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INACTIVATION OF VACCINIA H1 RELATED PHOSPHATASE IN THE PRESENCE OF PHOSPHONOMETHYLATING AGENTS

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Abstract

Vaccinia H1-related (VHR) phosphatase was inactivated with phosphonomethyl trifluoromethanesulfonate (PMT), phosphonomethyl pentafluorobenzenesulfonate (PMFB), or phosphonomethyl trifluoroethylsulfonate (PMTres). PMTres produced only about 10% loss of activity; however, both PMT and PMFB produced well over 50% inactivation. Both arsenate and phosphate slowed the inactivation from PMFB in a concentration-dependent manner, suggesting that PMFB modifies a residue at the active site. These results are guiding further studies toward the synthesis of a stable analog of the transiently phosphorylated cysteinyl residue in VHR phosphatase.

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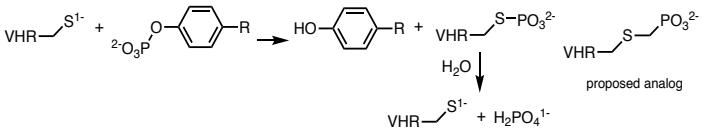
Introduction

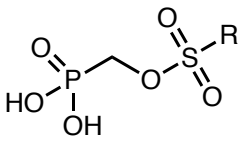
Vaccinia H1-related (VHR) phosphatase is a member of the dual-specificity phosphatase subfamily of the protein tyrosine phosphatase (PTPase) family. VHR phosphatase participates in various signal transduction processes and is a target for disease intervention (1). The active sites of dual-specificity phosphatases have the conserved sequence motif (VXVHCXXGXXRS(X)$_5$AY(L/I)M) (2). In order to hydrolyze phosphotyrosine residues, Cys-124, the cysteinyl residue in the motif above, attacks the phosphorus atom creating a transiently phosphorylated cysteinyl residue. In the second step water attacks the phosphorus atom and the cysteinyl residue departs (Scheme 1). Although the outline of the mechanism is similar for all PTPases, some details differ or remain obscure, especially regarding the general base. There are two possible general base catalysts for the centrosome-associated PTPase VHZ (3). For CDC25, a key regulator in the cell cycle, the identity of the general acid/general base has been debated (4).

Scheme 1: Mechanism of VHR phosphatase comparing the phosphoenzyme to the proposed analog



In our attempt to construct a nonhydrolyzable analog of the phosphorylated cysteinyl intermediate via covalent modification of Cys-124, we synthesized three molecules bearing various sulfonyl leaving groups on the phosphonomethyl moiety (Chart 1), phosphonomethyltrifluoromethanesulfonate (PMT), phosphonomethyltrifluoroethanesulfonate (PMTres) and phosphonomethylpentafluorobenzenesulfonate (PMFB). The pK_a value of Cys-124 in VHR phosphatase is 5.6, suggesting that it will be more nucleophilic at pH 6 than protonated cysteinyl residues (5). The high reactivity of Cys-124 facilitates constructing an analog by alkylation of this residue. We obtained rates of inactivation of VHR phosphatase by these sulfonates. We found that arsenate and phosphate reduce the rate of inactivation by one of them, indicating that the sulfonates react at the active-site, an initial step toward creating an analog.

Chart 1: Structures of the irreversible inhibitors in this study



R = CF_3 (PMT)
R = CH_2CF_3 (PMTres)
R = C_6F_5 (PMFB)

Experimental

General.

Linear regression using in Excel 16.16.9 was used to find the values of k_{obs} (defined below). Regression using ProStat 6.5 was used to obtain the best-fit values of other kinetic parameters. NMR data were collected on a Bruker 400 MHz AMX spectrometer. Syntheses were performed under N_2 using oven-dried glassware.

Reagents.

Pentafluorobenzenesulfonylchloride was purchased from Lancaster Synthesis. 2,2,2-Trifluoroethanesulfonylchloride and 4-Nitrophenyl phosphate di(2-amino-2-ethyl-1,3-propanediol) salt were purchased from Sigma-Aldrich. Triethylamine was stirred over calcium hydride and distilled. Bis(2-mercaptoethyl)sulfone and BisTris (Ultrol grade) were purchased from Calbiochem. Molecular biology certified glycerol was purchased from Shelton Scientific. $Na_2HAsO_4•7H_2O$ was purchased from Alfa Aesar. D_2O was purchased from Cambridge Isotope Laboratories. Other reagents were purchased from commercial sources and were used without further purification.

Synthesis of PMT.

Phosphonomethyltrifluoromethanesulfonate was synthesized as described (6). After this work was completed, syntheses of di-tert-butylphosphonomethyltrifluoromethanesulfonate employed a flash silica column (7).

Synthesis of di-tert-butylphosphonomethyltrifluoroethanesulfonate.

Di-*tert*-butylhydroxymethylphosphonate (4.98 mmol, 1.12 g) and triethylamine (5.98 mol, 0.605 g) dissolved in dry DCM (5 mL) were stirred at -12 to -10 °C. Trifluoroethanesulfonyl chloride (5.48 mmol; 1.00 g) in DCM (5 mL) was added dropwise over 5 min. The reaction was warmed to -6 °C. Dry DCM (15 mL) was added, followed by two washes with cold, saturated $NaHCO_3$ and one wash with cold brine. The solution was dried with $MgSO_4$, and the solvent was removed by rotary evaporation. Yield was 36%.

Synthesis of PMTres.

The di-*tert*-butylester was dissolved in 4 M HCl in dioxane (2 mL) and stirred for 4 hours. The volatiles were removed with rotary evaporation, followed by high vacuum, leaving a near-white solid.

Synthesis of di-tert-butylphosphonomethylpentafluorobenzenesulfonate.

Di-*tert*-butylhydroxymethylphosphonate (6.69 mmol, 1.50 g) and triethylamine (8.02 mol, 0.812 g) dissolved in dry DCM (6 mL) were stirred at -16 to -9 °C. Pentafluorobenzenesulfonyl chloride (7.36 mmol, 1.961 g) in DCM (6 mL) was added dropwise over 30 min. The reaction was warmed to -5 °C. Dry DCM (15 mL) was added, followed by two washes with saturated $NaHCO_3$ and one wash with brine.

Synthesis of PMFB.

The di-*tert*-butylester was dissolved in 4 M HCl in dioxane and stirred for four hours. The volatiles were removed with rotary evaporation, followed by high vacuum, leaving a near-white semi-solid.

Purification of VHR phosphatase.

The purification of VHR phosphatase was performed in a similar manner to Dixon and coworkers with modifications (8). All steps were performed near 4 °C. A plasmid bearing the gene for VHR phosphatase was transformed into E. coli strain B834(DE3). Transformed bacteria were grown in 2xYT medium plus 100 µM ampicillin with shaking at 37 °C, and when A_{600} of 0.80 was reached, protein production was induced by the addition of 0.4 mM isopropylthiogalactopyranoside. After about eight hours the cells were centrifuged at 5000 RPM in a JA-10 rotor for twenty minutes then sonicated in 50 mM succinate, 1 mM EDTA, 14 mM 2-ME, 0.2 mM PMSF, pH 6.0. Polyethyleneimine was added to a final concentration of 0.5%; then the mixture was stirred for 15 minutes and centrifuged for 40 minutes at 5000 RPM in a JA-10 rotor. Solid ammonium sulfate was added to a final concentration of 65%; then the mixture was stirred for 15 minutes and centrifuged for 45 min at 5000 RPM in a JA-10 rotor. The pellet was either frozen or dissolved in 15 mL of 20 mM MES, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, pH 6.0 or in 75 mL of 50 mM succinate, 1 mM EDTA, 14 mM 2-ME, 5% glycerol, pH 6.0. The solution was dialyzed against the same buffer. In the case of the MES buffer, the protein was diluted to 100 mL and glycerol added to 10% final concentration to minimize precipitation. When hazy precipitation occurred, the mixture was clarified by centrifugation at 27,000 x g for twenty minutes.

A 50-mL column SP-Sepharose Fast Flow (Sigma; Na^+-form) was equilibrated in 20 mM MES, 1 mM EDTA, 1 mM DTT, 2 mM PMSF pH 6.00 or in 50 mM succinate, 1 mM EDTA, 14 mM 2-ME, 5% glycerol, pH 6.0. The protein was loaded onto the column at about 1 mL/min, and it was eluted with a 600 mL gradient of 0-600 mM NaCl with a flow rate of 0.5 ml/min. Fractions were analyzed on the basis of phosphatase activity, and the pooled fractions were concentrated to about 3 mL. A 2.5 by 100 cm column of Sephadex G-75 or BioGel P-30 was equilibrated in 50 mM Tris, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, pH 7.4. The concentrated protein was loaded, and the flow rate was maintained near 0.5 mL/min. Fractions were pooled on the basis of activity assays and SDS PAGE. In some cases TCEP and BMS were added to selected fractions to a final concentration of 0.5 mM each. For some preparations a 22-mL Cibacron Blue column was equilibrated with 50 mM succinate, 1 mM EDTA, 14 mM 2-ME, 5% glycerol, pH 6.0. The column was eluted with 4-5 column volumes of this buffer; then the phosphatase was eluted with the same buffer plus 100 mM phosphate.

Reversible inhibition.

The concentration of 4-nitrophenylphosphate (4-NPP) was varied from 6 to 80 mM; the concentration of arsenate was varied from 0 to 90 µM; or the concentration of phosphate was varied from 0 to 15 mM. The concentration of VHR phosphatase was 1.5 to 3.0 µg/mL. Stop-time assays were performed using 4-NPP as the substrate. Typical conditions were 750 mM Bis-Tris, pH 6.0, 5% glycerol, 0.015% TRX-100, 0.2 mM EDTA, 0.5 mM bis(2-mercaptoethylsulfone) in a 0.200-mL volume. Reactions were stopped with 1.00 mL of 1.0 M NaOH after 20 minutes at 25 °C and read at 405 nm.

Inactivations involving phosphonomethylsulfonates.

In a typical assay VHR phosphatase (0.1 mg/mL) was incubated at 30 °C in the presence of the inactivating agent for varying lengths of time. The buffer was 0.75 M Bis-Tris pH 6.0, 5% glycerol, 0.015% Triton X-100 (TRX-100), 0.20 mM EDTA, 0.5 mM BMS in a final volume of 75 µL. A 9.1-µL aliquot was diluted 100-fold in 50 mM Bis Tris pH 6.0, 5% glycerol, 0.015% TRX-100, 0.2 mM EDTA, 0.5 mM BMS and left on ice for 45 minutes then brought to room temperature for 5 minutes. A 20-µL aliquot of this mixture was assayed in a 180-µL aliquot of the same buffer with 10 mM 4-NPP for 100 minutes at 30 °C. The reaction was stopped with a 1000-µL aliquot of 1.0 M NaOH and read at 405 nm. A control experiment using 120 mM ethylphosphonate showed little change in activity over four hours. Protection studies were carried out in a similar manner to the inactivation studies, except for the addition of varying concentrations of arsenate or phosphate.

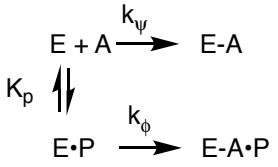
Data analysis of inactivation.

Data points of $\ln((Abs_t - Abs_{blank})/(Abs_0 - Abs_{blank}))$ versus time were plotted, where Abs is the absorbance at 405 nm in the phosphatase activity assay. The negative of the slope is the observed pseudo first-order rate constant, k_{obs}. The blanks were run in the absence of enzyme. The slopes of plots of k_{obs} vs. A (the inactivator) are the second order rate constants for inactivation, k_i (9). In the presence of a protecting agent P (Scheme 3),

$$k_{obs} = (k_\psi + k_\phi \bullet P/K_p)/(1 + P/K_p) \qquad (1)$$

k_ψ and k_ϕ are the pseudo first-order rate constants of inactivation for the unliganded and liganded forms of the enzyme E, respectively,

Scheme 2: Inactivation of enzyme (E) by irreversible inhibitor (A) in the presence of a reversibly bound inhibitor (P)



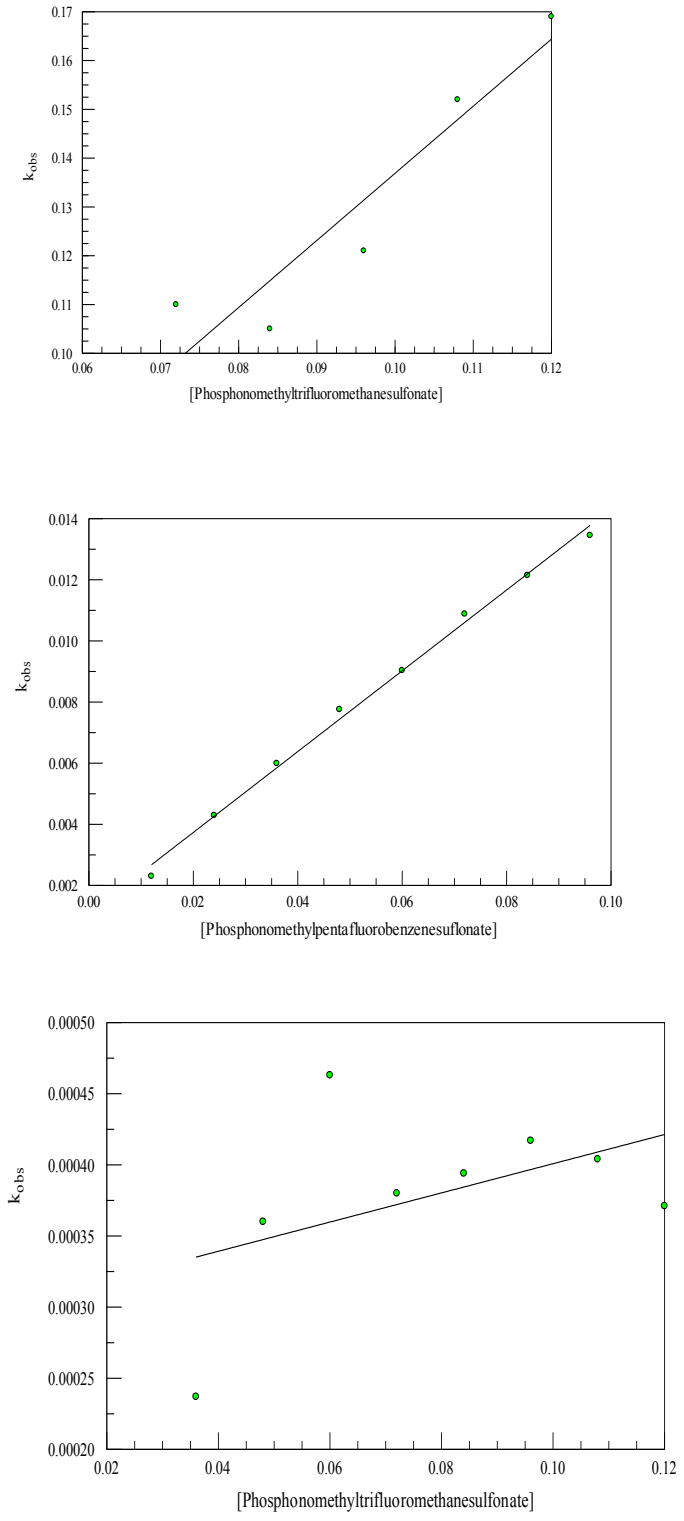


Figure 1: Pseudo-first order inactivation rate constants versus molar concentration of phosphonomethyltrifluoromethanesulfonate (A, top), phosphonomethylpentafluorobenzenesulfonate (B, middle), or phosphonomethyltrifluoroethanesulfonate (C, bottom). Seven to nine aliquots of VHR phosphatase incubated with alkylating reagent were removed at various times and residual phosphatase activity determined using 4-nitrophenylphosphate. Each data point represents a single determination of k_{obs} in min^{-1}, found from a plot of the natural logarithm of residual activity vs. time. A (top). The enzyme was incubated for up to three minutes with 0.072-0.120 M PMT. B (middle). The enzyme was incubated for up to 240 minutes in the presence of 0.012-0.096 M PMFB. C (bottom). The enzyme was incubated for up to 240 minutes with 0.036-0.120 M PMTres.

and K_P is the dissociation constant of P.

Solvolyses.

A 700-µL portion of 70 mM phosphonate in 0.75 M BisTris pH 6.0, 5% glycerol, 1 mM EDTA, and 10% D_2O was prepared and a 10-µL aliquot of triethylphosphate (TEP) was added. The logarithms of integrals of ^{31}P NMR signals relative to the integral from TEP were plotted versus time to provide the pseudo-first order rate constant.

Results

Inactivation.

The rate of inactivation of VHR phosphatase by PMT follows pseudo first-order kinetics over a short timespan (to minimize hydrolysis of PMT) and follows second-order kinetics (Figure 1A). The rate of inactivation of VHR phosphatase by PMFB follows pseudo first-order kinetics over four hours and follows second-order kinetics over a broad range of concentrations (Figure 1B). The inactivation of VHR phosphatase by PMTres proceeded roughly to 10% completion, and the plot of k_{obs} versus the concentration of PMTres was noisier (Figure 1C) than for PMT or PMFB (Figure 1). The rate constants for inactivation by phosphonomethylsulfonates are collected in Table 1. Additional inactivation data are collected in Table S1.

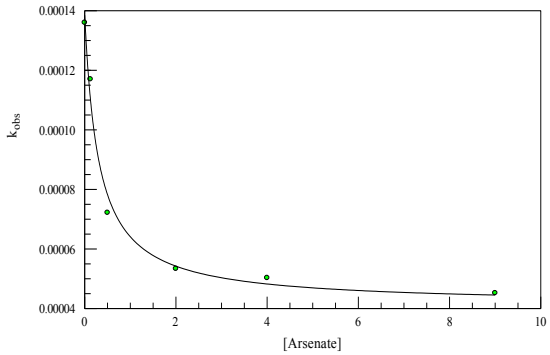
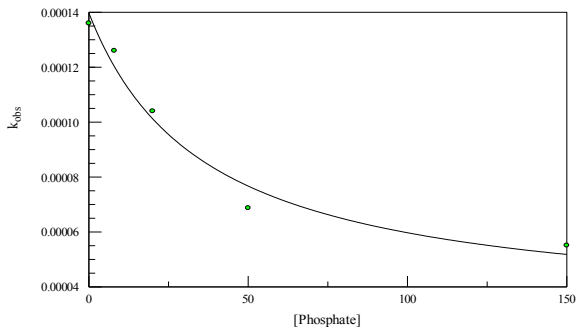


Figure 2: Pseudo-first order rate constant for inactivation of VHR phosphatase by 48 mM phosphonomethylpentafluorobenzenesulfonate as a function of the molar concentration of arsenate (A above) or phosphate (B below). Nonlinear regression was used to find the best-fit values of the parameters in equation (1). Each point represents a single determination of k_{obs} in min^{-1}.



Inhibition.

The inhibitory effects of arsenate and phosphate on the rate of hydrolysis of 4-NPP were studied. A competitive inhibition model was used to obtain the kinetic constants K_M and K_i (10). As seen in Table 2, our values for 4-NPP, phosphate, and arsenate are larger than the K_M and K_i values obtained at lower ionic strength by previous workers (8,10,11).

Protection.

The ability of arsenate ions and phosphate ions to protect against inactivation of VHR phosphatase by PMFB was examined (Table 3). Both arsenate (Figure 2A) and phosphate (Figure 2B) protected in a concentration-dependent manner, and the protection appears to be incomplete even when the concentration of the anion was much greater than the value of K_p (Scheme 2). Each protecting agent was studied in the presence of both 48 mM and 96 mM PMFB, and the pseudo-first order rate constants for inactivation in the absence and presence of the protecting agent, k_ψ and k_ϕ, were roughly double at the higher concentration of PMFB, as expected.

Discussion

The rates of inactivation of PMTres, PMFB, and PMT are qualitatively in accord with the leaving group abilities (Table 1) of the three compounds in solvolytic reactions (12). A combination of steric hindrance and electrostatic repulsion from the three oxygen atoms of the phosphoryl group may be responsible for the low reactivity of PMTres observed in this work. The greater leaving group ability of the trifluoromethanesulfonyl group within PMT also makes it labile to solvolysis, which limits the fraction of modified enzyme produced.

Reversible inhibitors are expected to protect against active-site directed modifying agents. If the concentration of the protecting agent is varied, its protection constant is expected to be similar in magnitude to its inhibitory constant. The magnitudes of the inhibitory constants for arsenate and phosphate and the Michaelis constant for 4-NPP in initial velocity experiments were larger than previously observed under different conditions of much lower ionic strength (Table 2). We speculate that solution anions compete for binding to the enzyme versus these three species. However, the inhibitory constant of arsenate is similar in magnitude to its corresponding protection constant (Table 4), suggesting that PMFB reacts with an active-site nucleophile. The incompleteness of protection by arsenate may be explained in two ways. Either arsenate fails to shield an active-site nucleophile fully, or a second nucleophile elsewhere within VHR phosphatase reacts more slowly. Whether or not protection was complete in the presence of phosphate is less clear. The relative errors in the values of k_ϕ are larger than were the case for arsenate, and the highest concentration of phosphate was only about 3-fold greater than K_p vs. about 30-fold greater than K_p for arsenate. The greater relative errors in K_p and k_φ for phosphate versus arsenate may reflect this difference. The protection studies suggest that PMFB is reacting with an active site nucleophile but may also be reacting with a second nucleophile.

Reversible inhibitors should protect against inactivation by affinity labels (13). Moreover, affinity labels typically show saturation kinetics; however, neither PMT nor PMFB shows saturation behavior. PMFB fulfills the first of these two criteria for being an affinity label but having only a phosphoryl group, it may not resemble a sufficient portion of the substrate to have affinity for the active site. Experiments which characterize the chemical nature of VHR phosphatase modified by PMT or PMFB are currently being analyzed.

In conclusion we partially inactivated VHR phosphatase with two related reagents. On the basis of protection studies, it is probable that PMFB modified Cys124, although a second nucleophile may also become modified (protection studies involving PMT will be reported in a future publication). Both groups are expected to donate a phosphonomethyl group to Cys124, producing a nonhydrolyzable analog of the intermediate phosphoenzyme. Because inactivation is incomplete, structural studies will benefit from the separation of modified from unmodified VHR phosphatase, which will be reported in a future publication.

Table 1: Second order rate constants for the inactivation of VHR phosphatase by phosphonomethylsulfonates

Compound	k_i, M^{-1} min^{-1}	Relative reactivity[a]	Half-life, min[b]
PMTres	0.00103±0.00081	240	38,000
PMFB	0.132±0.0035	330	6500
PMT	1.375±0.30	83000	13

[a] Relative solvolysis rate constants vs. a mesylate ester; data from Crossland 1971.
[b] At 21 °C. Conditions were 0.75 M BisTris pH 6.0, 5% glycerol, 1 mM EDTA.

Table 2: Kinetic constants from reversible inhibition studies

	Arsenate	Phosphate
K_M 4-NPP (mM)	17.8	13.8
K_i (mM)	0.25	9.0
K_M 4-NPP (mM)	1.59[a]	
K_i (mM)	0.021±0.001[b]	0.97±0.12[c]

[a] Zhou et al., 1994.
[b] Denu et al., 1995.
[c] Denu and Dixon, 1995.

Table 3: Arsenate or phosphate protection against inactivation of VHR phosphatase by PMFB

	$k\psi$ (sec^{-1})[a]	$k\phi$ (sec^{-1})[a]	K_p (mM)
Arsenate			
48 mM PMFB	1.38 ± 0.05 e-04	4.1 ± 0.4 e-05	0.31 ± 0.08
Arsenate			
96 mM PMFB	2.90 ± 0.08 e-04	6.9 ± 0.6 e-05	0.30 ± 0.05
Phosphate			
48 mM PMFB	1.40 ± 0.07 e-04	3.0 ± 1.7 e-05	37 ± 18
Phosphate			

[a] As shown in equation (1) and Scheme 2.

Acknowledgments

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Supplemental

NMR data
di-tert-butylphosphonomethyltrifluoroethanesulfonate. ^{1}H-NMR ($CDCl_3$): 4.40 ppm (d, $^{2}J_{PH}$ = 8.0 Hz, 2H, PCH_2), 4.17 (q, $^{3}J_{FH}$ = 8.8 Hz, 2H, CF_3CH_2), 1.54 ppm (s, 18H, $-C(CH_3)_3$). ^{31}P-NMR ($CDCl_3$) 6.9 ppm.
PMTres. ^{1}H-NMR (d_6-DMSO) 5.04 (q, $^{3}J_{FH}$ = 9.6 Hz, 2H, CF_3CH_2), 4.36 ppm (d, $^{2}J_{PH}$ = 9.4 Hz, 2H, PCH_2). ^{31}P-NMR (d_6-DMSO) 10.3 ppm.
di-tert-butylphosphonomethylpentafluorobenzenesulfonate. ^{1}H-NMR ($CDCl_3$) 4.34 ppm, (d $^{2}J_{PH}$ = 8.9 Hz, 2H, $-CH_2P$), 1.52 ppm (s, 18H, $-CH_3$). ^{31}P-NMR ($CDCl_3$) 5.7 ppm.
PMFB. ^{1}H-NMR (d_6-DMSO) 4.31 ppm, (d, $^{2}J_{PH}$ = 9.3 Hz, 2H, PCH_2). ^{31}P-NMR (d_6-DMSO) 9.3 ppm.
Inactivations using biotinylation reagents
EZ-Link™ Iodoacetyl-PEG2-Biotin and EZ-Link™ Maleimide-PEG2-Biotin were purchased from ThermoFisher Scientific. The conditions were 50 mM succinate, 1 mM EDTA pH 6, 0.015% Triton X-100, 5% glycerol at 30 °C. Stock solutions of the biotinylation reagents were prepared at concentrations of 50 mM. The rate constants for inactivation by biotinylation reagents and related electrophiles are collected in Table S1.

Table S1: Pseudo-first order rate constants for the inactivation of VHR phosphatase by biotinylation reagents and related compounds at 30 °C and pH 6.

Reagent	Concentration, mM	k_{obs}, min^{-1}
Iodoacetamide	3.0	0.0191
EZ-Link™ Iodoacetyl-PEG2-Biotin	4.2	0.0091
EZ-Link™ Maleimide-PEG2-Biotin	0.166	0.108
Biotin HPDP	2.5	0.352
Iodoacetic acid[a]	3.0	0.18[a]