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Potent Non-peptidyl Inhibitors of Protein Tyrosine Phosphatase 1B

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Abstract—The development of inhibitors of protein tyrosine phosphatases (PTPs) has recently been the subject of intensive investigation due to their potential as chemotherapeutics and as tools for studying signal transduction pathways. Here we report the evaluation of a variety of small molecule, non-peptidyl inhibitors of protein tyrosine phosphatase 1B (PTP1B), bearing the α,α-difluoromethylenephosphonic acid (DFMP) group, a non-hydrolyzable phosphate mimetic. A series of phenyl derivatives bearing a single DFMP group were initially surveyed. In general, these were not significantly more potent inhibitors than the parent compound, α,α-difluorobenzylphosphonic acid, with the exception being the *meta*-phenyl substituted species which decreased the IC_{50} by approximately 17-fold relative to α,α-difluorobenzylphosphonic acid. However, certain compounds bearing two DFMP moieties were very potent inhibitors. Some of these are among the most potent small molecule inhibitors of any PTP reported to date with the best one exhibiting a K_i of 1.5 μM. The structural basis for these results are discussed. One of the bis-DFMP inhibitors was examined in detail and it was found that the fluorines were essential for potent inhibition. Inhibition was independent of pH between pH 5.5–7.2 suggesting that both the mono and dianionic forms of the individual DFMP groups bind equally well. The trends observed in the inhibitory potency of these compounds with PTP1B were very similar to the trends observed by other workers on the K_m's of the analogous phenylphosphate substrates with rat PTP1. This indicates that studies of non-peptidyl substrates with rat PTP1 can be used as a guide for the development of human PTP1B inhibitors.

Introduction

The phosphorylation and dephosphorylation of tyrosine residues in proteins is now recognized as an important cellular regulatory mechanism.[1,2] The phosphorylation event is catalyzed by protein tyrosine kinases (PTKs) while the dephosphorylation is catalyzed by protein tyrosine phosphatases (PTPs). It has been known for several years now that the disruption of this process is at the root of a variety of disease states including several human cancers. Consequently, there has been much interest in the development of therapeutic agents that target these enzymes. Until fairly recently, most of the work in this area has focussed on preparing agents that target the PTKs[3,4] while much less effort has been directed towards the PTPs. However, recent reports that over expression of certain PTPs occurs in a number of disease states[1,5–8] have resulted in an increase in research in this area, and a number of reports describing PTP inhibitors have recently appeared.[9–27]

The most common approach to designing PTP inhibitors has been to incorporate non-hydrolyzable phosphotyrosine (pTyr) mimetics in place of pTyr in synthetic peptide substrates.[9–13] Among the most potent of this class of inhibitors were those containing difluoromethylenephosphonyl phenylalanine[9] (F_2Pmp, **1**). Certain peptides bearing **1** bind up to 10^3 times better than the analogous peptide bearing methylenephosphonyl phenylalanine (Pmp). Some selectivity between

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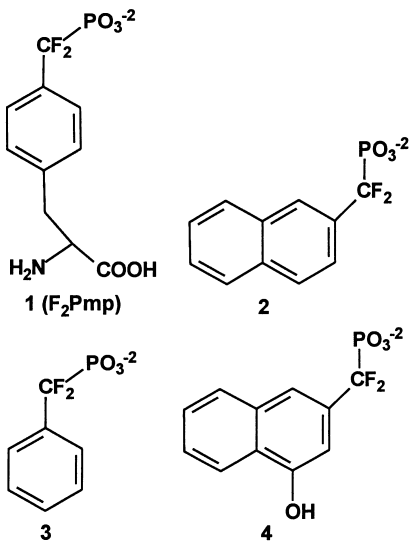
PTPs could be obtained by varying the amino acid sequence around the F_2Pmp group.[13] Although this approach has produced potent and relatively selective PTP inhibitors, there are a number of drawbacks to using peptidyl frameworks. Perhaps the most serious of these shortcomings is their poor bioavailability: peptidyl inhibitors are usually susceptible to proteolytic degradation and often exhibit poor cellular uptake. Another potential problem is the possibility that not all PTPs recognize their in vivo substrates based solely on the amino acid sequences that surrounds the pTyr residue.

Several irreversible, non-peptidyl inhibitors of PTPs have been reported, however, these are not specific for any particular PTP[14–19] and some also inhibit other types of phosphatases. A number of metal-containing reversible inhibitors such as gallium nitrate[20] and vanadate[19,21] have been reported. These species, which most likely function as mimics of inorganic phosphate or as transition state analogues, are also known to inhibit many other enzymes including several involved in signal transduction pathways.[28,29]



There have been only a handful of reports describing reversible, organic, small molecule inhibitors of PTPs. Frechette and co-workers reported that certain functionalized benzylic α-hydroxyphosphonic acids are good inhibitors of CD45, the best of which exhibited an IC_{50} of 1.2 μM.[24] Researchers at Merck have reported that adrenolate (4-amino-1-hydroxybutylidene-1.1-bisphos phonate), a potent inhibitor of bone resorption, is an inhibitor of PTPε (IC_{50} = 2 μM).[25] Nornuciferene, an aporphine alkaloid has been reported to be an inhibitor of CD45 with an IC_{50} of 5.3 μM.[26]

We are specifically interested in designing reversible, small molecule inhibitors of PTP1B. PTP1B is a cytosolic PTP consisting of a single catalytic domain. It is anchored by its C-terminus to the cytoplasmic face of the ER. This is one of the most studied of all the PTPs and several crystal structures of this enzyme complexed with various ligands[30,31] including the naphthyl derivative **2**,[23] have been reported. Although the precise in vivo function of PTP1B has not yet been established, there is now considerable evidence that this enzyme may be involved in the down-regulation of insulin signaling by dephosphorylating specific phosphotyrosine residues on the insulin receptor kinase.[32–39] PTP1B is now being considered as a potential therapeutic target to help combat certain forms of diabetes. For example, vanadate, a phosphate analogue and a potent competitive inhibitor of PTP1B[19,21] has been shown to be an insulin-mimetic and in human clinical trials to be potentially useful in treating certain forms of diabetes.[40] It has been suggested that part of vanadate's insulin-mimetic effect is due to its inhibition of PTPs.[41] However, vanadate is a non-specific phosphatase inhibitor and has a wide variety of effects on biological systems.[42] Consequently, there has been much interest in obtaining specific inhibitors of this enzyme since they could have potential both as therapeutics and as tools for studying insulin-dependent signal transduction pathways.

Reports describing reversible, organic, small molecule inhibitors of PTP1B are sparse. Sulfircin, a sulfate-bearing marine natural product, was shown to be a non-specific inhibitor of certain phosphatases such as Cdc25A (IC_{50} = 7.8 μM), V HR (IC_{50} = 4.7 μM) and PTP1B (IC_{50} = 29.8 μM).[27] A variety of sulfircin analogues have been synthesized and examined for PTP inhibition, the best of which exhibit IC_{50}'s of 2.8 μM and 4.4 μM with Cdc25A and PTP1B, respectively.[27] Burke and co-workers examined a series of benzylic phosphonates as reversible inhibitors of rat PTP1, the rat analogue of human PTP1B.[22] From this series, the naphthyl derivative, **2** bearing the difluoromethylenephosphonic acid (DFMP) moiety, exhibited a K_i value of 179 μM. However, **2** was also a good inhibitor of the serine/threonine phosphatase PP2A, but was not examined with other PTPs. Surprisingly, the phenyl derivative **3** was a poor inhibitor. On the basis of molecular modeling studies with **2** and PTP1B, Burke and co-workers determined that the addition of a hydroxyl group at the 4-position of **2** would yield a more potent inhibitor.[23] Thus, **4** was constructed via a 12-step synthesis,[43] and, consistent with the modeling predictions, found to be a better inhibitor than **2**. The effect, however, was very modest, lowering the K_i by only twofold (93 μM) compared to **2**. More recently, we have shown that naphthyl derivatives bearing two DFMP groups at positions 2 and 7 or positions 2 and 6 in the naphthyl ring are

approximately sixfold more potent inhibitors of PTP1B than **2**.[44]

In this report, we examine a series of phenyl derivatives bearing the DFMP moiety as inhibitors of PTP1B. In general, we have found that the majority of phenyl derivatives bearing a single DFMP group were not good inhibitors of PTP1B and were not significantly more effective than the parent compound **3**. However, certain compounds bearing two aryl DFMP moieties are good inhibitors of PTP1B and some of these are among the most potent small molecule inhibitors of PTP1B reported to date.

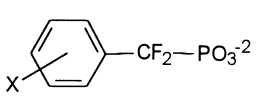
Results and Discussion

As a starting point for the development of non-peptidyl PTP1B inhibitors, we consulted the existing literature regarding the active site specificity of this enzyme or related PTPs with both peptidyl and non-peptidyl substrates and ligands. The presence of a phosphate group appears to be essential for binding of tyrosyl-containing peptides and proteins: PTPs do not bind tyrosine-containing peptides or proteins that lack a phosphate group[45,46] nor do they bind *O*-methylphosphotyrosine-containing peptides.[45] Consequently, it is not surprising that the majority of reversible, organic, PTP inhibitors reported to date have incorporated into them a non-hydrolyzable pTyr mimetic such as the DFMP moiety or others such as thiophosphate,[12] sulfate,[11] and methylenephosphonyl[9] groups. However, the DFMP group appears to be the most effective.[9,13] Montserat et al. and Zhang have studied the active site specificity of rat PTP1 with a number of non-peptidyl substrates.[47,48] Rat PTP1 is the rat homologue of human PTP1B and the catalytic domain of this enzyme (residues 1–322) has 97% sequence homology to the corresponding 322 residues in human PTP1B.[49] In general, aromatic phosphates are significantly better substrates than aliphatic ones in terms of both higher k_{cat}'s and lower K_m's. β-naphthylphosphate has a lower K_m than phenylphosphate (approximately two to ninefold, depending on the pH). However, substitution of the phenylphosphate ring with certain substituents can result in a lowering of the K_m to levels considerably less than that of β-naphthylphosphate.[47,48] In general, substituents on the phenyl ring *ortho* and to a lesser extent *meta* to the phosphate group have higher K_m's compared to their *para*-substituted counterparts, and this has been attributed to steric hindrance.[47] Negatively charged substituents remote from the phosphate group often significantly increase the affinity of the enzyme for the substrate.

In light of the above substrate and inhibitor studies, we decided to focus our efforts on aryl derivatives bearing the DFMP group. Although Burke and co-workers had shown that compounds **2**–**4** were not particularly potent PTP1B inhibitors, Frechette's studies[24] with benzylic α-hydroxyphosphonates and CD45 indicate that relatively simple aryl phosphonates could be effective inhibitors of PTPs. In addition, there have been a number of reports describing small molecule aryl-bearing phosphonates as excellent inhibitors of other enzymes that are capable of utilizing phosphotyrosine such as osteoclastic acid phosphatase[50] and human prostatic acid phosphatase.[51,52] We therefore reasoned that an appropriately functionalized aryl DFMP derivative may exhibit potent inhibition of PTP1B.

Since the above mentioned studies with phenyl phosphate substrates and rat PTP1 indicated that *ortho* and to a lesser extent *meta* substitution interferes with ligand binding, we focussed mainly upon *para*-substituted phenyl DFMP derivatives. The first series of compounds studied were phenyl DFMP derivatives substituted mainly at the *para*-position with simple aromatic and non-aromatic moieties (Table 1). An initial assessment of the inhibitory effects of these derivatives was performed using 500 μM inhibitor with PTP1B and FDP as substrate at a concentration equal to its K_m value (20 μM) at pH 6.2. The results, in terms of percent inhibition, are summarized in Table 1. Consistent with Burke's studies,[22] the unsubstituted compound, **3**, is a very poor inhibitor of PTP1B, resulting in only 41% inhibition at 500 μM and has an IC_{50} of 610 μM with PTP1B. Substitution at the *para*-position with non-aromatic moieties (**5**–**8**) did not significantly improve the inhibition and, in one instance (**5**) yielded a worse inhibitor. Similarly, substitution at the *para*-position with aromatic groups (**9**, **10** and **13**) did not significantly improve the inhibition. However, addition of a phenyl group at the *meta*-position (**12**) significantly improved

Table 1. Percent inhibition of PTP1B with 500 μM, α,α-difluoromethylenephosphonates, **3**, and **5**–**13**



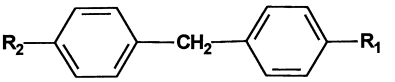
	% Inhibition[a]
3, X = H	41 ± 2
5, X = *p*-COOH	32 ± 2
6, X = *p*-Br	64 ± 2
7, X = *p*-N_3	71 ± 1
8, X = *p*-NO_2	73 ± 2
9, X = *p*-$COOCH_2Ph$	68 ± 1
10, X = *p*-COPh	66 ± 2
11, X = *o*-Ph	25 ± 2
12, X = *m*-Ph	94 ± 1
13, X = *p*-Ph	67 ± 1

[a]Errors are reported as ± SD.

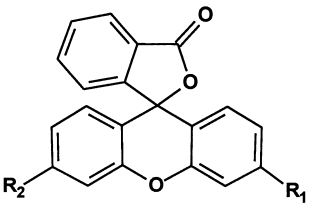
the inhibitor potency. This appeared to be entirely unique to the *meta*-substitution since the *para*-phenyl and *ortho*-phenyl substituted derivatives (**11** and **13**) were moderate (*para*-substituted) to very poor (*ortho*-substituted) inhibitors. The *meta*-phenyl derivative (**12**) has an IC_{50} of 35 μM and is therefore more potent than naphthyl derivatives **2** and **4**. The IC_{50} for the *para*-phenyl derivative (**13**) was only 210 μM. Since the percent inhibition of compounds **6**–**10** was very similar to **13**, it is likely that they would exhibit similar IC_{50}'s.

We noted that the results in Table 1 followed the same trend as that observed by Montserat et al. for the K_m's of the analogous phosphate substrates with rat PTP1.[47] For example, these workers have reported that *p*-carboxyphenylphosphate exhibits a higher K_m (2.1 mM) than *p*-nitrophenyl phosphate (0.68 mM) with rat PTP1, while our studies show that the *para*-carboxy-α,α-difluorobenzylphosphonic acid **5** is a poorer inhibitor of human PTP1B than the *para*-nitro-α,α-difluorobenzylphosphonic acid **8**. Although *meta*-substituted phenylphosphates were usually poorer substrates than their *para*-substituted analogues with rat PTP1,[47] *meta*-phenyl phenylphosphate was an exception. The K_m reported for *para*-phenyl phenylphosphate, 2.4 mM, is approximately seven times greater than that reported for *meta*-phenyl phenylphosphate (0.32 mM).[47] Similarly, the IC_{50} determined for *para*-phenyl-α,α-difluorobenzylphosphonic acid **13** (210 μM) is six times greater than *meta*-phenyl-α,α-difluorobenzylphosphonic acid **12** (35 μM). However, non-peptidyl substrates *meta*-substituted with aliphatic hydrocarbons (ethyl, isopropyl) exhibited higher K_m's than *meta*-phenyl phenylphosphate with rat PTP1.[47] This suggests that the effect observed with the *meta*-phenyl-substituted compounds may involve more than just simple hydrophobic interactions and may possibly involve pi-stacking interactions with an aromatic residue located close to the active site.

The above results suggest that studies using phenyl-based, non-peptidyl substrates with rat PTP1 could be used as a guide for the development of human PTP1B inhibitors bearing the DFMP group. We therefore decided to examine additional DFMP-bearing structures based on substrate studies with rat PTP1. An important observation made by Montserat et al. was that phenylphosphate substrates bearing an additional charged moiety (phosphate or carboxylate) remote from the phosphate group exhibited very low K_m's with rat PTP1.[47] This was especially evident for certain symmetrical substrates bearing two phosphate groups and oneof these (**14**) is the most potent low molecular weight substrate ever reported for a PTP and exhibits a K_m (16 μM) 19 times lower than its mono-phosphorylated analogue (**15**) and is almost as low as the best peptidyl



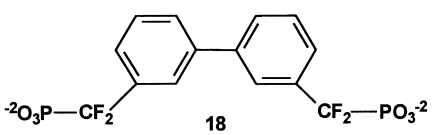
14, $R_1 = R_2 = OPO_3^{-2}$
15, $R_1 = H$, $R_2 = OPO_3^{-2}$



16, $R_1 = R_2 = OPO_3^{-2}$
17, $R_1 = H$, $R_2 = OPO_3^{-2}$

substrates. These results are consistent with our earlier studies where we demonstrated that fluoresceindiphosphate (**16**) has a significantly lower K_m and hydrolysis of the first phosphate group occurs much faster than fluoresceinmonophosphate (**17**) with PTP1B.[53] Consequently, we reasoned that an additional DFMP group on inhibitors such as **12** and **13** may yield very potent PTP1B inhibitors.

We initially tested this hypothesis with compound **12** since it was the most potent inhibitor amongst those reported in Table 1. However, the symmetrical bis-DFMP derivative **18** was a slightly poorer inhibitor than the mono-DFMP compound **12** (Table 2). This was not



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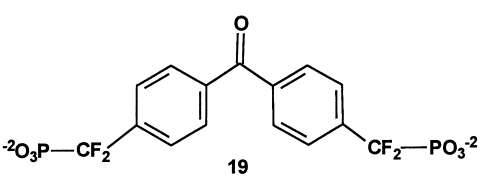

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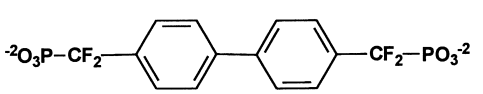

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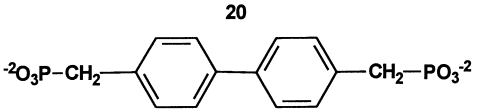

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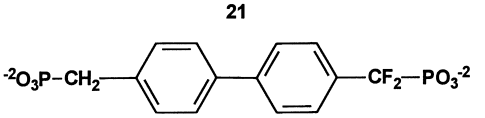

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Table 2. IC_{50} Values for phosphonates, **12**, **13** and **18**–**22**, with PTP1B

Phosphonate	IC_{50} (μM)
12	35 ± 1
13	210 ± 5
18	45 ± 1
19	15 ± 1
20	2025 ± 15
22	51.5 ± 0.3

[a]Errors are reported as ± SD.

the case with the symmetrical bis-DFMP analogues of **10** and **13** (compounds **19** and **20**) which completely inhibited PTP1B at 500 μM and exhibited IC_{50}'s of 9.9 μM and 15 μM, respectively (Table 2). Thus, in the case of the biphenyl derivative (**20**) the additional DFMP group decreased the IC_{50} by about 14-fold. The inhibition was competitive and a K_i of 4.5 ± 1 μM (Fig. 1(a) and (b)) was determined for **20**.

To determine whether the presence of the fluorines was absolutely necessary for potent inhibition, the non-fluorinated analogue of **20**, compound **21**, was examined. **21** was a very poor inhibitor of PTP1B and exhibited an IC_{50} of about 2 mM (Table 2) approximately 200-fold greater than that of **20**. To ascertain whether the α-fluorines were essential on both phosphonate groups the bis-phosphonate **22**, in which only one of the benzylic phosphonates was α-fluorinated, was examined. This compound exhibited an IC_{50} of 51 μM, which is only fivefold greater than the fully fluorinated species **20** but is approximately 40-fold less than the IC_{50} obtained with the non-fluorinated species **21**. Thus, for this biphenyl series of bis-phosphonates, two fluorines alpha to just one of the phosphonate groups enhance the enzymes affinity for the bis-phosphonate by approximately 40-fold. However, the addition of two more α-fluorines on the other phosphonate group results in a mere fivefold affinity enhancement.

The enhanced binding effect of the fluorines alpha to the phosphonate group in peptidyl and non-peptidyl PTP1B and rat PTP1 inhibitors varies depending on the inhibitor. With certain F_2Pmp-bearing peptides this effect is dramatic and can result in an over 1000-fold decrease in K_i compared to their Pmp analogues.[9] The effect seen here with compounds **19**–**22** is less dramatic but nevertheless quite significant (approximately 200-fold difference in IC_{50} between **20** and **21**). Why are α-fluorophosphonates significantly better inhibitors than their non-fluorinated counterparts? Two possible explanations have been put forward.[13] One is that the fluorines may increase the affinity by H-bonding with specific residues. The alternative explanation is that the PTPs

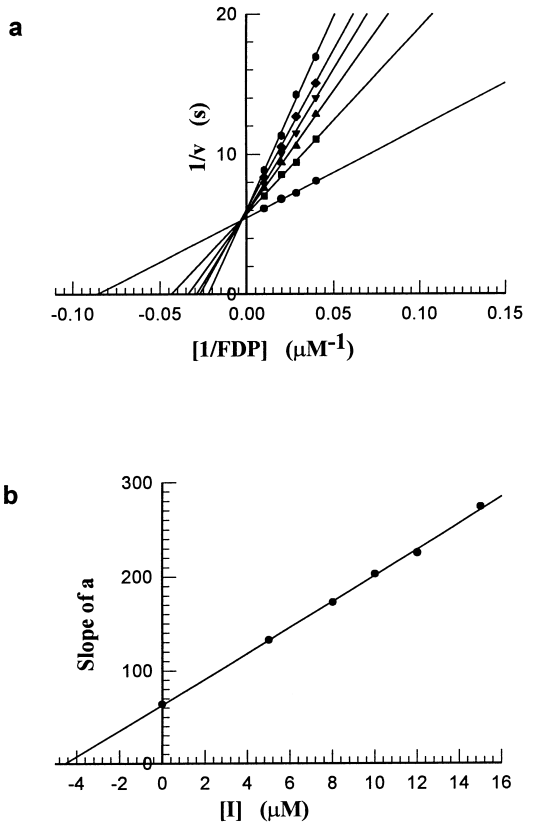


Figure 1. Inhibition of PTP1B by compound **20**. (a) The activity of PTP1B (0.15 μg/mL) was measured at pH 6.2 as described under 'Experimental Procedures' in the presence of the following concentrations of **20**: (●), 0 μM; (■), 5 μM; (▲), 8 μM; (▼), 10 μM; (◆), 12 μM; (●), 15 μM. (b) Replot of the slope from the double-recipricol plot versus concentrations of compound **20**.

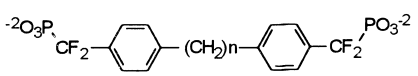
require the dianionic form of the phosphonates for binding. At pH 6–7, the non-fluorinated phosphonates exist only partially as the dianions while the fluorinated derivatives have almost completely ionized[13] and therefore bind tighter. However, Chen et al. have shown that F_2Pmp-bearing peptides inhibit rat PTP1-catalyzed dephosphorylation of pTyr-containing peptides in a pH independent manner between pH 5.0–7.0.[13] This indicates that the dianionic and monoanionic forms of these inhibitors bind equally well and that inhibition is not due to pK_a effects. The crystal structure of **2** complexed with PTP1B has recently been reported.[23] The two fluorine atoms interact with the phenyl ring of Phe182 via Van der Waals contacts and the Pro-R fluorine forms H-bonds with the amido group of Phe182. To determine whether the fluorines in **20** enhance binding via a pK_a effect or some other type of interaction such as H-bonding, we measured the inhibition constant of **20** as a function of pH. The second ionization constant

(pK_a) of α,α-difluorobenzylphosphonic acid has been determined[54] to be 5.71 and it is likely that the second ionization constants of the individual DFMP groups on **20** will be similar. Thus, we determined the K_i of **20** with PTP1B between pH 5.5 and 7.2. We found that the K_i of **20** changed very little in this pH range (data not shown) which suggests that both the monoanionic and dianionic forms of the individual DFMP groups on **20** bind to PTP1B equally well suggesting that the fluorines in **20** enhance binding by interacting with specific residues on the enzyme.

Montserat et al. reported that diphenylmethyl derived phosphates exhibited lower K_m's than biphenylphosphate derivatives.[47] Thus, we examined the bis-DFMP diphenylmethyl derivative (**23**) as a PTP1B inhibitor (Table 3) and obtained an IC_{50} (23 μM) that was slightly greater than that of **20**. The length of the hydrocarbon chain linking the two aryl groups was increased and these species were examined (**24**–**26**, Table 3) for PTP1B inhibition. The 1,2-diphenylethane derivative (**24**) was a better inhibitor than **20** yielding an IC_{50} of 6.8 μM. Any further increase in the length of the hydrocarbon linker arm resulted in only slightly better inhibitors than (**24**) with the 1,4-diphenylbutane derivative (**26**) being the best, exhibiting an IC_{50} of 4.4 μM and was a potent competitive inhibitor with a K_i of 1.5 μM (Fig. 2(a) and (b)).

It is remarkable that although α,α-difluorobenzylphosphonic acid **3** is a very poor inhibitor of PTP1B, simply by joining two such derivatives together, much more potent inhibitors of PTP1B can be obtained. Why does the presence of more than one phosphonate or phosphate group enhance the binding of both substrates and inhibitors to PTPs? Very recently, X-ray crystallographic analysis of PTP1B grown in the presence of compound **14** or saturating pTyr revealed a second aryl phosphate-binding site.[55] This is a low-affinity, non-catalytic binding site adjacent to the active site, with Arg-24 and Arg-254 forming important ionic interactions with the phosphate group. These studies[55] also reveal that the high affinity of **14** is not a result of one of the phosphate groups occupying the catalytic site and the other occupying the low affinity non-catalytic site. Instead, **14** binds in two mutually exclusive modes. One of the phosphates of **14** binds in either the catalytic site or the non-catalytic site while the second phosphate group is bound by a combination of electrostatic, hydrophobic, aromatic-aromatic and water-mediated hydrogen bonds in an area slightly removed from both the catalytic and non-catalytic sites. It is possible that one of the DFMP groups in compounds **19**, **20** and **23**–**26** occupies the high affinity catalytic site while the other DFMP group occupies the low affinity site. However, this is unlikely for all six of these inhibitors since our results indicate that increasing the distance between the two DFMP groups does not dramatically increase or decrease their inhibitory potency relative to one another. Studies using synthetic peptidyl substrates indicate that a number of PTPs, including PTP1B, have

Table 3. IC_{50} Values for bis-phosphonates, **23**–**26**, with PTP1B

	IC_{50} (μM)
23, $n=1$	23 ± 2
24, $n=2$	6.8 ± 0.2
25, $n=3$	5.8 ± 0.3
26, $n=4$	4.4 ± 0.4

[a]Errors are reported as ±SD.

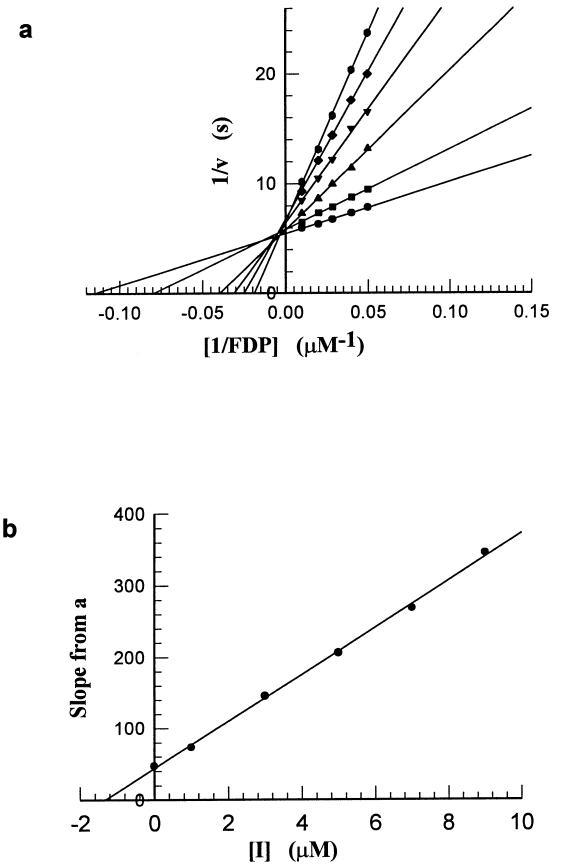


Figure 2. Inhibition of PTP1B by compound **26**. (a) The activity of PTP1B (0.15 μg/mL) was measured at pH 6.2 as described under 'Experimental Procedures' in the presence of the following concentrations of **26**: (●), 0 μM; (■), 1 μM; (▲), 3 μM; (▼), 5 μM; (◆), 7 μM; (⬢), 9 μM. (b) Replot of the slope from the double-reciprocal plot versus concentrations of compound **26**.

a strong preference for acidic residues N-terminal to phosphotyrosine and other physiologically occurring pTyr proteins exhibit this motif.[56–59] The crystal structure of the Cys215Ser PTP1B mutant complexed with DADE(pY)L, a high affinity peptide substrate, reveals that the preference for acidic residues at positions P-1 and P-2 is mainly a result of salt bridges between Arg47 and the side chains of the Asp and Glu at the P-1 and P-2 positions in the peptide.[30] It is also likely that there are additional residues besides Arg47 that are capable of interacting with anionic groups since it is known that the presence of acidic residues at positions P-3 and P-4 in peptidyl substrates also enhances their affinity for rat PTP1 and PTP1B.[56,58] Thus, the high affinity of inhibitors **19**, **20** and **23**–**26** may be a result of interactions of the second DFMP moiety with residues that do not constitute either phosphate-binding sites as was the case with one of the phosphate groups on **14**.

A number of compounds reported in this study (**12**, **19**, **20**, **23**–**26**) are good inhibitors of PTP1B and are significantly better inhibitors of PTP1B than naphthyl DFMP inhibitors **2** and **4**. Under our assay conditions, naphthyl derivative **2** exhibits an IC_{50} of $95 \pm 10\,\mu M$ with PTP1B. This is approximately 24-fold greater than that exhibited by **26**. It should also be pointed out that Burke and co-workers have reported that **2** is a good inhibitor of the serine/threonine phosphatase PP2A.[22] We have also examined **2** with PP2A and found that, under our assay conditions, it has an IC_{50} of 753 μM which indicates that **2** is approximately eight times less potent with PP2A than with PTP1B. We also examined **26** with PP2A and found that it has an IC_{50} of $200 \pm 8\,\mu M$ which is approximately 45 times greater than **26** with PTP1B. Thus, **26** is considerably more potent and selective than **2** in terms of selectivity between PP2A and PTP1B. Unfortunately, inhibition studies with **4** and PP2A were not reported. It is also important to note that the inhibitors obtained in this study are readily accessible, starting from commercially available starting materials via synthetic routes that require only three–five steps.

Conclusion

In summary, we have examined a series of phenyl DFMP derivatives as inhibitors of PTP1B. Several of the inhibitors examined in this study exhibit K_i values in the low μM range and are among the most potent, reversible, organic, non-peptidyl PTP inhibitors reported to date. The most potent of these compounds were those bearing two DFMP groups and the fluorines were essential for potent inhibition. In addition to having two DFMP groups, the presence of a phenyl group *meta* to a phenylDFMP moiety significantly enhances binding to PTP1B. Combining these two observations in a single compound should produce highly potent PTP1B inhibitors. The trends we observed in the inhibitory potency of the DFMP compounds with PTP1B were very similar to the trends observed by other workers[47,48] on the K_m's of the analogous phenylphosphate substrates with rat PTP1. This indicates that studies with non-peptidyl substrates with rat PTP1 can be used as a guide for the development of human PTP1B inhibitors. Studies by Kole et al. suggest that inhibitors bearing the DFMP group are incapable of penetrating cellular membranes.[22] Consequently, we are in the process of converting our most potent inhibitors (such as **26**) into cell permeable, fully substituted phosphate esters that can be activated by light or intracellular enzymes and these 'caged' compounds will be used for cellular studies to study kinase-dependent signal transduction pathways. Although we have established that many of the low molecular weight compounds described here can serve as potent PTP inhibitors, it still remains to be seen if inhibitors such as **20** and **23**–**26** are selective for PTP1B compared to other PTPs. The crystal structures of PTP1B complexed with inhibitors **20** and **26** is currently underway[60] and these studies will allow us to determine how these inhibitors bind to PTP1B and will be invaluable for the further development of yet more potent PTP1B inhibitors.

Experimental

General. Unless otherwise noted, all starting materials for syntheses were obtained from commercial suppliers (Aldrich or Lancaster) and were used without further purification. Tetrahydrofuran (THF) and diethylether (ether) were distilled from sodium/benzophenone ketyl under argon. Dichloromethane was distilled from calcium hydride under argon. DMF was distilled under reduced pressure from calcium hydride and stored over 4-Å sieves under argon. Reactions involving moisture-sensitive reagents were executed under an inert atmosphere of dry argon. Flash chromatography was performed using silica gel 60 (Toronto Research Chemicals, 230–400 mesh ASTM). ^1H, ^{31}P, and ^{19}F NMR spectra were recorded on a Varian 200-Gemini NMR machine at approximately 200, 80, and 188 MHz, respectively. Unless stated otherwise, all ^{13}C spectra were recorded on a Varian-500 or Varian-400 at approximately 125 and 100 MHz, respectively. For ^1H NMR spectra run in $CDCl_3$, chemical shifts (δ) are reported in parts per million relative to the internal standard tetramethylsilane (TMS). For ^1H NMR spectra run in D_2O, chemical shifts (δ) are reported in parts per million relative to the HDO peak at δ 4.68. For ^{13}C spectra run in $CDCl_3$, chemical shifts are reported in parts per million relative to the $CDCl_3$ residual carbons (δ 77.0 for

the central peak). For ^{13}C spectra run in D_2O, chemical shifts are reported in parts per million relative to the CH_3 peak of 3-(trimethylsilyl)-1-propanesulfonic acid (external). For ^{31}P NMR spectra, chemical shifts are reported in parts per million relative to 85% phosphoric acid (external). For ^{19}F NMR, chemical shifts are reported in parts per million relative to trifluoroacetic acid (external). Electron impact (EI) and fast atom bombardment (FAB) mass spectra (negative ion) were obtained on a Micromass 70-S-250 mass spectrometer. All inhibitors described in this report were examined for purity by HPLC (Waters Prep LC 4000 System equipped with a Vydac 218TP54 C-18 reverse phase column using 0.1% TFA in water/acetonitrile) and exhibited a single peak in the HPLC chromatogram. Fluorescein diphosphate (FDP) substrate was synthesized as described.[61] Buffer chemicals and bovine serum albumin (BSA) were obtained from Sigma Chemical Company. PP2A was purchased from Upstate Biotechnology. Expression and purification of FLAG-PTP1B has been described elsewhere.[19] Enzyme assay solutions were prepared with deionized/distilled water.

Inhibitor syntheses. The synthesis of inhibitors **3**, **6**, **8**–**13**, **19**, **20**, **23**, **25** and **26**, have been reported elsewhere.[62] The synthesis of inhibitors **5**, **7**, **18**, **21**, **22** and **24** are described below.

4-[(Dimethylphosphono)difluoromethyl]benzoic acid (27). A mixture of **9**[62] (300 mg, 0.81 mmol) and approximately 15 mg of 10% Pd/C, in methanol (5 mL), was stirred overnight under H_2 (1 atm). The mixture was filtered through Celite and concentrated by rotary evaporation to give **27** (90% yield) as a white solid (mp = 124–126 °C). No further purification was necessary. ^1H NMR ($CDCl_3$): δ 8.18 (d, J = 8.8 Hz, 2H, aryl), 7.74 (d, J = 8.8 Hz, 2H, aryl), 3.87 (d, J = 10.3 Hz, 6H, 2(CH_3)); ^{31}P NMR ($CDCl_3$): δ 5.99 (t, J_{PF} = 114.5 Hz); ^{19}F NMR ($CDCl_3$): δ −29.82 (d, J_{FP} = 114.5 Hz); ^{13}C NMR ($CDCl_3$): δ 167.6 (s, COOH), 136.8 (td, J_{CF} = 21.8 Hz, J_{CP} = 13.7 Hz, aryl), 133.9 (s, aryl), 130.1 (s, aryl), 126.5 (td, J_{CF} = 6.6 Hz, J_{CP} = 2.2 Hz, aryl), 118.3 (td, J_{CF} = 262.9 Hz, J_{CP} = 219.7 Hz, CF_2), 55.2 (d, J_{CP} = 7.3 Hz, 2(CH_3)); EIMS: m/z 280 (M^+), 171 (M^+-$PO(OMe)_2$); HRMS(EI): calcd for $C_{10}H_{11}F_2O_5P$ (M^+) m/z 280.0302, found 280.0312.

4-[(Phosphono)difluoromethyl]benzoic acid, ammonium salt (5). Trimethylsilylbromide (TMSBr, 0.24 mL, 4 equiv) was added to **27** (130 mg, 0.46 mmol) dissolved in anhydrous CH_2Cl_2 (5 mL) and stirred for 6 h. The mixture was then concentrated down and placed under high vacuum for 2 h. The product was then re-dissolved in CH_2Cl_2 (3 mL) and 4 equiv NH_4HCO_3 (0.146 g in 3 mL H_2O) was added. The mixture was stirred vigorously for 30 min at which time a white precipitate formed. The organic layer was evaporated and the aqueous layer lyophilized several times leaving **5** as a white solid in near quantitative yields. ^1H NMR (D_2O): δ 7.89 (d, J = 8.8 Hz, 2H, aryl), 7.63 (d, J = 8.8 Hz, 2H, aryl); ^{31}P NMR (D_2O): δ 4.94 (t, J_{PF} = 97.7 Hz); ^{19}F NMR (D_2O): δ 8.08 (d, J_{FP} = 97.7 Hz); ^{13}C NMR (D_2O): δ 175.7 (s, CO), 139.0 (td, J_{CF} = 22.0 Hz, J_{CP} = 11.7 Hz, aryl), 138.4 (s, aryl), 129.6 (s, aryl), 126.8 (t, J_{CF} = 6.6 Hz, aryl), 121.9 (td, J_{CF} = 261.8 Hz, J_{CP} = 189.2 Hz, CF_2); FABMS: m/z 251 ($M^{-3}+2H^+$).

4-[(Diethylphosphono)difluoromethyl]azidobenzene (28). A mixture of 4-[(diethylphosphono)difluoromethyl]-nitrobenzene[62] (0.53 g, 1.71 mmol) and zinc dust (5 equiv) in TFA (25 mL) was stirred overnight. The reaction was filtered, cooled to 0 °C in an ice bath, $NaNO_2$ (0.24 g, 2 equiv) was added and the mixture stirred in the dark for 30 min. NaN_3 (1.11 g, 10 equiv) was added over a period of 2 min and the mixture stirred for an additional 15 min. Ether (50 mL) was added and the mixture stirred for 1 h. The reaction mixture was then washed with water (1×50 mL) and the organic layer dried (Na_2SO_4) and concentrated leaving an oily yellow residue. Silica gel chromatography (EtOAc/CH_2Cl_2, 1:9, R_f = 0.9) gave pure **28** (76% yield) as a yellow oil. ^1H NMR ($CDCl_3$): δ 7.59 (d, J = 8.1 Hz, 2H, aryl), 7.09 (d, J = 8.4 Hz, 2H, aryl), 4.20 (m, 4H, 2(CH_2)), 1.32 (t, J = 7.0 Hz, 6H, 2(CH_3)); ^{31}P NMR ($CDCl_3$): δ 3.86 (t, J_{PF} = 117.0 Hz); ^{19}F NMR ($CDCl_3$): δ −31.9 (d, J_{FP} = 117.0 Hz); ^{13}C NMR ($CDCl_3$): δ 142.7 (d, J = 2.2 Hz, aryl), 129.0 (m, aryl), 127.9 (td, J_{CF} = 6.6 Hz, J_{CP} = 2.2 Hz, aryl), 118.9 (s, aryl), 117.7 (td, J_{CF} = 262.9 Hz, J_{CP} = 219.7 Hz, CF_2), 64.7 (d, J_{CP} = 6.6 Hz, 2(CH_2)), 16.3 (d, J_{CP} = 5.1 Hz, 2(CH_3)); IR (neat): 2149.1 cm^{-1}. EIMS: m/z 306 (MH^+), 169 (MH^+-$PO(OEt)_2$), 109 (MH^+-197); HRMS(EI): calcd for $C_{11}H_{14}F_2N_3O_3P$ (M^+) m/z 305.0747, found 305.0741.

4-[(Phosphono)difluoromethyl]azidobenzene, ammonium salt (7). TMSBr (0.32 mL, 3 equiv) was added to a solution of **28** (250 mg, 0.82 mmol) in anhydrous CH_2Cl_2 (5 mL) and refluxed for 36 h. The reaction was concentrated by rotary evaporation and the residual oil placed under high vacuum for 2 h. The product was then re-dissolved in CH_2Cl_2 (3 mL) and 3 equiv NH_4HCO_3 (0.20 g in 3 mL H_2O) was added. The biphasic mixture was rapidly stirred for 30 min at which time a white precipitate formed. The organic and aqueous layers were separated and the aqueous layer lyophilized several times yielding pure **7** as a white solid. ^1H NMR (D_2O): δ 7.60 (d, J = 8.4 Hz, 2H, aryl), 7.18 (d, J = 8.5 Hz, 2H, aryl); ^{31}P NMR (D_2O): δ 4.62 (t, J_{PF} = 104.5 Hz); ^{19}F NMR (D_2O): δ −29.2 (d, J_{FP} = 104.5 Hz); ^{13}C NMR (D_2O): δ (142.6, 131.6, 128.4, 119.7 {s, aryl}), 120.8 (td,

J_{CF} = 256.6 Hz, J_{CP} = 199.2 Hz, CF_2); IR (KBr disc): 2147.3 cm^{-1}; FABMS: *m/z* 248 ($M^{-2}+1H^+$).

3,3′-Bis(phosphonomethyl)biphenyl (29). A solution of 3,3′-bis(bromomethyl)biphenyl[63] (1.0 g, 2.9 mmol) and trimethylphosphite (3.5 mL, 29.4 mmol, 10 equiv) in dry benzene (4 mL) was refluxed for 12 h. The excess trimethyl-phosphite and dimethylmethylphosphonate (formed during the reaction) were removed by vacuum distillation and the residue was purified by silica gel chromatography (MeOH:$CHCl_3$, 5:95, R_f = 0.3) to give pure **29** as a clear, colorless oil (85% yield). ^{1}H NMR ($CDCl_3$): δ 7.35 (m, 8H, aryl), 3.66 (d, *J* = 11.7 Hz, 12H, 4(OCH_3)), 3.19 (d, *J* = 20.5 Hz, 4H, CH_2P); ^{31}P NMR ($CDCl_3$): δ 26.75 (s); ^{13}C NMR ($CDCl_3$): δ 141.0 (s, aryl), 131.9 (d, J_{CP} = 9.1 Hz, aryl), 128.6 (m, aryl), 125.6 (d, J_{CP} = 3.7 Hz, aryl), 52.6 (d, J_{CP} = 6.4 Hz, -OCH_3), 32.8 (d, J_{CP} = 138.2 Hz, -CH_2P); δ EI(MS) *m/z* 398 (M^+), 289 (M^+- $PO(OMe)_2$); HRMS (EI) calcd for $C_{18}H_{24}O_6P_2$ (M^+) *m/z* 398.1048, found 398.1044.

3,3′-Bis[(dimethylphosphono)difluoromethyl]biphenyl (30). To a solution of sodium hexamethyldisilazide (NaHMDS, Aldrich, 4.15 mL, 1.0 M in THF, 5.5 equiv) in dry THF (4.0 mL) at −78 °C was added a solution of **29** (0.3 g, 0.754 mmol, 1.0 equiv) in dry THF (30 mL) over a period of 2 min. The resulting orange to dark red solution was stirred for 1 h at −78 °C and then a solution of *N*-fluorobenzenesulfonimide (NFBS, 1.73 g, 7.3 equiv) in dry THF (4.0 mL) was added over a period of 2 min, during which time the solution turned from dark red to light yellow. After addition, the solution was stirred for 1.5 h and then allowed to warm to −30 °C during which time a precipitate formed. The reaction was quenched with 0.01 N HCl (50 mL) and the resulting solution (precipitate dissolves) was extracted with EtOAc (3×50 mL). The organics were combined and washed with 5% $NaHCO_3$ (1×75 mL), brine (1×75 mL), dried ($MgSO_4$), and concentrated by rotary evaporation to give a yellow oil that was purified via silica gel chromatography (EtOAc:hexanes, 4:1, R_f = 0.3) to give **30** as a pale-yellow oil (42% yield). ^{1}H NMR ($CDCl_3$): δ 7.30 (m, 8H, aryl), 3.87 (d, *J* = 10.3 Hz, 12H, 4(OCH_3)); ^{31}P NMR ($CDCl_3$): δ 6.60 (t, J_{PF} = 116 Hz); ^{19}F NMR ($CDCl_3$): δ −31.5 (d, J_{FP} = 116 Hz); ^{13}C NMR ($CDCl_3$): δ 140.6 (s, aryl), 136.0 (m, aryl), 129.7 (s, aryl), 129.1 (s, aryl), 125.5 (btd, aryl), 124.9 (btd, aryl), 118.1 (td, J_{CF} = 263.5 Hz, J_{CP} = 215.9 Hz, CF_2), 54.6 (d, J_{CP} = 7.3 Hz, OCH_3); EI(MS) *m/z* 470 (M^+), 361 (M^+-$PO(OMe)_2$); HRMS (EI) calcd for $C_{18}H_{20}F_4O_6P_2$ (M^+) *m/z* 470.0671, found 470.0671.

3,3(-Bis[(phosphono)difluoromethyl]biphenyl, ammonium salt (18). TMSBr (0.19 mL, 8.5 equiv) was added to a solution of **30** (0.08 g, 0.170 mmol) in anhydrous CH_2Cl_2 (2 mL) and stirred for 12 h. The mixture was then concentrated down and placed under high vacuum for 1.5 h. The product was then re-dissolved in CH_2Cl_2 (2 mL) and 6.2 equiv NH_4HCO_3 (0.083 g in 2 mL H_2O) was added. The biphasic mixture was rapidly stirred for 30 min, the organic layer was removed by rotary evaporation and the aqueous layer lyophilized several times to remove excess NH_4HCO_3, yielding pure **18** as a white powder (99% yield). ^{1}H NMR (D_2O): δ 7.91 (s, 2H, aryl), 7.77 (s, 2H, aryl), 7.58 (m, 4H, aryl); ^{31}P NMR (D_2O): δ 6.90 (bs); ^{19}F NMR (D_2O): δ −27.3 (d, J_{FP} = 94.6 Hz);^{13}C NMR: δ 140.78 (s, aryl), 137.40 (btd, aryl), 129.83 (s, aryl), 129.23 (s, aryl), 126.19 (bt, aryl), 125.33 (bt, aryl); FABMS: *m/z* 413 ($M^{-4}+3H^+$).

4,4′-bis(phosphonomethyl)biphenyl, ammonium salt (21). TMSBr (0.33 mL, 6 equiv) was added to 4,4′-bis(phosphonomethyl)biphenyl[62] (200 mg, 0.425 mmol) dissolved in anhydrous CH_2Cl_2 (3 mL) and stirred for 12 h. The mixture was then concentrated down and placed under high vacuum for 1.5 h. The product was then re-dissolved in CH_2Cl_2 (3 mL) and 8 equiv NH_4HCO_3 (0.209 g in 3 mL H_2O) was added. The biphasic mixture was rapidly stirred for 30 min, the organic layer was removed by rotary evaporation and the aqueous layer lyophilized several times to remove excess NH_4HCO_3, yielding pure **21** as a white powder (99% yield). ^{1}H NMR (D_2O): δ 7.69 (d, *J* = 7.7 Hz, 4H, aryl), 7.45 (d, *J* = 8.0 Hz, 4H, aryl), 3.10 (d, *J* = 20.8 Hz, 4H, CH_2P); ^{31}P NMR (D_2O): δ 21.00 (s); ^{13}C NMR (D_2O): δ 151.6 (d, J_{CP} = 2.9 Hz, aryl), 148.2 (d, J_{CP} = 8.6 Hz, aryl), 143.7 (d, J_{CP} = 5.7 Hz, aryl), 140.2 (s, aryl), 49.1 (d, J_{CP} = 128.6 Hz, CH_2P); FABMS: *m/z* 341 ($M^{-4}+3H^+$).

4-[4-((Diethylphosphono)difluoromethyl)benzene]toluene (31). To 4-[(diethylphosphono)difluoromethyl]bromobenzene[62] (410 mg, 1.21 mmol) in deaerated ethanol (10 mL), was added 4-methylbenzeneboronic acid (0.25 g, 1.5 equiv), $Pd(OAc)_2$ (0.011 g, 0.042 equiv), and solid Na_2CO_3 (0.19 g, 1.5 equiv). The dark brown reaction mixture was stirred at room temperature for 36 h, under argon, diluted with ether (100 mL) and filtered. The filtrate was washed with 1 N NaOH (1×100 mL), brine (1×100 mL), dried ($MgSO_4$) and concentrated down by rotary evaporation. The product was purified using silica gel chromatography (EtOAc:hexane, 3:7, R_f = 0.3) to give **31** as a white solid (71% yield). ^{1}H NMR ($CDCl_3$): δ 7.67 (s, 4H, aryl), 7.51 (d, *J* = 7.3 Hz, 2H, aryl), 7.27 (d, *J* = 5.9 Hz, 2H, aryl), 4.21 (m, 4H, 2(CH_2)), 2.41 (s, 3H, CH_3Ar), 1.34 (t, *J* = 6.6 Hz, 6H, 2(CH_3)); ^{31}P NMR ($CDCl_3$): δ 4.39 (t, J_{PF} = 117.5 Hz); ^{19}F NMR ($CDCl_3$): δ −32.2 (d, J_{FP} = 117.5 Hz); ^{13}C NMR ($CDCl_3$): δ (143.7, 137.8, 137.2, 129.6, 127.0, 126.8, 126.7, 126.6 {s, aryl}), 118.3 (weak td, CF_2), 64.6 (d, J_{CP} = 7.4 Hz, CH_2), 20.93 (s, CH_3Ar), 16.2 (d, J_{CP} = 4.6 Hz, CH_3); EIMS: *m/z* 354 (M^+), 217 (M^+

-PO(OEt)$_2$); HRMS(EI): calcd for $C_{18}H_{21}O_3F_2P$ (M^+) m/z 354.1194, found 354.1196.

4-[4-((Diethylphosphono)difluoromethyl)benzene]diethyl benzylphosphonate (32). *N*-Bromosuccinimide (1 equiv, 0.14 g) was added in several portions to a solution of **31** (275 mg, 0.78 mmol) and benzoyl peroxide (0.0075 equiv, 0.0014 g) in anhydrous CCl_4 (25 mL). Another portion of benzoyl peroxide (0.0014 g) was added towards the final addition of NBS. The mixture was refluxed for 2 h, cooled to room temperature, washed with water (2×15 mL), dried ($MgSO_4$), and concentrated down by rotary evaporation. The crude reaction mixture was passed through a silica gel column (hexane:EtOAc, 6:4, R_f = 0.4) yielding a partially purified mixture of monobrominated (~80% of the total) and dibrominated (~5% of the total) as a colorless oil. This crude mixture (170 mg) was then added to excess triethyl phosphite (10 mL) and refluxed for 10 h. The excess phosphite and diethyl ethylphosphonate (formed during the reaction) was removed by vacuum distillation and the product was purified via silica gel chromatography (EtOAc, R_f = 0.5) to give **32** as a yellow oil (73% yield). ^{1}H NMR ($CDCl_3$): δ 7.68 (s, 4H aryl), 7.56 (d, J = 7.4 Hz, 2H, aryl), 7.39 (d, J = 8.8 Hz, 2H, aryl), 4.14 (m, 8H, 4(CH_2)), 3.21 (d, J_{HP} = 22.0 Hz, 2H, CH_2P), 1.31 (m, 12H, 4(CH_3)); ^{31}P NMR ($CDCl_3$): δ 23.88 (s), 4.30 (t, J~117 Hz); ^{19}F NMR ($CDCl_3$): δ −32.1 (d, J_{FP}~117 Hz); ^{13}C NMR ($CDCl_3$): δ 143.1 (s, aryl), 138.5 (d, J_{CP} = 3.7 Hz, aryl), 131.6 (d, J_{CP} = 9.6 Hz, aryl), 131.4 (m, aryl), 130.3 (d, J_{CP} = 6.6 Hz, aryl), 127.3 (d, J_{CP} = 3.6 Hz, aryl), 126.9 (s, aryl), 126.7 (td, J_{CF} = 6.6, J_{CP} = 2.2 Hz, aryl), 118.1 (weak td, J_{CF} = 263.3, J_{CP} = 218.9 Hz, CF_2), 64.7 (d, J_{CP} = 7.4 Hz, CH_2), 62.1 (d, J_{CP} = 6.6 Hz, CH_2), 33.5 (d, J_{CP} = 138.4 Hz, CH_2P), 16.4 (d, J_{CP} = 5.1 Hz, 2(CH_3)), 16.3 (d, J_{CP} = 5.1 Hz, 2(CH_3)); EIMS: m/z 490 (M^+), 353 (M+ -PO(OEt)$_2$); HMRS(EI): calcd for $C_{22}H_{30}F_2O_6P_2$ (M^+) m/z 490.1472, found 490.1486.

4-[4-((Phosphono)difluoromethyl)benzene]benzylphosphonic acid, ammonium salt (22). TMSBr (0.08 mL, 6 equiv) was added to **32** (50 mg, 0.10 mmol) dissolved in anhydrous CH_2Cl_2 (4 mL) and refluxed for 36 h. The mixture was then concentrated down and placed under high vacuum for 2 h. The product was then re-dissolved in CH_2Cl_2 (3 mL) and 8 equiv NH_4HCO_3 (0.065 g in 3 mL H_2O) was added. The mixture was stirred for 30 min at which time a white precipitate formed. The organic and aqueous layers were separated and the aqueous layer lyophilized several times to remove excess NH_4HCO_3, yielding pure **22** as a white powder (88% yield). ^{1}H NMR (D_2O): δ 7.66 (bs, 6H, aryl), 7.39 (bs, 2H, aryl), 3.03 (d, J_{HP} = 20.5 Hz, 2H, CH_2P); ^{31}P NMR (D_2O): δ 21.21 (s), 6.24 (bs); ^{19}F NMR (D_2O): δ −37.14 (d, J_{FP} = 94.6 Hz); ^{13}C NMR (D_2O): δ 142.3 (s, aryl), 138.5 (s, aryl), 136.5 (d, J_{CP} = 9.6 Hz, aryl), 136.5 (m, aryl), 131.2 (d, J_{CP} = 5.9 Hz, aryl), 127.9 (s, aryl), 127.6 (bt, aryl), 127.3 (s, aryl), 123.0 (weak td, CF2), 36.6 (d, J_{CP} = 126.7 Hz, CH_2P); FABMS: m/z 377 (M^{-4} + 3H^+).

1,2(-Bis[4-((dimethylphosphono)methyl)benzene]ethane (33). 1,2-Bis[4-(bromomethyl)benzene]ethane[64] (0.8 g, 2.17 mmol) was added to trimethylphosphite (2.6 mL, 21.7 mmol, 10 equiv) in benzene (5 mL). The resulting solution was refluxed for 12 h. Unreacted phosphite and dimethyl methylphosphonate (formed during the reaction) were removed by vacuum distillation and the residue was subjected to silica gel chromatography (MeOH/$CHCl_3$ 4:96, R_f = 0.4) to give pure **33** as a white solid (75% yield). ^{1}H NMR ($CDCl_3$): δ 7.15 (m, 8H, aryl), 3.66 (d, J_{HP} = 10.6 Hz, 12H, 4(OCH_3)), 3.13 (d, J_{HP} = 21.6 Hz, 4H, 2(CH_2P)), 2.88 (s, 4H, aryl-(CH_2)$_2$-aryl); ^{31}P NMR ($CDCl_3$): δ 26.8 (s); ^{13}C NMR ($CDCl_3$): δ 140.35 (d, J_{CP} = 3.6 Hz, aryl), 129.61 (d, J_{CP} = 6.6 Hz, aryl), 128.72 (d, J_{CP} = 2.9 Hz, aryl), 128.62 (s, aryl), 52.81 (d, J_{CP} = 7.3 Hz, OCH_3), 37.32 (s, aryl-(CH_2)$_2$-aryl), 32.45 (d, J_{CP} = 138.4 Hz, CH_2P); EIMS m/z 426 (M^+), 213 (M^+ -213); HRMS (EI) calcd for $C_{20}H_{28}O_6P_2$ (M^+) m/z 426.1361, found 426.1366.

1,2′-Bis[4-((dimethylphosphono)difluoromethyl)benzene]-ethane (34). To a solution of NaHMDS (Aldrich, 1.0 M in THF, 4.26 mL, 4.26 mmol, 5.5 equiv) in dry THF (5 mL) was added a solution of **33** (0.33 g, 0.775 mmol, 1 equiv) in dry THF (7 mL), dropwise at −78 °C over a period of 2 min. The color of the solution changed from light yellow to red, and a fine suspension formed. After 1 h of stirring at −78 °C, a solution of NFBS (1.91 g, 5.65 mmol, 7.3 equiv) in dry THF (4.3 mL) was added over a period of 2 min, during which time the suspension became a solution and the color changed from red to yellow. After addition, the solution was stirred for 1–2 h and was allowed to warm to −30 °C, at which point a precipitate formed. The reaction was quenched with 0.01 N HCl (50 mL, precipitate dissolves) and was extracted with EtOAc (3×50 mL). The organics were combined and washed with 5% $NaHCO_3$, brine, dried ($MgSO_4$) and concentrated by rotary evaporation to give a yellow oil which was purified via flash chromatography (hexane:EtOAc, 1:4, R_f = 0.3) to give pure **34** as a white solid (37% yield). ^{1}H NMR ($CDCl_3$): δ 7.50 (d, J = 8.0 Hz, 4H, aryl), 7.23 (d, J = 8.0 Hz, 4H, aryl), 3.79 (d, J_{HP} = 10.6 Hz, 12H, 4(OCH_3)), 2.97 (s, 4H, aryl-(CH_2)$_2$-aryl); ^{31}P NMR ($CDCl_3$): δ 6.64 (t, J_{PF} = 117.5 Hz); ^{19}F NMR ($CDCl_3$): δ −31.5 (d, J_{FP} = 117.5 Hz); ^{13}C NMR ($CDCl_3$): δ 144.20 (s, aryl), 130.10 (btd, aryl), 128.62 (s, aryl), 126.13 (bt, aryl), 118.16 (weak td, CF_2), 54.87 (d, J_{CP} = 5.9 Hz, OCH_3), 37.15 (s, aryl-(CH_2)$_2$-aryl); EIMS m/z 498 (M^+), 389 (M^+ -PO(OMe)$_2$), 249 (M^+ -249); HRMS (EI) calcd for $C_{20}H_{24}F_4O_6P_2$ (M^+) m/z 498.0984, found 498.0973.

1,2′-Bis[4-((phosphono)difluoromethyl)benzene]ethane, ammonium salt (24). To a solution of 0.633 g (0.127 mmol, 1 equiv) of **34** in dry CH_2Cl_2 (2 mL) was added TMSBr (0.1 mL, 0.763 mmol, 6 equiv). The solution was stirred at room temperature for 12 h. Following removal of CH_2Cl_2 the residue was subjected to high vacuum for several hours. The oil was re-dissolved in CH_2Cl_2 (2 mL) and a solution of NH_4HCO_3 (0.062 g in 3 mL water, 6.2 equiv) was added. The biphasic mixture was stirred vigorously for 30–60 min and the CH_2Cl_2 layer was removed by rotary evaporation. The aqueous layer was then lyophilized three to four times, yielding **24** as a white powder in near quantitative yield. ^{1}H NMR (D_2O): δ 7.5 (d, J = 8.0 Hz, 4H, aryl), 7.31 (d, J = 8.1 Hz, 4H, aryl), 2.99 (s, 4H, aryl-$(CH_2)_2$-aryl); ^{31}P NMR (D_2O): δ 5.84 (t, J_{PF} = 93.9 Hz); ^{19}F NMR (D_2O): δ −27.41 (d, J_{FP} = 96.1 Hz); ^{13}C NMR (D_2O): δ 144.06 (s, aryl), 134.87 (btd, aryl), 129.17 (s, aryl), 126.82 (bt, J_{CF} = 6.4 Hz, aryl), 122.71 (weak td, CF_2), 36.99 (s, aryl-$(CH_2)_2$-aryl); FABMS: m/z 441 ($M^{-4} + 3H^+$).

Kinetic studies with PTP1B. Rates of PTP1B-catalyzed dephosphorylation in the presence or absence of inhibitors were determined using FDP as substrate[53] in assay buffer containing 50 mM bis-Tris, 2 mM EDTA, 5 mM DTT and 0.2 mg/mL BSA, at 25 °C. Stock solutions of inhibitors were prepared in assay buffer and found to be stable under these conditions indefinitely. Assays were carried out in 1 mL cuvettes with total volumes of 700 μL. Reactions were initiated by the addition of PTP1B (final concentration 0.15 μg/mL). Rates were obtained by continuously monitoring the production of FMP at 450 nM using a Varian Cary 1 spectrophotometer. The percentage inhibition in the presence of inhibitors were performed in duplicate using FDP at K_m concentration (20 μM) with 500 μM inhibitor at pH 6.2. IC_{50} determinations were determined in duplicate at five or six different inhibitor concentrations with FDP at K_m concentration at pH 6.2. K_i's for **20** and **26** were determined by measuring the initial rate (v) using various FDP concentrations (20, 25, 35, 50 and 100 μM FDP for **20** and 25, 35, 50 and 100 μM FDP for **26**) at various fixed concentrations of inhibitor (0, 5, 8, 10, 12 and 15 μM of **20** and 0, 1, 3, 5, 7 and 9 μM of **26**). 1/v versus 1/[S] was plotted as shown in the following equation:

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

The slopes of these plots ($(K_m/V_{max})(1 + [I]/K_i)$) were determined using the program SigmaPlot. These slopes were replotted against the concentration of inhibitor using the SigmaPlot program and the K_i's were obtained from the x-intercepts of these replots. All K_i studies were determined using bis-Tris as buffer and were performed in duplicate.

Kinetic studies with PP2A. Kinetic studies with PP2A were performed using FDP as substrate in 25 mM Tris–HCl, pH 6.5, containing 5 mM DTT, 0.5 mM $MnCl_2$ and 0.1 mg/mL of BSA. Assay were carried out in 96-well plates with total volume of 200 μL and rates were obtained by continuously monitoring the fluorescence from the production of FMP with excitation at 440 nm (slit width 20 nm) and emission at 530 nm (slit width 25 nm) using a Cytofluor II plate reader (Perseptive Biosystems). Reactions were initiated by the addition of PP2A (final concentration 0.13 μg/mL based on the commercially reported protein content). Under these conditions, the FDP has a K_m of 35 μM and V_{spec} of 0.35 μmol/mg/min. The V_{spec}/K_m of 0.01 μmol/mg/min/μM is slightly lower than what was reported for the best known phosphorylated protein substrate.[65] The IC_{50}'s of **2** and **26** were determined in duplicate at 10 different inhibitor concentrations with FDP at K_m concentration (35 μM).

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