Substituent Effects on the Mechanism-Based Inactivation of Prostatic Acid Phosphatase

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Abstract: The mechanism of the inactivation of prostatic acid phosphatase (PAP) by 4-halomethylaryl phosphates, such as 4-(fluoromethyl)phenyl phosphate (FMPP), was probed by varying the benzylic leaving group and adding a nitro group to the 2 position. These studies demonstrate that both the rate and efficiency of inactivation are dependent on the nature of the leaving group in the benzylic position, with the brominated inhibitor being the most effective ($t_{1/2}$ = 11 s), the chloride being less effective ($t_{1/2}$ = 200 s), and the fluoride showing little or no inhibition. The addition of a nitro group to the 2 position of the benzene ring of the inhibitor results in high selectivity for the inactivation of prostatic acid phosphatase over PTPases like YOP51*. This pattern of selectivity contrasts with that of FMPP which quickly inactivates both prostatic acid phosphatase and YOP51*. The potential uses of these types of mechanism-based inactivators are discussed.

Introduction

Phosphate esters are found in all living organisms and are involved in an important array of biological processes as varied as the transmission of genetic information, signal transduction, and the metabolism of small molecules. The synthesis and hydrolysis of phosphate esters in proteins is a common post-translational modification that can regulate both protein structure and activity.[1] Although the phosphorylation of protein tyrosine residues is a relatively uncommon cellular event compared to protein serine/threonine phosphorylation, it is an important biological control mechanism. Tyrosine phosphorylation is known to play a role in metabolic regulation, cell growth and proliferation, cytoskeletal structure, and development.[1,2] *In vivo*, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases) control the level of protein tyrosine phosphorylation. Overexpression of PTKs has been shown to lead to cell transformation, and it has been suggested that PTPases may function as tumor suppressers.[3] The roles that kinases and phosphatases play in cell transformation may be quite complex, however. For example, several cancers including breast cancer and head and neck squamous cell carcinoma show increased levels of PTPase activity.[4] Whether the PTPase activity serves to augment or inhibit the growth of these cancers remains unknown.

Prostatic acid phosphatase, a broad specificity phosphatase, may play a role in the development of cancer in the prostate.[5,6] Prostatic acid phosphatase expression is decreased in many prostate cancers, while excretion into the blood stream increases. The resultant increase in the serum prostatic acid phosphatase level is commonly used as a diagnostic marker for prostate cancer.[7] Prostatic acid phosphatase displays a modest preference for the hydrolysis of aromatic phosphate esters vs aliphatic phosphate esters[8] and certain phosphoproteins are exceptionally good substrates for this enzyme.[9]

Despite a great deal of work on the chemistry and biological role of phosphatases, there has been little parallel progress in the development of potent inhibitors of these enzymes. In particular, the development of rationally designed inhibitors has been almost nonexistent. We have sought to ameliorate this situation by developing new strategies for the mechanism-based inhibition of phosphatases.[10−12] One such strategy is typified by 4-(fluoromethyl)phenyl phosphate (FMPP), which inhibits prostatic acid phosphatase,[10] calcineurin,[11] YOP 51*,[13] and SH2-containing PTPase SHP.[14] Mechanism-based inhibitors (MBIs) have several properties that make them attractive tools for the study of enzymes. First, they covalently inactivate their target enzyme, providing a means to specifically label the active site of the enzyme. Second, since MBIs must be a substrate for the enzymes they inactivate, they are usually very specific inhibitors of only those enzymes. A probable mechanism for the inactivation of prostatic acid phosphatase by FMPP is shown in Scheme 1. After the binding of FMPP to the enzyme, enzyme-catalyzed cleavage of the phosphate ester bond results in the formation of an enzyme-bound 4-(fluoromethyl)phenol. Such compounds are known to eliminate fluoride ion to generate quinone methides. The enzyme probably actively assists in this elimination (k_{elim}) since the spontaneous formation of quinone methides from phenols is usually fairly slow.[15] The quinone methide thus generated can then alkylate an active site nucleophile (k_{alk}), leading to enzyme inactivation.

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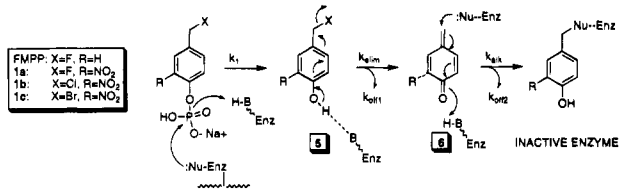
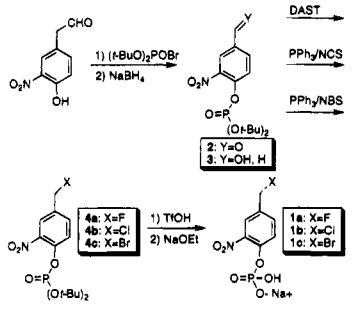
Scheme 1



Scheme 2



A number of factors may affect the inactivation process shown in Scheme 1. The acidity of the phenol intermediate, k_{elim}, k_{alk}, k_{off1}, and k_{off2}, may all affect the efficiency of the inactivation process. In an effort to understand, and perhaps improve on, the inactivation of prostatic acid phosphatase by FMPP, we wished to test the possibility that changing the leaving group (thereby speeding up or slowing down k_{elim}) would affect the inactivation process. As FMPP is solvolytically labile in water ($t_{1/2} \sim 6$ h) and even less stable in buffer, it was expected that related benzylic halides with better leaving groups (i.e., chloride or bromide) would be too labile for study. Therefore, several modified inactivators were designed that retained the functionality necessary for inactivation but would be stable toward solvolysis. This was accomplished by addition of a nitro group to the 2-position of the inhibitors. The resulting inactivators, 4-halomethyl-2-nitrophenyl phosphates (**1a–c**), were sufficiently stable so that even the benzylic bromide could be synthesized and tested.

Results and Discussion

Synthesis of Inactivators. Phosphatase inactivators **1a–c** were synthesized as shown in Scheme 2. Commercially available 4-hydroxy-2-nitrobenzaldehyde was phosphorylated with *tert*-butylphosphorobromidate[16] in the presence of DBU and DMAP. The resultant aldehyde, **2**, is very unstable and was used directly in the next reaction. Reduction of the aldehyde with sodium borohydride gave alcohol **3** (69% for two steps), which was then halogenated with DAST (45%), NCS/PPh_3 (53%), or NBS/PPh_3 (69%) to give the corresponding benzylic halides, **4a–c**. Deprotection of the *tert*-butyl groups with 2 mol % triflic acid in THF followed by treatment with sodium ethoxide gave the desired monosodium salts (78% for **1a** and **1b**, 86% for **1c**) contaminated with a small amount of sodium triflate. The use of TFA[17] in the deprotection reaction resulted in decomposition of the compounds. Clean deprotection of the inhibitors could be achieved by prolonged (3 days) treatment with 0.5 equiv of methane sulfonic acid in THF. However, neutralization of the reaction mixture gave a mixture of sodium methane sulfonate and the desired phosphate salt that was difficult to purify. By using triflic acid, the amount of sulfonate salt in the final product was reduced to a level that did not interfere with enzymatic assays.

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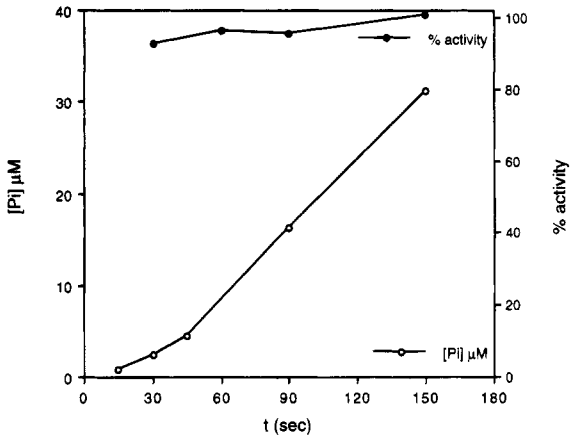

Figure 1. Fluoride **1a** is a substrate for PAP but not an inactivator. Treatment of PAP with **1a** (1 mM) leads to a rapid release of inorganic phosphate (open circles) but no decrease in PAP activity (closed circles).

Table 1. Kinetic Constants for the Reaction of Prostatic Acid Phosphatase with Nitrohalides **1a–c**

inactivator	K_i (K_m) μM	k_{inact} (k_{cat}) s^{-1}	partition ratio
1a	(39)	(260)	
1b		0.0035	104 000 ± 9000
1c	9.1 ± 2.1	0.063 ± 0.005	5700 ± 100

Stability of Inactivators. Enhancing the leaving group ability of the benzylic substituent on the inhibitors should result in increased rates of elimination to the quinone methide. We wanted to determine if this would enhance the efficiency of mechanism-based inactivation. Unfortunately, the chloride and bromide corresponding to FMPP were difficult to synthesize and assay because of their lability. The presence of an electron withdrawing group on the aromatic ring was expected to stabilize the benzylic halide with respect to solvolysis, making the inactivators easier to synthesize and assay. Indeed, compounds **1a–c** showed a substantial increase in stability in D_2O relative to the unnitrated inhibitors. As monitored by proton NMR, bromide **1c** showed only 35% decomposition after 8 h in D_2O at room temperature, while **1a** has a half-life of more than a month at pD 5.4. These compounds are therefore more stable toward solvolysis than the parent inhibitor FMPP.

Inactivation of Prostatic Acid Phosphatase. We have previously demonstrated that FMPP is a mechanism-based inactivator of prostatic acid phosphatase.[10] We anticipated that the nitrohalides would inactivate the enzyme in the same manner. To test this hypothesis, we performed several assays that differentiate among mechanism-based inactivation, slow tight binding inactivation, affinity inactivation, and metabolic inactivation. Since a mechanism-based inactivator is a substrate for an enzyme, it should show time dependent and concentration dependent inactivation that is saturable and is slowed by the addition of competitive inhibitors. In addition, the demonstration that exogenous nucleophiles do not reduce the rate of enzyme inactivation provides evidence that inhibition is not caused by metabolic inactivation. Moreover, a change in the concentration of the enzyme should not change the rate of inactivation if the inactivation is mechanism-based.

Nitrofluoride **1a** is a good substrate for prostatic acid phosphatase but does not inactivate the enzyme (Figure 1, Table 1). However, incubation of prostatic acid phosphatase with chloride **1b** or bromide **1c** leads to rapid inactivation (Figures 2 and 3). The inactivation is first order and saturable (Figure 4),[18] and inorganic phosphate protects the enzyme against inactivation (Figures 5 and 6). Cysteine, a good scavenger of

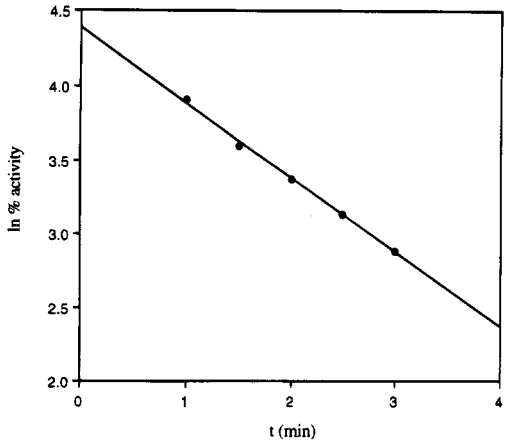

Figure 2. Time dependence of the inactivation of PAP with chloride **1b**. Residual PAP activity after incubating with **1b** (500 μM).

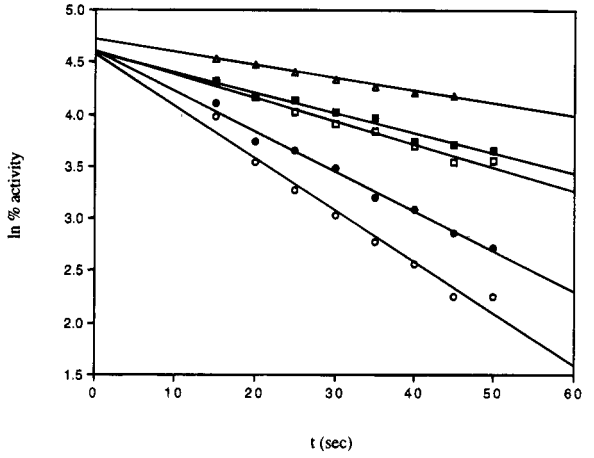

Figure 3. Time dependence and concentration dependence of the inactivation of PAP with bromide **1c**. Residual PAP activity after incubating with the following concentrations of **1c**: 60 μM (open circles), 20 μM (closed circles), 10 μM (open squares), 5 μM (closed squares), and 3 μM (open triangles).

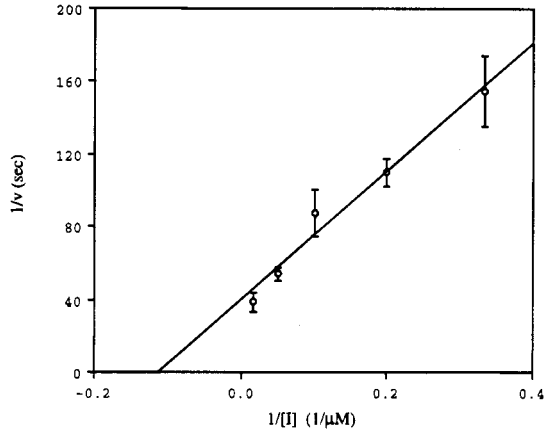

Figure 4. Double reciprocal plot of the inactivation of PAP with **1c**. Error bars represent the standard deviation of four experiments.

quinone methides,[19] does not slow the rate of inactivation, indicating that the enzyme is not inactivated by electrophiles that are released into solution (metabolic inactivation, Figures 5 and 6).[20] Also, doubling or halving the enzyme concentration does not change the rate of inactivation indicating that the enzyme is not being indiscriminately labeled by electrophilic

(18) The K_i for **1b** could not be determined accurately because of the high partition ratio. We estimate that it is in the range of 20–40 μM.

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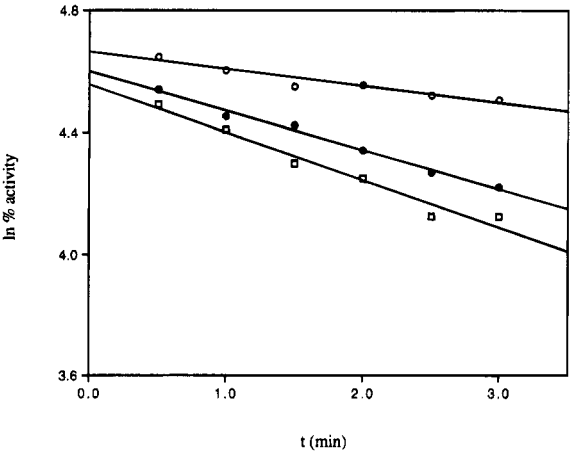


Figure 5. Inactivation of PAP with chloride **1b** (100 μM) in the presence of 10 mM P_i (open circles), with no additives (closed circles), and in the presence of 5 mM cysteine (open squares).

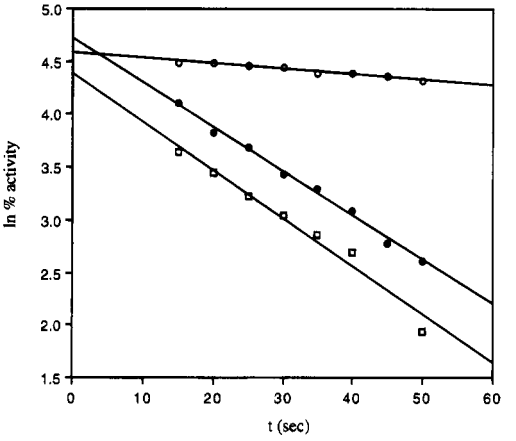


Figure 6. Inactivation of PAP with bromide **1c** (30 μM) in the presence of 10 mM P_i (open circles), with no additives (closed circles), and in the presence of 5 mM cysteine (open squares).

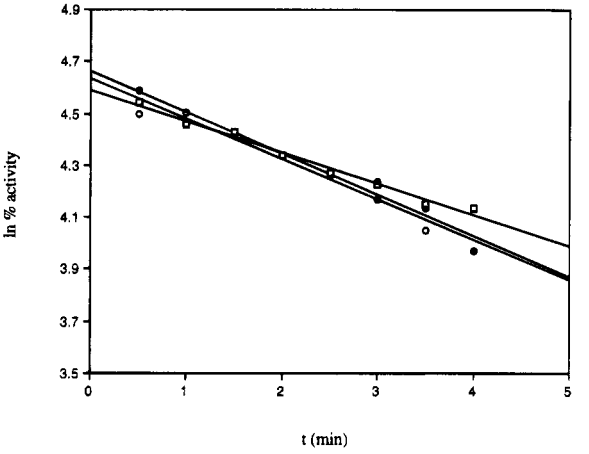


Figure 7. Inactivation of PAP with chloride **1b** (100 μM) as a function of [PAP]: 25 ng/mL (open squares), 50 ng/mL (closed circles), and 100 ng/mL (open circles).

compounds released to solution (Figures 7 and 8). We have previously shown that a phosphonate analogue of FMPP does not inactivate prostatic acid phosphatase, indicating that these inactivators are not functioning as affinity reagents.[10] A likely

(20) Cysteine has the effect of slightly increasing the rate of inactivation. The rate of substrate hydrolysis is not changed by the addition of 5 mM cysteine indicating that it is not intercepting the phosphoenzyme intermediate to any great extent. This phenomenon is general to these types of inhibitors. (See data in ref 10).

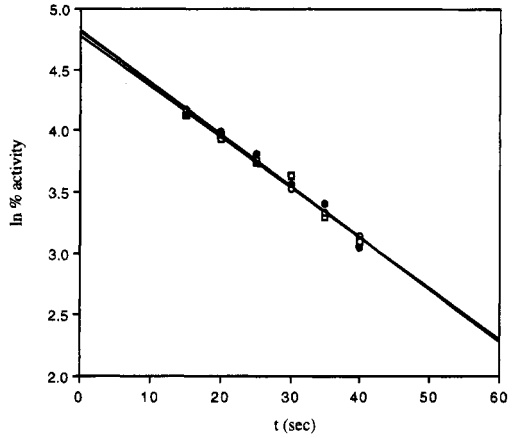


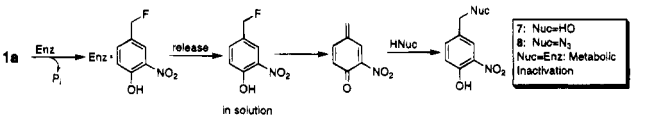
Figure 8. Inactivation of PAP with bromide **1c** (30 μM) as a function of [PAP]: 25 ng/mL (open squares), 50 ng/mL (closed circles), and 100 ng/mL (open circles).

mechanism for inactivation is shown in Scheme 1. Hydrolysis of the inactivator by prostatic acid phosphatase (k_1) initially results in the production of a 4-hydroxy-2-nitrobenzylic halide **5**. This intermediate can either be released into solution (k_{off1}) or eliminate HCl or HBr (k_{elim}) to give a quinone methide **6**. Quinone methide formation is accelerated many orders of magnitude upon deprotonation of the phenol,[21] so the enzyme may catalyze the elimination if, as is likely,[22] there is an appropriately positioned base in the active site. The quinone methide can also be released into solution (k_{off2}) or it can inactivate the enzyme by reacting with an active site nucleophile (k_{alk}).

An enzyme may turn over many inactivator molecules before becoming covalently labeled. The ratio of the rate of turnover to the rate of inactivation is called the partition ratio. The partition ratio can be approximated by the number of moles of inactivator that are required to inactivate 1 mol of enzyme. Each molecule of prostatic acid phosphatase turns over on the average of 5700 molecules of **1c** before becoming inactivated and 104 000 molecules of **1b** before becoming inactivated (Table 1). For prostatic acid phosphatase to turn over an inactivator and not be inactivated, either the phenol or quinone methide must be released into solution (k_{off1} and k_{off2}). The partition ratio is dependent on the ratio of k_{off1} to k_{elim} and the ratio of k_{off2} to k_{alk}. Since changing the leaving group from chloride to bromide greatly decreases the partition ratio, the release of the intermediate phenol must be a factor that reduces the efficiency of this type of inactivation. Based on this premise, modifying the inactivator to slow k_{off1} (by improving the binding of the phenol), or speeding up k_{elim} (by insertion of a better leaving group), should increase the efficiency of inactivation.

To determine the fate of intermediates derived from the enzyme catalyzed hydrolysis of **1a**, **1a** was incubated with PAP, and the reaction was followed by ^{1}H NMR. In acetate buffer, the major product of PAP catalyzed hydrolysis of **1a** was the benzylic alcohol **7** (confirmed by independent synthesis). In the presence of an excess of sodium azide the benzylic azide **8** was formed exclusively, demonstrating that azide is a good scavenger of quinone methides (Scheme 3). In contrast to the kinetic assays described earlier, these NMR assays require high concentrations of enzyme. Under these conditions, it was observed that PAP was inactivated by **1a**. It is likely that this inactivation is caused by the reaction of the enzyme with free

Scheme 3



quinone methide formed in solution, a reaction whose rate should show a dependence on the concentration of enzyme. In the absence of azide 50% turnover of the inhibitor took 20 h, while in the presence of azide 50% turnover was observed in under 10 min, indicating that azide slows enzyme inactivation. Since **1a** does not react significantly with azide in the absence of PAP, the azide must be reacting with an intermediate formed after hydrolysis of the phosphate ester. This result demonstrates that trapping by azide effectively reduces metabolic inactivation. No signals consistent with phenol **5** or quinone methide **6** were observed, demonstrating that even though elimination of fluoride from **5** is too slow to allow for inactivation of the enzyme, elimination from **5** and trapping of **6** are too fast for these intermediates to build up in solution.

The same experiment was repeated with alkaline phosphatase at pD 9.8 in tris buffer. In the absence of azide, alcohol **7** and a second product (presumably the tris adduct) were formed, while in the presence of azide the benzylic azide **8** is formed exclusively.

Reaction with PTPases. Another goal of our research has been to develop a family of inactivators that display a range of selectivities in the inactivation of different enzymes. The selectivity of these types of inactivators should be controllable by varying the structure of the inactivator. An inactivator that binds poorly to an enzyme will likely inactivate poorly since the partition ratio will be high. Moreover, a poor substrate is also likely to have a reduced rate of inactivation. For example, while bromide **1c** is a potent inactivator of prostatic acid phosphatase, it is a much poorer inactivator of YOP 51* [23] (15% inactivation after 3 min at 200 μM inactivator). This lack of reactivity toward YOP 51* is a reflection of the fact that *o*-nitrophenyl phosphate is a poor substrate for this enzyme. At pH 5.5, the K_m of *o*-nitrophenylphosphate is 1.2 mM compared to 300 μM for *p*-nitrophenyl phosphate and k_{cat} is 60 s^{-1} compared with 320 s^{-1} for *p*-nitrophenyl phosphate.

Since *o*-nitrophenyl phosphate is a good substrate for prostatic acid phosphatase (K_m 40 μM, k_{cat} 570 s^{-1}), it is not obvious why fluoride **1a** does not inactivate prostatic acid phosphatase while FMPP does. The nitrobromide, nitrochloride, and nitrofluoride all give rise to the same highly reactive quinone methide. Since both the bromide and chloride inactivate well, the lack of inactivation in the case of the fluoride must be related to the rate at which it suffers elimination. Since the nitro group is a strongly electron withdrawing group and a quinone methide is itself electron deficient, the elimination to a quinone methide should be slowed by the nitro group, even though the *o*-nitrophenol is more acidic than the phenol derived from FMPP.[21] However, the quinone methide resulting from hydrolysis of **1a**–**c** should be a remarkably reactive alkylating agent. Whether partitioning of the quinone methide off the enzyme is an important element in the determining potency of the inactivation brought about by compounds **1b** and **1c** has yet to be ascertained.

Summary and Conclusion. We have developed a family of new mechanism-based phosphatase inactivators, the 4-halomethyl-2-nitrophenyl phosphates. These compounds were synthesized to investigate whether the rate and efficiency of inactivation of prostatic acid phosphatase is dependent on the nature of the leaving group. Indeed, both of these parameters

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are highly dependent on the nature of the leaving group. This result suggests that elimination of HX from phenol **5** is in competition with its release into solution. From this observation, it is predicted that inhibitors that have enhanced binding would also inactivate the enzyme faster and more efficiently, since the partitioning of enzyme-bound intermediates determines the efficiency of inactivation. We have not yet determined whether quinone methide **6** can also be released into solution, but if it can, then enhanced binding of an inactivator should also improve the efficiency of the alkylation step by improving the ratio of k_{alk} to k_{off2} (Scheme 1).

The nitrohalide inactivators also demonstrate that simple changes in structure can lead to drastic changes in the selectivity of enzyme inactivation. While FMPP rapidly inactivates both YOP 51* and PAP, the nitrohalides are much better inactivators of PAP than YOP 51*. This result is consistent with what is known about the substrate specificities of the two enzymes. PAP is a relatively broad specificity phosphatase, while PTPases are very selective for phosphotyrosine containing peptides or proteins. The introduction of a nitro group *ortho* to the phosphate ester affects the binding and selectively deactivates these molecules as both substrates and inhibitors of phosphotyrosine phosphatases. This suggests that it may be possible to develop inactivators that are specific for one or a small group of phosphatases by taking advantage of their different substrate specificities.

Experimental Section

General Methods. All reactions were conducted in flame dried glassware under an atmosphere of dry argon or nitrogen. Ether and THF were distilled from sodium benzophenone ketyl. Methylene chloride, toluene, and acetonitrile were distilled from calcium hydride. Phosphorus trichloride was distilled prior to use and *tert*-butyl alcohol was dried with magnesium sulfate and then distilled. Triethylamine was distilled from KOH. DBU, DMAP, $NaBH_4$, DAST, PPh_3, and triflic acid were commercial products and used without purification. NCS and NBS were crystallized from water before use, and sodium ethoxide was prepared fresh for each reaction by reacting sodium pellets with absolute ethanol.

Di-*tert*-butyl-4-formyl-2-nitrophenyl Phosphate (2). *tert*-Butylphosphorobromidate (2.1 g, 7.8 mmol) in THF (5 mL) was added dropwise to a solution of 4-hydroxy-2-nitrobenzaldehyde (650 mg, 3.9 mmol), DBU (550 μL, 4 mmol), and DMAP (50 mg, 0.4 mmol) in THF (15 mL) at 0 °C. The reaction mixture was warmed to ambient temperature and maintained for 1 h. Ether (60 mL) was added, and the resulting solution was washed with 5% sodium bicarbonate (2 × 30 mL) and water (1 × 30 mL). The organic extract was dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was used directly in the next reaction. An analytical sample was prepared by flash column chromatography (ether containing 1% triethylamine): ^{1}H NMR ($CDCl_3$) δ 10.00 (s, 1H), 8.34 (s, 1H), 8.08 (dd, J = 8.8 Hz, 2.4 Hz, 1 H), 7.88 (d, J = 8.8 Hz, 1H), 1.53 (s, 18H); ^{13}C NMR ($CDCl_3$) δ 188.66 (s), 148.28 (d, J = 5.3 Hz), 141.84 (d, J = 6 Hz), 133.97 (s), 132.03 (s), 126.34 (s), 122.54 (s), 85.97 (d, J = 8.4 Hz), 29.66 (d, J = 4.5 Hz); ^{31}P NMR ($CDCl_3$) δ −16.52 (s); IR (neat) 2984, 2735, 1705, 1610, 1543, 1371, 1257, 1043 cm^{-1}.

Di-*tert*-butyl-4-(hydroxymethyl)-2-nitrophenyl Phosphate (3). Crude aldehyde **2** (3.9 mmol theoretical yield) in methylene chloride (5 mL) was added to 1:1 MeOH:CH_2Cl_2 (20 mL) at −78 °C. Sodium borohydride (300 mg, 8 mmol) was added in portions and the reaction was maintained at −78 °C for 1 h. The reaction mixture was poured into dichloromethane (100 mL), and the resulting suspension was carefully quenched with water (30 mL). The aqueous layer was separated, and the organic phase was washed with 5% sodium bicarbonate (2 × 30 mL) and water (1 × 30 mL). The organic extract was dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude product was then purified by flash column chromatography (1:1 hexane: ethyl acetate containing 1% triethylamine). The desired alcohol (**3**) was obtained as a pale yellow oil (970 mg, 69% for two steps): ^{1}H NMR ($CDCl_3$) δ 7.79 (s, 1H), 7.45 (m, 2H), 4.64 (s, 2 H), 1.47 (s, 18H); ^{13}C NMR ($CDCl_3$) δ 142.43 (d, J = 6.1 Hz), 141.44 (d, J = 8.3 Hz), 138.66 (s), 131.49 (s), 122.96 (s), 121.88 (s), 85.46 (d, J = 8.4 Hz), 62.88 (s), 29.67 (d, J = 3.8 Hz); ^{31}P NMR ($CDCl_3$) δ −16.14 (s); IR (neat) 3408, 2984, 1537, 1358, 1261, 1005, 939 cm^{-1}.

Di-*tert*-butyl-4-(fluoromethyl)-2-nitrophenyl Phosphate (4a). Diethylamino sulfurtrifluoride (550 μL, 4.17 mmol) was added dropwise to a solution of alcohol **3** (630 mg, 1.75 mmol) in dichloromethane (20 mL) at 0 °C. The reaction was maintained for 1 h and then warmed to ambient temperature and diluted with dichloromethane (30 mL). The resulting solution was washed with 5% sodium bicarbonate (2 × 20 mL) and water (1 × 20 mL). The organic extract was dried over Na_2SO_4, filtered, and concentrated in vacuo. The product was purified by flash column chromatography (2:1 hexane:ethyl acetate containing 1% triethylamine). The desired fluoride was obtained as a pale yellow oil (286 mg, 45%): ^{1}H NMR ($CDCl_3$) δ 7.84 (s, 1H), 7.68 (d, J = 8.8 Hz, 1 H), 7.54 (d, J = 8.4 Hz, 1H), 5.38 (d, J = 47.2 Hz, 2 H), 1.50 (s, 18H). ^{13}C NMR ($CDCl_3$) δ 144.02 (s), 141.55 (d, J = 8.4 Hz), 132.73 (d, J = 18.2 Hz), 132.15 (d, J = 5.3 Hz), 123.83 (d, 6.1 Hz), 122.49 (s), 85.31 (d, J = 8.4 Hz), 82.53 (d, J = 169.2 Hz), 29.70 (d, J = 3.8 Hz). ^{31}P NMR ($CDCl_3$) δ −15.931 (s); ^{19}F NMR ($CDCl_3$) δ −210.10 (t, J = 48 Hz); IR (neat) 2984, 2937, 1620, 1539, 1371, 1286, 1263, 1170, 1012, 939, 900 cm^{-1}; HRMS (*m/z*) 364.1320 MH$^+$ (calculated for $C_{15}H_{24}FNO_6P$, 364.1325).

Di-*tert*-butyl-4-(chloromethyl)-2-nitrophenyl Phosphate (4b). NCS (247 mg, 1.85 mmol) was added in portions to a solution of alcohol **3** (652 mg, 1.81 mmol) and triphenylphosphine (498 mg, 1.9 mol) in dichloromethane (5 mL) at 0 °C. The reaction was allowed to warm to room temperature and was maintained there for 30 min. The crude reaction mixture was loaded directly onto a silica gel column eluted with 2:1 hexane:ethyl acetate containing 1% triethylamine. The desired chloride was obtained as a pale yellow oil (362 mg, 53%): ^{1}H NMR ($CDCl_3$) δ 7.84 (s, 1H), 7.63 (d, J = 8.4 Hz, 1 H), 7.54 (d, J = 8.8 Hz, J = 2 Hz, 1H), 4.56 (s, 2 H), 1.48 (s, 18H); ^{13}C NMR ($CDCl_3$) δ 143.71 (d, J = 5.3 Hz), 141.38 (d, J = 7.6 Hz), 133.90 (s), 133.53 (s), 125.04 (s), 122.45 (d, J = 2.3 Hz), 85.27 (d, J = 7.6 Hz), 44.06 (s), 29.65 (d, J = 4.5 Hz); ^{31}P NMR ($CDCl_3$) δ −15.926 (s); IR (neat) 2984, 2936, 1618, 1537, 1500, 1371, 1252, 1168, 1003, 920 cm^{-1}; HRMS (*m/z*) 379.0959 M$^+$ (calculated for $C_{15}H_{23}ClNO_6P$, 379.0952).

Di-*tert*-butyl-4-(bromomethyl)-2-nitrophenyl Phosphate (4c). NBS (415 mg, 2.33 mmol) was added in portions to a solution of alcohol **3** (800 mg, 2.22 mmol) and triphenylphosphine (640 mg, 2.44 mmol) in dichloromethane (5 mL) at 0 °C. The reaction was allowed to warm to room temperature and was maintained there for 30 min. The crude reaction mixture was loaded directly onto a silica gel column eluted with 2:1 hexane:ethylacetate containing 1% triethylamine. The desired bromide was obtained as a pale yellow oil (650 mg, 69%): ^{1}H NMR ($CDCl_3$) δ 7.85 (s, 1H), 7.63 (dd, J = 8.8 Hz, J = 0.8 Hz, 1 H), 7.56 (d, J = 8.8 Hz, J = 1.6 Hz, 1H), 4.46 (s, 2 H), 1.51 (s, 18H); ^{13}C NMR ($CDCl_3$) δ 143.67 (d, J = 5.3 Hz), 141.36 (d, J = 6.1 Hz), 134.25 (s), 134.04 (s), 125.48 (s), 122.49 (s), 85.29 (d, J = 8.4 Hz), 30.61 (s), 29.65 (d, J = 4.8 Hz); ^{31}P NMR ($CDCl_3$) δ −15.972 (s); IR (neat) 2982, 1618, 1539, 1369, 1253, 1010, 912 cm^{-1}; HRMS (*m/z*) 367.9718 M$^+$ − *t*Bu (calculated for $C_{11}H_{14}BrNO_6P$, 367.9722).

Sodium 4-(Fluoromethyl)-2-nitrophenyl Hydrogen Phosphate (1a). Trifluoromethanesulfonic acid (1.1 μL, 0.012 mmol) was added to a solution of fluoride **4a** (186 mg, 0.59 mmol) in THF (1 mL). The reaction was maintained at room temperature for 16 h and then diluted with THF (5 mL). Ethanolic sodium ethoxide was added (464 μL, 1.26 M, 0.59 mmol), and the solvent was evaporated. The crude material was triturated with acetonitrile and THF to give the desired salt as a tan solid (124 mg, 78%) ^{1}H NMR (CD_3OD:D_2O, 1:1) δ 7.93 (s, 1H), 7.67 (d, J = 8.4 Hz, 1H), 7.62 (d, J = 8.4 Hz, 1H), 5.42 (d, J = 47.6 Hz, 2H); ^{13}C NMR (CD_3OD:D_2O, 1:1) δ 147.25, 142.59, 134.58, 132.90 (d, J = 17.4Hz), 125.18 (d, J = 32.8Hz), 84.29 (d, J = 162.4Hz); ^{31}P NMR (CD_3OD:D_2O, 1:1) δ −3.60 (s); ^{19}F NMR (CD_3OD: D_2O, 1:1) δ −205.68 (t, J = 47 Hz); IR (KBr pellet) 3599 (br), 3430 (br), 3092, 2966, 2901, 2339 (br), 1628, 1541, 1348, 1265, 1084, 935, 885 cm^{-1}. FAB mass spectrum (Ar, negative ion mode), *m/z* 249.3 [M − Na − H]$^-$.

Sodium 4-(Chloromethyl)-2-nitrophenyl Hydrogen Phosphate (1b). Trifluoromethanesulfonic acid (1.3 μL, 0.015 mmol) was added

to a solution of chloride **4b** (251 mg, 0.75 mmol) in THF (1 mL). The reaction was maintained at room temperature for 16 h and then diluted with THF (5 mL). Ethanolic sodium ethoxide was added (595 μL, 1.26 M, 0.75 mmol), and the solvent was evaporated. The crude material was triturated with acetonitrile and THF to give the desired salt as a tan solid (124 mg, 78%): ^{1}H NMR (CD_3OD:D_2O, 1:1) δ 7.95 (s, 1H), 7.68 (d, J = 8.4 Hz, 1 H), 7.57 (d, J = 8.4 Hz, 1H), 4.69 (s); ^{13}C NMR (CD_3OD:D_2O, 1:1) δ 146.71, 142.56, 135.47, 134.77,126.19, 124.11, 45.24; ^{31}P NMR (CD_3OD:D_2O, 1:1) δ −3.97; IR (KBr pellet) 3582 (br), 3427 (br), 3084, 2855(br), 2357 (br), 1626, 1537, 1348, 1267, 1095, 926, 710 cm^{-1}; FAB mass spectrum (Ar, negative ion mode), m/z 265.2 [M − Na − H]$^-$.

Sodium 4-(Bromomethyl)-2-nitrophenyl Hydrogen Phosphate (1c). Trifluoromethanesulfonic acid (2.3 μL, 0.026 mmol) was added to a solution of triester **4c** (501 mg, 1.32 mmol) in THF (2 mL). The reaction was maintained at room temperature for 16 h and then diluted with THF (5 mL). Ethanolic sodium ethoxide was added (1050 μL, 1.26 M, 1.32 mmol), and the solvent was evaporated. The crude material was triturated with acetonitrile and THF to give the desired salt as a tan solid (380 mg, 86%): ^{1}H NMR (CD_3OD:D_2O, 1:1) δ 7.95 (d, J = 2 Hz, 1H), 7.60 (dd, J = 8.8, 2 Hz, 1 H), 7.57 (d, J = 8.8 Hz, 1H), 4.60 (s, 2 H); ^{13}C NMR (CD_3OD:D_2O, 1:1) δ 146.78, 142.51, 135.86, 135.05, 126.56, 124.11, 32.26; ^{31}P NMR (CD_3OD:D_2O, 1:1) δ −3.77 (s); IR (KBr pellet) 3600 (br), 3430 (br), 3080, 2860(br), 2300 (br), 1624, 1537, 1350, 1263, 1080, 933, 897 cm^{-1}; FAB mass spectrum (Ar, negative ion mode), m/z 309.4, 311.4 [M − Na − H]$^-$.

4-Hydroxy-3-nitrobenzyl Alcohol (7). Sodium borohydride (76 mg, 2 mmol) was added in small portions to a solution of 4-hydroxy-3-nitrobenzaldehyde (130 mg, 0.78 mmol) in methanol (2 mL) at 0 °C. The reaction was maintained at 0 °C for 30 min and then diluted with chloroform (20 mL) and poured into 1 N HCl (10 mL). The organic layer was separated and washed twice with water (10 mL). The organic extract was dried over Na_2SO_4, filtered, and concentrated in vacuo to yield the desired alcohol as a yellow solid (128 g, 98%): ^{1}H NMR (CD_3OD) δ 8.05 (d, J = 2.4 Hz, 1H), 7.58 (dd, J = 2.4 Hz, 8.8 Hz, 1H), 7.20 (d, J = 8.8 Hz, 1H), 4.57 (s, 2H); ^{13}C NMR (CD_3OD) δ 154.80, 136.93, 135.40, 124.09, 120.99, 63.76; IR (neat) 3242 (br), 2928, 2878, 1630, 1579, 1533, 1427, 1334, 1251, 1170, 1016, 833, 682 cm^{-1}.

4-Hydroxy-3-nitrobenzyl Azide (8). Diethyl azodicarboxylate (65 mg, 0.37 mmol) was added slowly to a suspension of 4-hydroxy-3-nitrobenzyl alcohol (25 mg, 0.15 mmol), triphenylphosphine (76 mg, 0.29 mmol), and zinc azide pyridine complex[24] (34 mg, 0.11 mmol) in toluene (2 mL). The reaction was maintained at room temperature for 3 h. The reaction was diluted with chloroform (10 mL) and washed twice with 1 N HCl (5 mL) and twice with water (5 mL). The organic extract was dried over Na_2SO_4, filtered, and concentrated in vacuo. The resulting material was purified by flash chromatography (85:15 hexane:ethyl acetate). The desired azide was obtained as a yellow oil (7 mg, 24%): ^{1}H NMR (CD_3OD) δ 8.08 (d, J = 2 Hz, 1H), 7.61 (dd, J = 2 Hz, 8.8 Hz, 1H), 7.18 (d, J = 8.8 Hz, 1H), 4.41 (s, 2H); ^{13}C NMR (CD_3OD) δ 179.82, 155.37, 138.01, 129.66, 125.88, 121.60, 54.23; IR (neat) 3354 (br), 2922, 2852, 2095, 1626, 1583, 1533, 1427, 1284, 1032, 821 cm^{-1}.

Standard Inactivation of PAP. A solution of appropriately diluted inactivator in water (10 μL) was added to PAP (50 ng/mL) in buffer A (100 mM sodium acetate buffer pH 5.0, 500 μL) at 25 °C. At the indicated time, the inactivation was stopped, and the residual PAP activity was determined by diluting the inactivation reaction with 20 mM p-nitrophenyl phosphate in buffer A (500 μL). After 10 min, the reaction was quenched with 1 N NaOH (100 μL), and the absorbance at 405 nm was determined.

Active Site Protection with Inorganic Phosphate (P_i). Inorganic phosphate protection was performed using the standard inactivation procedure with buffer A containing 10 mM sodium phosphate.

Inactivation in the Presence of Cysteine. Inactivation in the presence of cysteine as a scavenger was performed using the standard inactivation procedure with buffer A containing 5 mM cysteine.

Determination of the Partition Ratio of 1b. PAP (50 ng/mL, 1 mL) was inactivated with chloride **1b** (500 μM). Aliquots (100 μL) were removed at 1, 4, 7, 11, 17, and 20 min and assayed for inorganic phosphate by the method of Lanzetta.[25] The partition ratio was calculated by dividing the [Pi] released from the inactivator by the [PAP] that had been inactivated at that time as determined in a standard inactivation.

Determination of the Partition Ratio of Bromide 1c. PAP (150 ng/mL, 1 mL) was inactivated with bromide **1c** (100 μM). Aliquots (100 μL) were removed every 15 s for 2 min and assayed for inorganic phosphate by the method of Lanzetta.[25] The partition ratio was calculated by dividing the [Pi] released from the inactivator by the [PAP] that had been inactivated at that time as determined in a standard inactivation.

Determination of Kinetic Constants (K_i and k_{inact}). The K_i and k_{inact} were determined by the method of Kitz and Wilson.[26] Rates were determined from the slope of the ln % activity vs time, and these rates were plotted as a double reciprocal vs 1/[I]. This line can be fitted to eq 1 to determine the kinetic constants.

$$1/v = (K_i/k_{inact})1/[I] + 1/k_{inact} \quad (1)$$

Dependence of the Rate of Inactivation on the Concentration of Enzyme. The standard inactivation was carried out at 25, 50, and 100 ng/mL prostatic acid phosphatase.

Determination of the Products of the PAP Catalyzed Hydrolysis of 1a. PAP (210 μg/mL, 50 μL) was added to a solution of **1a** in 50 mM NaOAc, 1 mg/mL of BSA pD 5.5 (950 μL, [**1a**]$_{final}$ = 13.7 mM). NMRs were taken at intervals, and the percentage of products were determined by integration of the benzylic signals (3.9−4.5 ppm relative to DHO at 4.67).

The assay was repeated with buffer containing 50 mM NaN_3 and a final concentration of **1a** of 14 mM.

Determination of the Products of the Alkaline Phosphatase Catalyzed Hydrolysis of 1a. Alkaline phosphatase (328 μg/mL, 400 μL) in 40 mM TrisDCl, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mg/mL BSA pD 9.8 was added to a solution of **1a** in the same buffer (600 μL, [**1a**]$_{final}$ = 8.8 mM). NMRs were taken at intervals and the relative concentrations of products were determined by integration of the benzylic signals (3.9−4.5 relative to DHO at 4.67 ppm).

The assay was repeated with buffer containing 277 mM NaN_3 and a final concentration of **1a** of 11.4 mM.

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