Activation Mechanisms of Nucleoside Phosphoramidate Prodrugs

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A series of thymidine and tetrahydrofurfuryl phosphoramidates bearing haloethyl or piperidyl substituents was synthesized and used to investigate the activation mechanisms of nucleoside phosphoramidate prodrugs. Structure assignments for the tetrahydrofurfuryl reaction products were confirmed by comparison to authentic samples. Structural assignments for thymidine phosphoramidate reaction products were made by analogy to the tetrahydrofurfuryl products. Generation of the phosphoramidate anion leads to cyclization and subsequent nucleophilic attack at carbon and phosphorus of the resulting aziridinium ion intermediate to give the observed products. Nucleophilic attack by water at carbon and phosphorus occurs without selectivity, supporting a mechanism of action of haloethylamine nucleoside prodrugs involving intracellular release of the nucleotide. Activation of the benzotriazolyl piperidyl phosphoramidates is followed by P−N bond hydrolysis; this reaction is subject to specific acid catalysis and to nucleophilic catalysis by 1-hydroxybenzotriazole. These results suggest that the mechanism of action of the piperidyl nucleoside phosphoramidates involves the intracellular release of the active nucleotide following P−N bond cleavage, presumably by the action of an endogenous phosphoramidase.

Introduction

The development of nucleoside prodrugs capable of undergoing intracellular activation to the corresponding nucleotide has become an area of intense interest.[1] Our interest in developing new methods for the intracellular delivery of nucleotides has resulted in the synthesis of several biologically active nucleoside phosphoramidates that act as prodrugs for the known thymidylate synthase inhibitor, 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP).[2] These nucleotide prodrug analogues exploit the reactivity of a haloethyl 5′-phosphoramidate moiety that undergoes intracellular activation to the haloethyl phosphoramidate anion followed by rapid P−N bond hydrolysis to liberate the corresponding nucleotide.

The haloethyl nucleoside phosphoramidates prepared in this laboratory bear structural resemblance to the known anticancer agent phosphoramide mustard.[3] The use of haloethyl phosphoramidates as alkylating agents for the treatment of cancer is well-documented.[4] Ludeman and others[5] have carried out extensive investigations of the activation mechanisms of phosphoramide mustard and related phosphorodiamidates that are capable of undergoing cyclization to the corresponding aziridinium ion intermediate. In these cases, the desired reaction at carbon of the aziridinium ion by nucleophiles present in DNA results in alkylation of DNA, although significant hydrolysis of the P−N bond is known to occur as well.[5] It was anticipated that the haloethyl moiety of our nucleoside prodrug analogues would undergo P−N bond hydrolysis following activation and, as a consequence, serve as a masking group for the desired 5′-phosphate.[2] However, little is known about the reactivity of haloethyl phosphoramidate anions bearing ester substituents.

Thus, activation studies were carried out in this laboratory on model haloethyl and piperidyl phosphoramidates for which the parent nucleoside phosphoramidate prodrugs are known to have growth inhibitory activity.[2] The purpose of these studies was to provide convincing evidence that the activation process involves hydrolysis of the P−N bond, supporting a mechanism of action involving intracellular release of the desired nucleotide. The complexity of the results obtained in studies carried out on model haloethyl phosphoramidates raised numerous questions about the proposed activation mechanisms of the parent nucleotide phosphoramidate prodrugs.[2] Furthermore, the piperidyl phosphoramidate analogues, which were assumed to be stable analogues, displayed surprising growth inhibitory activity in L1210 cells. These perplexing results prompted a closer investigation of the activation mechanisms of nucleotide phosphoramidate prodrugs. We report herein the synthesis and activation mechanisms of model thymidine and tetrahydrofurfuryl phosphoramidates.

Results and Discussion

Synthesis of Haloethyl Phosphoramidates. The reaction profiles observed for the nucleoside phosphoramidate analogues were complex. Therefore, tetrahydrofurfuryl phosphoramidates were synthesized and used as models for the investigation of the activation processes. The facile synthesis and characterization of authentic tetrahydrofurfuryl phosphoramidates have allowed us to confirm the structures of reaction products in this system. Confirmation of the structurally related but more complex nucleoside phosphoramidate reaction products was then accomplished by analogy to the tetrahydrofurfuryl model system.

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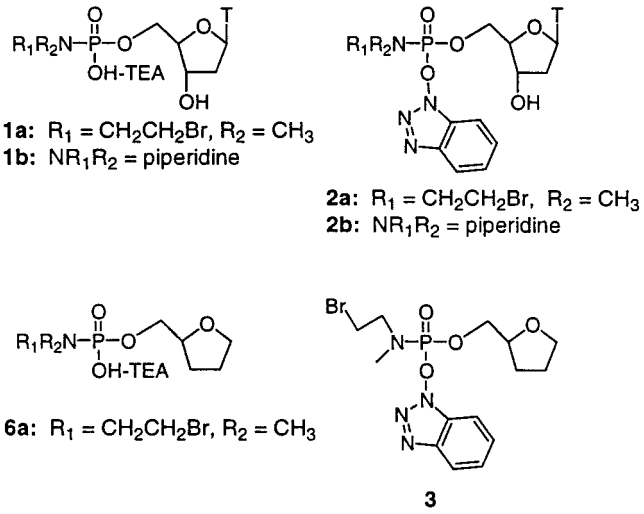
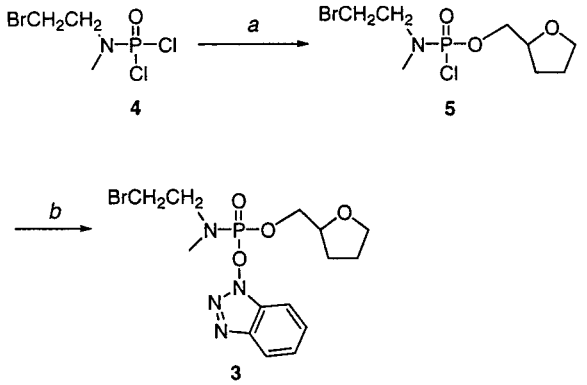
Chart 1



Scheme 1[a]

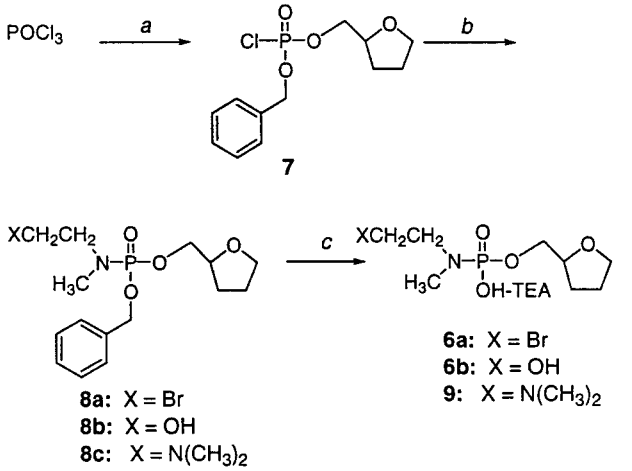


[a] Reagents and conditions: (a) tetrahydrofurfuryl alcohol, LiHMDS, THF, −78 °C to rt, 45 min; (b) HOBT, TEA, THF, rt, 1 h 15 min.

Thymidyl phosphoramidate anion **1a** and benzotriazolyl thymidyl phosphoramidate **2a** (Chart 1) were prepared as described in the accompanying paper.[2] Benzotriazolyl tetrahydrofurfuryl *N*-methyl-*N*-(2-bromoethyl)phosphoramidate (**3**) was prepared as a 1:1 mixture of diastereomers as shown in Scheme 1. Treatment of phosphoryl dichloride **4**[6] with the lithium alkoxide of tetrahydrofurfuryl alcohol in THF afforded the tetrahydrofurfuryl phosphoryl monochloride **5** in 87% yield. Reaction of **5** with 1-hydroxybenzotriazole (HOBT) in the presence of triethylamine resulted in the formation of the desired benzotriazolyl phosphoramidate **3** in 67% yield.

Preparation of haloethyl phosphoramidate anion **6a** was accomplished as shown in Scheme 2. Phosphorus oxychloride was reacted with tetrahydrofurfuryl alcohol in the presence of triethylamine, and the resulting intermediate was treated with benzyl alcohol in triethylamine to form the phosphodiester monochloride **7** in 55% yield as a 1:1 mixture of diastereomers. Compound **7** was treated with *N*-methyl-*N*-(2-bromoethyl)amine hydrobromide in the presence of triethylamine in THF to give benzyl ester **8a** in 70% yield. Catalytic hydrogenolysis in THF resulted in quantitative conversion of benzyl ester **8a** to phosphoramidate anion **6a** (as determined by ^{31}P NMR). Haloethyl phosphoramidate anion **6a** is unstable and was therefore used im-

Scheme 2[a]



[a] Reagents and conditions: (a) i. tetrahydrofurfuryl alcohol, TEA, CH_2Cl_2, −10 °C to rt, 20 min, ii. benzyl alcohol, TEA, CH_2Cl_2, 0 °C, 1 h; (b) $CH_3NHCH_2CH_2X$, TEA, THF or CH_3CN, rt, 1 h; (c) i. H_2, Pd/C, THF, rt, 5 min, ii. TEA.

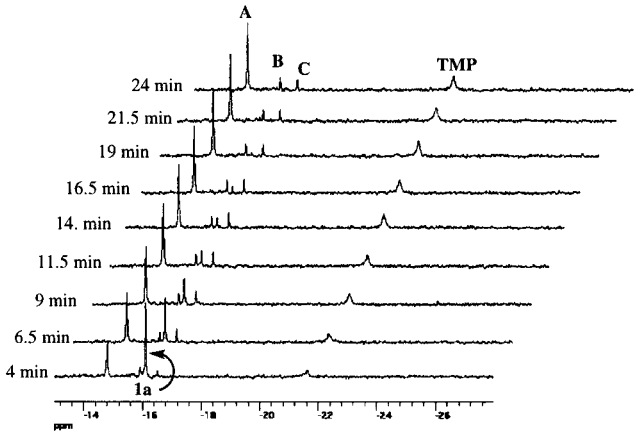
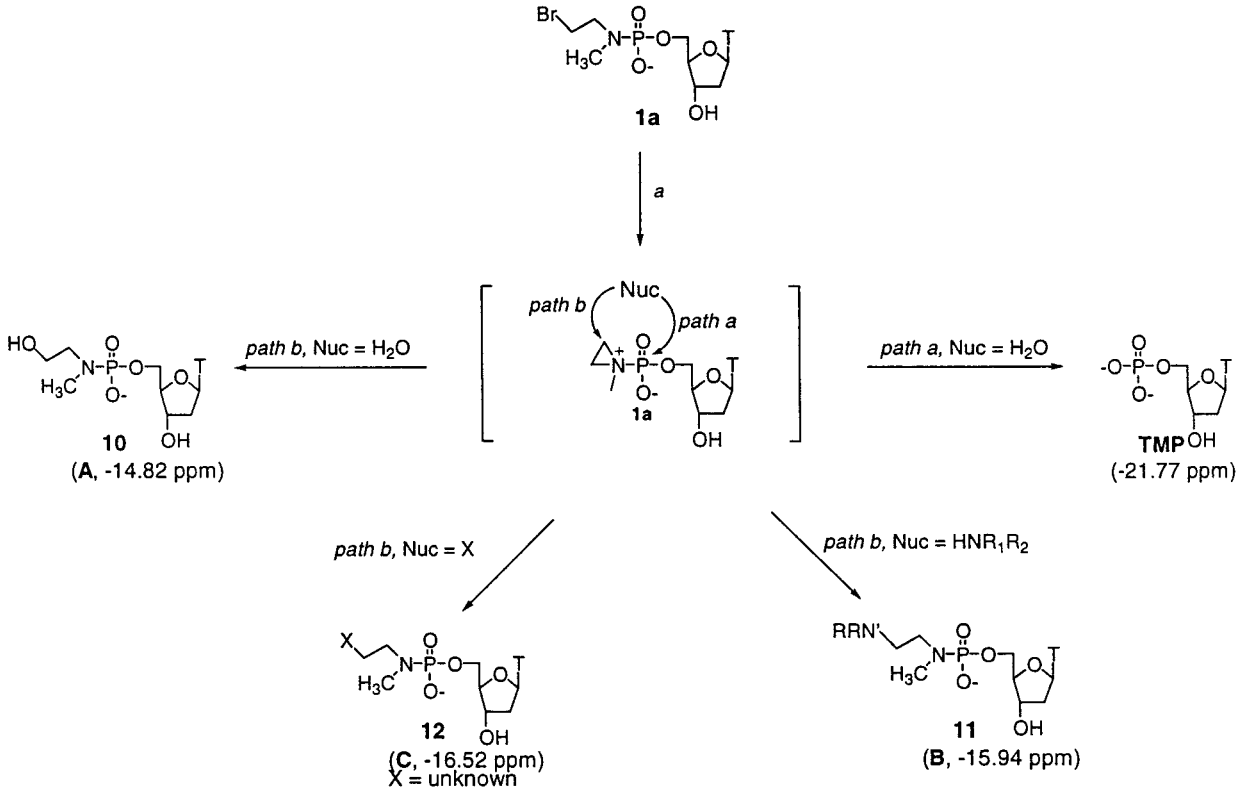


Figure 1. Reaction of phosphoramidate anion **1a** in cacodylate buffer (ca. 100 mM, pH 7.4, 37 °C): **A**, solvolysis product **10**; **B**, amine-trapped product **11**; **C**, buffer product **12**; TMP, thymidine 5′-monophosphate. Chemical shifts are reported relative to triphenylphosphine oxide reference. See Results and Discussion section for details.

mediately in the ^{31}P NMR kinetics experiments described below.

^{31}P NMR Kinetics of *N*-Methyl-*N*-(2-bromoethyl)-phosphoramidates. The reactions of haloethyl phosphoramidates (Chart 1) were studied by using ^{31}P NMR under model physiologic conditions (4.5:1 0.4 M cacodylate buffer/CH_3CN, 37 °C, pH 7.4). ^{31}P Chemical shifts are reported in ppm using 1% triphenylphosphine oxide in benzene-d_6 as the coaxial reference. A kinetic analysis of the reaction was performed using the Quattro Pro optimization routine as described in the Experimental Section. The results for compound **1a** are shown in Figure 1. Thymidine phosphoramidate **1a** (−16.12 ppm) disappeared with a rate constant $k = 0.19 \pm 0.02$ min^{-1} ($t_{1/2}$ = 3.7 min). Disappearance of **1a** resulted in the formation of four products (Scheme 3): solvolysis product **10** (**A**, −14.82 ppm), amine-trapped product **11** (**B**, −15.94 ppm), transient unknown product **12** (**C**, −16.52 ppm), and thymidine monophosphate (TMP, −21.77 ppm). The steady-state concentrations of products **10** and TMP were ca. 43% and 43%, respectively, of the

Scheme 3[a]



[a] Reagents and conditions: (a) 4.5:1 0.4 M cacodylate buffer/acetonitrile, pH 7.4, 37 °C.

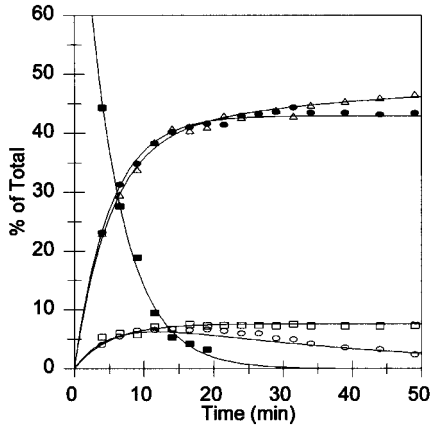


Figure 2. Reaction of phosphoramidate anion **1a** in cacodylate buffer (ca. 100 mM, pH 7.4, 37 °C). Data points were measured from ^{31}P NMR peak areas; the solid lines represent the best-fit values calculated from least-squares minimization using the Quattro Pro optimization routine: (■) phosphoramidate anion **1a**; (●) solvolysis product **10**; (△) hydrolysis product TMP; (□) amine-trapped product **11**; (○) buffer-trapped product **12**.

total mixture. Minor products **11** and **12** made up <13% of the total product composition.

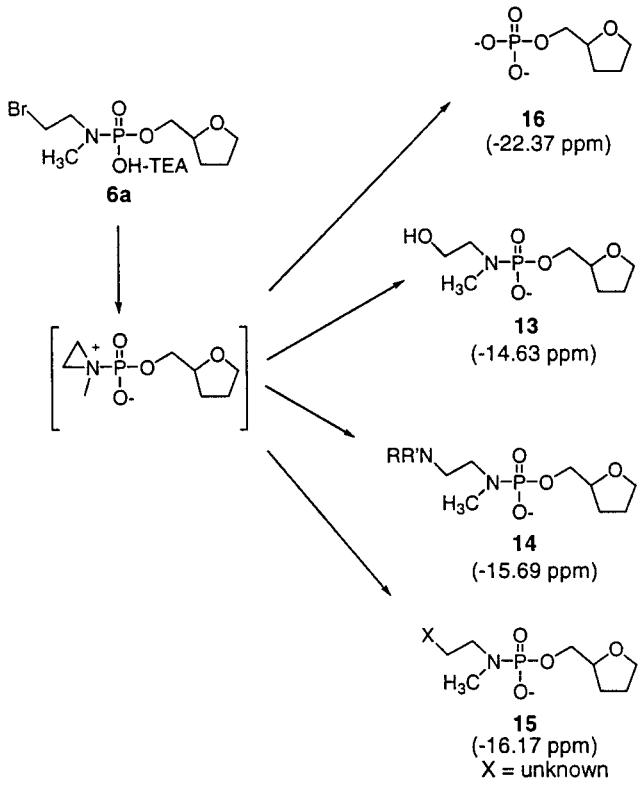
The kinetic analysis of the data obtained in the reaction of thymidine phosphoramidate anion **1a** is shown in Figure 2. The analysis revealed that the rates of formation for all reaction products were identical, suggesting that the rate-limiting step for the conversion of phosphoramidate anion to trapped and P−N bond-cleaved products is the cyclization of anion **1a** to the aziridinium ion intermediate. The concentrations of solvolysis product **10** and amine-trapped product **11** remained constant after phosphoramidate anion **1a** was consumed. Slow conversion of unknown product **12**, presumably to TMP, was also observed.

The complex mixture of phosphate and phosphoramidate anion products observed in the reaction of phosphoramidate **1a** made product isolation and characterization difficult as a method for structure verification. An alternative strategy for product characterization was sought using ^{31}P NMR. Characterization of the peak corresponding to TMP (Figure 1) was accomplished by ^{31}P NMR comparison to authentic TMP. Addition of commercially available TMP to the NMR tube containing the reaction mixture of compound **1a** resulted in an increase in the resonance at −21.77 ppm, confirming the structure assignment for the reaction product TMP. Reaction products **10**−**12** were subsequently characterized by comparison to reaction products formed in the structurally analogous tetrahydrofurfuryl model system.

Tetrahydrofurfuryl phosphoramidate **6a** was synthesized (Scheme 2) and monitored under model physiologic conditions by ^{31}P NMR. The experiment carried out on compound **6a** revealed a reaction profile nearly identical to that observed for thymidine phosphoramidate **1a** (Scheme 3). The resonance for tetrahydrofurfuryl phosphoramidate anion **6a** disappeared (k = 0.34 ± 0.02 min^{-1}, $t_{1/2}$ = 2.0 min) to give four products (Scheme 4): solvolysis product **13** (−14.63 ppm), a small amount of amine-trapped product **14** (−15.69 ppm), transient unknown product **15** (−16.17 ppm), and hydrolysis product, tetrahydrofurfuryl monophosphate **16** (−22.37 ppm). The reaction products were characterized by ^{31}P NMR comparison to authentic phosphate and phosphoramidates as described below.

Synthesis of Authentic Products. Hydroxyethyl phosphoramidate anion **6b** was prepared as described

Scheme 4

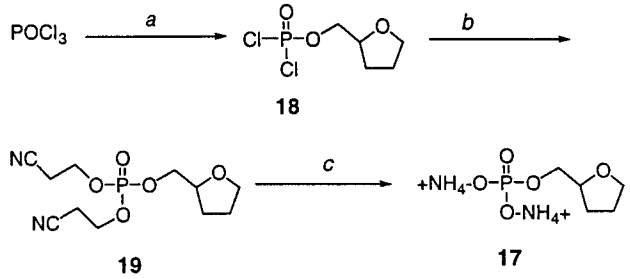


for phosphoramidate anion **6a** in Scheme 2. Following catalytic hydrogenolysis, phosphoramidate anion **6b** was added to the NMR tube containing the reaction mixture of haloethyl phosphoramidate anion **6a**. The resulting increase in the ^{31}P resonance at −14.63 ppm (**13**, Scheme 4) verified the peak assignment for solvolysis product **13** in the reaction of compound **6a**. By analogy, it was concluded that the structure assignment for solvolysis product **10** in the reaction of thymidine phosphoramidate **1a** (Scheme 3) was correct.

Authentic tetrahydrofurfuryl monophosphate **17** was synthesized as shown in Scheme 5. Phosphorus oxychloride was treated with tetrahydrofurfuryl alcohol in the presence of triethylamine to give phosphoryl dichloride **18** in 94% yield. Subsequent treatment with 3-hydroxypropionitrile in the presence of triethylamine afforded phosphotriester **19** in 54% yield. Removal of the cyanoethyl protecting groups in aqueous ammonium hydroxide followed by lyophilization of the resulting aqueous mixture gave authentic monophosphate **17** in 97% yield. Addition of authentic tetrahydrofurfuryl monophosphate **17** to the NMR tube containing the reaction mixture of phosphoramidate **6a** resulted in an increase in the resonance at −22.37 ppm (**16**, Scheme 4). This result confirmed the peak assignment for hydrolysis product **16**.

It was hypothesized that ring opening of *N*-methylaziridine released by nucleophilic displacement at phosphorus could result in the formation of a variety of secondary amines capable of trapping the aziridinium ion present in the reaction mixture to form the phosphoramidate product **14** (Scheme 4). The plausibility of amine trapping at carbon of the aziridinium ion was tested in an experiment in which the reaction of compound **6a** was carried out in the presence of excess dimethylamine (5 equiv) at room temperature. Disappearance of phosphoramidate anion **6a** resulted in the formation of solvolysis product **13** and monophosphate **16** (Scheme 4) as expected. In addition, the formation of a new product **9** (−15.61 ppm) was observed. Confirmation of the structure assignment for this reaction product was accomplished by ^{31}P NMR comparison to authentic *N*-methyl-*N*-(2-dimethylaminoethyl)phosphoramidate anion **9**. Authentic compound **9** was prepared from phosphodiester **7** as shown in Scheme 2. Addition of authentic phosphoramidate anion **9** to the NMR tube containing the reaction mixture resulted in an increase in the ^{31}P NMR resonance at −15.61 ppm. This observation was sufficient to confirm the occurrence of amine attack on the carbon of the aziridinium ion in the reaction of tetrahydrofurfuryl phosphoramidate anion **6a** and thymidine phosphoramidate anion **1a**.

Scheme 5[a]



[a] Reagents and conditions: (a) tetrahydrofurfuryl alcohol, TEA, CH_2Cl_2, −40 °C to rt, 20 min; (b) 3-hydroxypropionitrile, TEA, THF, −10 °C to rt, overnight; (c) concd NH_4OH, rt, 24 h.

The ^{31}P chemical shift of **15** (Scheme 4) indicates that it is most likely a phosphoramidate ester. Disappearance of this ^{31}P NMR resonance, ostensibly to monophosphate **16**, was also observed in the reaction of compound **6a** at 37 °C. It was conceivable that a component of the buffer might participate in the reaction of phosphoramidate anion **6a**.[5] Thus, the reaction of phosphoramidate **6a** was carried out in distilled water over 30 min at room temperature. A ^{31}P NMR spectrum of the sample revealed the presence of unreacted phosphoramidate anion **6a**, solvolysis product **13**, amine-trapped product **14**, and monophosphate **16** as before (Scheme 4). However, the unknown product **15** was not observed in the absence of buffer. This result implicated the participation of buffer in the formation of **15**, and efforts toward further characterization of this peak were abandoned. It should be noted that amine-trapped product **14** and buffer product **15** are simply artifacts of the closed-system environment in these ^{31}P NMR experiments and contribute <15% to the total product composition.

Kinetics of Benzotriazolyl Haloethyl Phosphoramidate Esters. The reaction profiles exhibited by phosphoramidate anions **1a** and **6a** provided a foundation for additional mechanistic studies carried out on the benzotriazolyl phosphoramidate esters **2a** and **3** (Chart 1). It was anticipated that activation of the benzotriazolyl phosphoramidate esters to the corresponding phosphoramidate anions would occur via hydrolysis of the benzotriazolyl group.

A ^{31}P NMR experiment was carried out on benzotriazolyl thymidine phosphoramidate **2a** under model physiologic conditions. Phosphoramidate ester **2a** ap-

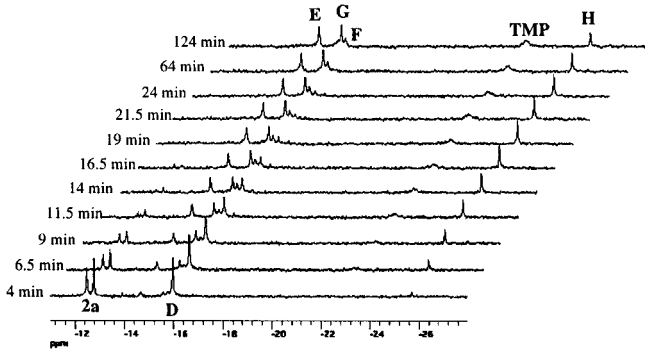


Figure 3. Activation of benzotriazolyl phosphoramidate **2a** in 4.5:1 cacodylate buffer/CH_3CN (ca. 100 mM, pH 7.4, 37 °C): **D**, phosphoramidate anion **20**; **E**, solvolysis product **10**; **F**, amine-trapped product **11**; **G**, OBT-trapped product **21**; **H**, OBT phosphate **22**; TMP, thymidine 5′-monophosphate.

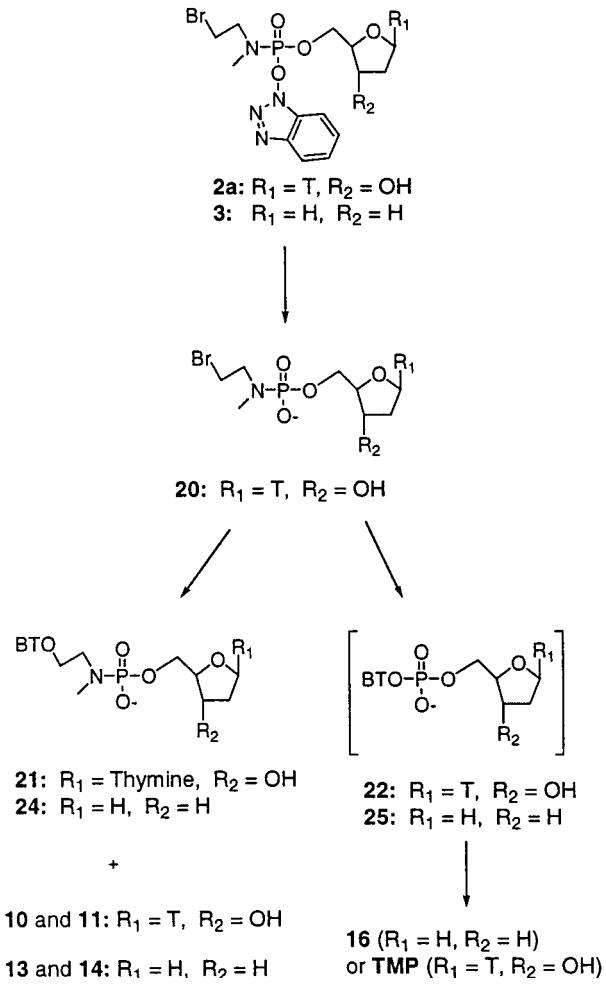
peared as two resonances (−12.50, −12.78 ppm) characteristic of a mixture of diastereomers (Figure 3). Apparent first-order hydrolysis of **2a** ($k = 0.15$ min$^{-1} \pm 0.02$, $t_{1/2} = 4.7$ min) resulted in the formation of phosphoramidate anion **20** (**D**, −15.99 ppm) which appeared as a single resonance (Scheme 6). The observed conversion of two resonances to a single phosphoramidate anion resonance indicated a collapse of the diastereomeric mixture to a single isomer. The short half-life of the OBT ester suggests that nucleoside phosphoramidate prodrugs containing this delivery group are unlikely to be clinically useful.

As shown in Scheme 6, phosphoramidate anion **20** was converted with a rate constant $k = 0.20 \pm 0.01$ min^{-1} ($t_{1/2} = 3.4$ min) to solvolysis product **10** (**E**), amine-trapped product **11** (**F**), and TMP as observed in the reaction of phosphoramidate anion **1a** (Scheme 3). However, little or no buffer product **12** was observed, and two new resonances appeared: a new product **21** (**G**, −15.59 ppm) and P−N bond-cleaved product **22** (**H**, −25.73 ppm). A kinetic analysis of the data obtained indicated that the concentrations of solvolysis product **10**, new product **21**, and amine-trapped product **11** remained constant after phosphoramidate anion **20** was consumed, and product **22** underwent slow conversion to TMP ($k = 0.0042$ min$^{-1} \pm 0.001$ min^{-1}, $t_{1/2} = 164$ min).

The ^{31}P NMR resonances corresponding to reaction products **21** (−15.59 ppm) and **22** (−25.73 ppm) appeared only in the reaction of benzotriazolyl phosphoramidate **2a** (Figure 3) and not in the reaction of the phosphoramidate anion itself (**1a**, Figure 1). On the basis of this observation, it was hypothesized that products **21** and **22** arise from nucleophilic attack by HOBT at carbon and phosphorus of the aziridinium ion, respectively. Confirmation of these structure assignments was sought by comparison to the structurally analogous reaction products formed in the reaction of tetrahydrofurfuryl phosphoramidate ester **3** (Scheme 1).

A ^{31}P NMR experiment carried out on benzotriazolyl tetrahydrofurfuryl phosphoramidate **3** (Scheme 6) revealed a reaction profile nearly identical to that observed for thymidine phosphoramidate **2a** (Scheme 6). Apparent first-order hydrolysis of **3** ($k = 0.094$ min^{-1}, $t_{1/2} = 7.4$ min) resulted in the formation of the corresponding phosphoramidate anion (−15.89 ppm) which was converted with a rate constant $k = 0.43$ min^{-1} ($t_{1/2}$

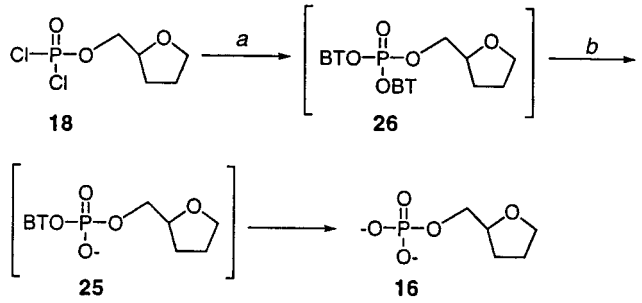
Scheme 6



= 1.6 min) to solvolysis product **13**, amine-trapped product **14**, and tetrahydrofurfuryl monophosphate **16** as observed in the reaction of phosphoramidate anion **6a** (Scheme 4). In addition, a new product **24** (−15.54 ppm) and P−N bond-cleaved product **25** (−25.25 ppm) were observed. As expected, the concentrations of solvolysis product **13**, OBT-trapped product **24**, and amine-trapped product **14** remained constant after phosphoramidate anion was consumed, and product **25** underwent slow conversion to tetrahydrofurfuryl monophosphate **16** ($k = 0.011$ min^{-1}, $t_{1/2} = 61$ min).

The structure assignments for products **24** and **25** are supported by data obtained in an experiment of phosphoramidate anion **6a** carried out in the presence of excess HOBT (5 equiv). In addition to the formation of reaction products **13**−**16** (Scheme 4), the resonances corresponding to products **24** and **25** were observed as the major products. The resonance at −15.54 ppm is consistent with trapping at carbon of the aziridinium ion by the nucleophile HOBT. More interestingly, the resonance at −25.25 ppm is thought to arise from nucleophilic attack by HOBT at phosphorus; cleavage of the P−N bond by nucleophiles other than water has not been observed previously in these systems. Ideally, confirmation of the structure of product **25** might be accomplished by ^{31}P NMR comparison to authentic compound. However, the inherent reactivity of this intermediate made isolation and characterization very difficult. Numerous attempts to prepare authentic OBT

Scheme 7[a]



[a] Reagents and conditions: (a) HOBT, pyridine, THF, 0 °C to rt; (b) 4.5:1 0.4 M cacodylate buffer/THF, pH 7.4, 37 °C.

phosphate were unsuccessful. Thus, an alternative strategy for the structural verification of OBT phosphate **25** was sought.

The characterization of OBT phosphate **25** was ultimately accomplished in a ^{31}P NMR experiment in which the conversion of OBT phosphate **25** to tetrahydrofurfuryl monophosphate **16** was reproduced. This strategy (Scheme 7) involved the hydrolysis of the highly reactive bis(benzotriazolyl) intermediate **26** generated in situ from phosphoryl dichloride **18**. Intermediate **26** was prepared by reaction of **18** with HOBT (2 equiv) in the presence of pyridine (Scheme 7). Following removal of pyridine hydrochloride, the reaction mixture was used immediately in a ^{31}P NMR hydrolysis experiment. Complete conversion of the presumed bis(benzotriazolyl) intermediate **26** to OBT phosphate **25** (−25.17 ppm) was essentially instantaneous. OBT phosphate **25** underwent conversion to tetrahydrofurfuryl phosphate **16** (−22.39 ppm) with a rate constant $k = 0.011 \pm 0.01$ min^{-1} ($t_{1/2} = 64$ min), a value in excellent agreement with that obtained in the reaction of benzotriazolyl tetrahydrofurfuryl phosphoramidate **3** ($k = 0.011$ min^{-1}). The structure assignment is also supported by a peak at *m*/*z* 300 corresponding to **25** in the mass spectrum of a sample taken early in the hydrolysis of **26**.

Nucleophilic Trapping of the Aziridinium Ion. The product distributions in the reactions of phosphoramidates **1a**, **2a**, **3**, and **6a** (Chart 1) are governed largely by the reactions of the aziridinium ion involving water as the nucleophile. In all cases, water reacts without selectivity at carbon and phosphorus. Trapping of the aziridinium ion at carbon and cleavage of the P−N bond by nucleophiles other than water was observed as well. Reaction of HOBT at carbon and phosphorus also occurred without selectivity. On the contrary, amines were found to react only at carbon of the aziridinium ion intermediate. No P−N bond aminolysis was observed in the reactions of phosphoramidates **1a**, **2a**, **3**, and **6a**.

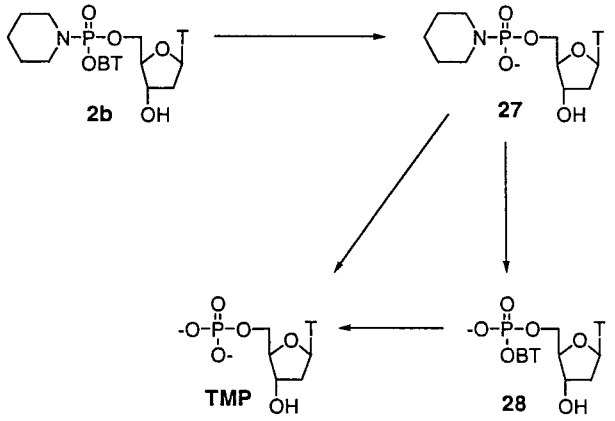
This evidence of exclusive nucleophilic attack at carbon of the aziridinium ion prompted a closer look at some puzzling results obtained in activation studies performed on nucleoside phosphoramidate prodrugs containing a nitrofuryl delivery moiety.[2] Chemical activation of nucleoside phosphoramidates bearing a nitrofuryl group can be carried out using sodium dithionite as a chemical reducing agent. As expected, treatment of the nitrofuryl nucleoside phosphoramidate with aqueous sodium dithionite results in immediate expulsion of the phosphoramidate anion, presumably via the reduction of the nitro group.[4b] However, in this case the phosphoramidate anion was converted exclusively to a single trapped product; no P−N bond cleavage occurred. Bisulfite liberated during the reaction presumably traps the aziridinium ion more effectively than water and predominates over hydrolysis of the P−N bond. This hypothesis was confirmed in an experiment carried out on compound **6a** in which excess bisulfite was added. In this case, the resonance for phosphoramidate anion **6a** disappeared to give a single resonance (−15.81 ppm) corresponding to the bisulfite-trapped product.

This result prompted the consideration of glutathione (GSH), the most abundant intracellular thiol (intracellular concentrations 1−10 mM), in this reaction. An experiment was carried out on phosphoramidate **6a** in which excess GSH (5 equiv, ca. 400 mM) was added. Phosphoramidate anion **6a** disappeared to form solvolysis and hydrolysis products **13** and **16** in addition to the presumed GSH-trapped phosphoramidate. No P−N bond cleavage by GSH was observed, and the overall contribution of GSH-trapped phosphoramidate was <50% of the total composition. Furthermore, the conversion of phosphoramidate anion to hydrolysis product was reduced by <50% in the presence of 400 mM GSH.

In general, polarizable nucleophiles (i.e. nitrogen and sulfur nucleophiles) show a preference for reaction at the more polarizable electrophilic center, in this case, carbon of the aziridinium ion. These data are consistent with experimental data obtained in studies of the reactivities of various nucleophiles on a series of phosphoryl substrates.[7] In general, sulfur nucleophiles are not reactive at phosphorus while oxygen nucleophiles are known to react at both phosphorus and saturated carbon centers. Reactions of amine nucleophiles at phosphorus are highly dependent upon nitrogen basicity and steric effects.

Synthesis and Kinetics of Piperidyl Phosphoramidate Analogues. The piperidyl nucleoside phosphoramidate prodrugs prepared in this laboratory[2] exhibited significant growth inhibitory activity, although they were expected to be "stable" analogues. Thus, the activation mechanism of these compounds was explored using the model thymidine phosphoramidates **1b** and **2b** shown in Chart 1. Phosphoramidates **1b** and **2b** were prepared as described in the accompanying paper.[2] The behavior of the piperidyl phosphoramidate anion was studied in a ^{31}P NMR kinetics experiment carried out on compound **1b** under model physiologic conditions. Phosphoramidate **1a** (−15.55 ppm) hydrolyzed slowly ($t_{1/2} \sim 11$ days) at pH 7.4 resulting in the formation of TMP. Additional studies were performed in which the rate of P−N bond hydrolysis was measured as a function of both pH and buffer concentration. On the basis of data obtained in these studies, it was concluded that the P−N bond hydrolysis reaction is specific acid-catalyzed but not subject to general acid catalysis. The mechanistic data obtained in these studies suggested that, although the biological activity of piperidyl phosphoramidate prodrug analogues[2] is consistent with intracellular liberation of the nucleotide, this most likely occurs by the action of endogenous phosphoramidase. Protonation of the piperidyl nitrogen at physiologic pH

Scheme 8



followed by hydrolysis of the P−N bond cannot account for the biological activity observed for these analogues.

A ^{31}P NMR kinetics experiment carried out on benzotriazolyl phosphoramidate **2b** under model physiologic conditions revealed a slightly different reaction profile. As expected, the resonances for ester **2b** were replaced by a resonance corresponding to the piperidyl phosphoramidate anion **27** ($t_{1/2}$ = 9.7 min, Scheme 8). Apparent nucleophilic displacement of the piperidyl group by HOBT resulted in the formation of covalent intermediate **28**, which then hydrolyzed to give the nucleotide, TMP. Additional studies showed a strong dependence upon HOBT concentration for the rate of conversion of piperidyl phosphoramidate anion **1b** to TMP; a 3-fold increase in the rate of conversion to TMP was observed when the reaction was carried out in the presence of 2 equiv of HOBT. These data suggest that P−N bond hydrolysis of piperidyl phosphoramidates is subject to nucleophilic catalysis by HOBT.

Summary

A series of thymidine and tetrahydrofurfuryl phosphoramidates was synthesized and used as chemical models for the analogous nucleoside phosphoramidate prodrugs.[2] These analogues were studied using ^{31}P NMR for the purpose of increasing our understanding of the reactivity of phosphoramidate anions and for clarifying the proposed mechanism of action for the 5-fluoro-2′-deoxyuridine phosphoramidate prodrugs reported in the accompanying paper.[2]

The structure assignments of phosphate and phosphoramidate products in complex phosphoramidate reaction mixtures were accomplished by comparison of the products to synthesized authentic materials. A knowledge of the reactivity of the aziridinium ion intermediate at carbon and phosphorus toward different nucleophiles was obtained, providing insight into the inherent problems associated with certain types of chemical activation of phosphoramidate prodrugs containing a haloethylamine substituent. Studies carried out on the P−N bond hydrolysis reactions of piperidine phosphoramidates were instrumental for the resolution of the mechanism of activation of phosphoramidates incapable of forming the aziridinium ion intermediate.

The mechanistic results obtained have provided a foundation on which to draw conclusions regarding the mechanism of action of nucleoside phosphoramidates containing either haloethylamine or piperidine substituents. We conclude that the mechanism of action of nucleoside phosphoramidates containing the *N*-methyl-*N*-(2-bromoethyl) substituent proceeds through an aziridinium ion intermediate followed by hydrolysis of the P−N bond to release the active nucleotide intracellularly. The mechanism of action of the piperidyl nucleoside phosphoramidates is most likely the intracellular release of the active nucleotide. However, simple protonation on nitrogen followed by hydrolysis of the P−N bond does not account for the observed growth inhibition at physiologic pH. Cleavage of the P−N bond, in this case, presumably takes place by the action of an endogenous phosphoramidase.

Experimental Section

Materials and Methods. All ^{31}P and ^{1}H NMR spectra were recorded on a 250 MHz Bruker instrument. 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/toluene-d_8 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 μm). 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 diisopropylethylamine were distilled prior to use. Tetrahydrofurfuryl alcohol and benzyl alcohol were distilled from sodium prior to use. Piperidine, *N*,*N*,*N*′-trimethylethylenediamine and 3-hydroxypropionitrile were distilled from calcium hydride prior to use. HOBT was dried in a 90 °C oven. *N*-Methyl-*N*-(2-bromoethyl)amine hydrobromide and 2-(methylamino)ethanol were dried by coevaporation with acetonitrile prior to use.

^{31}P NMR Kinetics Experiments. ^{31}P NMR kinetics experiments were carried out at 37 °C (or ambient temperature, 24−26 °C) on a 250 MHz Bruker NMR using the Bruker variable-temperature unit to control the probe temperature. The actual sample temperature was taken manually before and after each experiment using a Fluke digital thermometer and thermocouple.

Typically, the benzotriazolyl phosphoramidate or phosphoramidate anion (0.015−0.076 mmol) was dissolved in a small amount of organic solvent (ca. 90 μL CH_3CN or THF) and diluted to 0.50 mL with buffer (0.1−0.4 M cacodylate or acetate buffer, ca. 490 μL). The reaction start time was noted at the time of dilution. The homogeneous reaction mixture was adjusted to the appropriate pH with dilute NaOH or HCl and transferred to a 5-mm NMR tube at room temperature. The sample was inserted into the AC 250 Bruker NMR probe and allowed to equilibrate for about 5 min prior to the start of acquisition. The elapsed time was noted at the start of acquisition. Data were collected for 1 or 2 h (longer experiments were followed manually by ^{31}P NMR). Spectra were acquired every 2.5 min for 30 min, then every 5 min for 30 min. For 2-h experiments, spectra were acquired every 10 min for an additional hour. The pH of the reaction mixture was recorded at the end of the experiment. In general, a pH range

of 7.0−8.0 was maintained for experiments whose target pH was 7.4. The relative concentrations of reaction intermediates were determined by measuring the peak areas. The relative concentration for each reactant or product at any given time is represented as a percent of the total.

Reactions involving the addition of a nucleophile (i.e. HOBT, DDTC or dimethylamine) were carried out in the following manner. The nucleophile (5−15 equiv) was added to a small amount of buffer (0.4 M cacodylate or acetate buffer, ca. 490 μL) and the resulting solution was adjusted to the appropriate pH using dilute NaOH or HCl. The mixture was added to a vial containing the phosphoramidate in CH_3CN (ca. 90 μL). The experiment proceeded as described above.

Kinetic Analyses. The kinetic analyses for the reactions described were carried out using the optimization routine in the Quattro Pro Program. The ^{31}P NMR peak areas were measured, and the product composition was determined at each time point as a percentage of the total material. Rate expressions were derived for each reaction, and the rate constants were determined by minimization of the least-squares difference between observed and calculated product composition at each time point. The rate of disappearance of phosphoramidate anion (k_{dis}, min^{-1}) was also determined by performing a linear regression of the ^{31}P NMR peak areas.

Tetrahydrofurfuryl *N*-Methyl-*N*-(2-bromoethyl)phosphoramidochloridate (5). A solution of tetrahydrofurfuryl alcohol (0.81 g, 7.80 mmol) in THF (20 mL) was cooled to −78 °C under an atmosphere of argon. LiHMDS (8.60 mL of 1.0 M solution in THF, 8.60 mmol) was added dropwise. The reaction mixture was stirred at −78 °C for 5 min. Phosphoramidic dichloride **4** (2.00 g, 7.80 mmol) was dissolved in THF (5 mL) and added in one portion to the alkoxide at −78 °C. The reaction mixture was warmed to room temperature over 45 min and quenched with saturated NH_4Cl (10 mL). The reaction mixture was added to EtOAc (25 mL) in a separatory funnel. The aqueous layer was extracted with EtOAc, and the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel chromatography (100:10:0.5 $CHCl_3$:EtOAc:MeOH) to yield **5** (2.19 g, 87%) as a clear oil: R_f = 0.44 (100:10:0.5 $CHCl_3$:EtOAc:MeOH); ^{1}H NMR ($CDCl_3$) δ 4.14 (m, 3H), 3.85 (m, 2H), 3.49 (m, 4H), 2.82 (d, 3H, J = 12.9 Hz), 2.02 (m, 1H), 1.93 (m, 2H), 1.69 (m, 1H); ^{31}P NMR ($CDCl_3$) δ −8.39.

Tetrahydrofurfuryl 1-Benzotriazolyl *N*-Methyl-*N*-(2-bromoethyl)phosphoramidate (3). HOBT (65.4 mg, 0.50 mmol) and triethylamine (0.074 mL, 0.53 mmol) were dissolved in THF (1 mL) and added dropwise to phosphoramidic chloride **5** (141 mg, 0.44 mmol) in THF (4 mL) at room temperature. The reaction mixture was stirred at room temperature under argon for 1 h 15 min. Triethylamine hydrochloride was removed by filtration and the filtrate was concentrated. The residue was purified by silica gel chromatography (4:1 $CHCl_3$:EtOAc) to give benzotriazolyl phosphoramidate **3** (124 mg, 67%) as a clear oil that was a 1:1 mixture of diastereomers (as determined by ^{31}P NMR): R_f = 0.52 (4:1 $CHCl_3$:EtOAc); ^{1}H NMR ($CDCl_3$) δ 7.99 (d, 1H, J = 8.4 Hz), 7.73 (d, 1H, J = 8.2 Hz), 7.53 (t, 1H, J = 8.2 Hz), 7.39 (t, 1H, J = 7.3 Hz), 4.24 (m, 3H), 3.83 (m, 2H), 3.41 (m, 4H), 2.92 (d, 3H, J = 10.2 Hz), 1.99 (m, 1H), 1.91 (m, 2H), 1.69 (m, 1H); ^{31}P NMR ($CDCl_3$) δ −15.84, −16.02 (1:1 mixture); HRMS ($C_{14}H_{20}N_4O_4BrP$) calcd 419.0484 (M + H)$^+$, found 419.0486.

Tetrahydrofurfuryl Benzyl Phosphorochloridate (7). Tetrahydrofurfuryl alcohol (0.50 mL, 5.16 mmol) was dissolved in CH_2Cl_2 (20 mL) and cooled to −10 °C under argon. Phosphorus oxychloride (0.48 mL, 5.16 mmol) was added in one portion followed by the dropwise addition of triethylamine (0.79 mL, 5.68 mmol), and the reaction was warmed to room temperature over 20 min. The reaction mixture was then cooled to 0 °C and benzyl alcohol (0.53 mL, 5.16 mmol) was added dropwise followed by the dropwise addition of triethylamine (0.79 mL, 5.68 mmol). Stirring was continued at 0 °C for 1 h. The viscous reaction mixture was poured into brine (25 mL), and the aqueous layer was extracted with $CHCl_3$ (2 × 20 mL). The combined organic layers were dried over Na_2SO_4 and concentrated. Purification of the crude product by silica gel chromatography (3:1 hexanes:EtOAc) afforded benzyl ester **7** as a clear oil (819 mg, 55%). The product was isolated as a 1:1 mixture of diastereomers (as determined by ^{31}P NMR): R_f = 0.20 (3:1 hexanes:EtOAc); ^{1}H NMR ($CDCl_3$) δ 7.39 (s, 5H), 5.22 (dd, 2H, J_{HCNP} = 8.9 Hz), 4.15 (m, 3H), 3.83 (m, 2H), 1.99 (m, 1H), 1.90 (m, 2H), 1.69 (m, 1H); ^{31}P NMR ($CDCl_3$) δ −21.25, −21.33 (1:1 mixture).

Tetrahydrofurfuryl Benzyl *N*-Methyl-*N*-(2-bromoethyl)phosphoramidate (8a). *N*-Methyl-*N*-(2-bromoethyl)amine hydrobromide (159 mg, 0.73 mmol), which was prepared according to Fries,[6] was suspended in THF (3 mL) under an atmosphere of argon. Phosphoryl monochloride **7** (211 mg, 0.73 mmol) was dissolved in THF (2 mL) and added to the reaction mixture in one portion. Triethylamine (0.12 mL, 0.87 mmol) was added dropwise at room temperature, and the reaction mixture was stirred for 45 min. Triethylamine hydrochloride was removed by filtration and the filtrate was concentrated. The residue was purified by silica gel chromatography (1:1 EtOAc:hexanes → 100% EtOAc) to give **8a** (204 mg, 70%) as a clear oil: R_f = 0.18 (1:1 EtOAc:hexanes); ^{1}H NMR ($CDCl_3$) δ 7.37 (m, 5H), 5.03 (d, 2H, J = 7.7 Hz), 4.12 (m, 1H), 3.96 (m, 2H), 3.82 (m, 2H), 3.41 (m, 4H), 2.69 (d, 3H, J = 9.6 Hz), 1.98 (m, 1H), 1.89 (m, 2H), 1.66 (m, 1H); ^{31}P NMR ($CDCl_3$) δ −16.09; HRMS ($C_{15}H_{23}NO_4BrP$) calcd 392.0626 (M + H)$^+$, found 392.0636.

Tetrahydrofurfuryl Benzyl *N*-Methyl-*N*-(2-hydroxyethyl)phosphoramidate (8b). 2-(Methylamino)ethanol (0.041 mL, 0.52 mmol) was dissolved in THF (1.5 mL) under an atmosphere of argon. Triethylamine (0.082 mL, 0.58 mmol) was added in one portion followed by the addition of molecular sieves (4 Å, 0.5 mL). Phosphoryl monochloride **7** was dissolved in THF (1 mL) and added dropwise at room temperature. The reaction mixture was stirred at room temperature for 1 h and then decanted from the molecular sieves. The sieves were washed with $CHCl_3$ (4 × 2 mL). The combined organic solutions were concentrated and purified by silica gel chromatography (95:5 $CHCl_3$:MeOH) to afford **8b** (88 mg, 78%) as a clear oil. The product was isolated as a 1:1 mixture of diastereomers (as determined by ^{1}H and ^{31}P NMR): R_f = 0.41 (95:5 $CHCl_3$:MeOH); ^{1}H NMR ($CDCl_3$) δ 7.36 (m, 5H), 5.02 and 5.03 (d, 2H, J = 7.8 Hz, 1:1 mixture), 4.10 (m, 1H), 3.97 (m, 2H), 3.82 (m, 2H), 3.68 (t, 2H, J = 5.0 Hz), 3.16 (m, 2H), 2.67 and 2.68 (d, 3H, J = 9.8 Hz, 1:1 mixture), 2.57 (d, 1H), 1.97 (m, 1H), 1.89 (m, 2H), 1.64 (m, 1H); ^{31}P NMR ($CDCl_3$) δ −15.06, −15.09 (1:1 mixture); HRMS ($C_{15}H_{24}NO_5P$) calcd 330.1470 (M + H)$^+$, found 330.1469.

Tetrahydrofurfuryl Benzyl *N*-Methyl-*N*-(2-dimethylaminoethyl)phosphoramidate (8c). Benzyl ester **7** (210 mg, 0.72 mmol) was dissolved in anhydrous CH_3CN (6 mL) under argon. Freshly distilled *N*,*N*,*N*′-trimethylethylenediamine (0.080 mL, 1.08 mmol) was dissolved in CH_3CN (3 mL) and added dropwise over 5 min. The homogeneous reaction mixture was stirred at room temperature for 1 h and then concentrated under reduced pressure. The residue was dissolved in water (25 mL) and the aqueous solution was adjusted to pH 12 with 1 M NaOH. The solution was extracted with $CHCl_3$ (8 × 10 mL). The combined organic layers were dried over Na_2SO_4 and concentrated. The residue was chromatographed on silica gel (EtOH) to afford **8c** (193 mg, 75%) as a clear oil. It should be noted that attempts to purify the crude product by reverse-phase chromatography using CH_3CN/H_2O (0.1% TFA) resulted in decomposition of the product: R_f = 0.14 (EtOH); ^{1}H NMR ($CDCl_3$) δ 7.37 (m, 5H), 5.02 (d, 2H, J = 7.4 Hz), 4.12 (m, 1H), 3.95 (m, 2H), 3.81 (m, 2H), 3.12 (m, 2H), 2.66 (d, 3H, J = 9.8 Hz), 2.41 (t, 2H, J = 7.2 Hz), 2.23 (s, 6H), 1.99 (m, 1H), 1.88 (m, 2H), 1.68 (m, 1H); ^{31}P NMR ($CDCl_3$) δ −16.08; HRMS ($C_{17}H_{29}N_2O_4P$) calcd 357.1943 (M + H)$^+$, found 357.1925.

Tetrahydrofurfuryl *N*-Methyl-*N*-(2-bromoethyl)phosphoramidate Triethylammonium Salt (6a). Phosphoramidate **8a** (7.0 mg, 0.018 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 **8a**. The flask was equipped with a balloon filled with hydrogen, and the

reaction mixture was stirred for 5 min at room temperature. Triethylamine (2.6 μL, 0.019 mmol) was added and stirring was continued for 1−2 min. The catalyst was filtered and the filtrate was concentrated to 0.50 mL. Complete conversion to phosphoramidate anion **8a** was observed by ^{31}P NMR. The remaining THF was removed by rotary evaporation and the unstable product **6a** was immediately analyzed by mass spectrometry or used for a kinetics experiment: ^{31}P NMR (THF) δ −17.41; HRMS ($C_8H_{17}NO_4BrP$, free acid) calcd 302.0157 $(M + H)^+$, found 302.0157.

Tetrahydrofurfuryl *N*-Methyl-*N*-(2-hydroxyethyl)phosphoramidate Triethylammonium Salt (6b). Phosphoramidate anion **6b** was prepared from benzyl ester **8b** (5.7 mg, 0.017 mmol) as described for the preparation of compound **6a**. Quantitative conversion of benzyl ester **8b** to phosphoramidate anion **6b** was observed by ^{31}P NMR. Phosphoramidate anion **6b** was concentrated for mass spectral analysis and used without further purification: ^{31}P NMR (0.4 M cacodylate buffer/CH_3CN, pH 7.4, 37 °C) δ −14.63; HRMS ($C_8H_{18}NO_5P$, free acid) calcd 240.1001 $(M + H)^+$, found 240.1007.

Tetrahydrofurfuryl *N*-Methyl-*N*-(2-dimethylaminoethyl)phosphoramidate Triethylammonium Salt (9). Authentic phosphoramidate **9** was prepared as described for compound **6a** from benzyl ester **8c** (6.6 mg, 0.018 mmol). Quantitative conversion of benzyl ester **8c** to phosphoramidate anion **9** was observed by ^{31}P NMR. Phosphoramidate anion **9** was concentrated for mass spectral analysis and used without further purification: ^{31}P NMR (0.4 M cacodylate buffer/CH_3CN, pH 7.4, 37 °C) δ −15.55; HRMS ($C_{10}H_{23}N_2O_4P$, free acid) calcd 267.1474 $(M + H)^+$, found 267.1476.

Tetrahydrofurfuryl Phosphorodichloridate (18). Phosphorus oxychloride (0.91 mL, 9.79 mmol) was added in one portion to tetrahydrofurfuryl alcohol (1.00 g, 9.79 mmol) dissolved in CH_2Cl_2 (25 mL) at −40 °C under an atmosphere of argon. Triethylamine (1.50 mL, 10.77 mmol) was added dropwise and the reaction mixture was warmed to room temperature over 20 min. The viscous mixture was poured over ice and the water layer was extracted with CH_2Cl_2 (2 × 25 mL). Combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel chromatography (3:1 hexanes:EtOAc) to give **18** (2.01 g, 94%) as a clear oil: R_f = 0.30 (3:1 hexanes:EtOAc); ^{1}H NMR ($CDCl_3$) δ 4.27 (m, 3H), 3.88 (m, 2H), 2.07 (m, 1H), 1.96 (m, 2H), 1.75 (m, 1H); ^{31}P NMR ($CDCl_3$) δ −17.14. HRMS ($C_5H_9O_3Cl_2P$) calcd 218.9745 $(M + H)^+$, found 218.9756.

Tetrahydrofurfuryl Bis(2-cyanoethyl) Phosphate (19). Phosphoryl dichloride **18** (100 mg, 0.46 mmol) was dissolved in THF (3 mL) and cooled to −10 °C under an atmosphere of argon. 3-Hydroxypropionitrile (0.062 mL, 0.91 mmol) and triethylamine (0.130 mL, 0.96 mmol) were dissolved in THF (2 mL) and added dropwise at −10 °C. The reaction mixture was warmed to room temperature and stirred overnight. Triethylamine hydrochloride was removed by filtration, and the filtrate was concentrated under reduced pressure. Purification of the crude product by silica gel chromatography (1:1 EtOAc:$CHCl_3$) afforded **19** (71 mg, 54%) as a colorless oil: R_f = 0.15 (EtOAc:$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 4.31 (m, 4H), 4.10 (m, 3H), 3.85 (m, 2H), 2.80 (t, 4H, J = 6.2 Hz), 2.02 (m, 1H), 1.93 (m, 2H), 1.65 (m, 1H); ^{31}P NMR ($CDCl_3$) δ −29.80; HRMS ($C_{11}H_{17}N_2O_5P$) calcd 289.0953 $(M + H)^+$, found 289.0939.

Tetrahydrofurfuryl Phosphate Diammonium Salt (17). Phosphotriester **19** (10.0 mg, 0.035 mmol) was dissolved in CH_3CN (90 μL) and added to concentrated ammonium hydroxide (500 μL) at room temperature. The mixture was transferred to an NMR tube, and the reaction was monitored by ^{31}P NMR for 30 min. Instantaneous removal of the first cyanoethyl group followed by slow removal of the second cyanoethyl group was observed. The half-life for removal of the second cyanoethyl group was calculated ($t_{1/2}$ = 194 min), and the reaction mixture was transferred to a vial and allowed to stir for 24 h (>7 half-lives). The aqueous mixture was frozen and lyophilized without further purification to give monophosphate **17** (7.3 mg, 97%) as a waxy white solid. Authentic compound **17** was used without further purification: ^{31}P NMR (0.4 M cacodylate buffer/CH_3CN, pH 7.4, 37 °C) δ −22.37; HRMS ($C_5H_9O_5P$) calcd 183.0422 $(M + H)^+$, found 183.0425.

Tetrahydrofurfuryl 1-Benzotriazolyl Phosphate (25). HOBT (324 mg, 2.40 mmol) and pyridine (0.21 mL, 2.60 mmol) were dissolved in THF (3.5 mL) and cooled to −10 °C under an atmosphere of argon. Phosphoryl dichloride **18** (250 mg, 1.14 mmol) was dissolved in THF (1.5 mL) and added dropwise. The reaction mixture was warmed to room temperature and stirred for 2 h. Pyridine hydrochloride was removed by passing the reaction mixture through a plug of cotton. A portion of the filtered reaction mixture (90 μL) was diluted with distilled water (500 μL) and immediately analyzed by electrospray ionization mass spectrometry. Mass spectral data confirmed the presence of OBT phosphate **25**.^{31}P NMR data obtained on an analogous mixture in 0.4 M cacodylate buffer/THF (4.5:1) confirmed the quantitative conversion of bis-benzotriazolyl phosphate **26** to OBT phosphate **25**. OBT phosphate **25** was used without further purification in a kinetics experiment carried out on a buffered mixture (4.5:1 0.4 M cacodylate buffer/ THF) containing **25**: ^{31}P NMR (4.5:1 0.4 M cacodylate buffer/THF, pH 7.4, 37 °C) δ −25.25; HRMS ($C_{11}H_{14}N_3O_5P$, free acid) calcd 300.0749 $(M + H)^+$, found 300.0734.

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