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Why Is Phosphonodifluoromethyl Phenylalanine a More Potent Inhibitory Moiety Than Phosphonomethyl Phenylalanine toward Protein-Tyrosine Phosphatases?

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Summary: The phosphonodifluoromethyl phenylalanine (F_2Pmp) is superior to phosphonomethyl phenylalanine (Pmp) as a non-hydrolyzable phosphotyrosine (pTyr) mimetic. The difluoromethyl moiety increases the inhibitory potency of a F_2Pmp-containing peptide over a Pmp-containing counterpart by 1000-fold toward the protein tyrosine phosphatase (PTPase), PTP1. Fluorine substitution at the methylene carbon have the double effect of lowering the phosphonate pK_{a2} as well as introducing hydrogen bonding interactions similar to the phosphate ester oxygen in pTyr. The inhibition of PTP1-catalyzed dephosphorylation reaction by both the F_2Pmp and Pmp-containing peptides did not vary as a function of pH. The data indicate that both the monoanion and the dianion forms of the phosphonate bind PTP1 with equal efficiency. Thus, the better binding by the F_2Pmp-peptide as compared to the Pmp-peptide is not due to the difference in pK_{a2}. Taken together, these results offer an explanation for the increased affinity of F_2Pmp for PTP1. The two fluorine atoms in F_2Pmp may be able to interact with active site residues in PTP1 in a fashion analogous to that involving the phenolic oxygen and side chains in the active site of PTP1. K_i measurements for a simple phosphonic acid, Pmp- and F_2Pmp-containing peptides suggest that although the principal recognition element is F_2Pmp itself, the surrounding amino acids are required for high affinity binding. Comparative analysis of the inhibition of PTP1, PTPα and LAR by F_2Pmp-containing peptides suggests that selective, tight-binding PTPase inhibitors can be developed.

Tyrosine phosphorylation of proteins is a fundamental mechanism for the control of cell growth and differentiation. *In vivo*, this process is reversible and dynamic; the phosphorylation states of proteins are governed by the opposing actions of protein tyrosine kinases (PTKs), which catalyze protein tyrosine phosphorylation, and protein tyrosine phosphatases (PTPases), which are

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responsible for dephosphorylation (1). PTPases constitute a growing family of transmembrane (receptor-like) and intracellular enzymes that rival the PTKs in terms of structural diversity and complexity (2). There is mounting evidence that PTPases may either potentiate or antagonize the action of PTKs. Hundreds of protein kinases and protein phosphatases and their substrates are integrated within an elaborate signal transducing network. The defective or inappropriate operation of this network is at the root of widespread diseases such as cancers (3, 4). Consequently, the characterization of PTKs and PTPases is an important focus in biomedical research.

Specific and selective inhibitors of PTPases would be useful in assessing their physiological function. Activation or deactivation of a particular pathway could be achieved by designing compounds that specifically disrupt one of the signalling pathways. Since amino acids flanking the phosphotyrosyl residue (pTyr) contribute to high affinity substrate binding (5), one current approach toward the design of potent and selective PTPase inhibitors relies on the incorporation of a nonhydrolyzable analog of pTyr into specific, optimal phosphopeptide templates. This is because pTyr itself is absolutely essential for PTPase recognition of peptide/protein-based substrates. For example, PTPases do not bind tyrosine-bearing peptides that lack the phosphate or phosphate mimicking functionality (6). Furthermore, O-methylation of pTyr in a peptide suppresses its ability to bind PTPase (7). This implies that a tyrosine moiety, in conjunction with the negatively charged phosphate group, is crucial for PTPase recognition. Based upon these structural considerations, several nonhydrolyzable analogs of pTyr have been prepared and inserted into PTPase-targeted peptides. Phosphonomethyl phenylalanine (Pmp) is a phosphonate-based surrogate of pTyr in which the phosphate ester oxygen has been replaced by a methylene unit (Fig. 1) (8). Indeed, Pmp-containing peptides have been shown to be effective, reversible inhibitors of PTPases (5, 9). Peptides containing sulfotyrosyl (10) or malonyl derivatives (11) are also shown to be effective inhibitors of PTPases. Surprisingly, substitutions by two fluorines at the methylene position in Pmp (F_2Pmp, Fig. 1, (12)) brings about a three orders of magnitude improvement in the IC_{50} value for PTP1B (13). It was not clear however, why phosphonodifluoromethyl phenylalanine (F_2Pmp) is superior to Pmp.

In this paper we have examined several Pmp and F_2Pmp-containing peptides in terms of their ability to inhibit the mammalian PTPases, PTP1, LAR and PTPα. We found significant differences in the active sites of these PTPases for inhibitor recognition. We have also studied the pH-dependency of PTP1 inhibition by Pmp- and F_2Pmp-containing peptides. The results from these experiments suggest that the reason F_2Pmp is a better inhibitory motif than Pmp is most likely due to the ability of these fluorine atoms to interact specifically with amino acid side chains in the PTP1 active site.

Materials and Methods

Materials. *p*-Nitrophenyl phosphate and 4-aminobenzylphosphonic acid were purchased from Sigma. The hexapeptide Ac-Asp-Ala-Asp-Glu-(Pmp)-Leu-NH_2 was a generous gift from Dr. Mark Saper from the University of Michigan. Solutions were prepared using deionized and distilled water.

Peptide Synthesis. Derivatized pTyr mimicking amino acids Fmoc-D/L-F_2Pmp$(OEt)_2$-OH (14), or Fmoc-L-F_2Pmp$(OEt)_2$-OH (15), were incorporated into the various peptides using manual solid phase peptide synthesis (SPPS) methodology with Fmoc chemistry. The peptides were

prepared using PAL resin (16), DIPCDI/HOBt coupling reagents and 20% piperidine/DMF for Fmoc deprotection. The resin bound peptides were acetylated with 4% 1-acetylimidazole/DMF. Manual SPPS methodology including resin cleavage/side chain deprotection conditions for the preparation of F_2Pmp peptides Ac-Asp-Ala-Asp-Glu-(F_2Pmp)-Leu-NH_2, Ac-Asp-(F_2Pmp)-Val-Pro-Met-Leu-NH_2, Ac-Gln-(F_2Pmp)-Glu-Glu-Ile-Pro-NH_2, Ac-Asn-(F_2Pmp)-Ile-Asp-Leu-Asp-NH_2, and Ac-Asn-(D-F_2Pmp)-Ile-Asp-Leu-Asp-NH_2 were described previously (13, 17, 18). The 9-mer peptide, Ac-Leu-Asn-(F_2Pmp)-Ile-Asp-Leu-Asp-Leu-Val-NH_2 was prepared using the Fmoc compatible chemistry cited above, with side chain protected amino acids, Fmoc-L-F_2Pmp$(OEt)_2$-OH, Fmoc-Asp(OtBu)-OH, and Fmoc-Asn(Trt)-OH. The peptide was cleaved from the PAL resin and the Asn and Asp side chains deprotected with TFA:thioanisole:EDT:m-cresol:H_2O (80:5:5:5:5, v/v), followed by TMS-triflate/dimethyl sulfide deprotection to remove the ethyl protective groups from the F_2Pmp amino acid side chain, based on methodology we developed earlier (17). The peptides were purified by reverse phase HPLC chromatography (Vydac C18), and structurally characterized by FAB mass spectrometry (VG-7070E-HF) and by amino acid analysis.

Recombinant Enzymes. The recombinant catalytic domain of rat PTP1 was created by inserting a stop codon at residue 323, yielding PTP1U323, in order to eliminate the hydrophobic "targeting" domain of the molecule. Homogeneous recombinant catalytic domain PTP1U323, from here on referred as PTP1, was purified as described (19). The first catalytic domain of PTPα was expressed in *E. coli* and purified to homogeneity as described previously (20). The catalytic domains of LAR was purified as described (21).

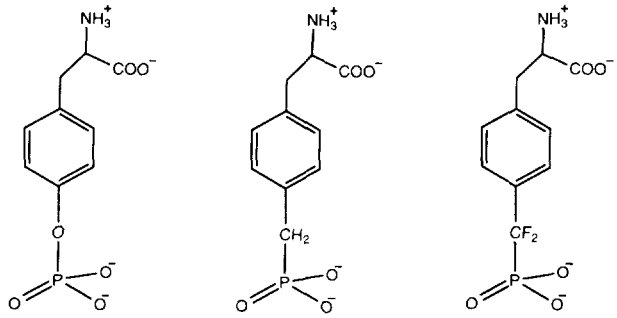
Enzyme Assay. The PTPase activity was usually assayed at 30 °C in a reaction mixture (0.2 ml) containing appropriate concentrations of *p*-nitrophenyl phosphate as substrate. Buffers used were as follow: pH 5.0 - 5.5, 100 mM acetate; pH 6.0, 50 mM succinate; pH 7.0, 50 mM 3,3-dimethylglutarate, and pH 8.0, 50 mM tris. All of the buffer systems contained 1 mM EDTA and the ionic strength of the solutions were kept at 0.15 M using NaCl. The reaction was initiated by addition of enzyme and quenched after 2-3 min by addition of 1 ml of 1 N NaOH. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without the addition of enzyme. The amount of product *p*-nitrophenol was determined from the absorbance at 405 nm using a molar extinction coefficient of 18,000 $M^{-1}cm^{-1}$. Michaelis-Menten kinetic parameters were determined from a direct fit of the v vs. [S] data to the Michaelis-Menten equation using the nonlinear regression program GraFit (Erithacus Software).

Inhibition by the Pmp and F_2Pmp-containing peptides. The inhibition constants for the Pmp and F_2Pmp-containing peptides were determined for homogeneous PTP1, LAR and PTPα in the following manner. At various fixed concentrations of inhibitors, the initial rate at various *p*-nitrophenyl phosphate concentrations was measured as described (22). The inhibition was competitive with respect to the substrate. The data were fit to equation 1 using KINETASYST (IntelliKinetics, State College, PA) to obtain the inhibition constant (K_i).

$$v=V_{max}\cdot S/(K_m\cdot[1+I/K_i]+S) \qquad (1)$$

Results and Discussion

Phosphonic acid derivatives are non-hydrolyzable phosphate mimetics that have found wide use in biological systems (23, 24). They are isosteric with parent phosphates yet are resistant to the action of phosphatases. For example, benzyl phosphonic acid was shown to be a competitive inhibitor for the low molecular weight PTPase with a K_i value of 4.6 mM (25). This value is higher than that of inorganic phosphate which is 2 mM. In order to build tighter-binding inhibitors, additional molecular features have to be incorporated into the phosphonate functionality. Pmp is a nonhydrolyzable phosphonate analog of pTyr in which the phosphate ester oxygen has been replaced by a methylene unit (Fig. 1). Indeed, Pmp-containing peptides have been shown to act as competitive PTPases inhibitors with inhibition constants in the micromolar range (5, 9).



pTyr **Pmp** **F_2Pmp**

Figure 1. Structures of phosphotyrosine (pTyr) and phosphonate pTyr mimetics, phosphonomethyl phenylalanine (Pmp) and phosphonodifluoromethyl phenylalanine (F_2Pmp).

Pmp-containing peptides also bind to src homology 2 (SH2) domains, but with reduced affinity compared to native pTyr-containing peptides (26). Interestingly, substitution at the methylene position in Pmp by two fluorine atoms (to generate F_2Pmp) improves the SH2 domain binding affinities of these peptides so that they are comparable to those of the pTyr-containing peptides (18). An F_2Pmp-containing peptide is also a much more potent PTP1B inhibitor than its Pmp-containing counterpart (13).

There are two possible explanations for the reduced affinity of the Pmp-containing peptides in comparison with the pTyr and F_2Pmp-containing peptides (13). The pK_{a2} values of the phosphonate or phosphate moieties in Pmp, F_2Pmp and pTyr are different. The pK_{a2} values for phenyl phosphate (6.22), benzyl phosphonate (7.72), and difluorobenzyl phosphonate (5.71) have been determined (27). The pK_{a2} values for Pmp (7.1) and pTyr (5.7) have also been determined (26). A comparison (Table 1) between these pK_{a2} values gives an approximate value of 5.1 for

Table 1

Comparison of the second pK_a values for phosphate monoesters and phosphonic acids

Compounds	pK_{a2}	Compounds	pK_{a2}
Phenyl phosphate	6.22[a]	pTyr	5.7[b]
Benzyl phosphonic acid	7.72[a]	Pmp	7.1[b]
α,α-Difluorobenzyl phosphonic acid	5.71[a]	F_2Pmp	5.1[c]

[a]These values are taken from (27). [b]These values are taken from (26). [c]Estimated by comparison between the pK values of phosphates and phosphonic acids.

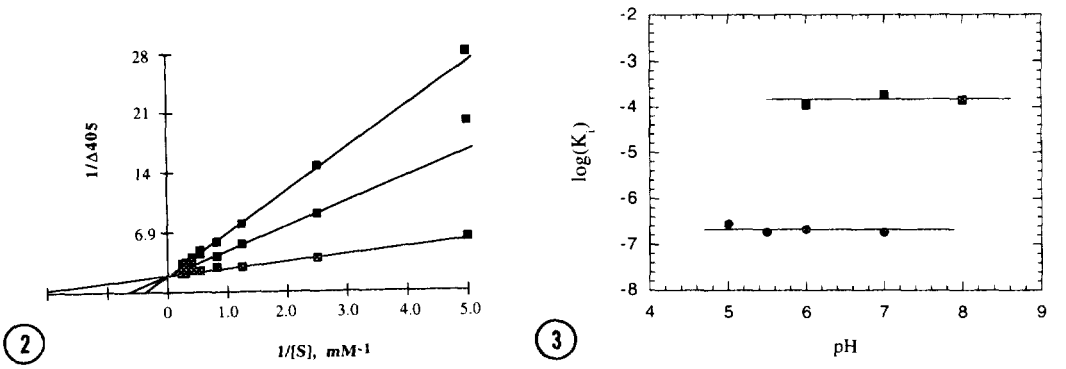


Figure 2. Effect of Ac-Asp-Ala-Asp-Glu-(F_2Pmp)-Leu-NH_2 on the PTP1-catalyzed hydrolysis of *p*-nitrophenyl phosphate. The experiment was performed at pH 7.0 and 30 °C, with 0, 0.485, and 0.975 μM inhibitor.

Figure 3. pH-dependencies of inhibition of PTP1 by Ac-Asp-Ala-Asp-Glu-(Pmp)-Leu-NH_2 (■) and Ac-Asp-Ala-Asp-Glu-(F_2Pmp)-Leu-NH_2 (●).

F_2Pmp. If high affinity interaction with the PTPases or the SH2 domains requires the dianionic form of phosphonates or phosphates, the reduced affinity of Pmp peptides can be explained by the fact that at neutral pH the phosphate group in pTyr and the phosphonate group in F_2Pmp carry a -2 charge whereas only 50% of the phosphonate groups in Pmp have a -2 charge. On the other hand, substitution of the ester oxygen with a CH_2 unit may also lead to the loss of potential hydrogen-bonding interaction(s) between the ester oxygen and PTPases or SH2 domains which may be important for high affinity binding. Fluorine substitutions at the methylene unit have the double effect of lowering the phosphonate pK_{a2} as well as introducing hydrogen bonding interactions similar to the ester oxygen in pTyr.

In order to differentiate these two possibilities, we have measured the inhibition constants of Ac-Asp-Ala-Asp-Glu-(Pmp)-Leu-NH_2 and Ac-Asp-Ala-Asp-Glu-(F_2Pmp)-Leu-NH_2 as a function of pH against the mammalian PTPase, PTP1. PTP1 (28) is the rat homologue of the human PTP1B. The catalytic domain of PTP1 resides between residues 1 to 322 and is 97% identical to the corresponding 322 residues of the human PTP1B. We found that both Asp-Ala-Asp-Glu-(Pmp)-Leu-NH_2 and Ac-Asp-Ala-Asp-Glu-(F_2Pmp)-Leu-NH_2 were competitive inhibitors of PTP1 using *p*-nitrophenyl phosphate as a substrate (Fig. 2) with K_i values of 181 and 0.18 μM respectively at pH 7.0. Thus, the F_2Pmp peptide binds PTP1 three orders of magnitude stronger than the Pmp peptide. If the dianion of the phosphonates are the correct form for binding, the inhibition should be enhanced as one raises the pH above those of the pK_{a2} values. We measured the pH dependence of the PTP1 inhibition by these peptides (Fig. 3). The K_i values were constant for Asp-Ala-Asp-Glu-(Pmp)-Leu-NH_2 from pH 6 to 8. Similarly, the K_i values for Ac-Asp-Ala-Asp-Glu-(F_2Pmp)-Leu-NH_2 were constant from pH 5 to 7. Furthermore, inhibition of PTP1 by competitive inhibitors such as inorganic phosphate, arsenate, and 4-aminobenzyl phosphonate did not show any pH dependence (Z.-Y. Zhang, unpublished results). These results

suggest that both the monoanion and the dianion forms of the phosphate analogs bind PTP1 equally well, and the reason that F_2Pmp-containing peptides bind PTP1 better than Pmp-containing peptides is not due to the difference in their pK_{a2} values.

Based on the results described above, we propose the reason that the F_2Pmp-containing peptide is superior to Pmp-containing peptide may be that the two fluorine atoms in F_2Pmp can restore or enhance the hydrogen-bonding interactions normally between the phenolic oxygen in pTyr and side chains in the active site of PTP1. This hypothesis is supported by biochemical and structural data. PTPases effect catalysis through a covalent cysteinyl phosphoenzyme intermediate (29). In addition to nucleophilic catalysis, it appears that all PTPases also utilize a general acid to facilitate the departure of the phenolic leaving group by protonation of the bridging oxygen (30). Indeed, the structure of PTP1B (with Cys215 to Ser substitution) complexed with a peptide substrate (31) reveals that the phenolic oxygen of pTyr forms a net work of hydrogen bonds with the side chain of the corresponding general acid Asp181 and a buried water molecule. Interestingly, the phenolic oxygen of pTyr also forms a hydrogen bond with Ser177 of the Src SH2 domain (32). It is tempting to suggest that the higher affinity of F_2Pmp-containing peptides toward SH2 domains may also be due to the ability of the fluorine atom(s) to form a hydrogen with the hydroxyl group of Ser177. This needs to be confirmed by experimentation.

We then measured the inhibition constants of several other F_2Pmp-containing peptides (Table 2). Although peptides Ac-Asp-Ala-Asp-Glu-(F_2Pmp)-Leu-NH_2, Ac-Asp-(F_2Pmp)-Val-Pro-Met-Leu-NH_2, Ac-Gln-(F_2Pmp)-Glu-Glu-Ile-Pro-NH_2, Ac-Asn-(F_2Pmp)-Ile-Asp-Leu-Asp-NH_2, and Ac-Leu-Asn-(F_2Pmp)-Ile-Asp-Leu-Asp-Leu-Val-NH_2 vary in amino acids flanking the F_2Pmp and the positioning of the F_2Pmp moiety in the peptide, their displayed K_i values toward PTP1 do not differ greatly from each other. This indicates that PTP1 may possess a broad

Table 2

K_i values for PTP1 at pH 7.0 and 30 °C

Inhibitors	K_i, (μM)
4-aminobenzyl phosphonate	25,000 ± 5000
Ac-Asp-Ala-Asp-Glu-(Pmp)-Leu-NH_2	181 ± 11
Ac-Asp-Ala-Asp-Glu-(F_2Pmp)-Leu-NH_2	0.18 ± 0.02
Ac-Asp-(F_2Pmp)-Val-Pro-Met-Leu-NH_2	0.12 ± 0.01
Ac-Leu-Asn-(F_2Pmp)-Ile-Asp-Leu-Asp-Leu-Val-NH_2	0.16 ± 0.01
Ac-Gln-(F_2Pmp)-Glu-Glu-Ile-Pro-NH_2	2.1 ± 0.19
Ac-Asn-(F_2Pmp)-Ile-Asp-Leu-Asp-NH_2	0.42 ± 0.02
Ac-Asn-((*D*)-F_2Pmp)-Ile-Asp-Leu-Asp-NH_2	4.5 ± 0.16

substrate specificity. As shown in the three-dimensional structure of the catalytic domain of human PTP1B (33), the protein surface surrounding the catalytic cleft is relatively open and consists of a number of depressions and protrusions. This may allow numerous modes of peptide recognition and is consistent with the ability of PTP1 to hydrolyze a wide variety of pTyr-containing substrates with nearly equal efficiency (5, 6, 34).

We also determined whether PTP1 is sensitive to stereochemistry at the α-position of the F_2Pmp residue. It was previously shown that PTPases catalyzed the hydrolysis of free D-pTyr and L-pTyr with similar efficiency (35). Interestingly, Ac-Asn-(D-F_2Pmp)-Ile-Asp-Leu-Asp-NH_2 exhibited a K_i value that was 10-fold larger than that of Ac-Asn-(F_2Pmp)-Ile-Asp-Leu-Asp-NH_2 (Table 2). It is clear that PTP1 prefers the naturally occurring L-stereoisomer when the F_2Pmp is constrained within a peptide-based environment. Overall, the fact that modifications at the F_2Pmp site (e.g. fluorine substitutions and changes in stereochemistry) greatly affect the affinity of these peptides toward PTP1 suggests that the principal interactions of PTP1 are with the F_2Pmp motif itself. In the structure of PTP1B complexed with a pTyr-containing peptide substrate, the recognition pocket for pTyr represents the dominant driving force for peptide binding since pTyr contributes about 53% of the peptide solvent-accessible surface area (31). However, it is important to point out that high affinity binding also requires the presence of amino acid residues adjacent to the pTyr/F_2Pmp motif. For example, 4-aminobenzyl phosphonate inhibited PTP1 at pH 7 with a K_i value of 25 mM (Table 2) while the Pmp-containing peptides Ac-Asp-Ala-Asp-Glu-(Pmp)-Leu-NH_2 and Asp-Ala-Asp-Glu-(Pmp)-Leu-Ile-Pro-Gln-Gln-Gly inhibited PTP1 with K_i values of 181 and 10 μM (5) respectively. Furthermore, we have shown that PTP1 displays a k_{cat}/K_m value for Asp-Ala-Asp-Glu-(pTyr)-Leu-Ile-Pro-Gln-Gln-Gly that is 3200-fold higher than that of pTyr alone (5). Together, these results suggest that the binding of peptide substrate/inhibitor is a cooperative event that involves the recognition of both pTyr/F_2Pmp functional groups as well as the structural features from surrounding residues.

Finally, we compared the relative potencies of two F_2Pmp peptides toward PTP1, PTPα and LAR. PTPα is a receptor-like PTPase that has a small, highly glycosylated extracellular segment, a transmembrane region, and an intracellular segment possessing two active PTPase domains. It is expressed ubiquitously. Evidence suggests that it is involved in the activation of c-*src* by dephosphorylation of Tyr527 (36, 37). Most recent evidence indicates that PTPα is also involved in the down-regulation of insulin receptor signalling (38). LAR (Leukocyte Common Antigen Related) is a widely distributed receptor-like transmembrane PTPase (39). It is composed of two cytoplasmic PTPase domains, a transmembrane region, and an extracellular segment which resembles cell adhesion molecules. Interestingly, both LAR and PTP1B are implicated as negative regulators of the insulin action pathway (40, 41). We wanted to determine if these F_2Pmp peptides show any selectivities toward these PTPases. Selective, tight-binding PTPase inhibitors may have beneficial effects in the treatment of human diseases such as diabetes. The average sequence identity among mammalian PTPase catalytic domains is about 40%, and most of the conserved amino acid residues are located in and around the active site where catalysis occurs (33). Consequently, it has been assumed that much of what we learn about one PTPase should prove

applicable to other PTPase family members as well. In constrast, we found there is a remarkable variability in the degrees of inhibition of PTPases by the F_2Pmp peptides. Thus, Ac-Asp-Ala-Asp-Glu-(F_2Pmp)-Leu-NH_2 is a potent inhibitor for PTP1 ($K_i = 0.18 \pm 0.02$ μM), but a significantly poorer one for LAR ($K_i = 376 \pm 43$ μM). Similarly, Ac-Asp-(F_2Pmp)-Val-Pro-Met-Leu-NH_2 is a potent PTP1 inhibitor ($K_i = 0.12 \pm 0.01$ μM), but a much poorer one for PTPα ($K_i = 465 \pm 130$ μM). It appears that significant differences exist within the active sites of various PTPases. Additional studies are required to further our understanding of PTPase substrate specificity. Incorporation of a nonhydrolyzable pTyr mimetic such as F_2Pmp that retains the interaction between the phenolic oxygen and PTPase active site into a PTPase directed peptide could provide selective, tight-binding inhibitors of PTPases.

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