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Articles

Synthesis and Biological Activity of *N*-Sulfonylphosphoramidates: Probing the Electrostatic Preferences of Alkaline Phosphatase

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N-Sulfonylphosphoramidates have been synthesized to investigate the electrostatic requirements for binding to alkaline phosphatase. Alkyl- and aryl *N*-benzylated sulfonamides were phosphorylated with bromophosphates or synthesized via phosphoramidite chemistry in moderate yields (44−77%.) The resulting tribenzylated *N*-sulfonylphosphoramidates may be deprotected in one step to give the free acids in quantitative yields. Physical data of *N*-sulfonylphosphoramidates, including pK_a's and stability toward hydrolysis, were determined. Inhibition data suggests that AP does not bind trianionic *N*-sulfonylphosphoramidates better than dianionic *N*-sulfonylphosphoramidates, although *N*-sulfonylphosphoramidates are bound tighter than *N*-phenylphosphoramidate. k_{cat} for the hydrolysis of *N*-sulfonylphosphoramidates by bovine and *E. coli* alkaline phosphatases is 10−60% that of *p*-nitrophenyl phosphate.

Introduction

Two families of enzymes, kinases and phosphatases, play important roles in maintaining the phosphorylation state of proteins and small molecules vital for a wide range of cellular processes.[1] About half of all phosphatases are metallophosphatases. Alkaline phosphatase (AP), a well-studied member of this group, has served as a model for the study of metallophosphatases. AP has a shallow, positively charged active site containing an arginine reside, two zinc ions, and a magnesium ion.[2] Along with the arginine residue, the metal ions serve the dual purpose of facilitating the binding of the negatively charged phosphate moiety and activating the substrate for attack by a nucleophilic serine residue (Figure 1).

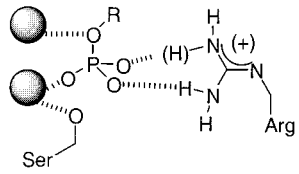

Figure 1. Schematic representation of phosphate monoester binding in the active site of alkaline phosphatase. The spheres represent active site zinc atoms.

Because the active site is shallow, the enzyme binds only the phosphate moiety of the substrate, and recognition depends little on the alcohol portion of the substrate.[3] This fact makes it difficult to design inhibitors for this enzyme. Because electrostatic interactions of the phosphate moiety with the enzyme are the main binding elements in the active site of AP, a more detailed picture

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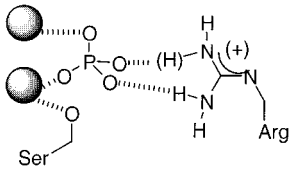

Figure 2. Schematic representation of inorganic phosphate binding in the active site of alkaline phosphatase. The spheres represent active site zinc atoms.

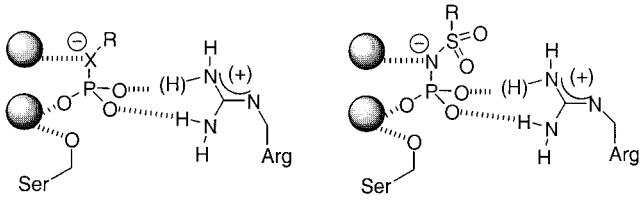

Figure 3. A potential motif for alkaline phosphatase inhibitors involves a bridging atom (X) capable of bearing a negative charge. *N*-Sulfonylphosphoramidates fit this motif.

of these interactions is critical if we are to develop a full understanding of the enzyme and improve our ability to design inhibitors of it.

The binding of inorganic phosphate by alkaline phophatase provides a model for electrostatic active site interactions. Inorganic phosphate is one of the best inhibitors of AP, with a K_i of about 5 μM for *E. coli* AP.[4] In fact, inorganic phosphate is bound tighter than a phosphate monoester substrate under conditions of high pH. Figure 2 depicts a putative model for inorganic phosphate binding to AP. It is unclear, however, whether inorganic phosphate is bound as the di- or trianion. A recent study suggests that an active site arginine present in AP may be deprotonated and thus capable of accepting a hydrogen bond from an H-bond donor.[5] Accordingly, the enhanced binding of inorganic phosphate, as compared to a phosphate monoester, may be due to a hydrogen bond between the neutral arginine and a protonated phosphate oxygen. On the other hand, it is possible that inorganic phosphate binds as the trianion and thus takes maximum advantage of electrostatic interactions with positively charged groups in the active site. In an effort to gain further information about the effect of substrate/inhibitor charge on binding to AP, we sought to design synthetic molecules that would predominately exist either in the dianionic or trianionic form as a function of substitution pattern.

A phosphate monoester mimic with the potential to be trianionic may be obtained by replacing the bridging oxygen of the ester with a nitrogen atom (Figure 3). Phosphoramidates have been tested as substrates for *E. coli* AP, and previous reports suggest that they are hydrolyzed with a k_{cat} value 30−60% that of phosphate monoesters.[6] However, at biologically relevant pH's, alkyl or aryl phosphoramidates are not sufficiently acidic to exist predominately as the trianion. Attaching a strongly electron-withdrawing substituent to the nitrogen lowers the third pK_a of the phosphoramidate. *N*-Sulfonylphosphoramidates (Figure 3) have recently been tested as inhibitors of carbonic anhydrase.[7] They possess a third

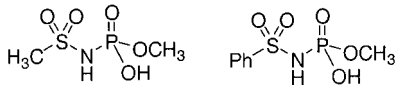

Figure 4. *N*-Sulfonylphosphoramidate monoesters.

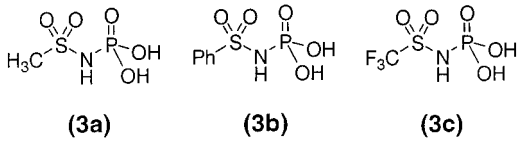

Figure 5. *N*-Sulfonylphosphoramidates.

pK_a on the order of 7.5−10, depending on the nature of the appended substituent. This range of pK_a's suggests that a series of *N*-sulfonylphosphoramidates could be constructed, some being primarily dianionic and others being primarily trianionic at pH 9, the pH optimum of AP.

Sometime ago, *N*-sulfonylphosphoramidate monoesters (Figure 4) were tested as substrates of AP, but function only as weak inhibitors.[8] In light of more recent structural knowledge of the active site of AP, it appears that *N*-sulfonylphosphoramidate monoesters may be sterically precluded from binding productively to the shallow active site. On the other hand, *N*-sulfonylphosphoramidate diacids, which more closely resemble phosphate monoesters (the natural substrate), may not have this difficulty. To test the binding of *N*-sulfonylphosphoramidates to AP, three target compounds were chosen (Figure 5). *N*-(Methylsulfonyl)phosphoramidate (**3a**) is the smallest possible *N*-sulfonylphosphoramidate, while *N*-(phenylsulfonyl)phosphoramidate (**3b**) is a representative aryl *N*-sulfonylphosporamidate. The additional strongly electron-withdrawing group of *N*-(trifluoromethylsulfonyl)phosphoramidate (**3c**) ensures that this compound will have significantly different ionization properties than either an alkyl- or aryl *N*-sulfonylphosphoramidate and will allow us to test the binding of a trianionic molecule to AP.

Results

A number of methods for the synthesis of *N*-sulfonylphosphoramidate esters have been developed.[9] Early attempts at the synthesis of *N*-sulfonylphosphoramidic acids and esters involved the reaction of sulfonamides with PCl_5, followed by hydrolysis or alcoholysis.[10] This hydrolysis may be affected by careful addition of formic acid, although the product is susceptible to acid cleavage of the P−N bond, releasing inorganic phosphate. This four-step procedure was recently used to synthesize inhibitors of carbonic anhydrase, though no yields were reported.[11] Alternate methods for the synthesis of *N*-sulfonylphosphoramidate esters include a derivative of the Arbuzov reaction, reaction of phosphite with *N*-sulfonyl azides, and a variation of the Atherton−Todd

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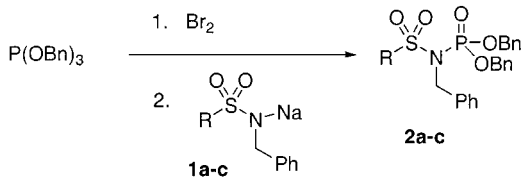
Scheme 1



a. R = CH_3, 63%, two steps
b. R = Ph, 77%, two steps
c. R = CF_3, 44%, two steps

approach.[12] A series of *N*-(fluoroalkyl)sulfonylphosphoramidatic acids were recently constructed by reaction of sulfonamide anions with chlorophosphates, though this multistep scheme produced low overall yields.[13]

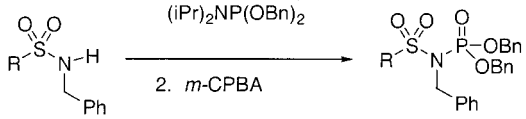
We reasoned that the synthesis of *N*-sulfonylphosphoramidates might be greatly simplified by adoption of the proper protecting group strategy. The use of the benzyl (Bn) protecting group offers a number of advantages. A benzyl ester may be removed via hydrogenolysis to yield a free acid in quantitative yield with no need for further purification. Furthermore, because benzyl esters are removed under mild, neutral conditions, the P−N bond scission in *N*-sulfonylphosphoramidates, observed under the acidic conditions employed in other syntheses, may be avoided.

Scheme 1 depicts the synthesis of tribenzyl *N*-sulfonylphosphoramidates via phosphorylation of a sulfonamide, adapted from a similar procedure developed by us and others for the synthesis of phosphate esters.[14] Oxidation of tribenzyl phosphite with bromine gives dibenzyl bromophosphate in situ. Reaction of *N*-benzylsulfonamides (**1a**−**c**) with dibenzyl bromophosphate gives the fully benzylated *N*-sulfonylphosphoramidates (**2a**−**c**) in reasonable yields (44−77%.) The protected *N*-sulfonylphosphoramidates (**2a**−**c**) may be deprotected by hydrogenolysis to give *N*-sulfonlyphosphoramidates (**3a**−**c**) as either the free acids or triethylammonium salts. This facile synthesis is accomplished in just two steps from the sulfonamide.

Tribenzyl *N*-sulfonylphosphoramidates may also be synthesized using phophoramidate chemistry (Scheme 2). Treatment of a sulfonamide with dibenzyl diisopropylphosphoramidite, followed by oxidation with *m*-CPBA gives **2a**,**b** in yields comparable to those obtained via the bromophosphate approach. Using this method, the alkyl and aryl *N*-sulfonlyphosphoramidates (**2a**,**b**) were obtained. However, synthesis of the trifluoromethyl derivative (**2c**) failed.

Using the tribenzyl phosphite approach, *N*-alkyl- and *N*-arylphosphoramidate esters may also be synthesized

Scheme 2



a. R = CH_3, 64%
b. R = Ph, 61%
c. R = CF_3, 0%

Scheme 3

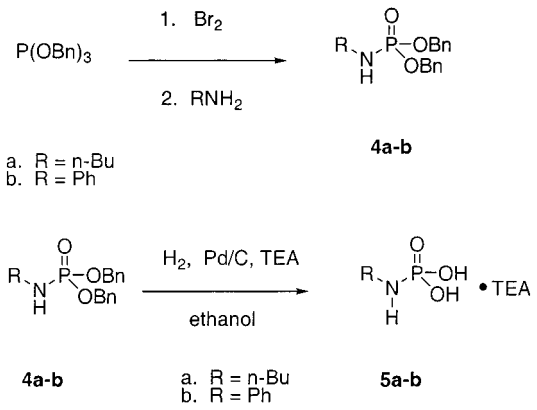


Table 1. pKa Values of *N*-Sulfonylphosphoramidates

	pK_{a_2}	pK_{a_3}
3a	5.1	10.3
3b	5.2	9.4
3c	∼3	∼7

conveniently and in high yield (Scheme 3). Aniline and *n*-butylamine were phosphorylated with dibenzyl bromophosphate to give the *N*-phenyl- and *N*-butylphosphoramidates (**4a**,**b**) respectively. Because of the acid lability of phosphoramidic acids, direct hydrogenolysis of the benzyl-protected substituents could not be affected in protic or aprotic solvents without significant product decomposition. When carried out in ethanol, this reaction provided a side product whose NMR spectra is consistent with ethyl phosphate, presumably arising from the ethanolysis of the phosphoramidates. Hydrogenolysis could be achieved, however, by addition of triethylamine to the reaction mixture prior to hydrogenolysis (Scheme 3). Under these conditions, **4b** was cleanly deprotected to give *N*-phenylphosphoramidic acid (**5b**), though minor impurities were observed when (**4a**) was subjected to hydrogenolysis.

Chemical Reactivity *N*-Sulfonylphosphoramidic acids are strong triacids, with a first pK_a on the range of 2−3 in water at 25 °C.[7] Second and third pK_a values of *N*-sulfonylphosphoramidates **3a**−**c** were determined by ^{31}P NMR titration.[15] Approximately 2 mg of compounds **3a**−**c** were dissolved in buffers with pH's ranging from 2 to 10. The phosphorus chemical shift of each sample was plotted as a function of buffer pH, and the pK_a was determined by logistic regression (SigmaPlot) as the point of inflection of the sigmoidal curves.[15] Table 1 lists the 2nd and 3rd pK_a's of the *N*-sulfonylphosphoramidates (**3a**−**c**). *N*-(Trifluoromethyl)sulfonylphosphoramidate (**3c**) showed only a gradual change in phosphorus chemical

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Table 2. Relative Rates of Hydrolysis of *N*-Sulfonylphosphoramidates and *N*-Phenylphosphoramidate

substrate	V_o[a] (B AP)[b]	V_o[a] (EC AP)[c]
*p*NPP	100	100
3a	28	57
3b	12	16
3c	39	37
5b	21	19

[a] Relative to pNPP. [b] Bovine alkaline phosphatase. [c] *E. coli* alkaline phosphatase.

shift at varying pH, and the point of inflection of the curves could not be mathematically fit. Approximate pK_a's were therefore determined graphically.

Under acidic conditions, *N*-sulfonylphosphoramidates are known to suffer hydrolysis of the P−N bond. To determine the stability of **3a**−**c** toward hydrolysis at higher pH's, degradation of the *N*-sulfonylphosphoramidates was followed over time by ^{31}P NMR. In buffers of pH 5 and 6, **3a** and **3b** have a half-life of approximately 24 h (data not shown). At pH 9, however, no hydrolysis product was observed. In contrast, no hydrolysis was detected for **3c** at pH 5, 6, or 9. These data suggests that *N*-sulfonylphosphoramidates are not susceptible to hydrolysis at pH's significantly above their pK_{a2}.

Alkaline Phosphatase Recognition of *N*-Sulfonylphosphoramidates. To determine how well alkaline phosphatase utilizes *N*-sulfonylphosphoramidates as substrates, relative rates of hydrolysis were determined. The rate of phosphate release was measured by the Lanzetta assay[16] for the AP-catalyzed hydrolysis of *N*-sulfonylphosphoramidates, and the rate was normalized relative to the catalyzed hydrolysis of *p*-nitrophenyl phosphate (*p*NPP.) Table 2 lists the relative initial rates of hydrolysis. At pH 9, *N*-sulfonylphosphoramidates are hydrolyzed by bovine AP at 12−39% the rate of phosphate monoester. The rate of catalyzed hydrolysis of **5b** was also measured to be 19% that of *p*NPP. Literature values for the relative rate of hydrolysis of *N*-(phenyl)phosphoramidate are on the range of 29−37%.[17]

The inhibitory abilities of **3a**−**c** were measured for four phosphatases: bovine AP, *E. coli* AP, purple acid phosphatase (PuAP), and small tyrosine phosphatase 1 (Stp1). IC_{50}'s for *N*-sulfonylphosphoramidate were determined with enzymes using *p*NPP as a substrate[18] (Table 3). *N*-Sulfonylphosphoramidates inhibit AP at significantly lower concentrations than they inhibit PuAP or Stp1. Furthermore, the three *N*-sulfonylphosphoramidates tested possess significantly lower IC_{50} values than phenylphosphoramidate.

Table 3. IC_{50}'s of *N*-Sulfonylphosphoramidates for Four Phosphatases

	IC_{50} (μM)			
substrate	B AP[a]	EC AP[b]	PuAP[c]	Stp1[d]
3a	60	52	1800	10000
3b	9	10	630	2500
3c	16	73	770	≫10000
5b	370	770		
*p*NPP	$K_m = 40$	$K_m = 10$	$K_m = 700$	$K_m = 200$

[a] Bovine alkaline phosphatase. [b] *E. coli* alkaline phosphatase. [c] Kidney bean purple acid phosphatase. [d] Small tyrosine phsophatase 1.

Discussion

To investigate the effects of charge on substrate binding to AP, a series of *N*-sulfonylphosphoramidates were investigated as inhibitors of AP. Under the assay conditions, two of the *N*-sulfonylphosphoramidates (**3a**,**b**) exist primarily as dianions while the third (**3c**) is trianionic. The inhibitory constants of **3a**−**c** are all within the same order of magnitude. These data suggests that AP does not discriminate between dianionic and trianionic inhibitors. If the enzyme were to bind trianions significantly tighter than dianions, *N*-(trifluoromethylsulfonyl)phosphoramidate (**3c**) would be expected to be a better inhibitor of AP than **3a**,**b**. On the other hand, if dianions bound more tightly to AP, **3c** would not be expected to inhibit the enzyme as effectively as dianionic compounds such as **3a**,**b**.

Both bovine AP and *E. coli* AP recognize *N*-sulfonylphosphoramidates as substrates and catalyze their hydrolysis at a rate 2−10 times slower than that observed for *p*NPP. The hydrolysis of **3a**,**b** was not unexpected. Hydrolysis of dianionic *N*-sulfonylphosphoramidate requires the expulsion of a sulfonamide anion. These anions are less basic than the alkoxide ions that serve as leaving groups in the hydrolysis of phosphate esters. In contrast, the ability of trianionic *N*-(trifluoromethyl)sulfonylphosphoramidate to serve as a substrate was surprising, because this hydrolysis requires the expulsion of a sulfonamide dianion.

One explanation for the observation that **3c** acts as a substrate for AP is that the dianionic form of **3c** is acting as the substrate in this reaction. Even though **3c** exists in primarily the trianionic state, a small portion of the dianion will be present at pH 9. If **3c** were to bind as the dianion, it would be hydrolyzed in the same way as **3a**,**b**. Another possibility is that the trianion may be reactive enough to undergo enzyme-catalyzed hydrolysis. For this hydrolysis to occur, the leaving sulfonamide would have to form a relatively stable dianion. The first pK_a of trifluoromethanesulfonamide is 6.3, and evidence suggests that trifluoromethylsulfonamide may be doubly deprotonated in aqueous solution at high pH.[19] It therefore seems possible that the dianion of trifluoromethylsulfonamide may function as a leaving group in this reaction, and that trianionic *N*-(trifluoromethylsulfonyl)-phosphoramidate may serve as a substrate for AP.

While the greater ionization ability of *N*-sulfonylphosphoramidates does not seem to enhance the inhibitory capacity of these molecules, the sulfonyl group of *N*-sulfonylphosphoramidate does seem to affect binding. *N*-phenylsulfonylphosphoramidate has a significantly

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(18) Because *N*-sulfonylphosphoramidates are sustrates of phosphatases, their binding ability could, in principle, be determined as K_m values. In practice, however, K_m values for these substrates cannot be determined accurately because of the difficulty in measuring the concentration of inorganic phosphate released in assays performed at low substrate concentration. A measurement of inhibition, such as an IC_{50} value, also provides the pertinent binding information without the technical difficulties associated with the direct measurement of K_m.

(19) ^{19}F NMR spectra were obtained for aqueous samples of trifluoromethanesulfonamide at various pHs. When dissolved in aqueous buffers below its first pK_a, 6.33, trifluoromethanesulfonamide has a chemical shift of −80.3, and above this pH, it has a chemical shift of −80.6. When dissolved in 10 M NaOH, a new shift (−79.3 ppm) occurs. The trifluoromethanesulfonamide dianion is a species consistent with this significant chemical shift at high pH.

lower IC_{50} (50−55-fold) than *N*-phenylphosphoramidate. At the same time, *N*-phenylsulfonylphosphoramidate is hydrolyzed by *E. coli* AP at a slower rate than is *N*-phenylphosphoramidate. These data suggest that the sulfonyl group is playing some role in interacting with the protein active site. Ligation of the sulfonyl group to the active site metal might be one possible explanation. Such a phenomenon has been employed in the design of other inhibitors of AP,[20] and crystal structures indicate that such interactions may be significant in determining the mode of binding.[5] The sulfonyl group may also confer some selectivity among phosphatase targets. *N*-Sulfonylphosphoramidates selectively inhibit alkaline phosphatases as compared to an acid phosphatase (PuAP) and Stp1, a nonmetallophosphatase (Table 3.)

Conclusions

While phosphorylation of alcohols with bromo- and iodophosphates has been previously recognized as a useful means to form phosphate triesters, this methodology can be extended to the formation of phosphoramidate and *N*-sulfonylphosphoramidate esters as well. *N*-Sulfonylphosphoramidates were shown to be slow substrates and moderate inhibitors of AP. Triply anionic *N*-sulfonylphosphoramidates do not appear to bind tightly in the alkaline phosphatase active site, suggesting AP does not prefer to bind trianionic molecules in preference to dianions, in spite of the plethora of positive charges in the active site of AP.

Experimental Section

Diethyl ether (Et_2O) and tetrahydrofuran (THF) were distilled under nitrogen from sodium−benzophenone ketyl. Methylene chloride (CH_2Cl_2) was distilled from calcium hydride. Pyridine (pyr.) was distilled from barium oxide. Butylamine was distilled out of magnesium sulfate. Aniline was distilled from crushed potassium hydroxide under reduced pressure. Reactions were carried out under nitrogen atmosphere. Biochemical assays were carried out in doubly distilled and deionized waster. Bovine alkaline phosphatase, *E. coli* alkaline phosphatase, and *p*-nitrophenyl phosphate were purchased from Sigma. Kidney bean purple acid phosphatase stock was from a previous study.[20] Stp-1 was prepared according to a literature procedure.[21]

Analytical thin-layer chromatography (TLC) was performed on precoated (0.25 mm thickness) glass plates (E. Merck, silica gel 60 F-254). Components were visualized by UV light (254 nm) if possible and by staining plates with either an acidic solution of $(NH_4)_6Mo_7O_{24}$ monohydrate and ceric sulfate or an acidic solution of *p*-anisaldehyde, followed by heating. Column chromatography was performed using Kieselgel-60 230−400 mesh silica gel (E. Merck.) Bulk solvent removal was carried out on a rotary evaporator at water aspirator pressure. All nonvolatile compounds were routinely dried under high vacuum.

Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on a 400 MHz spectrometer. The chemical shifts are reported in parts per million (δ, ppm) relative to an internal tetramethylsilane standard. Coupling constants are reported in hertz (Hz), and the data are presented in the following form: chemical shift (multiplicity, coupling constants, number of protons.) Multiplicities are recorded by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; sx, sextet; *J*, coupling constant (Hz). Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded at 101 MHz. Chemical shifts are reported relative to residual solvent peaks. Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectra were recorded at 121.5 MHz. Chemical shifts are reported relative to an external neat H_3PO_4 sample (0 ppm). Fluorine-19 nuclear magnetic resonance (^{19}F NMR) spectra were recorded at 282 MHz. Chemical shifts are reported relative to an external neat CF_3COOH standard (−78.5 ppm). Infrared (IR) spectra (thin film, KBr pellet, or single beam) were recorded by FT-IR. Mass spectra were determined by high resolution (HRMS) or high-resolution fast-atom bombardment mass spectra (HRFABMS). Melting points were determined in open capillary tubes and are uncorrected.

Inhibition of Bovine Alkaline Phosphatase. Assays were conducted in 50 mM Tris HCl buffer at pH 9.0 with 1 mM $MgCl_2$. The initial rate (∼5%) of product formation was measured for the following ranges of inhibitor concentration: **3a**, 0−100 μM; **3b**, 0−35 μM; **3c**, 0−67 μM; **5b**, 0−2.2 mM. A solution of substrate and inhibitor (590 μL of 175 μM pNPP) was incubated in a cuvette in a temperature-controlled jacket at 25 °C for 2 min. Enzyme solution was added (10 μL, 395 ng/mL), and the cuvette was inverted until the solution was properly mixed. The absorbance was measured continuously at 405 nm for one minute, and the initial rate was obtained as the slope of the resulting plot of absorbance change versus time. Percent activity was calculated by comparing the rates of product release at various concentrations of inhibitor to the rate of product release in the absence of inhibitor. The inverse of the initial rate of reaction was plotted as a function of inhibitor concentration. In this plot, IC_{50} is equal to the opposite of the *x*-intercept.

Inhibition of *E. coli* Alkaline Phosphatase. Assays were conducted in 50 mM Tris HCl buffer at pH 9.0 with 1 mM $MgCl_2$. The initial rate (∼5%) of product formation was measured for the following ranges of inhibitor concentration: **3a**, 0−133 μM; **3b**, 0−33 μM; **3c**, 0−133 μM; **5b**, 0−2.0 mM. A solution of substrate and inhibitor (590 μL of 50 μM pNPP) was incubated in a cuvette in a temperature-controlled jacket at 25 °C for 2 min. Enzyme solution was added (10 μL, 1.88 U/mL), and the cuvette was inverted until the solution was properly mixed. The absorbance was measured continuously at 405 nm for one minute, and the initial rate was obtained as the slope of the resulting plot of absorbance change versus time. Percent activity was calculated by comparing the rates of product release at various concentrations of inhibitor to the rate of product release in the absence of inhibitor. The inverse of the initial rate of reaction was plotted as a function of inhibitor concentration. In this plot, IC_{50} is equal to the opposite of the *x*-intercept.

Inhibition of Purple Acid Phosphatase. Assays were conducted in 100 mM NaOAc buffer at pH 5.0 containing 50 mM NaCl. Product formation (∼5%) was measured at the following ranges of inhibitor concentration: **3a**, 0−4.0 mM; **3b**, 0−2.8 mM; **3c**, 0−1.4 mM. To a solution of substrate and inhibitor (590 μL of 3.0 mM pNPP) was added enzyme solution (10 μL of 4.6 μg/mL enzyme in buffer contain 50 μg BSA/mL.) After 5 min, the reaction was quenched by the addition of 100 μL of 1.0 M NaOH. The absorbance of the resulting solution was measured at 405 nm. Percent activity was calculated by comparing the amount of product release at various concentrations of inhibitor to the rate of product release in the absence of inhibitor. The inverse of the initial rate of reaction was plotted as a function of inhibitor concentration. In this plot, IC_{50} is equal to the opposite of the *x*-intercept.

Inhibition of Stp1. Assays were conducted in 50 mM succinate buffer at pH 6.0 containing 150 mM NaCl and 1 mM EDTA. Product formation (∼5%) was measured for the following ranges of inhibitor concentration: **3a**, 0−4.0 mM; **3b**, 0−2.7 mM; **3c**, 0−2.7 mM. To a solution of substrate and inhibitor (595 μL of 800 μM pNPP) was added 5 μL of enzyme solution (1 μg/mL). After 10 min, the reaction was quenched by the addition of 100 μL of 1.0 M NaOH. The absorbance of the resulting solution was measured at 405 nm. Percent activity was calculated by comparing the amount of product release at various concentrations of inhibitor to the rate of product release in the absence of inhibitor. The inverse of the initial

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rate of reaction was plotted as a function of inhibitor concentration. In this plot, IC_{50} is equal to the opposite of the *x*-intercept.

***N*-Sulfonylphosphoramidates as Substrates of Alkaline Phosphatase.** The relative rates of hydrolysis of *p*NPP and *N*-sulfonylphosphoramidates **3a**−**c** were followed by detection of inorganic phosphate release, detected by the method of Lanzetta et al.[15] To 1 mL of 500 μM substrate was added enzyme (20 μL of 395 nM/mL bovine AP, or 15 μL of 1.71 U/mL E. coli AP). At time points (0−60 min), 100 μL aliquots of the reaction solution were quenched into 800 μL of molybdenate color indicator (ϵ = 78 000 Abs/M). After 60 s, the color indicator was quenched with 100 μL of 34% sodium citrate solution. After 1 h, the absorbances of these solutions were measured at 660 nm. Blanks contained 100 μL of 500 μM substrate with no enzyme mixed with 800 μL of color indicator and quenched as above. Initial rates (∼10%) were determined from the slope of the line of a plot of the concentration of inorganic phosphate released as a function of time.

***N*-Phenylphosphoramidate (5b) as a Substrate of Alkaline Phosphatase.** The relative rates of hydrolysis of pNPP and *N*-phenylphosphoramidates **5b** was followed by detection of inorganic phosphate release, detected by a variation of the method of Lanzetta, et al.[15] To 1 mL of 500 μM substrate was added enzyme (20 μL of 395 nM/mL bovine AP, or 15 μL of 1.71 U/mL E. coli AP.) At time points (0−60 min), 100 μL aliquots of the reaction solution were quenched into 800 μL of molybdenate color indicator (ϵ = 70 000 Abs/M). After 6 s, the color indicator was quenched with 100 μL of 34% sodium citrate solution. (Because the substrate is not stable under the acidic assay conditions, the assay was quenched quickly, resulting in only 10% loss of sensitivity.) After 1 h, the absorbances of these solutions were measured at 660 nm. Blanks contained 100 μL of 500 μM substrate with no enzyme mixed with 800 μL of color indicator and quenched as above. Initial rates (∼10%) were determined from the slope of the line of a plot of the concentration of inorganic phosphate released as a function of time.

General Procedure for the Preparation of Dibenzyl *N*-Benzyl-*N*-sulfonylphosphoramidates (2). Method A: Bromophosphate. *N*-Benzylsulfonamide (**1a**−**c**) (1 mmol) was dissolved in 2 mL of dry tetrahydrofuran and added dropwise to sodium hydride (1.1 mmol) that had been prewashed in 5 mL of pentane. After stirring for 45 min, no gas evolution was visible. Tribenzyl phosphite[22] (1.5 mmol) was dissolved in 2 mL of dry methylene chloride at 0 °C. Bromine (1.45 mmol), dissolved in 3 mL of methylene chloride, was added to the phosphite with stirring over the course of 5 min. After 10 min, the solution was warmed to room temperature and stirred an additional 15 min. To the deprotonated sulfonamide solution at −30 °C was added the phosphorylating reagent, dropwise over 10 min. The reaction was warmed to room temperature and allowed to stir for 60 min. The solvent was removed in vacuo, and the residue was partitioned between 25 mL of ether and 25 mL of 1 M HCl. The ether layer was collected and washed with brine (25 mL) and then dried over magnesium sulfate. The ether was removed in vacuo, and the crude material was purified by silica gel chromatography (**2a**: 1:1 Hex:EtOAc; **2b**: 2:1 Hex:EtOAc; **2c**: 2:1 Hex:Et_2O). **Method B: Phosphoramidate.** *N*-Benzylsulfonamide(**1**) (1 mmol) and 1-*H*-tetrazole (1 mmol) were dissolved/suspended in 2 mL of dry methylene chloride. Dibenzyl diisopropylphosphoramidite[23] (1 mmol) was added to the reaction dropwise. Upon stirring for 1 h, the solution grew clear and then cloudy. The solution was cooled to −40 °C, and *m*-CPBA (2 mmol), in 3 mL of methylene chloride, was added dropwise. After stirring the reaction for 1 h, the reaction was allowed to warm to room temperature. The solvent was evaporated in vacuo, and the crude material was partitioned between ether (30 mL) and 10% aq sodium bicarbonate (30 mL). The ether layer was collected and washed with 30 mL of 10% aq sodium hydroxide followed by 30 mL of brine. The ether layer was dried over magnesium sulfate, and the ether was removed in vacuo. The crude material was purified by silica gel column chromatography (**2a**: 1:1 Hex:EtOAc; **2b:** 2:1 Hex:EtOAc).

Dibenzyl *N*-Benzyl-*N*-(methylsulfonyl)phosphoramidate (2a). Yield: Method A: 61% isolated, Method B: 64% colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.76 (s, 3H), 4.68 (d, J = 10.8 Hz, 2H), 5.04 (m, 4H), 7.2−7.35 (m, 15H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 42.21, 51.54, 69.75 (d, J = 5.3 Hz), 128.04, 128.16, 128.39, 128.49, 128.61, 128.89, 135.03 (d, 6.9 Hz), 136.21 ppm; ^{31}P NMR (121 MHz, $CDCl_3$): −0.14 ppm; IR (neat): 1165, 1275, 1355 cm^{-1}; HRFABMS (M + H) calcd for $C_{22}H_{25}NO_5PS$ 446.1191, found 446.1194.

Dibenzyl *N*-Benzyl-*N*-(phenylsulfonyl)phosphoramidate (2b). Yield: Method A: 77%, Method B: 61% white solid: mp 77−78 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.67 (d, J = 11.6 Hz, 2H), 4.93 (m, 4H), 7.20−7.76 (m, 15H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 52.07, 69.60 (d, J = 5.3 Hz) 127.77, 127.85, 128.18, 128.28, 128.51, 128.57, 128.62, 128,75, 133.06, 135.21 (d, J = 7.6 Hz), 136.34, 139.92 ppm; ^{31}P NMR (121 MHz, $CDCl_3$): 0.23 ppm; IR (thin film): 1010, 1173, 1276, 1367, 3066 cm^{-1}; HRMS (M + H) calcd for $C_{27}H_{27}NO_5PS$ 508.1348, found 508.1368.

Dibenzyl *N*-Benzyl-*N*-(trifluoromethylsulfonyl)phosphoramidate (2c). Yield: Method A: 44%, Method B: 0% colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.77 (d, J = 11.6 Hz, 4H), 4.90 (t, J = 10.4 Hz, 2H), 7.18−7.53 (m, 15H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.10, 70.59 (d, J = 5.3 Hz), 119.55 (q, J = 323 Hz), 128.12, 128.56, 128.61, 128.80, 129.35, 134.55 (d, J = 7.9 Hz), 135.20 ppm; ^{31}P NMR (121 MHz, $CDCl_3$): −2.75 ppm; ^{19}F NMR (282 MHz, $CDCl_3$): δ −74.56 ppm; IR (neat): 1015, 1139, 1230, 1288, 1403 cm^{-1}; HRMS (M − H) calcd for $C_{22}H_{20}F_3NO_5PS$ 498.0752, found 498.0750.

General Procedure for the Preparation of *N*-Sulfonylphosphoramidic Acids (3). Dibenzyl *N*-benzyl-*N*-sulfonylphosphoramidate (**2**) (0.5 mmol) was dissolved in 2 mL of ethanol. Approximately 50 mg of 10% activated palladium on carbon was added to the reaction. The flask was sealed and evacuated in vacuo, followed by introduction of an H_2 atmosphere. After 2 h, the reaction flask was purged with nitrogen and the solution was filtered through Celite. The ethanol was evaporated in vacuo to yield **3a**,**b** quantitatively, with no need for further purification. **3c** was not stable as the free acid, but could be isolated as the triethylammonium salt by dissolving the crude product in triethylamine/bicarbonate buffer and evaporating the buffer in vacuo.

***N*-(Methylsulfonyl)phosphoramidic Acid (3a).** White, highly hygroscopic solid: ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.13 (s) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 42.90 ppm; ^{31}P NMR (121 MHz, $CDCl_3$): −4.62 ppm; IR (neat): cm^{-1}; HRFABMS (M + H) calcd for CH_7NO_5PS 175.9781, found 175.9783.

***N*-(Phenylsulfonyl)phosphoramidaic Acid (3b).** White solid: mp147−148 °C (148−149 °C);[24] ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51−7.98 (m) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 128.20, 129.99, 133.87, 143.44 ppm; ^{31}P NMR (121 MHz, $CDCl_3$): −5.15 ppm; IR (KBr): 1182, 1215, 1332, 2363, 2413, 3229 cm^{-1}; HRMS (M + H) calcd for $C_6H_9NO_5PS$ 237.9939, found 237.9950.

Triethylammonium *N*-(Trifluoromethylsulfonyl)phosphoramidate (3c). White, hygroscopic solid: mp 81−83 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.27 (t, J = 6.4 Hz, 9H), 3.08 (q, J = 6.4 Hz, 6H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 8.03, 45.77, 121.41 (q, J = 322 Hz) ppm; ^{31}P NMR (121 MHz, $CDCl_3$): −2.33 ppm; ^{19}F NMR (282 MHz, $CDCl_3$): δ −79.87 ppm IR (KBr): 1165, 1206, 1266, 1476, 2491, 2677, 2976 cm^{-1}; HRFABMS (M + H) calcd for $C_{13}H_{34}F_3N_3O_5PS$ 432.1909, found 432.1900.

General Procedure for the Preparation of Dibenzyl Phosphoramidate (4). Tribenzyl phosphite[22] (2 mmol) was

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dissolved in 3 mL of methylene chloride at 0 °C. Bromine (1.95 mmol) dissolved in 2 mL of methylene chloride was added to the phosphite dropwise. The reaction was stirred for 20 min and then warmed to room temperature and stirred for an additional 20 min. In a separate flask, the amine (3 mmol) was dissolved in 5 mL of methylene chloride at −30 °C. The phosphorylating reagent was added to the amine dropwise over 10 min. The reaction was warmed to room temperature and stirred for 2 h. The solvent was evaporated in vacuo, and the residue was partitioned between 25 mL of ether and 25 mL of 2 M HCl. The ether layer was separated from the organic layer and washed again with 25 mL of 2 M HCl and then 25 mL of brine. The organic layer was collected and dried with magnesium sulfate, followed by evaporation of the solvent in vacuo. The crude product was purified by silica gel chromatography (1:1 Hex:EtOAc for both **4a** and **4b**.)

Dibenzyl *N*-(*n*-Butyl)phosphoramidate (4a). Yield: 80% white solid: mp 46−47 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.84 (t, J = 7.2 Hz, 3H), 1.26 (sx, J = 7.2 Hz, 2H), 1.39 (p, J = 7.2 Hz, 2H), 2.84 (d q, J = 9.6 Hz, 7.2 Hz, 2H), 3.06 (m, 1H), 5.04 (d, J = 5.6 Hz), 7.20−7.40 (m, 10H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.54, 19.57, 33.55 (d, J = 6.8 Hz), 40.97, 67.63 (d, 4.5 Hz), 127.57, 128.03, 128.34, 136.47 (d, 7.6 Hz) ppm; ^{31}P NMR (121 MHz, $CDCl_3$): 10.36 ppm; IR (thin film): 1000, 1456, 1548, 2959, 3218 cm^{-1}; HRFABMS (M + H) calcd for $C_{18}H_{25}NO_3P$ 134.1572, found: 134.1557.

Dibenzyl *N*-Phenylphosphoramidate (4b).[25] Yield: 97% white solid: mp 88−89 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.09 (m, 4H), 6.94−7.30 (m, 15H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 68.44 (d, 4.6 Hz), 117.60 (d, J = 7.6 Hz), 121.60, 127.95, 128.31, 128.41, 129.16, 135.69 (d, J = 8.3 Hz), 139.51 ppm; ^{31}P NMR (121 MHz, $CDCl_3$): 3.11 ppm; IR (thin film): 1001, 1227, 1422, 1500, 1605, 2912, 3093, 3169 cm^{-1}; HRFABMS (M + Na) calcd for $C_{20}H_{20}NO_3PNa$ 376.1079, found: 376.1072.

General Procedure for the Preparation of Triethylammonium Phosphoramidates (5). Dibenzyl phosphoramidate (**4**) (0.3 mmol) and triethylamine (0.75 mmol) were dissolved in 2 mL of ethanol. Approximately 40 mg of 10% activated palladium on carbon was added to the reaction. The flask was sealed and evacuated, followed by introduction of an H_2 atmosphere. After 0.5 h, the reaction flask was purged with nitrogen, and the solution was filtered through Celite. The ethanol was evaporated in vacuo to yield **5a**,**b**.

Triethylammonium *N*-(*n*-Butyl)phosphoramidate (5a). White solid, mp 74−76 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 0.97 (t, J = 9.6 Hz, 3H), 1.32 (t, 9.6 Hz, 4.5H), 1.42 (sx, J = 10.0 Hz, 2H), 1.67 (p, J = 10 Hz, 2H), 3.04 (m, 2H), 3.19 (q, J = 9.6 Hz, 3H) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ 9.26, 14.16, 21.22, 31.50 (d, J = 7.6 Hz), 44.15, 47.56 ppm; ^{31}P NMR (121 MHz, CD_3OD): 0.54 ppm; IR (single beam): 976, 1081, 1173, 1235, 1464, 2959 cm^{-1}; HRFABMS (M + H) calcd for $C_{10}H_{28}N_2O_3P$ 255.1838, found: 255.1829.

Triethylammonium Phenylphosphoramidate (5b). White solid: mp 102−104 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 1.25 (t, J = 7.2 Hz, 9H), 3.07 (q, J = 7.2 Hz, 6H), 6.74−7.13 (m, 5H) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ 9.16, 47.40, 118.01 (d, J = 6.9 Hz), 120.07, 129.82, 145.13 ppm; ^{31}P NMR (121 MHz, CD_3OD): 0.84 ppm; IR (KBr): 1082, 1154, 1313, 1501, 1604, 2359, 2677, 2979, 3218 cm^{-1}; HRFABMS (M + H) calcd for $C_{12}H_{24}N_2O_3P$: 275.1525, found: 275.1534.

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Supporting Information Available: ^{1}H NMR spectra of all compounds lacking elemental analysis (**2a**, **2b**, **2c**, **4a**, **5a**, **5b**). ^{13}C NMR spectra of **3a**. ^{31}P and ^{19}F NMR spectra of **3c**. ^{19}F NMR spectra of trifluoromethanesulfonamide in pH 4 buffer, pH 9 buffer, and 10 M NaOH. This material is available free of charge via the Internet at http://pubs.acs.org.

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