Design and Synthesis of Phosphotyrosine Peptidomimetic Prodrugs

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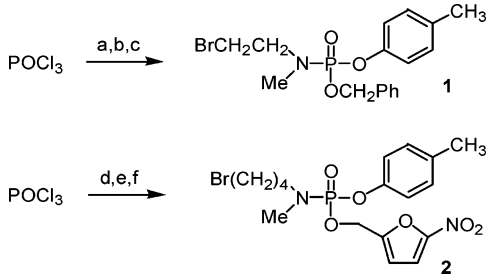
A novel approach to the intracellular delivery of aryl phosphates has been developed that utilizes a phosphoramidate-based prodrug approach. The prodrugs contain an ester group that undergoes reductive activation intracellularly with concomitant expulsion of a phosphoramidate anion. This anion undergoes intramolecular cyclization and hydrolysis to generate aryl phosphate exclusively with a $t_{1/2} = \sim 20$ min. Phosphoramidate prodrugs (**8**−**10**) of phosphate-containing peptidomimetics that target the SH2 domain were synthesized. Evaluation of these peptidomimetic prodrugs in a growth inhibition assay and in a cell-based transcriptional assay demonstrated that the prodrugs had IC_{50} values in the low micromolar range. Synthesis of phosphorodiamidate analogues containing a P−NH−Ar linker (**16**−**18**) was also carried out in the hope that the phosphoramidates released might be phosphatase-resistant. Comparable activation rates and cell-based activities were observed for these prodrugs, but the intermediate phosphoramidate dianion underwent spontaneous hydrolysis with a $t_{1/2} = \sim 30$ min.

Introduction

Protein tyrosine phosphorylation is a common intracellular signaling event that controls numerous signaling pathways. Specific sequences, contained within a protein that undergoes phosphorylation on tyrosine, can serve as recognition sites for other proteins. Src homology 2 (SH2) domains mediate these protein−protein interactions. SH2 domains are noncatalytic motifs of about 100 amino acids that are contained in a variety of proteins.[1,2] The ability of these SH2 domains to bind to phosphotyrosine-containing sequences is governed by the immediately adjacent C-terminal sequence to the phosphotyrosine residue. In particular, it has been found that the SH2 domains of the cytoplasmic tyrosine kinases Src and Lck bind with micromolar affinity to the peptide sequence pY-E-E-I.[3,4] Src and Lck have been implicated in a variety of disorders that include breast cancer, colon cancer, autoimmune diseases, and osteoporosis, and therefore they constitute good targets to develop new therapeutic agents.[5−8]

Several peptidomimetics that target the SH2 domain of Lck have been developed.[9−11] However, the presence of a highly ionizable phosphate group in these compounds compromises their cell membrane permeability. To overcome this problem, several less anionic phosphotyrosine isosteres have been developed and included in the design of SH2 domain inhibitors.[12−14] Another approach that has been used is the generation of phosphotyrosine prodrugs in the form of phosphoesters that are simply activated by enzymatic hydrolysis.[15] However, due to the ubiquitous presence of esterases, this type of approach cannot be considered selective. We have developed previously a series of cell membrane permeable nucleoside phosphoramidate prodrugs that deliver nucleotides intracellularly.[16−18] These phosphoramidates are composed of a phosphoester delivery group and a masking phosphoramide group. The release of the active nucleotide is triggered by the enzymatic transformation and elimination of the delivery group, which is followed by a spontaneous intramolecular cyclization and hydrolysis. Herein we report the application of this prodrug technology to the development of aryl phosphoramidates and apply it to the generation of cell membrane permeable phosphotyrosine mimetic prodrugs.

Scheme 1[a]



[a] Reagents: (a) *p*-cresol, Et_3N, CH_2Cl_2, −10° to 0 °C, 30 min; (b) benzyl alcohol, Et_3N, 0 °C, 1 h; (c) *N*-methyl-*N*-(2-bromo-ethyl)amine hydrobromide, Et_3N, THF, 20 min, rt; (d) *N*-methyl-*N*-(4-bromobutyl) amine hydrobromide, *i*-Pr_2NEt, CH_2Cl_2, −20 °C to rt, 4 h; (e) *p*-cresol, LiHMDS, THF; (f) nitrofurfuryl alcohol, LiHMDS, THF.

Results and Discussion

(a) Model Compounds. In an effort to simplify the reaction and experimental conditions, initial studies were carried out on model *p*-cresol phosphoramidates. Two different phosphoramidates were synthesized (Scheme 1). Phosphoramidate **1** contained a 2-bromoethyl masking group[19] and a benzyl ester as a conditional activating group.[20] Phosphoramidate **2** contained an extended 4-bromobutyl masking group and a nitrofurfuryl ester as an activating group.[17] The syntheses of phosphoramidates **1** and **2** are illustrated in Scheme 1. Phosphorus oxychloride ($POCl_3$) was consecutively reacted with *p*-cresol and benzyl alcohol in the presence of triethylamine followed by reaction with *N*-methyl-*N*-(2-bromoethylamine) hydrobromide to generate phosphoramidate **1**. For the synthesis of phosphoramidate **2**, $POCl_3$ was reacted with *N*-methyl-*N*-(4-bromobutylamine) hydrobromide in the presence of diisopropylethylamine to generate the phosphoramidic chloride which was then consecutively reacted with the lithium alkoxides of *p*-cresol and nitrofurfuryl alcohol.

On the basis of the results obtained from the study of nucleotide phosphoramidate prodrugs, it was expected that the aryl phosphoramidates would behave in a similar manner, i.e.,

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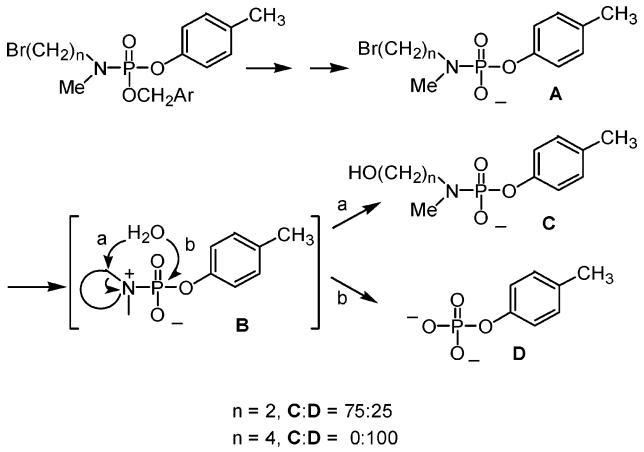
Scheme 2

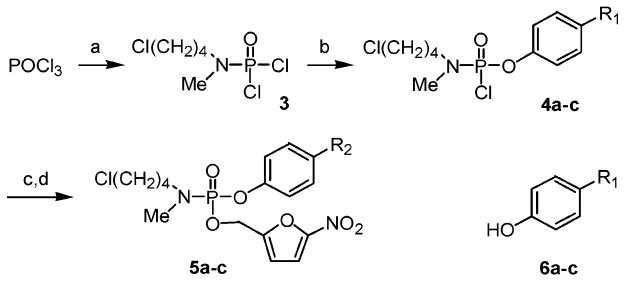


following delivery group activation and elimination, a phosphoramidate anion **A** would be formed (Scheme 2). The inductive effect of the oxygen anion would drive the intramolecular cyclization to yield the formation of the reactive intermediate **B**. This intermediate could react with water following pathway **a** and/or **b** to generate solvolysis product **C** and/or the hydrolysis product **D**, respectively.

To test this hypothesis, phosphoramidate **1** was hydrogenolyzed and its subsequent transformation was monitored by ^{31}P NMR. The analysis showed that when $n = 2$ the phosphoramidate anion **A** disappeared with a half-life of 60 min (0.4 M cacodylate buffer, pH ~ 7.4, 22 °C) and that the desired aryl phosphate **D** represented approximately 25% of the product. Attack of water at the carbon of the aziridinium ring clearly predominates in this system. In contrast, phosphoramidate **A** ($n = 4$) (generated by reduction of **2** with sodium dithionite) was converted to the aryl monophosphate **D** quantitatively with a half-life of <3 min (0.4 M cacodylate buffer, pH ~ 7.4, 22 °C). It is interesting to note that cyclization to the five-membered ring is much faster than to the three-membered ring in this phosphoramidate.

(b) Peptidomimetic Prodrugs. The results obtained from the model compounds prompted the application of this strategy to the synthesis of phosphotyrosine peptidomimetic prodrugs. Peptidomimetics targeted to the SH2 domain were selected to demonstrate the feasibility of the approach. However, it was discovered subsequently that the bromobutyl compound **2** was unstable during storage and appeared to decompose via a reaction initiated by intramolecular cyclization. In an attempt to enhance the stability of the phosphoramidate esters while retaining facile cyclization of the phosphoramidate anion intermediate, the bromine in the masking group was replaced with chlorine. Compound **11** (Scheme 4), which has low micromolar binding affinity to pp60^{src},[21] was selected as the initial peptidomimetic for the prodrug approach (e.g., **8**). By analogy, two additional phosphoramidate prodrugs **9** and **10** that differ in the spacer between the P and P+3 sites were synthesized (Scheme 4). To accomplish the synthesis of phosphoramidates **8**−**10**, a convergent approach was used involving preparation of two building blocks: (1) a carboxy-containing aryl phosphoramidate and (2) a benzylamine. The synthesis of the carboxy-containing building blocks is illustrated in Scheme 3. Reaction of $POCl_3$ and *N*-methyl-*N*-(4-chlorobutylamine) hydrochloride produced phosphoramidic dichloride **3**, which could be isolated and purified. We have shown previously that allyl esters can be used to protect carboxylic acids in the presence of phosphoramidates,[22] so allyl esters **6a**−**c**

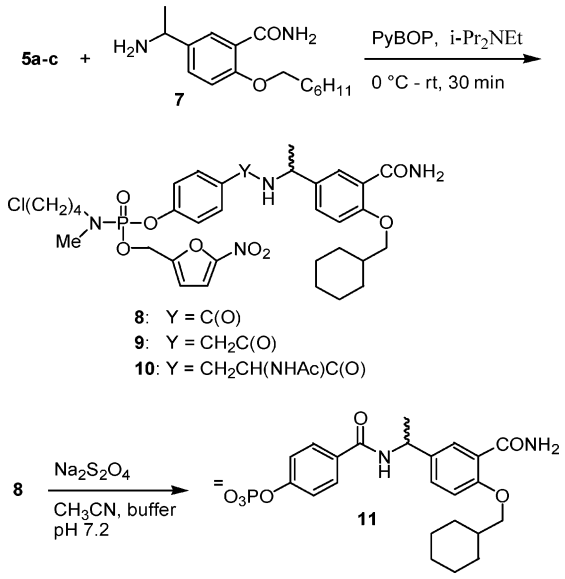
Scheme 3[a]



a, R_1 = COOAllyl; R_2 = COOH
b, R_1 = CH_2COOAllyl; R_2 = CH_2COOH
c, R_1 = CH_2CH(NHAc)COOAllyl; R_2 = CH_2CH(NHAc)COOH

[a] Reagents: (a) *N*-methyl-4-chlorobutylamine hydrochloride, *i*-Pr_2NEt, CH_2Cl_2, 0 °C to rt, 4 h; (b) **6a**−**c**, *i*-Pr_2NEt, CH_2Cl_2; (c) LiHMDS, nitrofurfuryl alcohol, THF, −30 °C, 5 h; (d) $Pd(PPh_3)_4$, $TolSO_2Na$, THF/H_2O.

Scheme 4

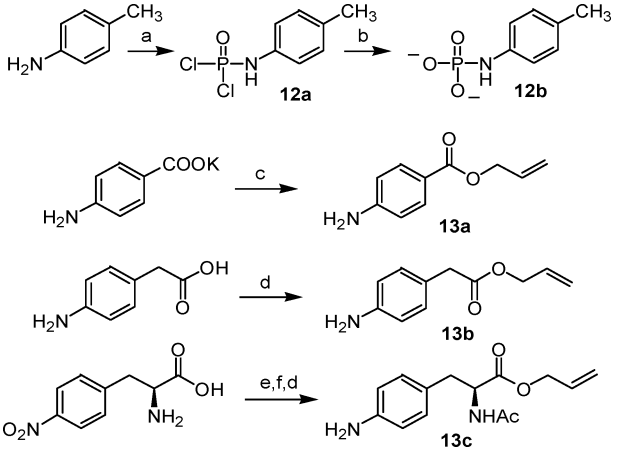


were prepared by refluxing the respective free acids in allyl bromide in the presence of diisopropylethylamine.[23] These allyl esters were then reacted with **3** in the presence of diisopropylethylamine to generate phosphoramidic monochlorides **4a**−**c**, respectively. Addition of preformed nitrofurfuryl lithium alkoxide to **4a**−**c** generated the corresponding phosphoramidates which were deallylated to give the carboxylic acids **5a**−**c**. These aryl phosphoramidates were then coupled to benzylamine **7** (synthesized according to the published procedure)[21] in the presence of PyBOP and diisopropylethylamine to generate phosphoramidate prodrugs **8**−**10** (Scheme 4).

To verify that the free phosphates could be released from their corresponding prodrugs, phosphoramidate **8** was subjected to reductive activation using sodium dithionite, the residue was dissolved in 0.4 M cacodylate buffer, final pH 7.2, and its transformation studied by ^{31}P NMR kinetics. The phosphoramidate anion derived from **8** generated phosphate **11** with a half-life of 20 min at 37 °C and 2.5 h at room temperature (Scheme 4).

(c) Phosphorodiamidates. Because both phosphate ionization and stability to phosphatase degradation can be modulated by replacement of the linking oxygen in phosphotyrosine mimetics,[24,25] the replacement of oxygen by NH was explored in these peptidomimetics. *p*-Toluidine phosphate **12b** (Scheme 5) was

Scheme 5[a]



[a] Reagents: (a) $POCl_3$, *i*-Pr_2NEt, CH_2Cl_2, 0 °C to rt; (b) Na_2CO_3 or NaOH, CH_3CN/H_2O; (c) allyl bromide, DMF; (d) allyl alcohol, cat. H_2SO_4, reflux; (e) Ac_2O, NaOH, H_2O/acetone; (f) H_2, Pd/C, NH.

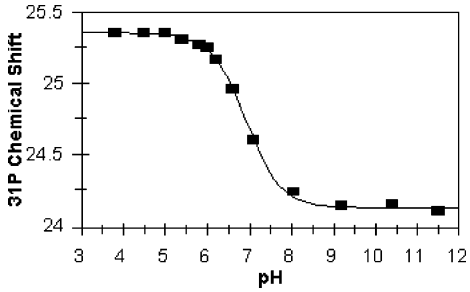


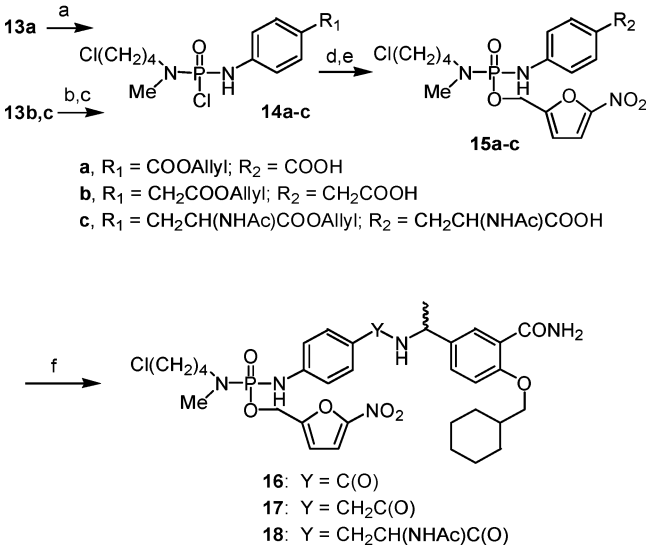
Figure 1. Chemical shift titration curve of *p*-toluidine phosphoramidate **12b**.

generated in situ from basic hydrolysis of phosphoramidic dichloride **12a** and explored as a simple model. The pK_{a2} for this compound was determined using the pH-dependent chemical shift change of the ^{31}P NMR resonance and found to be 6.9 (Figure 1). The overall ^{31}P NMR chemical shift change of about 1.25 ppm suggests that protonation of **12b** occurs on oxygen rather than nitrogen.[26,27]

Synthesis of the analogous phosphorodiamidate peptidomimetic prodrugs **16**−**18** is outlined in Schemes 5 and 6. Allyl ester **13a** was prepared by reaction of *p*-aminobenzoic acid potassium salt with allyl bromide in DMF. Attempts to prepare **13b** and **13c**[28] under similar conditions, however, resulted in allylation of the amino group. These compounds were prepared successfully using Fischer esterification. Phosphorylation of **13a** was slow and generated **14a** in poor yield even after reflux with phosphoramidic dichloride **3** for 10 days. Intermediates **14b** and **14c** were prepared in modest yield using a two-step procedure in which **13b** or **13c** was reacted with $POCl_3$ followed by reaction with *N*-methyl-*N*-(4-chlorobutylamine) hydrochloride. Addition of 5-nitrofurfuryl lithium alkoxide to the three different phosphorodiamidic chlorides **14a**−**c** generated the corresponding phosphorodiamidates which were cleaved to the corresponding acids **15a**−**c** by treatment with *p*-$TolSo_2Na$ and $Pd(PPh_3)$. Finally, the peptidomimetics **16**−**18** were prepared by coupling **15a**−**c** with benzylamine **7** in the presence of PyBOP and diisopropylethylamine.

To demonstrate that the phosphorodiamidates would undergo activation and release of the analogous phosphoramidate dianion, prodrug **16** was activated by sodium dithionite reduction and the subsequent reactions (Scheme 7) studied by ^{31}P NMR; the kinetic profile is shown in Figure 2. Although conversion of the phosphorodiamidate **19** (round symbols) to the phosphate

Scheme 6[a]



[a] Reagents: (a) **3**, *i*-Pr_2NEt, $(ClCH_2)_2$, reflux; (b) $POCl_3$, *i*-Pr_2NEt, CH_2Cl_2, −5 °C; (c) *N*-methyl-4-chlorobutylamine hydrochloride, *i*-Pr_2NEt, CH_2Cl_2, −5 °C; (d) LiHMDS, nitrofuryl alcohol, THF, −20 °C; (e) $Pd(PPh_3)_4$, $TolSO_2Na$, THF/H_2O; (f) PyBOP, *i*-Pr_2NEt, CH_2Cl_2.

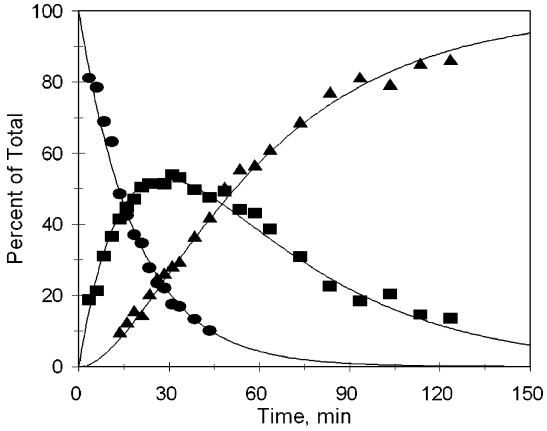
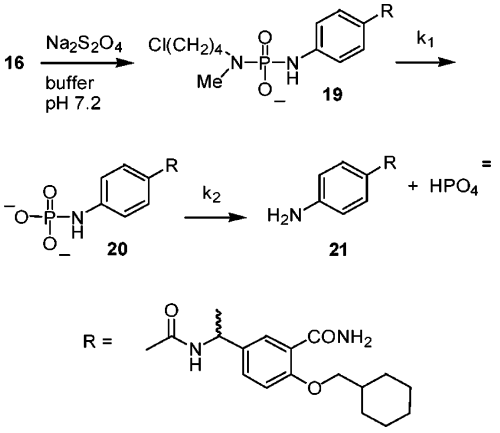


Figure 2. Formation and hydrolysis of NH-phosphate from phosphoramidate **19** at 37 °C, pH = 7.2. (●), phosphoramidate 19; (■), NH-phosphate 20; (▲), inorganic phosphate.

Scheme 7



analogue **20** (square symbols) is clearly evident at early time points, this product is unstable and proceeds to hydrolyze to the corresponding aniline **21** and inorganic phosphate (triangle symbols). Kinetic analysis (0.4 M cacodylate buffer, pH 7.2, 37 °C) gives rate constants $k_1 = 0.052$ and $k_2 = 0.022$ min^{-1},

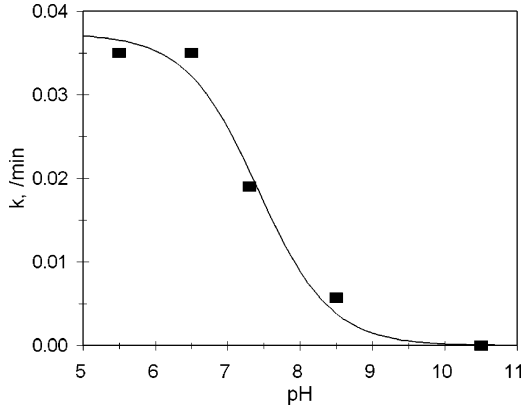


Figure 3. pH−rate profile for the hydrolysis of phosphoramidate **12b**, 37 °C.

Table 1. Response of Jurkat Cells and the NCI Tumor Cell Panel to Prodrugs[a]

cpd	NFAT IC_{50}, μM[b]	NCI panel GI_{50}, μM[c]
8	31 ± 3	12 ± 7
9	9 ± 5	3 ± 1
10	21 ± 4	6 ± 5
16	16 ± 5	18 ± 10
17	20 ± 4	ND[d]
18	28 ± 3	ND

[a] NFAT assay, cells exposed to drug for 6 h; NCI panel, cells exposed to drug for 48 h. See Experimental Section for details. [b] Mean ± SE, n = 4. [c] Mean ± SD for 62 cell lines in the panel. [d] Not determined.

corresponding to half-lives of 13 and 32 min, respectively. The pH dependence of the phosphoramide hydrolysis reaction was investigated using *p*-toluidine phosphate **12b**; the pH−rate profile (Figure 3) clearly demonstrates that this is an acid-catalyzed reaction and that the rate of hydrolysis is near half-maximal at physiologic pH.

Biological Evaluation

Although the chemical activation and ^{31}P analysis had provided insight into the ability of these prodrugs to generate aryl phosphates, there was the need to evaluate the feasibility of this approach in cell-based systems. Therefore, the phosphoramidate prodrugs **8**−**10** and **16**−**18** were evaluated in a cell-based assay that consists of a human leukemia Jurkat J77 T cell line transiently transfected with a luciferase reporter gene driven by four adjacent binding sites for the NFAT (nuclear factor of activated T cells) transcription factor. The activation of NFAT occurs in response to the mobilization of calcium following cross-linking of the T cell antigen receptor and is dependent on the expression of Lck with a functional SH2 domain.[8,29] Thus, inhibitors directed against the SH2 domain of Lck block both the mobilization of calcium and the subsequent activation of gene transcription.[12,30]

Transiently transfected Jurkat cells were incubated with phosphoramidates **8**−**10** and **16**−**18** for 1 h at concentrations from 0.1 to 1000 μM and then activated with a combination of anti-CD3 antibody and PMA. At the end of the 6 h incubation period the cells were lysed, supernatant was collected, and the luciferase activity was measured. The Src family selective tyrosine kinase inhibitor PP2[31] was also studied as a positive control; it showed an IC_{50} = 0.9 μM. The growth inhibitory activity of the compounds was also evaluated in the NCI tumor cell panel. The results from the cell-based assays are shown in Table 1. The prodrugs showed modest and similar activity in both the luciferase and growth inhibition assays. The cell-based IC_{50} values for prodrug **8** are in the same range as the published binding affinity (6 μM) for the free phosphate **11**,[21] consistent with intracellular prodrug activation and phosphate release. These results are also consistent with the targeting of phosphate **11** to the Lck SH2 domain, but it is also possible that binding of this phosphate to other sites could contribute to the reduced levels of luciferase expression. Although it was anticipated that the difference in length of the spacer between the P and P+3 groups would affect the activity of the prodrugs, it appears that all three structures can be accommodated at the binding site. It is surprising that the activities of prodrugs **16**−**18** are comparable to those of **8**−**10**, considering that the phosphorodiamidates released from the former compounds are hydrolytically unstable. However, it is likely that the phosphoramidates released from **8**−**10** will be substrates for intracellular phosphatases, so the intracellular lifetimes of the enzymatically labile phosphoramidates and hydrolytically labile phosphorodiamidates may in fact be comparable.

Conclusions

A novel approach for the intracellular delivery of aryl phosphate peptidomimetics has been developed. This approach consists of cell membrane permeable phosphoramidate prodrugs that undergo intracellular enzymatic activation followed by intramolecular cyclization and hydrolysis to generate the dianionic phosphate. Although the half-life for activation (cyclization) of the phosphoramidate is slower than that for aliphatic phosphoramidate prodrugs (20 min vs < 5 min, respectively), the slower activation rate is still adequate for the generation of a biological response. Attempts to utilize a phosphorodiamidate (by replacing the P(O)−O−Ar bond with P(O)−NH−Ar) that generates a presumed phosphatase-resistant ligand resulted in a prodrug of comparable activity, although NMR studies showed that the phosphoramide dianion undergoes spontaneous hydrolysis with a half-life of ∼30 min. Efforts to extend this work to phosphatase-resistant difluoromethylphosphonate analogues are underway and will be reported in due course.

Experimental Section

Materials and Methods. NMR spectra were recorded using a 250 MHz Bruker spectrometer equipped with a 5 mm multinuclear probe. ^1H chemical shifts are reported in parts per million using tetramethylsilane as an internal reference. ^{31}P NMR spectra were obtained using broadband ^1H decoupling, and chemical shifts are reported in parts per million using 1% triphenylphosphine oxide in benzene-d_6 as a coaxial insert. ^{31}P NMR kinetics carried out at 37 °C were conducted using a Bruker variable temperature unit. Silica gel grade 60 was used to carry out all chromatographic purifications. HPLC analysis was done using a Beckman System Gold equipped with a 168 detector set to 250 nm, a 126 solvent module, and an econosphere C18 column (5 μM, 4 × 250 mm) from Alltech Associates. Mass spectral analyses were obtained from the Mass Spectrometry Laboratory at Purdue University, West Lafayette, IN. All anhydrous reactions were carried out under argon, using flamed dried flasks, and all organic solvents were distilled prior to use.

^{31}P NMR Studies. Kinetics experiments were carried out as described previously.[17] Briefly, the compound (∼20 mg) was dissolved in acetonitrile (80 μL); a solution of the activating agent (sodium dithionite; 3 equiv) in cacodylate buffer (500 μL, 0.4 M, pH = 7.4) was added. The reaction mixture was transferred to a 5 mm NMR tube, and data acquisition was started with the probe maintained at 37 °C. Spectra were acquired every 2.5 min for 30 min and when necessary every 10 min for additional time. Time points were assigned to each data acquisition from the start of the reaction. The integration of the peak areas was used to determine the relative concentration of the intermediates and products.

Kinetic Analysis. The methodology has been described.[20] The ^{31}P peak areas were measured, and the product composition was determined at each time point as a percentage of the total material. For simple first-order reactions, the natural log of the peak area vs time was analyzed by linear regression and the rate constant obtained from the slope of the line. For multistep reactions, 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 using the Quattro Pro optimization routine.

Luciferase Assay. J77 Jurkat cells (2×10^7 cells in 500 μL of FBS-free RPMI media) were transiently transfected with 15 μg of NF-AT-luciferase plasmid by electroporation (300 V, 800 μF), transferred to a flask with 10 mL of FBS-containing RPMI media, and incubated for 24 h. Cells were then harvested, resuspended in 12 mL of FBS-containing RPMI media, and divided into a 6-well plate (2 mL/well). Drug stock solutions were prepared in DMSO, and each well was treated with a different concentration and incubated for 1 h. Each well was then treated with 10 μL of a solution of 1.25 μg of PMA (Calbiochem) in 115 μL of serum-free RPMI media, followed by the addition of 2 μg/well of anti-CD3 antibody (Pharmingen). The cells were incubated for 6 h, spun down, washed with PBS, lysed (1× Promega lysis buffer) for 15 min, and centrifuged. The supernatant was collected and stored at −78 °C overnight. Luciferase activity was measured, using a Lumat LB 9501 luminometer, from a mixture of 50 μL of luciferase substrate (Promega) and 10 μL of supernatant. The data obtained were analyzed by sigmoidal curve fit of relative luciferase units (RLU) vs log of drug concentration. The results are expressed as the drug concentration that generates a 50% decrease in RLU (IC_{50}) of the control value (DMSO).

NCI Human Tumor Cell Line Screen. Details of the methodology are described at http://dtp.nci.nih.gov/branches/btb/ivclsp.html. Briefly, cells are grown in supplemented RPMI 1640 medium for 24 h, then incubated with drug for 48 h at five concentrations from 10^{-8} to 10^{-4} M. The assay is terminated by addition of cold trichloroacetic acid, and the cells are fixed and stained with sulforhodamine B. Bound stain is solubilized, and the absorbance is read on an automated plate reader. Percentage growth inhibition is calculated from time zero, control growth, and the five concentration level absorbances.

***N*-Methyl-*N*-(4-bromobutyl)amine Hydrobromide.** Hydrobromic acid (20 mL, 48% by wt) was added slowly with stirring to *N*-methyl-*N*-4-hydroxybutylamine (4.0 g, 0.39 mmol) at 0 °C, and the reaction mixture was heated to reflux for 2 h. A distillation apparatus was attached, 10 mL of distillate was collected, and an additional 10 mL of 48% HBr was added. After the mixture was refluxed for 4 h, the procedure was repeated, and the reaction mixture was refluxed overnight. Approximately 20 mL of distillate was removed from the reaction mixture, and the still pot residue was poured into acetone that was cooled to −78 °C. A white precipitate formed that was collected by filtration (4.24 g, 42%), mp 86−88 °C. ^1H NMR (D_2O): δ 2.84 (t, 2H), 2.39 (t, 2H), 2.04 (s, 3H), 1.23 (m, 4H).

***N*-Methyl-*N*-(2-bromoethyl) *O*-Benzyl *O*-(4-Methyl)phenyl Phosphoramidate (1).** Triethylamine (0.37 mL, 2.6 mmol) was diluted in dry CH_2CL_2 (1 mL) and added slowly to a pre-cooled solution of *p*-cresol (0.25 mL, 2.4 mmol) and $POCl_3$ (0.22 mL, 2.4 mmol) in dry CH_2CL_2 (6 mL) at −10 °C. The reaction mixture was stirred and warmed to 0 °C over 30 min. Benzyl alcohol (0.25 mL, 2.4 mmol) was added, followed by triethylamine (0.37 mL, 2.6 mmol) diluted in dry CH_2CL_2 (1 mL). The reaction was stirred for 1 h at 0 °C, quenched with saturated ammonium chloride, and extracted with CH_2CL_2 (3 × 5 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield an oil. The crude product was subjected to column chromatography (3:1 hexanes/EtOAc) to give the phosphoryl monochloride (0.35 g, 50%) as an oil, which was used directly in the next step. $R_f = 0.49$ (3:1 hexanes/EtOAc). ^1H NMR ($CDCl_3$): δ 7.38 (s, 5H), 7.10 (m, 4H), 5.29 (m, 2H), 2.32 (s, 3H). ^{31}P NMR ($CDCl_3$): δ −24.0.

Triethylamine (0.090 mL, 0.78 mmol) was added dropwise to a solution of *N*-Methyl-*N*-2-bromoethylamine hydrobromide (0.070 g, 0.34 mmol) and the phosphoryl monochloride prepared above (0.10 g, 0.34 mmol) in dry THF (1.2 mL) at room temperature. The reaction mixture was stirred for 80 min and filtered through a plug of cotton, and the filtrate was evaporated to give an oil. The crude product was purified using column chromatography (10:1 $CHCl_3$/EtOAc) to yield **1** (60 mg, 47%) as an oil. $R_f = 0.56$ (10:1 $CHCl_3$/EtOAc). ^1H NMR ($CDCl_3$): δ 7.35 (s, 5H), 7.10 (m, 4H), 5.10 (d, 2H), 4.48−3.21 (m, 4H), 2.70 (d, 3H), 2.29 (s, 3H). ^{31}P NMR ($CDCl_3$): δ −19.7. MS (ESI) *m*/*z* 398/400 $(M + H)^+$.

***N*-Methyl-*N*-(4-bromobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-Methyl)phenyl Phosphoramidate (2).** Methyl bromobutyl phosphoramidic dichloride (1.0 g, 3.6 mmol) (prepared as described for the chlorobutyl compound **3**) was dissolved in dry THF (5 mL) and cannulated to a pre-cooled solution of *p*-cresol (0.38 g, 3.6 mmol) and LiHMDS (0.1 M solution in THF, 3.9 mL, 3.9 mmol) in dry THF (18 mL) at −78 °C. The reaction mixture was stirred and warmed to room temperature over 3 h. This mixture was then cannulated to a pre-cooled solution of nitrofurfuryl alcohol (0.51 g, 3.6 mmol) and LiHMDS (3.9 mL, 3.9 mmol) in dry THF (18 mL) at −78 °C. The reaction mixture was then warmed to −15 °C and stirred for 2.5 h. The reaction mixture was quenched with saturated ammonium chloride and extracted with EtOAc (2 × 10 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield a brown oil. The residue was purified using column chromatography to yield **2** (0.75 g, 46%) as an orange oil. $R_f = 0.5$ (100:10:0.5, $CHCl_3$/EtOAc/MeOH). ^1H NMR ($CDCl_3$): δ 7.26 (d, 1H), 7.09 (m, 4H,), 6.621 (d, 1H), 5.06 (d, 2H), 3.38 (t, 2H), 3.09 (m, 2H), 2.71 (d, 3H), 2.31 (s, 3H), 1.76 (m, 2H), 1.63 (m, 2H). ^{31}P NMR ($CDCl_3$): δ −19.0. MS (ESI) *m*/*z* 461/463 $(M + H)^+$.

***N*-Methyl-*N*-(4-chlorobutyl) Phosphoramidic Dichloride (3).** $POCl_3$ (2.4 mL, 25 mmol) was added to a pre-cooled solution of *N*-methyl-*N*-(4-chlorobutyl)amine hydrochloride (4.0 g, 25 mmol) in dry CH_2CL_2 (40 mL) at 0 °C, followed by the addition of triethylamine (7.1 mL, 51 mmol) diluted in dry CH_2CL_2 (20 mL). The reaction mixture was removed from the cooling bath, stirred overnight at room temperature, quenched with saturated ammonium chloride, and extracted with CH_2CL_2 (3 × 10 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to dryness to yield an oil. The crude product was purified using column chromatography (3:1 hexanes/EtOAc) to yield **12** (4.8 g, 80%) as a clear oil. $R_f = 0.45$ (3:1 Hex/EtOAc). ^1H NMR ($CDCl_3$): δ 3.58 (t, 2H), 3.29 (m, 2H), 2.84 (d, 3H), 1.79 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −6.6.

Allyl 4-Hydroxybenzoate (6a). Diisopropylethylamine (6.3 mL, 36 mmol) was added to a suspension of 4-hydroxybenzoic acid (5.0 g, 36 mmol) in allyl bromide (86 mL). The reaction mixture was refluxed for 2 h and cooled to room temperature. Excess allyl bromide was evaporated under reduced pressure, and the remaining oil was dissolved in EtOAc (50 mL) and washed with water (3 × 10 mL). The organic extract was washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield a yellow oil. The crude product was purified using column chromatography (10:1 $CHCl_3$/EtOAc) to yield **6a** (5.0 g, 78%) as a white solid, mp 100−102 °C. $R_f = 0.44$ (10:1 $CHCl_3$/EtOAc). ^1H NMR ($CDCl_3$): δ 7.99 (d, 2H), 6.86 (d, 2H), 6.02 (m, 1H), 5.30 (m, 2H), 4.80 (d, 2H). MS (ESI) *m*/*z* 179 $(M + H)^+$.

Allyl 4-Hydroxyphenylacetate (6b). Allyl ester **6b** was synthesized from phenylacetic acid (2.0 g, 13 mmol) as described for **6a** and obtained as an oil (2.3 g, 89%). $R_f = 0.31$ (10:1 $CHCl_3$/EtOAc). ^1H NMR ($CDCl_3$): δ 7.16 (d, 2H), 6.76 (d, 2H), 5.92 (m, 1H), 5.28 (m, 2H), 4.60 (d, 2H), 3.64 (s, 2H).

***N*-Acetyl-L-tyrosine Allyl Ester (6c).** Allyl ester **6c** was synthesized from Ac-Tyr-OH (10 g, 45 mmol) as described for **6a** and obtained as an oil (9.2 g, 80%). ^1H NMR ($CDCl_3$): δ 7.00 (d, 2H), 6.77 (d, 2H), 5.97 (m, 2H), 5.31 (m, 2H), 4.89 (m, 1H), 4.61 (d, 2H,), 3.13 (m, 2H), 2.00 (s, 3H).

***N*-Methyl-*N*-(4-chlorobutyl) *O*-4-Carboallyloxyphenyl Phosphoramidic Chloride (4a).** Diisopropylethylamine (0.73 mL, 4.2

mmol) was added neat to a pre-cooled solution of phosphoramidic dichloride 3 (0.50 g, 2.1 mmol) and allyl ester **6a** (0.37 g, 2.1 mmol) in dry CH_2CL_2 (8 mL) at −15 °C. The reaction mixture was stirred for 1 h at −15 °C and 3 h at −5 °C, then quenched with saturated ammonium chloride, and extracted with CH_2CL_2 (2 × 10 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to give an oil. The crude product was purified using column chromatography (10:1 $CHCl_3$/EtOAc) to yield **4a** (0.50 g, 63%) as a clear oil. R_f = 0.60 (10:1 $CHCl_3$/EtOAc). ^{1}H NMR ($CDCl_3$): δ 8.10 (d, 2H,), 7.34 (d, 2H), 6.09−5.98 (m, 1H), 5.45−5.28 (m, 2H), 4.84−4.81 (m, 2H), 3.57 (t, 2H), 3.35−3.17 (m, 2H), 2.84 (d, 3H), 1.82−1.79 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −13.1. MS (ESI) *m*/*z* 380/382 $(M + H)^+$.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(4-Carboallyloxymethyl)phenyl Phosphoramidic Chloride (4b).** Phosphoramidic chloride **4b** was synthesized from allyl ester **6b** (0.20 g, 10 mmol) as described for **4a**. The crude product was purified using column chromatography (10:1 $CHCl_3$/EtOAc) to yield **4b** a clear oil (0.13 g, 32%). R_f = 0.71 (10:1 $CHCl_3$/EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.32−7.19 (m, 4H), 5.98−5.82 (m, 1H), 5.31−5.20 (m, 2H), 4.60 (d, 2H), 3.64 (s, 2H), 3.58 (t, 2H), 3.25−3.18 (m, 2H), 2.83 (d, 3H), 1.80−1.78 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −12.7. MS (ESI) *m*/*z* 394/396 $(M + H)^+$.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(4-((*S*)-2-Acetylamino-2-carboallyloxyethyl))phenyl Phosphoramidic Chloride (4c).** Phosphoramidic chloride **4c** was synthesized from **6c** (0.50 g, 1.9 mmol) as described for **4a**, with the exception that the reaction mixture was stirred overnight. The crude product was purified using column chromatography (5% MeOH in CH_2CL_2) to yield **4c** (0.49 g, 55%) as an oil. R_f = 0.57 (5% MeOH in CH_2CL_2). ^{1}H NMR ($CDCl_3$:) δ 7.20−7.09 (m, 4H), 5.99−5.78 (m, 2H), 5.35−5.25 (m, 2H), 4.93−4.85 (m, 1H), 4.61 (d, 2H), 3.58 (t, 2H), 3.26−3.13 (m, 4H), 2.83 (d, 3H), 2.00 (s, 3H), 1.96−1.75 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −12.7. MS (ESI) *m*/*z* 465/467 $(M + H)^+$.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-Carboxy)phenyl Phosphoramidate (5a).** Phosphoramidic monochloride **4a** (0.63 g, 1.2 mmol) was dissolved in dry THF (2 mL) and cannulated to a pre-cooled solution of nitrofurfuryl alcohol (0.19 g, 1.3 mmol) and LiHMDS (1.0 M solution in THF, 1.5 mL, 1.5 mmol) in dry THF (2 mL) at −78 °C. The reaction was brought to −40 °C and stirred for 5.5 h. Saturated ammonium chloride was added, and the mixture was extracted with ethyl acetate (3 × 5 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield a dark oil. The crude product was purified by column chromatography (10:1 $CHCl_3$/EtOAc) to yield the allyl ester of phosphoramidate **5a** (0.30 g, 52%) as an orange oil. R_f = 0.25 (10:1 $CHCl_3$/EtOAc). ^{1}H NMR ($CDCl_3$): δ 8.05 (m, 2H), 7.26 (m, 3H), 6.64 (d, 1H), 6.05 (m, 1H), 5.31 (m, 2H), 5.09 (d, 2H), 4.82 (d, 2H), 3.53 (t, 2H), 3.1 (m, 2H), 2.72 (d, 3H), 1.67 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −21.7.

Sodium *p*-toluenesulfinate (0.29 g, 1.6 mmol) was dissolved in water (2.5 mL) and added to a solution of the allyl ester (0.71 g, 1.5 mmol) and $Pd(PPh_3)_4$ (80 mg, 73 μmol) in THF (6 mL). The reaction mixture was stirred for 30 min at room temperature, diluted with diethyl ether, and washed with water (3 × 5 mL). The aqueous extracts were combined, washed with ether (1 × 5 mL), acidified to pH 3 with 2% HCl, and extracted with EtOAc. The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield phosphoramidate **5a** (0.51 g, 78%) as an orange foam. ^{1}H NMR ($CDCl_3$): δ 8.08 (d, 2H), 7.31 (m, 3H), 6.66 (d, 1H), 5.11 (d, 2H), 3.53 (t, 2H), 3.13 (m, 2H), 2.74 (d, 3H), 1.71 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −20.4.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-Carboxymethyl)phenyl Phosphoramidate (5b).** Phosphoramidate **5b** (0.12 g, 36%) was synthesized in two steps from **4b** (0.20 g, 0.51 mmol) as described for **5a**. ^{1}H NMR ($CDCl_3$): δ 7.26−7.04 (m, 5H), 6.60 (d, 1H), 5.43−5.19 (m, 2H), 5.06 (d, 2H), 3.62 (s, 2H), 3.51 (t, 2H), 3.18−2.96 (m, 2H), 2.71 (d, 3H), 1.77−1.55 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −19.0.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-((*S*)-2-Acetylamino-2-carboxyethyl))phenyl Phosphoramidate (5c).** Phosphoramidate **5c** (0.15 g, 27%) was synthesized in two steps from **4c** (0.48 g, 1.0 mmol) as described for **5a**. R_f = 0.41 (10:1 $CHCl_3$/EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.30−7.25 (m, 1H), 7.23−6.83 (m, 4H), 6.80−6.47 (m, 1H), 5.09 (d, 2H), 5.01−4.59 (m, 2H), 3.76−3.33 (m, 2H), 3.36−2.98 (m, 4H), 2.98−2.55 (m, 3H), 2.14−1.77 (d, 3H), 1.89−1.55 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −19.3, −19.4 (1:1 mixture of diastereomers). MS (ESI) *m*/*z* 532/534 $(M + H)^+$.

5-(1-Aminoethyl)-2-cyclohexylmethoxybenzamide (7). Compound **7** was prepared from 5-acetylsalicylamide according to a three-step procedure described by Lunney et al. [21]

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-(1-(3-Carbamoyl-4-cyclohexylmethoxyphenyl)ethylcarbamoyl))-phenyl Phosphoramidate (8).** Diisopropylethylamine (0.11 mL, 0.62 mmol) was added neat to a pre-cooled solution of phosphoramidate **5a** (0.13 g, 0.28 mmol), amine **7** (0.090 g, 0.31 mmol), and PyBOP (0.16 g, 0.31 mmol) in dry CH_2CL_2 (2 mL) at 0 °C. The reaction was stirred for 10 min at 0 °C and 30 min at room temperature, then quenched with saturated ammonium chloride, and extracted with CH_2CL_2 (3 × 5 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield an oil. The crude product was purified using column chromatography (10% MeOH in EtOAc) to yield **8** (13 mg, 67%) as a white foam. ^{1}H NMR ($CDCl_3$): δ 8.25 (d, 1H), 7.72 (d, 2H), 7.50 (d, 1H), 7.24 (m, 3H), 6.95 (d, 2H), 6.63 (d, 1H), 5.28 (t, 1H), 5.07 (d, 2H), 3.93 (d, 2H), 3.52 (t, 2H), 3.09 (m, 2H), 2.70 (d, 3H), 1.87−1.58 (m, 14H), 1.48−0.85 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −21.2. HRMS (ESI) $C_{33}H_{42}ClN_4O_9P$ calculated 705.2456 $(M + H)^+$, found 705.2432. Anal. ($C_{33}H_{42}ClN_4O_9P\cdot H_2O$) C, H, N.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(1-(3-Carbamoyl-4-cyclohexylmethoxyphenyl)methyl)phenyl Phosphoramidate (9).** Compound **9** was obtained from **5b** (0.12 g, 0.25 mmol) as described for the preparation of **8**. The crude product was purified using column chromatography (5% MeOH in EtOAc) to yield **9** (0.10 g, 55%) as a brown foam. R_f = 0.44 (5% MeOH in EtOAc). ^{1}H NMR ($CDCl_3$): δ 8.10−8.00 (m, 1H), 7.99−7.86 (m, 1H), 7.45−7.09 (m, 7H), 6.98−6.85 (m, 1H), 6.69−6.56 (m, 1H), 6.05−5.89 (m, 1H), 5.87−5.69 (m, 1H), 5.19−4.95 (m, 3H), 4.02−3.81 (m, 2H), 3.64−3.29 (m, 4H), 3.26−2.92 (m, 2H), 2.87−2.52 (m, 3H), 2.02−1.57 (m, 11H), 1.54−0.89 (m, 6H). ^{31}P NMR ($CDCl_3$): δ −18.9, −19.0 (1:1 mixture of diastereomers). HRMS (ESI) $C_{34}H_{44}ClN_4O_9P$ calculated 719.2613 $(M + H)^+$, found 719.2600. Anal. ($C_{34}H_{44}ClN_4O_9\ P\cdot 2H_2O$) C, H, N. H: calcd, 6.41; found, 5.89.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-((*S*)-2-Acetylamino-2-(1-(3-carbamoyl-4-cyclohexylmethoxyphenyl)ethylcarbamoyl)ethyl))phenyl Phosphoramidate (10).** Compound **10** was obtained from **5c** (0.15 g, 0.27 mmol) as described for the preparation of **8**. The crude product was purified using column chromatography (10% MeOH in EtOAc) to yield **10** (0.13 g, 58%) as a brown foam. R_f = 0.34 (10% MeOH in EtOAc). ^{1}H NMR ($CDCl_3$): δ 8.26−8.11 (m, 1H), 8.09−7.99 (m, 1H), 7.95−7.76 (m, 1H), 7.24−7.09 (m, 2H), 7.08−6.99 (m, 2H), 6.96−6.82 (m, 1H), 6.72−6.55 (m, 1H), 6.50−6.20 (m, 1H), 6.18−6.54 (m, 1H), 5.16−4.82 (m, 3H), 4.79−4.46 (m, 1H), 4.03−3.78 (m, 2H), 3.62−3.43 (m, 2H), 3.15−2.81 (m, 5H), 2.80−2.51 (m, 3H), 2.04−1.89 (m, 3H), 1.87−1.56 (m, 10H), 1.49−0.90 (m, 8H). ^{31}P NMR ($CDCl_3$): δ −18.9. HRMS (ESI) $C_{37}H_{49}ClN_5O_{10}P$ calculated 790.2984 $(M + H)^+$, found 790.2981. Anal. ($C_{37}H_{49}ClN_5O_{10}P\cdot H_2O$) C, H, N.

***N*-(4-Methylphenyl)phosphoramidic Dichloride (12a).** A solution of diisopropylethylamine (0.81 mL, 4.7 mmol) in CH_2CL_2 (3 mL) was added slowly to a pre-cooled solution of *p*-toluidine (0.50 g, 4.7 mmol) and $POCl_3$ (0.43 mL, 4.7 mmol) in CH_2CL_2 (30 mL) at −10 °C. The cooling bath was removed, and the reaction was stirred overnight at room temperature. Saturated ammonium chloride was added, the layers were separated, and the aqueous layer was extracted with CH_2CL_2. The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and evaporated. The crude product was purified by column chromatography (10:1 $CHCl_3$:

EtOAc) to yield **12a** (0.72 g, 69%) as an oil. NMR ($CDCl_3$): δ 7.22 (d, 2H), 7.16 (d, 2H), 2.30 (s, 3H). ^{31}P NMR ($CDCl_3$): δ −16.6.

Kinetics. A solution of **12a** (20 mg) in acetonitrile (300 μL) was added to a solution of Na_2CO_3 (20 mg) in water (300 μL). The solution was mixed for 5 min, passed through a short plug of Celite, and diluted in cacodylate buffer (400 μL, 0.4 M). The pH was adjusted to 7.4, and the resulting solution was monitored by ^{31}P NMR at 37 °C.

***N*-(4-Methylphenyl)phosphoramidic Acid (12b).** A solution of **12a** (0.50 g, 2.2 mmol) in acetonitrile (5 mL) was added slowly to a solution of NaOH (0.18 g, 4.5 mmol) in H_2O (10 mL) at 0 °C. The pH was monitored near the end of the addition to ensure that the pH remained >11. The solution was then stirred for 30 min, warmed to room temperature, and filtered through Celite. This solution of **12b** as its disodium salt was suitable for all further experiments. ^{31}P NMR (D_2O): δ −24.8.

Allyl 4-Aminobenzoate (13a). Allyl bromide (0.99 mL, 11 mmol) was added to a suspension of potassium *p*-aminobenzoate (2.0 g, 11 mmol) in DMF (40 mL). The reaction mixture was stirred overnight, diluted with water, and extracted with EtOAc (3 × 15 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield an oil. The crude product was purified by column chromatography (10:1 $CHCl_3$/EtOAc) to yield **13a** (2.0 g, 70%) as a foam. ^{1}H NMR ($CDCl_3$): δ 7.99 (d, 2H), 7.08 (d, 2H), 6.05−5.97 (m, 1H), 5.51−5.30 (m, 2H), 4.82−4.78 (m, 2H).

Allyl 4-Aminophenylacetate (13b). A mixture of 4-aminophenylacetic acid (0.50 g, 3.3 mmol), allyl alcohol (20 mL), and concentrated H_2SO_4 (1 mL) was refluxed for 2 h and then allowed to cool to room temperature. The reaction mixture was poured over ice, basified (pH ∼ 10) with Na_2CO_3, and extracted with EtOAc (3 × 10 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to dryness to yield an oil. The crude product was purified using column chromatography (10:1 $CHCl_3$/EtOAc) to yield **13b** (0.63 g, 51%) as an oil. R_f = 0.34 (10:1 $CHCl_3$/EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.06 (d, 2H), 6.61 (d, 2H), 6.00−5.80 (m, 1H), 5.32−5.11 (m, 2H), 4.57 (d, 2H), 3.50 (s, 2H).

***N*-Acetyl-4-aminophenylalanine Allyl Ester (13c).** Ac_2O was added slowly to a solution of 4-nitrophenylalanine (3.0 g, 13 mmol) and NaOH (1.1 g, 26 mmol) in a 3:1 mixture of H_2O/acetone (120 mL). After the mixture was stirred for 1 h, acetone was removed under reduced pressure. The remaining aqueous solution was basified (pH ∼10) with saturated sodium bicarbonate and extracted with EtOAc (3 × 10 mL). The organic extracts were discarded. The aqueous layer was acidified (pH ∼ 3) with dilute HCl and extracted with EtOAc. The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to give *N*-acetyl-4-nitrophenylalanine (3.4 g, 74%) as a white solid. ^{1}H NMR (DMSO-d_6): δ 8.16 (d, 1H), 8.11 (d, 2H), 7.48 (d, 2H), 4.45 (m, 1H), 3.20−2.87 (m, 2H), 1.71 (s, 3H). A portion of this product (2.5 g, 9.6 mmol) was dissolved in ethanol (100 mL), 10% Pd/C (0.5 g) was added, and the mixture was agitated in a hydrogen atmosphere (60 psi) for 1 h. The reaction mixture was filtered and evaporated to give *N*-acetyl-4-aminophenylalanine (2.13 g, 100%) as a white solid. ^{1}H NMR (DMSO-d_6): δ 8.01 (d, 1H), 6.81 (d, 2H), 6.40 (d, 2H), 4.23 (m, 1H), 2.90−2.40 (m, 2H), 1.72 (s, 3H). This compound (5.19 g, 23.4 mmol) was converted to the allyl ester **13c** as described for the preparation of **13b**. The crude product was purified using column chromatography (5% MeOH in CH_2Cl_2) to yield **13c** (3.6 g, 60%) as an oil. ^{1}H NMR ($CDCl_3$): δ 6.86 (d, 2H), 6.60 (d, 2H), 6.00−5.79 (m, 1H), 5.40−5.21 (m, 2H), 4.82 (m, 1H), 4.60 (d, 2H), 3.02 (m, 2H), 1.98 (s, 3H).

***N*-Methyl-*N*-(4-chlorobutyl) *N*′-(4-Carboallyloxy)phenyl Phosphorodiamidic Chloride (14a).** Diisopropylethylamine (0.51 mL, 2.9 mmol) was added to a mixture of phosphoramidic dichloride **3** (0.70 g, 2.9 mmol) and allyl ester **13a** (0.52 g, 2.9 mmol) in dry dichloroethane (10 mL). The reaction mixture was refluxed for 10 days, allowed to cool room temperature, diluted with CH_2CL_2 (10 mL), and quenched with saturated ammonium chloride. The layers were separated, the organic layer was washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield an oil. The crude product was purified by column chromatography (10:1 $CHCl_3$/EtOAc) to yield **14a** (0.22 g, 20%) as an oil. ^{1}H NMR ($CDCl_3$): δ 7.99 (d, 2H), 7.08 (d, 2H), 6.05−5.97 (m, 1H), 5.57 (d, 1H), 5.51−5.30 (m, 2H), 4.82−4.78 (m, 2H), 3.54 (t, 2H), 3.36−3.13 (m, 2H), 2.74 (d, 3H), 1.87−1.64 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −11.3. MS (ESI) *m*/*z* 379/381 $(M + H)^+$.

***N*-Methyl-*N*-(4-chlorobutyl) *N*-(4-Carboallyloxymethyl)phenyl Phosphorodiamidic Chloride (14b).** Diisopropylethylamine (0.27 mL, 1.6 mmol) was added to a pre-cooled solution of **13b** (0.3 g, 1.6 mmol) and $POCl_3$ (0.2 mL, 1.6 mmol) in dry CH_2CL_2 (3 mL) at −5 °C. The reaction mixture was stirred for 10 min. *N*-Methyl-*N*-(4-chlorobutyl)amine hydrochloride (0.37 g, 2.4 mmol) was dissolved in dry CH_2CL_2 (2 mL) and added to the reaction mixture at −5 °C. Diisopropylethylamine (0.82 mL, 4.7 mmol) in dry CH_2CL_2 (1 mL) was then added slowly to the mixture. The reaction mixture was stirred and warmed to room temperature over 3 h, saturated ammonium chloride was added, and the aqueous layer was extracted with CH_2CL_2 (3 × 10 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield an oil. The crude product was purified by column chromatography (10:1 $CHCl_3$/EtOAc) to yield **14b** (0.62 g, 34%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 7.19 (d, 2H), 7.01 (d, 2H), 6.00−5.79 (m, 1H), 5.41−5.17 (m, 3H), 4.59 (d, 2H), 3.60 (s, 2H), 3.49 (bs, 2H), 3.27−3.09 (m, 2H), 2.71 (d, 3H), 1.84−1.59 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −10.6. MS (ESI) *m*/*z* 393/395 $(M + H)^+$.

***N*-Methyl-*N*-(4-chlorobutyl) *N*-(4-(2-((*S*)-Acetylamino-2-carboallyloxy)ethyl))phenyl Phosphorodiamidic Chloride (14c).** Phosphorodiamidic chloride **14c** was obtained from **13c** (0.50 g, 1.9 mmol) as described for the preparation of **14b**. The crude product was purified by column chromatography to yield **14c** (0.30 g, 33%) as a white foam. ^{1}H NMR ($CDCl_3$): δ 7.19−6.91 (m, 4H), 6.00−5.79 (m, 2H), 5.41−5.22 (m, 2H), 4.97−4.78 (m, 1H), 4.60 (d, 2H), 3.59−3.46 (m, 2H), 3.28−2.97 (m, 4H), 2.71 (d, 3H), 1.99 (s, 3H), 1.81−1.57 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −10.5. MS (ESI) *m*/*z* 464/466 $(M + H)^+$.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-Carboxy)phenyl Phosphorodiamidate (15a).** LiHMDS (1.0 M solution in THF, 0.65 mL, 0.65 mmol) was added slowly to a pre-cooled solution of nitrofurfuryl alcohol (0.08 g, 0.59 mmol) in dry THF (1 mL) at −78 °C. The mixture was then cannulated to a pre-cooled solution of **14a** (0.22 g, 0.59 mmol) in dry THF (1 mL) at −20 °C. The reaction was stirred for 4 h at −20 °C and then quenched with saturated ammonium chloride. The reaction mixture was extracted with ethyl acetate (3 × 5 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield an oil. The crude product was purified by column chromatography (10% MeOH in EtOAc) to give the allyl ester (0.11 g, 40%) as a brown foam. ^{1}H NMR ($CDCl_3$): δ 7.96 9 (d, 2H), 7.26 (d, 1H), 6.99 (d, 2H), 6.65 (d, 1H), 6.16−5.84 (m, 1H), 5.51−5.19 (m, 3H), 5.09 (d, 2H), 4.92−4.73 (m, 2H), 3.52 (t, 2H), 3.22−2.99 (m, 2H), 2.69 (d, 3H), 1.85−1.61 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −15.6. MS (ESI) *m*/*z* 486/488 $(M + H)^+$.

Sodium *p*-toluenesulfinate (45 mg, 0.25 mmol) was dissolved in water (0.6 mL) and added to a solution of the allyl ester (0.11 g, 0.23 mmol) and $Pd(PPh_3)_4$ (13 mg, 11 μmol) in THF (1.2 mL). The reaction mixture was stirred for 45 min at room temperature, and then diluted with diethyl ether and washed with water (3 × 2 mL). The aqueous extracts were combined, washed with ether (1 × 4 mL), acidified to pH 3 with 2% HCl, and extracted with EtOAc (3 × 3 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield **15a** (80 mg, 76%) as a yellow foam. ^{1}H NMR ($CDCl_3$, TMS) δ: 7.96 (d, 2H), 7.26 (d, 1H), 6.99 (d, 2H), 6.65 (d, 1H), 6.45−6.25 (m, 1H), 5.09 (d, 2H), 3.52 (t, 2H), 3.22−2.99 (m, 2H), 2.69 (d, 3H), 1.85−1.61 (m, 4H). ^{31}P NMR ($CDCl_3$) δ: −15.4. HPLC (gradient 30% to 100% CH_3CN/H_2O [0.1% TFA] over 35 min): 9.37 min, 100%. MS (ESI) *m*/*z* 444/446 $(M - H)^-$.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-Carboxymethyl)phenyl Phosphorodiamidate (15b).** Phosphorodiamidate **15b** was obtained as a yellow foam (0.10 g, 40%) from **14b** (0.21 g, 0.54 mmol) by the two step sequence described for the preparation of **15a**. ^{1}H NMR ($CDCl_3$): δ 7.19−7.06 (m, 3H), 7.88 (d, 2H), 6.51 (d, 1H), 5.82 (d, 1H), 5.01 (d, 2H), 3.51 (s, 2H), 3.48 (t, 2H), 3.11−2.97 (m, 2H), 2.61 (d, 3H), 1.79−1.51 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −14.4. HPLC (gradient 30% to 100% CH_3CN/H_2O [0.1% TFA] over 35 min): 11.48 min, 100%. MS (ESI) *m*/*z* 456/458 $(M - H)^-$.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-(2-((*S*)-Acetylamino-2-carboxy)ethyl))phenyl Phosphorodiamidate (15c).** Phosphorodiamidate **15c** was obtained as a foam (0.12 g, 38%) from **14c** (0.29 g, 0.63 mmol) by the two-step sequence described for the preparation of **15a**. ^{1}H NMR ($CDCl_3$): δ 7.00−6.81 (m, 3H), 6.75−6.62 (d, 3H), 6.28−6.15 9 (m, 2H), 5.21−5.00 (m, 2H), 4.92−4.61 (bs, 1H), 3.51 (bs, 2H), 3.19−2.92 (m, 4H), 2.62 (m, 3H), 1.99 (bs, 3H), 1.69−1.52 (m, 4H). ^{31}P NMR ($CDCl_3$): δ −15.1. HPLC (gradient 30% to 100% CH_3CN/H_2O [0.1% TFA] over 35 min): 10.12 min, 100%. MS (ESI) *m*/*z* 531/533 $(M + H)^+$.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-(1-(3-Carbamoyl-4-cyclohexylmethoxyphenyl)ethylcarbamoyl))-phenyl Phosphorodiamidate (16).** Diisopropylethylamine (0.07 mL, 0.38 mmol) was added to a pre-cooled solution of phosphoramidate **15a** (80 mg, 0.17 mmol), amine **7** (50 mg, 0.19 mmol), and PyBOP (90 mg, 0.31 mmol) in dry CH_2CL_2 (2 mL) at 0 °C. The reaction was stirred for 5 min at 0 °C and 30 min at room temperature. The reaction mixture was then quenched with saturated ammonium chloride and extracted with CH_2CL_2 (3 × 5 mL). The organic extracts were combined, washed with brine, dried over $MgSO_4$, filtered, and evaporated to yield an oil. The crude product was purified by column chromatography (10% MeOH in EtOAc) to yield **16** (70 mg, 60%) as a foam. ^{1}H NMR ($CDCl_3$): δ 8.22 (d, 1H), 7.96−7.81 (m, 1H), 7.61 (d, 2H), 7.56−7.40 (m, 1H), 7.25−7.13 (m, 1H), 6.92 (d, 2H), 6.82−6.66 (m, 1H), 6.66−6.53 (m, 1H), 6.27−6.00 (m, 2H), 5.35−5.10 (m, 1H), 5.03 (d, 2H), 3.90 (d, 2H), 3.48 (t, 2H), 3.18−2.92 (m, 2H), 2.64 (d, 3H), 1.94−1.47 (m, 10H), 1.38−0.98 (m, 7H). ^{31}P NMR ($CDCl_3$): δ −14.9. HPLC (gradient 30% to 100% CH_3CN/H_2O [0.1% TFA] over 35 min): 17.28 min, 100%. HRMS (ESI) *m*/*z* $C_{33}H_{43}ClN_5O_8P$ calculated 704.2616 $(M + H)^+$, found 704.2633. Anal. ($C_{33}H_{43}ClN_5O_8P\cdot 3H_2O$) C, H, N. H: calcd, 6.51; found, 5.90.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-(1-(3-Carbamoyl-4-cyclohexylmethoxyphenyl)ethylcarbamoyl)-methyl)phenyl Phosphorodiamidate (17).** Phosphorodiamidate **17** was obtained from **15b** (90 mg, 0.19 mmol) as described for the synthesis of **16**. The crude product was purified by column chromatography (10% MeOH in EtOAc) to yield **15b** (30 mg, 70%) as a yellow foam. ^{1}H NMR ($CDCl_3$): δ 8.08 (d, 1H), 7.87 (bs, 1H), 7.34 (dd, 1H), 7.20 (d, 1H), 7.11 (d, 2H), 6.99−6.81 (m, 4H), 6.60 (d, 1H), 6.13 (bs, 1H), 6.01 (d, 1H), 5.83 (d, 1H), 5.08 (d, 3H), 3.91 (d, 2H), 3.51−3.41 (m, 4H), 3.12−3.01 (m, 2H), 2.61 (d, 3H), 1.82−1.53 (m, 10H), 1.46−0.93 (m, 7H). ^{31}P NMR ($CDCl_3$): δ −15.0. HPLC (gradient 30% to 100% CH_3CN/H_2O [0.1% TFA] over 35 min): 19.27 min, 100%. HRMS (ESI) *m*/*z* $C_{34}H_{45}ClN_5O_8P$ calculated 718.2773 $(M + H)^+$, found 718.2766. Anal. ($C_{34}H_{45}ClN_5O_8P\cdot H_2O$) C, H, N.

***N*-Methyl-*N*-(4-chlorobutyl) *O*-(5-Nitrofuryl-2-methyl) *O*-(4-(2-((*S*)-Acetylamino-2-(1-(3-carbamoyl-4-cyclohexylmethoxyphenyl)ethylcarbamoyl)ethyl)))phenyl Phosphorodiamidate (18).** Phosphorodiamidate **18** was obtained from **15c** (0.12 g, 0.23 mmol) as described for the synthesis of **16**. The crude product was purified by column chromatography (10% MeOH in EtOAc) to yield **18** (0.14 g, 68%) as a foam. ^{1}H NMR ($CDCl_3$): δ 8.11 (bs, 1H), 8.02 (bs, 1H), 7.89−7.81 (m, 1H), 7.39−7.18 (m, 2H), 7.12−7.01 (m, 2H), 7.00−6.78 (m, 4H), 6.67−6.52 (m, 1H), 6.42 (bs, 1H), 6.31 (bs, 1H), 5.97 (d, 1H), 5.08−4.88 (m, 3H), 4.79−4.52 (m, 1H), 3.99−3.81 (m, 2H), 3.52−3.39 (m, 2H), 3.13−2.80 (m, 4H), 2.70−2.51 (t, 3H), 1.98−1.51 (m, 13H), 1.39−0.91 (m, 7H). ^{31}P NMR ($CDCl_3$): δ −14.2, −14.3 (1:1 mixture of diastereomers). HPLC (gradient 30% to 100% CH_3CN/H_2O [0.1% TFA] over 35 min): 18.25 min, 50%; 18.42 min, 50%. HRMS (ESI) *m*/*z* $C_{37}H_{50}ClN_6O_9P$ calculated 789.3147 $(M + H)^+$, found 789.3144. Anal. ($C_{37}H_{50}ClN_6O_9P\cdot 3H_2O$) C, H, N. H: calcd, 6.69; found, 6.13. N: calcd, 9.97; found, 9.36.

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Supporting Information Available: Elemental analysis data of **8**−**10** and **16**−**18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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