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# SUICIDE INACTIVATION OF HUMAN PROSTATIC ACID PHOSPHATASE AND A PHOSPHOTYROSINE PHOSPHATASE

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**SUMMARY:** 4-Difluoromethylphenyl bis(cyclohexylammonium) phosphate was synthesized in 4 steps starting from dibenzyl phosphite and shown to be a time-dependent suicide inactivator of human prostatic acid phosphatase and the SHP protein tyrosine phosphatase. The inactivation of human prostatic acid phosphatase followed pseudo-first-order kinetics with inactivation constants of $K_i$ = 1.0 mM; $k_i$ = 0.15 $min^{-1}$ ($t_{1/2}$ = 4.6 min at saturation). Phenyl phosphate protected the enzyme against inactivation, indicating that inactivation occurs in the active site. The inactivation of SHP also followed pseudo-first-order kinetics, with a $t_{1/2}$ = ~15 min in the presence of 8.2 mM inhibitor. The mechanism of inactivation likely involves the enzymatic release of difluoromethyl phenol which rapidly eliminates fluoride,generating a quinone methide. This potent electrophile then reacts with residues at the active site of the enzyme. This inhibitor and peptidic derivatives thereof have excellent potential for selective inactivation and labeling of protein tyrosine phosphatases.


Protein tyrosine phosphorylation plays a pivotal role within intracellular communication networks. By determining the phosphorylation state of specific intracellular proteins, protein tyrosine kinases[1] and protein tyrosine phosphatases[2] (PTPase) are able to regulate the activities of a host of different signaling pathways. The determination of the relative contributions of various kinases and phosphatases would be greatly aided by the availability of specific inhibitors. While much information exists regarding protein tyrosine kinase inhibitors, there has been little progress in the identification or design of PTPase inhibitors. Specific PTPase inhibitors would be invaluable tools, not only for study of the catalytic mechanism itself, but also for elucidating the function of individual tyrosine phosphatases within cells. Our approach to this problem was to design a mechanism based inactivator, 4-difluoromethylphenyl phosphate (DFPP) which is itself inert, but upon cleavage by the enzyme generates a reactive quinone methide in the active site. This species can then be attacked by nucleophilic residues at the enzyme active site, resulting in the inactivation

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Abbreviations: HPAP, human prostatic acid phosphatase; DFPP, 4-difluoromethylphenyl bis(cyclohexylammonium) phosphate; *p*-NPP, *p*-nitrophenyl phosphate, disodium salt; MFPP, 4-monofluoromethylphenyl phosphate; DAST, diethylaminosulfur trifluoride.

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of the enzyme, as shown in Scheme 1. A very similar strategy has been used previously for mechanism-based inactivation of glycosidases[3,4] as well as proteases and esterases[5,6].

This paper describes the synthesis of DFPP, a detailed kinetic evaluation of its activity as an inactivator of human prostatic acid phosphatase (HPAP), a relatively broad specificity phosphatase found in most body tissues, along with a demonstration of its efficacy against the tyrosine phosphatase SHP: SHP[7], also termed PTP1C[8], HCP[9] or SH-PTP1[10] is a cytoplasmic protein tyrosine phosphatase having two tandem *src* homology 2 (SH2) domains at the amino terminus.

## MATERIALS AND METHODS

*Materials*

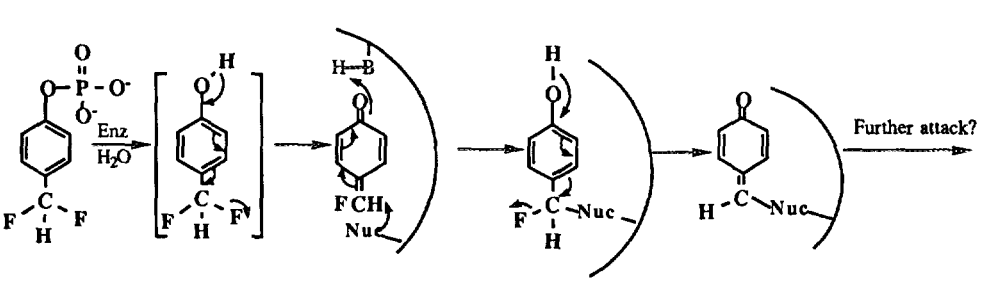
Buffer chemicals, *p*-nitrophenyl phosphate (*p*-NPP) disodium salt and bovine plasma thrombin were obtained from Sigma Chemical Company, as was Human Prostatic Acid Phosphatase (HPAP). Dibenzyl phosphite was purchased from Aldrich and glutathione Sepharose 4B from Pharmacia.

*Synthesis*

Melting points were determined on a Laboratory Devices Mel-Temp II melting point apparatus. $^{1}H$-, $^{31}P$-, and $^{19}F$- NMR spectra were acquired on a 200 MHz Bruker AC-200 instrument. Chemical shifts are given in the ($\delta$) scale referenced ($\delta$=0 ppm) to tetramethylsilane (TMS), phosphoric acid, and trifluoroacetic acid respectively. Mass spectra were recorded on an AEI-MS-9 spectrometer. All micro-analyses were performed in the microanalytical laboratory, Department of Chemistry, University of British Columbia.

4-Formylphenyl dibenzyl phosphate 2: A solution of freshly prepared dibenzyl phosphorochloridate[11,12] **1** (~9.5 mmol) in dry dichloromethane (10 mL) was added dropwise over a period of 40 min to a stirred suspension of 4-hydroxybenzaldehyde (1.17 g, 9.55 mmol) and imidazole (0.67 g, 9.55 mmol) in dry dichloromethane (50 mL) at room temperature, and stirred for 5 h. Precipitate was removed by filtration, washed with $CH_2Cl_2$, and the combined filtrates were concentrated by evaporation. The residue so obtained (TLC: $CH_3OH/CHCl_3$, 1:50, $R_f = 0.63$) was dissolved in $CHCl_3$ (50 mL), washed with 1M NaOH (3 x 50 mL), dried over $Na_2SO_4$ and then passed through a 2 cm layer of silica gel. After evaporation an oil-like material (1.85 g, 51%) was obtained, which solidified after leaving at -20 $^{0}C$ overnight. $^{1}H$-NMR ($CDCl_3$): $\delta$ 9.90 (s, 1 H), 7.81 (d, J = 9 Hz, 2 H), 7.32 (br.s, 10 H), 7.25 (d, J = 9 Hz, 2 H), 5.12 (d, J = 10 Hz, 4 H). $^{31}P$-NMR (81 MHz, $CDCl_3$): $\delta$ -5.97 ppm. MS (CI, $NH_3$): 400 ($M^+ + NH_4$, 56%). MS (EI): 291 ($M^+ - OCH_2C_6H_5$, 78%), 277 ($M^+ - C_6H_5CHO$, 18%).

4-Difluoromethylphenyl dibenzyl phosphate 3: Diethylaminosulfur trifluoride (DAST) (2.0 mL, 15 mmol) was added to a dry $CH_2Cl_2$ solution (15 mL) containing compound **2** (1.34g, 3.5 mmol) under $N_2$ and the reaction was stirred at room temperature for 12 h. After decomposition of the excess DAST with saturated aqueous $NaHCO_3$, the product was extracted with $CH_2Cl_2$ and the combined organic layer was dried ($Na_2SO_4$). Purification by column chromatography (silica gel,




Scheme 1

eluant $CHCl_3$) gave **3** as an oil (0.98 g, 69% yield) which solidified at -20 °C overnight. Recrystallization from $Et_2O$-pet. ether afforded a white solid, m.p. 40 °C. $^1$H-NMR ($CDCl_3$): δ 7.26 (m, 14 H), 6.61 (t, J = 56 Hz, 1 H), 5.13 (d, J = 9 Hz, 4 H). $^{19}$F-NMR (188 MHz, $CDCl_3$): δ -34.01 ppm. MS (CI, $NH_3$): 422 ($M^+ + NH_4$, 100%), 405 ($M^+ + 1$, 40%), 298 ($M^+ - OCH_2C_6H_5$, 80%). Analysis for $C_{21}H_{19}O_4F_2P$: calc: C, 62.38; H, 4.74. found: C, 62.53; H, 4.74.

4-Difluoromethylphenyl bis(cyclohexylammonium) phosphate **4**: Compound **3** (0.35 g, 0.87 mmol) and Pd/C (0.1 g) in EtOH (10 mL) were stirred under 1 atm. $H_2$ pressure at room temperature for 4 h. Pd/C was removed by filtration and cyclohexylamine was added to the filtrate until a pH value of 7 was attained, yielding a white precipitate which was collected and dried (0.29 g, 80%). The product was further purified by recrystallization from water/acetone/ethanol to yield white crystals of m.p.>192 °C (dec.). $^1$H-NMR ($D_2O$): δ 7.60 (d, J = 10 Hz, 2 H), 7.70 (d, J = 10 Hz, 2 H), 6.79 (t, J = 55 Hz, 1 H), 3.15-2.95 (m, 3 H), 1.5-2.0 (m, 11 H), 1.00-1.45 (m, 11 H). $^{19}$F-NMR (188 MHz, $D_2O$): δ -31.318 ppm. $^{31}$P-NMR (81 MHz, $D_2O$): δ +0.0015 ppm. MS (FAB): 423 ($M^+ + 1$, 33%), 100 (cyclohexylamine +1, 100%). Analysis for $C_{19}H_{33}O_4N_2P_1F_2$: calc: C, 54.03; H, 7.82; N, 6.64. found: C, 54.09; H, 7.95; N, 6.50.

*Bacterial Expression and Purification of the PTPase-Domain of SHP*

The plasmid carrying the cDNA for expression of the PTPase-domain of SHP was kindly provided by Matthew Thomas, Washington University School of Medicine, St. Louis. *E. coli* strain UT5600 (New England Biolabs) was transformed with plasmid pGEX-SHP(ΔSH2), encoding the PTPase-domain of SHP (amino acids 237 to 595). Glutathione S-transferase (GST) fusion proteins were expressed and purified as described[13]. Thrombin cleavage, used to obtain the isolated PTPase-domain, also resulted in a carboxy-terminal truncation that increased PTPase activity, as compared to the complete PTPase-domain. The characterization of this truncated enzyme (SHP(ΔSH2/ΔCT)) will be described elsewhere (Dechert et al., manuscript in preparation).

*Kinetics*

(i) HPAP: Inactivation experiments were performed by incubating HPAP (2 ng/μL) in 100 mM sodium acetate buffer (pH 5.0) containing bovine serum albumin (1.0 mg/mL) at 25 °C in the presence of different concentrations of DFPP (0.1, 0.3, 0.6, 1.0, 5.0 mM). Aliquots (20 μL) were removed at appropriate time intervals and assayed for residual enzyme activity by addition to 3 mM *p*-NPP solution (1 mL) in the same buffer. The reaction was quenched after 5 min by addition of 1.25 N NaOH (100 μL) and the absorption at 405 nm determined, from which residual rates were calculated using an extinction coefficient of $\varepsilon = 18{,}000\ M^{-1}cm^{-1}$). Pseudo-first-order rate constants at each inactivator concentration were calculated by non-linear regression analysis using the programme GraFit[14]. These rate constants were then fit directly to the equation shown below[14].

$$k_i = \frac{k_{obs}[I]}{K_i + [I]}$$

Protection against inactivation was demonstrated by measuring the rate of inactivation with 5.0 mM DFPP in the presence and absence of 50 mM phenyl phosphate.

(ii) SHP(ΔSH2/ΔCT): Two different assays were performed:

a) Using radioactive labeled synthetic peptide [$^{32}$P]-MB-1 (61 residues, corresponding to sequence of intracellular domain of mammalian Ig-α, doubly Y-phosphorylated): SHP(ΔSH2/ΔCT) (1 μg) was incubated with 2 μM [$^{32}$P]-MB-1 (specific activity 100 cpm/pmole) in 25 mM bis[2-hydroxyethyl]imino tris [hydroxymethyl] methane (Bis-Tris) buffer (pH 6.5) / 50 mM sodium chloride / 10 mM 2-mercaptoethanol in a final volume of 30 μL. After 25 min the reaction was stopped by spotting onto Whatman P81 paper and washing ten times with 0.5% phosphoric acid (400 mL). This removed free radiolabeled phosphate from immobilized radiolabeled phosphopeptide. The remaining [$^{32}$P]-phosphorylated peptide was determined by scintillation counting. The inactivation of SHP(ΔSH2/ΔCT) by DFPP was monitored by incubating the enzyme in Bis-Tris buffer, pH 6.5, at 0 °C in the presence of 5 mM DFPP and removing aliquots (28 μL) at different time points for assay of residual enzyme activity as described above. A simultaneous control experiment was carried out in the absence of DFPP.

(b) Using *p*-NPP as a substrate: The reaction was monitored by measuring rates of release of phenol products spectrophotometrically ($\lambda = 405nm$; $\varepsilon = 3.78mM^{-1}cm^{-1}$) in 25 mM Bis-Tris buffer, pH 6.5, containing 50 mM sodium chloride, 10 mM 2-mercaptoethanol at 25 °C. Values of

$K_m$ and $V_m$ for *p*-NPP were determined using substrate concentrations of 5-200 mM. The inactivation of SHP(ΔSH2/ΔCT) by DFPP was monitored by incubating the enzyme in the same buffer, but containing 50% glycerol, at 0 °C in the presence of DFPP (8.2 mM) and removing 5μL aliquots at different time points to assay with *p*-NPP (40 mM, 150 μL) as described above. A control experiment in the absence of DFPP was also performed.

## RESULTS AND DISCUSSION

Synthesis:

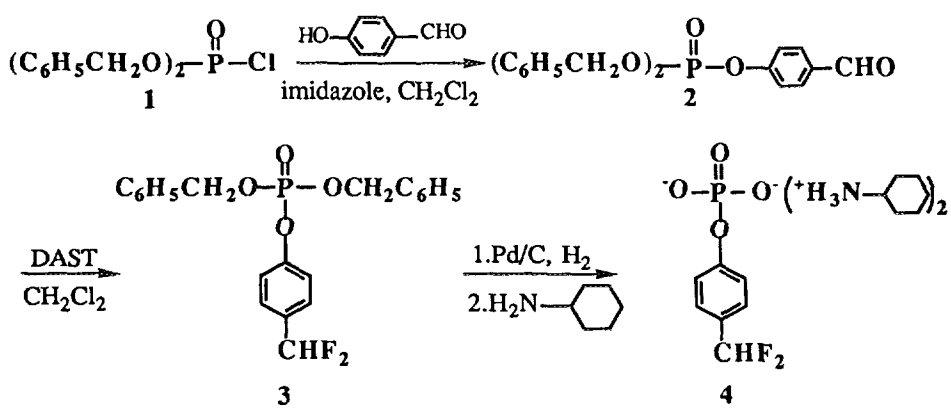
The synthesis of DFPP **4** was carried out according to Scheme 2. Starting from dibenzyl phosphite, compound **4** was simply prepared in four steps with a total yield of ~30%. Recrystallization from water/ethanol/acetone yielded analytically pure DFPP (**4**).

Kinetic Studies:

Prior to any kinetic studies, the stability of DFPP in aqueous solution was assessed. NMR analysis of samples of DFPP (**4**) dissolved in 100 mM sodium acetate buffer, pH 5.0, revealed minimal decomposition (~ 5%) after 3 days at room temperature. This level of decomposition is insignificant over the time periods studied (*vide infra*).

Incubation of HPAP with DFPP resulted in time-dependent inactivation according to pseudo-first order kinetics as shown in Figure 1a. Pseudo-first order rate constants obtained by direct fit of these data were found to exhibit a saturable dependence upon inactivator concentration as shown in Figure 1b, yielding an inactivation rate constant of $k_i = 0.15$ min$^{-1}$ ($t_{1/2} = 4.6$ min) and a dissociation constant of $K_i = 1.0$ mM. Incubation with DFPP (5 mM) in the presence of the poor substrate phenyl phosphate (50 mM) reduced the rate constant for inactivation from 0.123 min$^{-1}$ to 0.010 min$^{-1}$ (Figure 1c), suggesting that inactivation involves active site residues.

Two different assays were used to monitor inactivation of SHP(ΔSH2/ΔCT) by DFPP. One used *p*-NPP as substrate, and the other a radiolabeled phosphopeptide. Michaelis-Menten parameters of $K_m = 52$ mM, $k_{cat} = 9.8$ s$^{-1}$ were determined for SHP(ΔSH2/ΔCT) with *p*-NPP, indicating quite poor binding of simple aryl phosphate moieties. Incubation of SHP(ΔSH2/ΔCT)




Scheme 2

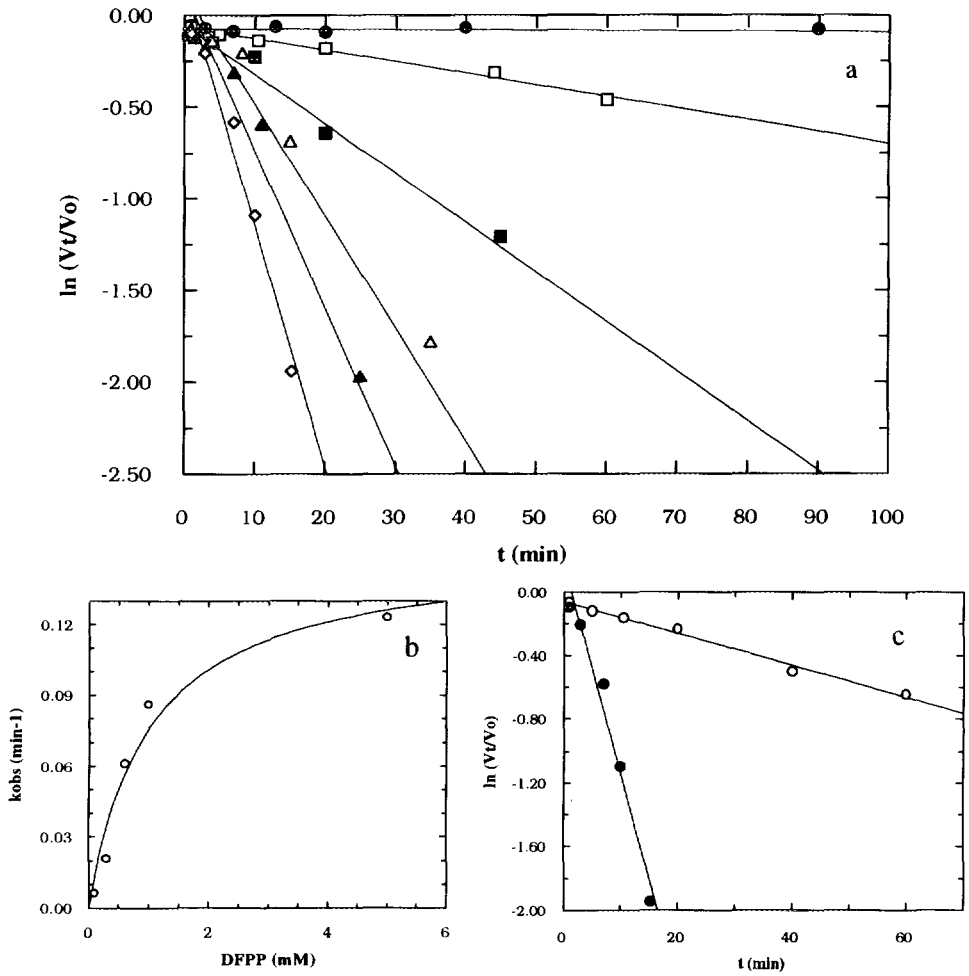



Figure 1. **Inactivation of HPAP by DFPP. a.** HPAP incubated with the following concentrations of DFPP and aliquots assayed with *p*NPP at the times shown: (●), 0.0 mM; (□), 0.1 mM; (■), 0.3 mM; (△), 0.6 mM; (▲), 1.0 mM; (◇), 5.0 mM. **b.** Replot of first order rate constants from **a** versus concentration of DFPP. **c.** Protection against inactivation of DFPP (5.0 mM) given by phenyl phosphate at (●), 0.0 mM; (○), 50 mM.

with DFPP (8.2 mM) resulted in time-dependent inactivation with $t_{1/2}$ ~15 min as shown in Figure 2a. When [$^{32}$P]-labeled peptide was used as a substrate, similar inactivation behaviour was observed, as shown in Figure 2b. Slight differences in the time courses are likely due to small differences in the concentration of inactivator employed in each case. Unfortunately, full kinetic parameters could not be determined due to the instability of the enzyme during long-term incubations, which resulted in unacceptable error levels, especially at low inactivator concentrations. Nonetheless, the data clearly indicate that DFPP is an effective, time-dependent inactivator of this important protein tyrosine phosphatase.

During the final stages of this work a similar inactivator, monofluoromethylphenyl phosphate (MFPP) was described[15]. However, no demonstration of its action on protein tyrosine

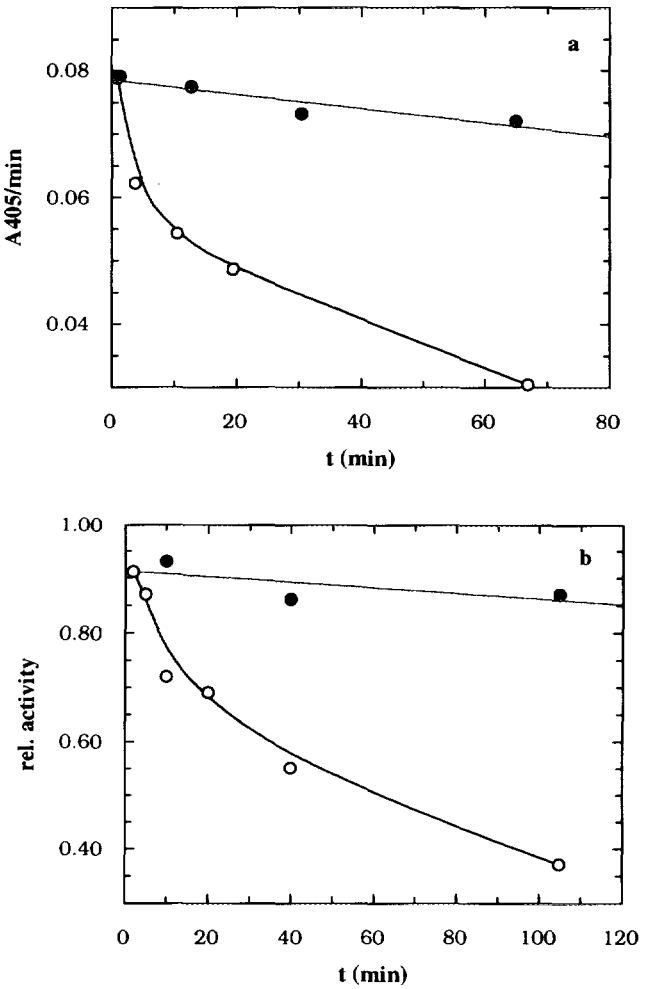



Figure 2. **Inactivation of SHP(Δ) by DFPP.** **a.** Assayed with *p*NPP; (○), inactivation curve; (●), control curve. **b.** Assayed using [$^{32}$P]-peptide; rel activity = ([$^{32}$P]$_{blank}$ - [$^{32}$P])/[$^{32}$P]$_{blank}$; (○), inactivation curve; (●), control curve.

phosphatases was provided. Further, the MFPP contained a benzylic monofluoride functionality which, in our experience, can be quite labile in aqueous solution, particularly in the presence of thiols. Such lability can be minimized by incorporating a second halogen functionality at the same benzylic center, as was done with DFPP. An additional advantage of DFPP as an inactivator is that a second quinone methide can be formed after elimination of the second fluoride (Scheme 1), giving an additional possibility of a reaction with a critical enzyme residue.

Comparison of the results on DFPP with those on MFPP with HPAP was of interest. MFPP exhibited an inactivation rate constant of $k_i$ = 1.2 min$^{-1}$ ($t_{1/2}$ = 35 s) and a dissociation constant of $K_i$ = 0.15 mM. It therefore inactivates 8 times faster and binds 7 times tighter than does DFPP. This is consistent with the greater stability of difluoromethyl phenol compared to the

monofluoro analogue, thus slower decomposition to yield the quinone methide. Interestingly no such differences were seen[3] in the rates of inactivation of β-glucosidase by *o*-methoxy-*p*-monofluoromethylphenyl β-D-glucoside and its difluoro derivative, suggesting that a different step in the inhibitory pathway was rate-determining in that case.

In conclusion, this paper describes the first example of mechanism-based inactivation of a protein tyrosine phosphatase, along with a detailed kinetic analysis of the equivalent inactivation of prostatic acid phosphatase. The inactivator employed, DFPP, is relatively stable, and its structure lends itself to the synthesis of phosphotyrosine analogs for use in peptide synthesis. The incorporation of the reactive functionality into the context of a peptide should considerably increase the affinity of this inhibitor for a given protein tyrosine phosphatase, thereby further increasing the probability of reaction between the quinone methide functionality and residues at the active site of the enzyme. Such a peptide-based approach may enable the selective inactivation of primary sequence-specific protein tyrosine phosphatases.

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