on the basis of previous findings[10] that, following generation of the phosphoramidate anion (**B**), they would cyclize to the aziridinium ion (**C**) and undergo P−N bond hydrolysis to liberate the corresponding nucleotide (**D**; Scheme 2). The piperidine analogues **1c** and **2c** (Scheme 1) were synthesized because, although the corresponding phosphoramidate anion is chemically stable, it presumably undergoes bioactivation resulting in significant growth inhibitory activity.[10] Finally, the 5′-amino analogue **10** (Scheme 3) was prepared to determine whether the corresponding nucleotide analogue might also show growth inhibitory properties; presumably this compound would not be a substrate for 5′-nucleotidase. We report herein the synthesis of nitrofuryl and benzotriazolyl nucleotide prodrugs and the growth inhibitory activity of these compounds.

Results and Discussion

Chemistry. Synthesis of Nucleoside Phosphoramidates. Target compounds **1** and **2** were synthesized according to the procedure outlined in Scheme 1. Phosphoryl dichlorides **7a**−**c** were prepared by the reaction of phosphorus oxychloride with the appropriate amine (**6a**−**c**) in the presence of triethylamine.[10] Reaction of **7a**−**c** with 2 equiv of 1-hydroxybenzotriazole in the presence of pyridine afforded the highly reactive bis(benzotriazolyl) phosphoramidate intermediates **8a**−**c**. Reaction of **8a**−**c** in situ with 5-FdU in the presence of 1-methylimidazole as a catalyst afforded diastereomeric mixtures of compounds **1a**−**c** in 43−60% yield. Nitrofuryl and benzyl nucleoside phosphoramidates **2a**−**c** were prepared in 70−75% yield as diastereomeric mixtures from the corresponding benzotriazolyl phos-

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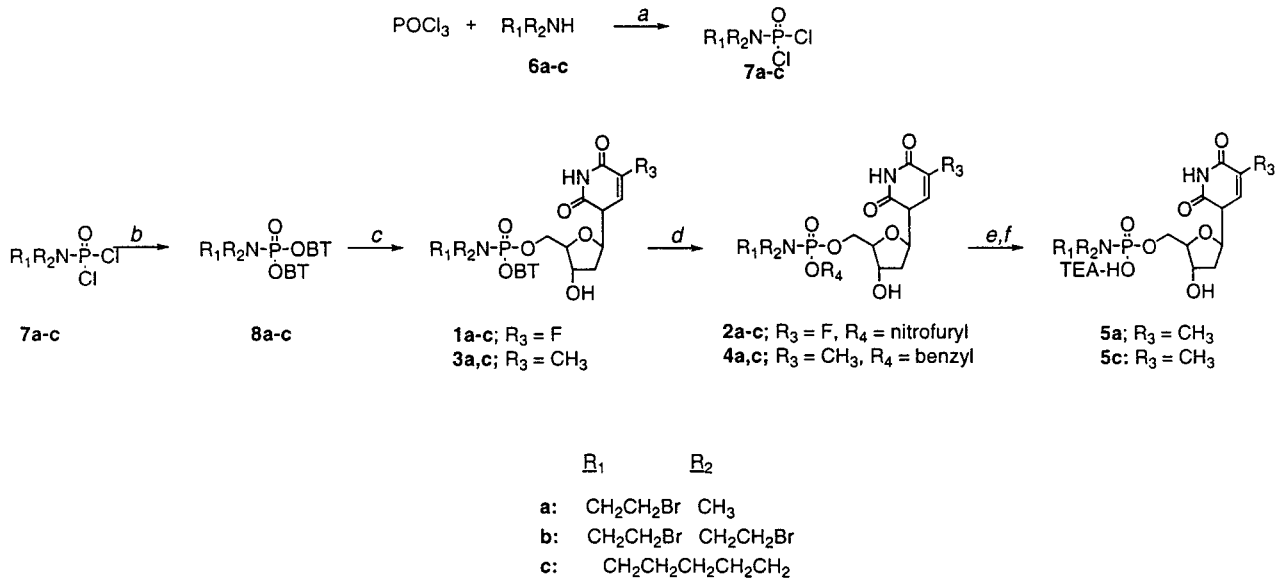
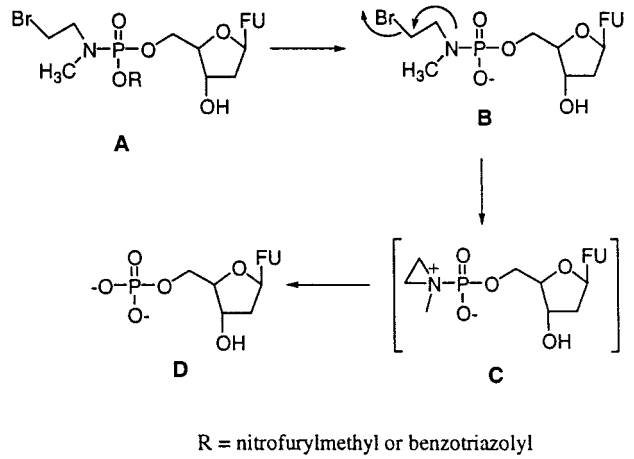
Scheme 1[a]



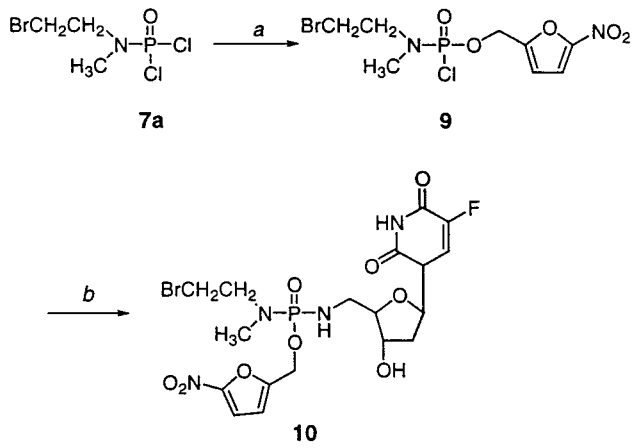
	R_1	R_2
a:	CH_2CH_2Br	CH_3
b:	CH_2CH_2Br	CH_2CH_2Br
c:	$CH_2CH_2CH_2CH_2CH_2$	

[a] Reagents and conditions: (a) triethylamine, CH_2Cl_2, −40 to 0 °C, 1 h; (b) HOBT, pyridine, 0 °C to rt, 2 h; (c) 5-fluorodeoxyuridine (or thymidine), *N*-methylimidazole, rt, overnight; (d) nBuLi (or RMgBr) and benzyl alcohol (or 1-hydroxymethyl-5-nitrofuran), THF, rt, then **1** (or **3**), −20 to 0 °C, 1 h; (e) H_2, 10% Pd/C, THF, 10 min, rt; (f) triethylamine, rt, 2 min.

Scheme 2



Scheme 3[a]



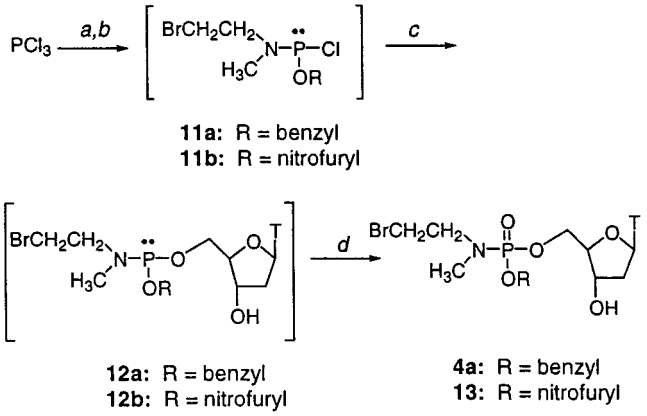
[a] Reagents and conditons: (a) LiHMDS, 2-hydroxymethyl-5-nitrofuran, THF, −7.8 to −10 °C, 3 h; (b) 5-fluoro-5′-amino-2′-deoxyuridine, PVP, EtOH, 0 °C to rt, 6 h.

phoramidates **1a**−**c** and either 2-hydroxymethyl-5-nitrofuran or benzyl alcohol in the presence of base.

5′-Amino nucleoside phosphoramidate analogue **10** was prepared as shown in Scheme 3. Treatment of **7a** with 2-hydroxymethyl-5-nitrofuran in the presence of LiHMDS afforded phosphoryl monochloride **9** in 81% yield. Subsequent reaction of **9** with 5-fluoro-5′-amino-2′-deoxyuridine (prepared according to Garnett et al.)[12] in the presence of poly(4-vinylpyridine) (PVP) provided the 5′-amino nucleoside phosphoramidate **10** in 61% yield.

Thymidine phosphoramidates **3** and **4** were synthesized to use as model compounds for ^{31}P NMR experiments; it was assumed that the 5-substituent on the pyrimidine ring would have little effect on the reaction rates of the phosphoramidate anion. Compound **13** was prepared for the purpose of conducting studies to ascertain the stability of the nitrofuryl analogues in serum-containing medium. Thymidine phosphoramidates **3** and **4** were prepared as described above (Scheme 1). Quantitative conversion of the benzyl nucleoside phosphoramidates **4a**,**c** to the corresponding phosphoramidate anions **5a**,**c** was accomplished by catalytic hydrogenolysis followed by treatment with triethylamine. The phosphoramidate anion prepared by this method was used without further purification in the ^{31}P NMR experiments discussed below. More recently, the development of a one-pot strategy for the synthesis of nucleoside phosphoramidate analogues was undertaken in an attempt to maximize the conversion of nucleoside. This alternative approach for the synthesis of phosphoramidates such as **4a** (Scheme 4) employs the in situ generation of a highly reactive phosphorus(III) chloride intermediate for the phosphorylation of the nucleoside. Phosphorus trichloride was reacted with benzyl alcohol in the presence of diisopropylethylamine followed by reaction with *N*-methyl-*N*-(2-bromoethyl)amine hydrobromide to generate monochloro intermediate **11**. Thymidine in pyridine was then titrated slowly with **11**; the rapid disappearance of nucleoside made it possible to monitor the reaction by TLC. It should be noted that fast addition of **11** resulted in the formation of 3′-

Scheme 4[a]



[a] Reagents and conditions: (a) benzyl alcohol or 1-hydroxymethyl-5-nitrofuran, diisopropylethylamine, CH_3CN/CH_2Cl_2 (1:3), −78 °C, 15 min; (b) *N*-methyl-*N*-(2-bromoethyl)amine hydrobromide, CH_3CN/CH_2Cl_2 (1:1.5), −78 to −60 °C, 20 min; (c) thymidine, pyridine, −50 °C; (d) *tert*-butyl hydroperoxide, −50 to 0 °C, 30 min.

Table 1. Growth Inhibition in L1210 Mouse Leukemia Cells

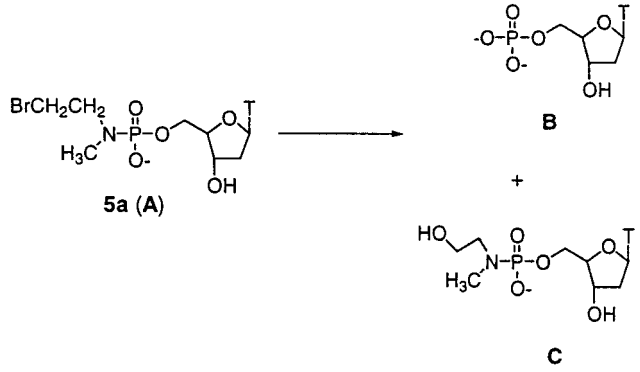
			IC_{50}, nM			
			treatment time, h			
compd	R_1	R_2	2	8	24	48
1a	CH_2CH_2Br	CH_3	429	178	22	3.3
1b	CH_2CH_2Br	CH_2CH_2Br	276	96	11	2.5
1c	$CH_2CH_2CH_2CH_2CH_2$		843	733	77	13
2a	CH_2CH_2Br	CH_3	1027	347	56	16
2b	CH_2CH_2Br	CH_2CH_2Br	2074	519	103	18
2c	$CH_2CH_2CH_2CH_2CH_2$		1592	292	100	36
10	CH_2CH_2Br	CH_3	1101	504	108	34
5-FU			2200	630	220	125

phosphorylated and 3′,5′-bisphosphorylated byproducts. Oxidation of phosphoramidite **12** with *tert*-butyl hydroperoxide afforded phosphoramidate **4a** in 68% yield. Phosphoramidate **6a** was prepared in 77% yield (Scheme 4) using this strategy. Phosphorylation of nucleosides using this approach has resulted in higher overall yields and is the method of choice for the preparation of nucleoside phosphoramidates.

Biological Activity. A. In Vitro Activity. Compounds **1a**−**c**, **2a**−**c**, and **10** were evaluated for growth inhibitory activity against L1210 mouse leukemia cells; the results are summarized in Table 1. All analogues exhibited potent inhibition of cell proliferation that was reversed by the addition of 5 μM thymidine, indicating that these compounds act via inhibition of thymidylate synthase. Compounds **1a,b** exhibited comparable potencies and were significantly more potent than 5-FU. Nitrofuryl analogues **2a,b** were less potent than benzotriazolyl analogues **1a**,**b** at all drug treatment times, presumably as a result of slower enzymatic activation of the nitrofuryl phosphoramidates. A similar trend was observed for the 5′-amino nucleoside phosphoramidate **10**. The data obtained for the haloethyl phosphoramidate series are consistent with a proposed mechanism of action involving intracellular generation of the phosphoramidate anion followed by cyclization to the aziridinium ion and subsequent hydrolysis of the P−N bond to liberate FdUMP.

Piperidyl phosphoramidates **1c** and **2c** are not capable of undergoing the same type of activation as the haloethyl nucleoside phosphoramidates and, as a result, were not expected to exhibit significant in vitro activity. However, potencies comparable to the corresponding haloethyl phosphoramidates were observed for **1a**,**c** at the longest drug exposure time (48 h). It was initially hypothesized that the activity observed in the piperidyl phosphoramidate series was consistent with a mechanism of action involving intracellular liberation of the phosphoramidate anion followed by protonation on the piperidyl nitrogen at physiologic pH and hydrolysis of the P−N bond to generate FdUMP. However, mechanistic studies carried out on model piperidyl phosphoramidates[13] indicate that hydrolysis is too slow to account for the observed inhibition of cell proliferation. Presumably, the piperidyl phosphoramidate anion undergoes enzymatic activation to FdUMP.

Scheme 5



To determine whether the nitrofuryl phosphoramidate analogues are stable under standard L1210 cell assay conditions (10% horse serum in Fischer's medium, pH 7.4, 37 °C), an experiment was performed in which HPLC was used to monitor the stability of thymidyl phosphoramidate **13**. Under these conditions, >95% of compound **13** remained after 8 h; **13** subsequently disappeared over a longer time period ($t_{1/2}$ = 18 h).

B. ^{31}P NMR Studies. A ^{31}P NMR study was carried out for the purpose of elucidating the chemical activation of this series of nucleoside phosphoramidates and to provide convincing evidence of P−N bond hydrolysis. The first NMR experiment was designed to provide information about the reactions of the phosphoramidate anion, a key precursor in this prodrug strategy. Thymidyl phosphoramidate anion **5a** was synthesized as described above, and its reactions were monitored by ^{31}P NMR under model physiologic conditions (ca. 100 mM in 0.4 M cacodylate buffer, pH 7.4, 37 °C). The resonance for **5a** (**A**, −16.12 ppm) disappeared ($t_{1/2}$ = 3.7 min) to give two major products (Scheme 5). The product of primary biological interest is the nucleotide (**B**, −21.77 ppm), arising from P−N bond hydrolysis of the aziridinium ion. A second product (**C**, −14.82 ppm) is also formed as a result of ring opening of the aziridinium ion. Two minor byproducts were also formed and accounted for <15% of the total product. Integration of the resonances corresponding to trapping of the aziridinium ion by water (solvolysis product, **C**) and the desired hydrolysis of the P−N bond (nucleotide, **B**) reveals a solvolysis-to-hydrolysis product ratio of 1:1. These data confirm the occurrence of P−N bond hydrolysis to liberate nucleotide in the activation of *N*-methyl-*N*-(2-bromoethyl) nucleoside phosphoramidate anion, but they also suggest that

≤50% of the prodrug is ultimately converted to the nucleotide.

A second ^{31}P NMR experiment was carried out on benzotriazolyl phosphoramidate **3a** to confirm that initial activation of the phosphoramidate prodrugs **1a**−**c** leads to the corresponding phosphoramidate anions. A solution of phosphoramidate **3a** was prepared (ca. 100 mM in 4.5:1 0.4 M cacodylate buffer/acetonitrile), and the mixture was monitored by ^{31}P NMR under physiologic conditions (pH 7.4, 37 °C). As expected, the resonances corresponding to the diastereomeric mixture of compound **3a** (−12.50, −12.78 ppm) were replaced ($t_{1/2}$ = 4.7 min) by a single resonance corresponding to the phosphoramidate anion (−15.99 ppm). The subsequent reactions of phosphoramidate anion resulted in the formation of a variety of products arising both from cleavage of the P−N bond and ring opening of the aziridinium ion.[13] Again, solvolysis product and the desired nucleotide were formed in a 1:1 ratio.

A similar experiment was carried out (ca. 100 mM, 0.4 M cacodylate buffer, pH 7.4, 37 °C) on the piperidyl phosphoramidate anion **5c**.[13] The resonance for piperidyl nucleoside phosphoramidate anion **5c** (−16.11 ppm) disappeared slowly ($t_{1/2} \sim$ 11 days) to give the corresponding nucleotide. The exceptionally slow conversion of piperidyl phosphoramidate anion to nucleotide under physiologic conditions suggests that protonation on nitrogen followed by P−N bond hydrolysis does not account for the observed growth inhibitory activity of phosphoramidate prodrugs **1c** and **2c**. Recent studies[8] suggest that endogenous phosphoramidase activity may account for the observed P−N bond hydrolysis in related phosphoramidate systems. Presumably **5c** undergoes a similar enzymatic conversion.

Summary

The synthesis of a new series of nucleoside phosphoramidate prodrugs bearing benzotriazolyl and nitrofuryl esters was accomplished. Potent inhibition of L1210 cell proliferation was observed for the 5-fluorodeoxyuridyl analogues that was reversed by the addition of thymidine. ^{31}P NMR activation studies in model systems confirm the release of nucleotide via P−N bond hydrolysis of the aziridinium ion intermediate. However, the data suggests that ≤50% of the prodrug is converted to FdUMP intracellularly. Finally, the piperidyl phosphoramidate anion is stable under model physiologic conditions; the activity of this compound presumably results from enzymatic conversion to FdUMP.

Experimental Section

Materials and Methods. All ^{31}P and ^{1}H NMR spectra were recorded on either a 250 or 270 MHz Bruker instrument unless specified otherwise. All ^{31}P NMR spectra were acquired using broadband gated decoupling. ^{31}P chemical shifts are reported in parts per million using 1% triphenylphosphine oxide in benzene-d_6 as the coaxial reference (triphenylphosphine oxide/benzene-d_6 has a chemical shift of +24.7 ppm relative to 85% phosphoric acid). Variable-temperature ^{31}P NMR kinetics experiments were controlled using the Bruker variable temperature unit. ^{1}H chemical shifts are reported in parts per million from tetramethylsilane. Flash chromatography using silica gel grade 60 (230−400 mesh) was carried out for all chromatographic separations. Thin-layer chromatography was performed using Analtech glass plates precoated with silica gel (250 microns). Visualization of the plates was accomplished using UV and/or the following stains: 1% 4-(*p*-nitrobenzyl)-pyridine in acetone followed by heating and subsequent treatment with 3% KOH in methanol (for detection of haloethyl functionality), 3% phosphomolybdic acid in methanol followed by heating, or *p*-anisaldehyde dip (1.85% *p*-anisaldehyde, 20.5% sulfuric acid, 0.75% acetic acid in 95% EtOH) followed by heating.

All reactions were carried out under an atmosphere of nitrogen or argon unless otherwise specified or reagents containing water were used. All organic solvents were distilled prior to use unless otherwise specified. Pyridine, triethylamine and diisopropylamine were distilled prior to use. 1-Hydroxybenzotriazole and all nucleosides were dried by coevaporation with pyridine prior to use.

In Vitro Growth Inhibition. Stock solutions of drugs were prepared in 95% ethanol, and serial dilutions of drug were prepared in ethanol such that 50 μL of drug solution added to 10 mL of cell suspension gave the desired final concentration. L1210 cells in exponential growth were suspended in Fischer's medium supplemented with 10% horse serum, 1% glutamine, and 1% antibiotic−antimycotic solution to give 10-mL volumes of cell suspension at a final density of 3−6 × 10^4/mL. Appropriate volumes of the drug solution were transferred to the cell suspensions, and incubation was continued for 2, 8, 24, or 48 h. The cells were spun down, resuspended in fresh drug-free medium, and returned to the incubator. Cell counts were determined 48 h after initiation of drug treatment. The procedure was repeated for a range of drug concentrations and the cell counts (reported as percent of control values) were plotted versus concentration for each drug treatment time (the control cell densities were in the range 5−7 × 10^5/mL). The data are reported as IC_{50} values in nM.

5′-Thymidyl 1-Benzotriazolyl *N*-Methyl-*N*-(2-bromoethyl)phosphoramidate (3a). Phosphoramidic dichloride **(7a)** (1.05 g, 4.13 mmol) was dissolved in THF (3 mL) and added dropwise to a stirred solution of 1-hydroxybenzotriazole (1.11 g, 8.24 mmol) and pyridine (0.67 mL, 8.24 mmol) in THF (20 mL) at 0 °C under an atmosphere of argon. The reaction mixture was removed from the 0 °C bath and stirred at room temperature for 2 h. The mixture was transferred to two Wheaton tubes, and the pyridine hydrochloride was removed by centrifugation under an atmosphere of argon. The supernatant containing the reactive bis(benzotriazolyl) intermediate **8a** was added in one portion to a stirred solution of thymidine (0.50 g, 2.06 mmol) in pyridine (2 mL) at room temperature. 1-Methylimidazole (0.20 mL, 2.47 mmol) was added dropwise, and stirring was continued at room temperature for 12 h. THF was removed under reduced pressure. The residue was diluted with $CHCl_3$ (50 mL) and washed with saturated $NaHCO_3$ (1 × 25 mL) to remove excess HOBT. The organic layer was washed with saturated NH_4Cl (2 × 25 mL) to remove 1-methylimidazole. The aqueous layers were combined and extracted with $CHCl_3$ (25 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel chromatography (75:115:10 EtOAc:$CHCl_3$:EtOH) to give **3a** (649 mg, 56%) as a white foam that was a 1:1.4 mixture of diastereomers (as determined by ^{1}H and ^{31}P NMR): R_f = 0.17 (75:115:10 EtOAc:$CHCl_3$:EtOH); ^{1}H NMR ($CDCl_3$) δ 9.18 and 9.10 (1H, s, 1:1.4 mixture), 8.00 (1H, t) 7.71 (1H, m) 7.60−7.29 (3H, m), 6.34 and 6.27 (1 H, t, 1.4:1 mixture), 4.98 (1H, s), 4.53 (3H, m), 4.25−4.05 (1H, m), 3.48 (4H, m), 2.94 (3H, d, J = 10.3), 2.52−2.15 (2H, m), 1.92 and 1.82 (3H, s, 1.4:1 mixture); ^{31}P NMR ($CDCl_3$) δ −14.56, −14.98 (1:1.4); HRMS ($C_{19}H_{24}N_6O_7BrP$) calcd 559.0706 (M + H)$^+$, found 559.0707

5-Fluoro-2′-deoxy-5′-uridyl 1-benzotriazolyl *N*-methyl-*N*-(2-bromoethyl)phosphoramidate (1a) was prepared from **7a** as described for **3a** in 60% yield. 5-FUdR (2 mmol) was used instead of thymidine: R_f = 0.41 (10% MeOH:$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 8.03−7.34 (5H, m), 6.23 (1H, m), 4.43 (3H, m), 4.12 (1H, m), 3.51 (4H, m), 3.01 (3H, d, J = 4.3 Hz), 2.49 (1H, m), 2.23 (1H, m); ^{31}P NMR (THF) δ −13.25, −13.71; HRMS ($C_{18}H_{21}BrFN_6O_7P$) calcd 563.0455 (M + H)$^+$, found 563.0444.

5-Fluoro-2′-deoxy-5′-uridyl 1-benzotriazolyl bis(2-bromoethyl)phosphoramidate (1b) was prepared as described for **3a** from **7b** in 58% yield. 5-FUdR (1.6 mmol) was used instead of thymidine: $R_f = 0.38$ (10% MeOH:$CHCl_3$); ^{1}H NMR (acetone-d_6) δ 8.08−7.41 (5H, m), 6.30 (1H, m), 4.61 (3H, m), 4.23 (1H, m), 3.62 (8H. m), 2.31 (2H, m); ^{31}P NMR (acetone) δ −13.60, −14.13; HRMS ($C_{19}H_{22}Br_2FN_6O_7P$) calcd $(M + H)^+$ 656.9696, found 656.9703.

5-Fluoro-2′-deoxy-5′-uridyl 1-benzotriazolyl piperidylphosphoramidate (1c) was prepared as described for **3a** from **7c** in 57% yield. 5-FUdR (1 mmol) was used instead of thymidine: $R_f = 0.33$ (10% MeOH:$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 8.05−7.35 (5H, m), 6.34 (1H, m), 4.60−4.13 (4H, m), 3.36 (4H, br), 2.47 (1H, br), 2.20 (1H, m), 1.65 (6H, br); ^{31}P NMR (THF, ref = H_3PO_4) δ −14.88, −15.30; HRMS ($C_{20}H_{24}FN_6O_7P$) calcd $(M + H)^+$ 511.1506, found 511.1506.

5′-Thymidyl 1-Benzotriazolyl Piperidylphosphoramidate (3c). Phosphoramidate **3c** was prepared from piperidyl dichloride **(7c)** (4.13 mmol) and thymidine as described for **3a** in 43% yield: $R_f = 0.47$ (9:1 $CHCl_3$:EtOH); ^{1}H NMR ($CDCl_3$) δ 9.03 and 8.96 (1H, s, 1:1.3 mixture), 8.05 (1H, m), 7.70 (1H, m), 7.56 (1H, m), 7.46−7.28 (2H, m), 6.37 and 6.26 (1H, t, 1.3:1 mixture), 4.48 (3H, m), 4.21−4.00 (1H, m), 3.76 (1H, s), 3.27 (4H, m), 2.45−1.99 (2H, m), 1.94 and 1.80 (3H, s, 1.3:1 mixture), 1.62 (6H, m); ^{31}P NMR ($CDCl_3$) δ −14.83, −15.05 (1:1.3 mixture); HRMS ($C_{21}H_{26}N_6O_7P$) calcd 506.1679 $(M + H)^+$, found 506.1670.

5′-Thymidyl Benzyl *N*-Methyl-*N*-(2-bromoethyl)phosphoramidate (4a). Method 1: Benzyl alcohol (0.056 mL, 0.54 mmol) was dissolved in THF (1 mL) under an atmosphere of argon, and a few crystals of 2,2′-dipyridyl indicator were added. Ethylmagnesium bromide (0.72 mL of ca. 0.75 M solution in THF, 0.54 mmol) was added dropwise to the mixture at room temperature until a pale pink color persisted. The resulting thick mixture containing the alkoxide was cooled to −20 °C. Phosphoramidate **3a** (100 mg, 0.18 mmol) was dissolved in THF (1 mL) and added dropwise to the alkoxide at −20 °C. A color change from pink to colorless was observed after the addition of **3a**. The reaction mixture was warmed to 0 °C over 1 h and then diluted with $CHCl_3$ (10 mL). The organic mixture was washed with saturated $NaHCO_3$ (1 × 25 mL), dried over Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography (5 → 10% MeOH:$CHCl_3$) to yield **4a** (67 mg, 70%, white foam) as a 1:2 mixture of diastereomers (as determined by ^{1}H and ^{31}P NMR): $R_f = 0.42$ (10% MeOH:$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 10.05 and 9.92 (1H, s, 2:1 mixture), 7.37 (5H, s), 6.28 (1H, t, $J = 10.4$ Hz), 5.04 (2 H, dd, $J_{HCNP} = 9.0$ Hz), 4.50 (1H, m), 4.23 (2H, dd, $J_{HCNP} = 7.5$ Hz), 4.08 (1H, m), 3.41 (4H, m), 3.05 (1H, s), 2.65 (3H, d, $J = 9.6$ Hz), 2.42 (1H, m), 2.12 (1H, m), 1.88 and 1.82 (3H, s, 2:1 mixture); ^{31}P NMR ($CDCl_3$) δ −14.47, −14.57 (1:2 mixture); HRMS ($C_{20}H_{27}N_3O_7BrP$) calcd 532.0848 $(M + H)^+$, found 532.0847.

Method 2: Benzyl alcohol (0.85 mL, 8.26 mmol) was dissolved in CH_3CN/CH_2Cl_2 (5:20 mL) and cooled to −78 °C. Phosphorus trichloride (4.13 mL, 2.0 M in CH_2Cl_2) was added slowly followed by the dropwise addition of diisopropylethylamine (2.16 mL, 12.4 mmol). The reaction mixture was allowed to stir at −78 °C for 15 min. *N*-Methyl-*N*-(2-bromoethyl)amine hydrobromide (1.81 g, 8.26 mmol) was dissolved in anhydrous CH_3CN (20 mL) and added to the reaction mixture dropwise. Diisopropylethylamine (4.32 mL, 24.8 mmol) was added dropwise and the reaction mixture was warmed to −60 °C and stirred for 20 min. Thymidine (1.0 g, 4.13 mmol) was coevaporated several times with anhydrous pyridine (6 × 30 mL) and then dissolved in pyridine (30 mL) and cooled to −45 °C. The mixture of thymidine in pyridine was then titrated with the reaction mixture containing intermediate **11a** until thymidine disappeared. The disappearance of thymidine was monitored by TLC (90:10 $CHCl_3$:MeOH). The reaction mixture was oxidized by the dropwise addition of *tert*-butyl hydroperoxide (1.65 mL, 5.0−6.0 M in decane) at −45 °C and warmed to 0 °C over 30 min. Saturated aqueous NH_4Cl (50 mL) was added, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated to a volume of 20 mL. Toluene (10 mL) was added, and the mixture was concentrated again to a volume of 20 mL. The coevaporation with toluene was repeated (×3) to remove pyridine. After the final coevaporation with toluene, the crude mixture was evaporated to dryness and the residue was passed through a short plug of silica gel (10:90 MeOH:$CHCl_3$) to remove any remaining amine hydrochloride salts and then purified by chromatography on silica gel (5:95 MeOH:$CHCl_3$) to afford compound **4a** as a white foam (1.48 g, 68%). This product was identical in all respects to that obtained from method 1.

5′-Thymidyl 5-nitro-2-furylmethyl *N*-methyl-*N*-(2-bromoethyl)phosphoramidate (13) was prepared as described for compound **4a** (method 2) on a 4.13-mmol scale in 77% yield: $R_f = 0.34$ (5:95 MeOH:$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 8.94 (1H, bs), 7.35 (1H, d, $J = 1$ Hz), 7.29 (1H, d, $J = 4$ Hz), 6.89 (1H, d, $J = 4$ Hz), 6.25 (1H, q, $J = 6$ Hz), 5.10 and 5.00 (2H, d, $J = 5$ Hz, 1:1 mixture), 4.54 (1 H, m), 4.26 (2H, m), 4.06 (1H, m), 3.48 (4H, m), 2.73 (3H, d, $J = 10$ Hz), 2.41 (1H, m), 2.22 (1H, m), 1.92 and 1.79 (3H, 1:1 mixture); ^{31}P NMR ($CDCl_3$) δ 14.65, −14.75 (1:1 mixture of diastereomers); MS ($C_{18}H_{24}BrN_4O_{10}P$) calcd $(M + H)^+$ 567.0492, found 567.0481.

5-Fluoro-2′-deoxy-5′-uridyl 5-nitro-2-furylmethyl *N*-methyl-*N*-(2-bromoethyl)phosphoramidate (2a) was prepared from **1a** on a 0.9-mmol scale as described for **4a** in 75% yield: $R_f = 0.29$ (10% MeOH:$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.80 (1H, m), 7.36 (1H, m), 6.74 (1H, m), 6.24 (1H, m), 5.15 (2H, d, $J = 9.0$ Hz), 4.58 (1H, m), 4.31 (2H, m), 4.09 (1H, m), 3.52 (4H, m), 2.77 (3H, d, $J = 9.3$ Hz), 2.48 (12H, m), 2.23 (1H, m); ^{31}P NMR (acetone) δ −14.64; HRMS ($C_{17}H_{21}BrFN_4O_{10}P$) calcd $(M + Na)^+$ 593.0060, found 593.0063.

5-Fluoro-2′-deoxy-5′-uridyl 5-nitro-2-furylmethyl bis(2-bromoethyl)phosphoramidate (2b) was prepared from **7b** on a 0.4-mmol scale as described for **4a** in 70% yield: $R_f = 0.32$ (10% MeOH:$CHCl_3$); ^{1}H NMR (acetone-d_6) δ 7.90 (1H, m), 7.49 (lH, d, $J = 3.6$ Hz), 6.95 (1H, d, $J = 3.5$ Hz), 6.23 (1H, rn), 5.21 (2H, m), 4.42 (1H, m), 4.28 (2H, m), 4.11 (1H, m), 3.42 (8H, m), 2.32 (2H, m); ^{31}P NMR (MeOH) δ −15.13. Anal. Calcd for $C_{18}H_{22}Br_2FN_4O_{10}P$: C 32.55, H 3.34, N 8.84. Found: C 32.64, H 3.34, N 8.46.

5-Fluoro-2′-deoxy-5′-uridyl 5-nitro-2-furylmethyl piperidylphosphoramidate (2c) was prepared on a 0.5-mmol scale from **1c** as described for **4a** in 71% yield: $R_f = 0.19$ (10% MeOH:$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.89 (1H, m), 7.30 (1H, m), 6.72 (1H, m), 6.25 (1H, m), 5.05 (2H, m), 4.58 (1H, m), 4.35 (2H, m), 4.16 (1H, m), 3.08 (4H, br), 2.48 (lH, m), 2.19 (lH, m), 1.54 (6H, br); ^{31}P NMR (CH_2Cl_2) δ −15.50, −15.71; HRMS ($C_{19}H_{24}FN_4PO_{10}$) calcd $(M + Na)^+$ 541.1112, found 541.1118.

5′-Thymidyl benzyl piperidylphosphoramidate (4c) was prepared from **3c** (210 mg, 0.42 mmol) as described above in 71% yield, and the product was isolated by silica gel chromatography (1:3:3 hexanes:EtOAc:acetone → 10% MeOH:$CHCl_3$) as a 2:1 mixture of diastereomers (as determined by ^{1}H NMR): $R_f = 0.25$ (1:3:3 hexanes:EtOAc:acetone); ^{1}H NMR ($CDCl_3$) δ 9.51 and 9.42 (1H, s, 2:1 mixture), 7.50 (1H, s), 7.36 (5H, m), 6.33 (1H, t), 5.01 (2H, m), 4.50 (1H, m), 4.20 (2H, m), 4.09 (1H, m), 3.29 (1H, s), 3.06 (4H, m), 2.41(1H, m), 2.07 (1H, m), 1.85 and 1.79 (3H, s, 2:1 mixture), 1.50 (6H, m); ^{31}P NMR ($CDCl_3$) δ −17.89; HRMS ($C_{22}H_{30}N_3O_7P$) calcd 480.1900 $(M + H)^+$, found 480.1922.

5′-Thymidyl *N*-Methyl-*N*-(2-bromoethyl)phosphoramidic Acid, Triethylammonium Salt (5a). Phosphoramidate **4a** (10.0 mg, 0.019 mmol) was dissolved in THF (1 mL). Pd/C (10%, 5 mg) was suspended in THF (1 mL) and transferred to the flask containing phosphoramidate **4a**. The flask was equipped with a balloon filled with hydrogen, and the reaction mixture was stirred for 10 min at room temperature. Triethylamine (2.8 μL, 0.020 mmol) was added and stirring was continued for 1−2 min. The catalyst was filtered, and the filtrate was concentrated to 0.50 mL and transferred to an NMR tube. Complete conversion to phosphoramidate anion **5a** was confirmed by ^{31}P NMR. The remaining THF was removed by rotary evaporation and the unstable product **5a** was immediately characterized by mass spectrometry or used in a

kinetics experiment. Further attempts to isolate the reaction product resulted in decomposition of the phosphoramidate anion: ^{31}P NMR (4.5:1 0.4 M cacodylate buffer:CH_3CN, pH 7.4, 37 °C) δ −15.99; HRMS ($C_{13}H_{21}N_3O_7BrP$) calcd 442.0379 $(M + H)^+$, found 442.0372.

5′-Thymidyl Piperidylphosphoramidic Acid, Triethylammonium Salt (5c). Phosphoramidate **4c** (7.0 mg, 0.015 mmol) was dissolved in THF (1 mL). Pd/C (10%, 5 mg) was suspended in THF (1 mL) and transferred to the flask containing phosphoramidate **4c**. The flask was equipped with a balloon filled with hydrogen, and the reaction was stirred for 10 min at room temperature. Triethylamine (2.2 μL, 0.016 mmol) was added and stirring was continued for 1−2 min. The catalyst was filtered and the filtrate was concentrated by rotary evaporation. Complete conversion to phosphoramidate anion **5c** was confirmed by ^{31}P NMR. Phosphoramidate anion **5c** was used without further purification for a kinetics experiment: ^{31}P NMR (4.5:1 0.4 M cacodylate buffer:CH_3CN, pH 7.4, 37 °C) δ −16.11; HRMS ($C_{15}H_{24}N_3O_7P$) calcd 390.1430 $(M + H)^+$, found 390.1428.

5-Nitro-2-furylmethyl *N*-Methyl-*N*-(2-bromoethyl)phosphoramidochloridate (9). To a solution of 2-hydroxymethyl-5-nitrofuran (1.08 g, 7.55 mmol) in THF (5 mL) at −78 °C was added LiHMDS (7.60 mL of 1 M solution in THF, 7.6 mmol) dropwise. The reaction was continued at −78 °C for 5 min. The resulting alkoxide was added to a solution of phosphoryl dichloride **7a** (2.02 g, 7.92 mmol) in THF (3 mL) at −78 °C. The reaction mixture was slowly warmed to −12 °C over 3 h, quenched with saturated aqueous NH_4CI, and extracted with EtOAc. The organic layer was dried and concentrated. The residue was purified by chromatography (0 to 1% MeOH:$CHCl_3$) to afford **9** (2.21 g, 81%) as a pale yellow oil: R_f 0.43 (0.7% MeOH:$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 7.32 (IH, d, J = 4.3 Hz), 6.77 (1H, d, J = 4.2 Hz), 5.20 (2H, m), 3.50 (4H, m), 2.84 (3H, d, J = 11.3 Hz); ^{31}P NMR (CH_2Cl_2) δ −7.71.

5-Fluoro-5′-amino-2′-deoxy-5′-uridyl 5-Nitro-2-furylmethyl *N*-Methyl-*N*-(2-bromoethyl)phosphoramidate (10). To a suspension of 5-fluoro-5′-amino-2′-deoxyuridine[12] (119 mg, 0.485 mmol) in anhydrous EtOH (2 mL) at 0 °C was added the phosphoramidic chloride **9** (184 mg, 0.509 mmol) in THF (1 mL) Cross-linked poly(4-vinylpyridine) (PVP; 500 mg, 25% cross-linked) was then added. The mixture was allowed to stir at room temperature for 6 h then filtered through a small pad of Celite to remove the PVP. The filtrate was concentrated and chromatographed (10% MeOH:$CHC1_3$) to yield **10** (168 mg, 61%) as a glassy solid: R_f = 0.35 (MeOH:$CHC1_3$ 1:5); ^{1}H NMR (300 MHz, CD_3OD) δ 7.92 (1H, dd, J = 6.8, 1.3 Hz), 7.43 (1H, d, J = 3.7 Hz), 6.80 (1H, d, J = 3.7 Hz), 6.16 (1H, m), 5.05 (2H, m), 4.35 (1H, m), 3.89 (1H, m), 3.54 (2H, m), 3.42 (2H, m), 3.19 (2H, m), 2.75 (3H, m), 2.23 (2H, m); ^{31}P NMR (EtOH) δ −6.85; HRMS ($C_{17}H_{22}BrFN_5O_9P$) calcd 574.0314 $(M + Na)^+$, found 574.0302.

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References

(1) *Cancer Chemotherapeutic Agents*; Foye, W. D., Ed.; American Chemical Society: Washington, DC, 1995; Chapter 3.

(2) Danenberg, P. V. Thymidylate synthetase-A target enzyme in cancer chemotherapy. *Biochim. Biophys. Acta* **1977**, *473*, 73−92.

(3) (a) Heidelberg, C.; Danenberg, P. V.; Langenbach, R. J. Thymidylate synthetase: Mechanism of inhibition by 5-fluoro-2′-deoxyuridylate. *Biochem. Biophys. Res. Commun.* **1972**, *48*, 1565−1571. (b) Santi, D. V.; McHenry, C. S.; Sommer, H. Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. *Biochemistry* **1974**, *13*, 471−481. (c) Heidelberger, C.; Hartmann, K.-U. Studies on fluorinated pyrimidines XIII. Inhibition of thymidylate synthase. *J. Biol. Chem.* **1961**, *236*, 3006−3013. (d) Cohen, S. S.; Flaks, J. G.; Barner, H. D.; Loeb, M. R.; Lichtenstein, J. The mode of action of 5-fluorouracil and its derivatives. *Proc. Natl. Acad. Sci. U.S.A.* **1958**, *44*, 1004−1012.

(4) (a) Santi, D. V. Perspectives on the design and biochemical pharmacology of inhibitors of thymidylate synthetase. *J. Med. Chem.* **1980**, *23*, 103−111. (b) Maggiora, L.; Chang, C. C. T.-C.; Torrence, P. F.; Mertes, M. P. 5-Nitro-2′-deoxyuridine 5′-phosphate. A mechanism-based inhibitor of thymidylate synthetase. *J. Am. Chem. Soc.* **1981**, *103*, 3192−3198.

(5) Inaba, M.; Naoe, Y.; Mitsuhashi, J. Mechanisms for 5-fluorouracil resistance in human colon cancer DLD-1 cells. *Biol. Pharm. Bull.* **1998**, *21*, 569−573.

(6) Farquhar, D.; Chen, R.; Khan, S. 5′-[4-(Pivaloyloxy)-1,3,2-dioxaphosphorinan-2-yl]-2′-deoxy-5-fluorouridine: A membrane-permeating prodrug of 5-fluoro-2′-deoxyuridylic acid (FdUMP). *J. Med. Chem.* **1995**, *38*, 488−495.

(7) (a) Winter, H.; Maeda, Y.; Uchida, H.; Mitsuya, H.; Zemlicka, J. Phosphodiester amidates of unsaturated nucleoside analogues: Synthesis and anti-HIV activity. *J. Med. Chem.* **1997**, *40*, 2191−2195. (b) Valette, G.; Pompon, A.; Girardet, J.-L.; Cappellacci, L.; Franchetti, P.; Grifantini, M.; La Colla, P.; 'Loi, A. G.; Périgaud, C.; Gosselin, G.; Imbach, J.-L. Decomposition pathways and in vitro HIV inhibitory effects of IsoddA pronucleotides: Toward a rational approach for intracellular delivery of nucleoside 5′- monophosphates. *J. Med. Chem.* **1996**, *39*, 1981−1990. (c) McGuigan, C.; Cahard, D.; Sheeka, H. M.; De Clercq, E.; Balzarini, J. Aryl phosphoramidate derivatives of d4T have improved anti-HIV efficacy in tissue culture and may act by the generation of a novel intracellular metabolite. *J. Med. Chem.* **1996**, *39*, 1748−1753. (d) McGuigan, C.; Pathirana, R. N.; Balzarini, J.; De Clerq, E. Intracellular delivery of bioactive AZT nucleotides by aryl phosphate derivatives of AZT. *J. Med. Chem.* **1993**, *36*, 1048−1052.

(8) Abraham, T. W.; Kalman, T. I.; McIntee, E. J.; Wagner, C. R. Synthesis and biological activity of aromatic amino acid phosphoramidates of 5-fluoro-2′-deoxyuridine and 1-B-arabinofuranosylcytosine: Evidence of phosphoramidase activity. *J. Med. Chem.* **1996**, *39*, 4569−4575.

(9) McIntee, E. J.; Remmel, R. P.; Schinazi, R. F.; Abraham, T. W.; Wagner, C. R. Probing the mechanism of action and decomposition of amino acid phosphomonoester amidates of antiviral nucleoside prodrugs. *J. Med.* Chem. **1997**, *40*, 3323−3331.

(10) Fries, K. M.; Joswig, C.; Borch, R. F. Synthesis and evaluation of 5-fluoro- 2′-deoxyuridine phosphoramidate analogues. *J. Med. Chem.* **1995**, *38*, 2672−2680.

(11) Borch, R. F.; Liu, J.; Schmidt, J. P.; Marakovits, J. T.; Joswig, C.; Gipp, J. J.; Mulcahy, R. T. Synthesis and evaluation of nitroheterocyclic phosphoramidates as hypoxia-selective alkylating agents. *J. Med.* Chem. **2000**, *43*, 2258−2265.

(12) Henn, T. F. G.; Garnett, M. C.; Chabra, S. R.; Bycroft, B. W.; Baldwin, R. W. Synthesis of 2′-deoxyuridine and 5-fluoro-2′-deoxyuridine derivatives and evaluation in antibody targeting studies *J. Med. Chem.* **1993**, *36*, 1570.

(13) Freel Meyers, C. L.; Borch, R. F. Activation mechanisms of nucleoside phosphoramidate prodrugs. *J. Med. Chem.* **2000**, *43*, 4319−4327 (accompanying manuscript).

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