The ability of protein-tyrosine phosphatases (PTPases) to catalyze the hydrolysis of simple aromatic phosphates has been recognized for some time. However, these compounds are significantly poorer substrates than their peptide-based counterparts containing phosphotyrosine. Consequently, the effort to create potent PTPase substrates has predominately focused on the use of peptidic carriers to deliver the phosphotyrosine moiety to the enzyme active site. We now report the synthesis and evaluation of several low molecular weight aromatic phosphates that serve as robust substrates for the rat PTPase, PTP1. We initially surveyed the ability of PTP1 to catalyze the hydrolysis of a variety of phenyl phosphate structural variants. Sterically demanding substituents positioned ortho and (to a lesser extent) meta to the phosphate group severely compromise the ability of these species to serve as phosphatase substrates. However, both benzylic and negatively charged substituents para to the hydrolyzable phosphate dramatically promote hydrolytic efficiency, which appears to be augmented through a dramatic enhancement in the affinity of the substrate for PTP1. The best substrate examined in this study exhibits a $K_m$ of $16 \pm 3 \mu M$. In addition, it serves as an inhibitor of the PTP1-catalyzed hydrolysis of $p$-nitrophenyl phosphate with a $K_i$ of $4.9 \pm 0.7 \mu M$. The extraordinary structural simplicity of this compound, as well as those of several others described herein, provides a promising starting point for the design of potent PTPase inhibitors.

The phosphorylation of tyrosine residues in proteins is one of several key molecular mechanisms by which cell growth and differentiation is regulated. Furthermore, the phosphorylation state of proteins is remarkably dynamic, which enables cellular behavior to respond rapidly to discrete changes in environmental conditions (1). This dynamic behavior is governed by the opposing actions of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPases).1 Protein-tyrosine kinases and PTPases, as well as their corresponding substrates, are integrated within an elaborate signal transducing network, an enzyme-based system which converts external environmental stimuli to internal cellular action. The defective or inappropriate operation of this network is at the root of a variety of disease states, such as cancer. Consequently, the characterization of the individual components and the delineation of the circuitry of this regulatory network has emerged as one of the most active fields in biomedical research. Ultimately, advances in this area may spawn enzyme-specific inhibitors, species that could serve as the basis for the creation of novel chemotherapeutic agents.

Clearly, a successful inhibitor of signal transducing enzymes must not only tenaciously bind to the specific target enzymes, but must do so without impeding the catalytic behavior of closely related species. Nature carefully regulates the activity of signaling enzymes by restricting their range of potential protein substrates to specific molecules containing the requisite structural recognition sites. In addition, substrate targeting is further enhanced by confining individual PTPases and protein-tyrosine kinases to specific cellular microenvironments. In much the same way, inhibitory agents must be engineered to recognize their intended target(s) in an exquisitely specific fashion. This has generally, although not exclusively, been approached by synthesizing peptides that encompass the site of phosphorylation (or dephosphorylation) in protein substrates. The inhibitor is simply obtained by replacing a key residue (e.g. a tyrosine phosphate) with an inert one (e.g. a tyrosine phosphonate) (2–5). These peptide-based inhibitors have been particularly useful in elucidating the molecular and catalytic characteristics of individual enzymes under in vitro conditions. Furthermore, these enzyme-specific inhibitors can be microinjected into cells to provide a biochemical context for the role of specific enzymes. Unfortunately, peptide-based species are less attractive as lead compounds for the generation of medicinally useful drugs.

We have found that PTPases will also utilize non-naturally occurring residues as substrates (6–8). Indeed, the "tyrosine-specific" PTPases from rat brain (PTP1) and Yersinia both catalyze the dephosphorylation of aromatic and aliphatic phosphates, even if these species are not contained within a peptide-based environment. However, in general, aromatic phosphates are significantly better substrates than their aliphatic counterparts (7). Unfortunately, even these aromatic phosphates are considerably poorer substrates than peptide-based systems (3). With these features in mind, we decided to explore, in greater detail, the ability of tyrosine-specific protein phosphatases to catalyze the hydrolysis of simple aromatic phosphates. Which aromatic substitution patterns are important for enzyme recognition? Is it possible to construct low molecular weight phosphates that are as efficiently hydrolyzed as phosphotyrosine-containing peptides? We have addressed these questions and have found that the peptidic environment can be replaced with appropriately positioned functionality to produce remarkably efficient PTPase substrates.

MATERIALS AND METHODS

All phenol derivatives and common reagents were obtained from commercial suppliers and used without further purification. Solvents were distilled and dried as required. All phosphorylated phenols were prepared by one of two general methods, except for compounds 1 and 19.

1 The abbreviations used are: PTPase, protein-tyrosine phosphatase; THF, tetrahydrofuran.

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(which were purchased), and 24 (which was prepared from the hydrolysis of commercially available 1,2-phenylene phosphorochloridite). All compounds, with the exception of 18 and 20, were protected by recrystallization as the cyclohexylammonium salt and were characterized as such. The structure of new compounds were confirmed by H NMR (400 MHz), 13C NMR (225.5 MHz), 31P NMR (161.9 MHz), and fast atom bombardment mass spectrometry (negative ion). Enzyme assay solutions were prepared with deionized/distilled water. The catalytic domain of rat PTP1 was expressed and purified as described previously (9).

Preparation of Aryl Phosphates

Note: The carboxylic acid functionalities in compounds 10-15 were protected prior to the phosphorylation step. A solution of tert-butylidemethyl-ethylsilyle chloride (2.1 mmol) and N-methylmorpholine (2.1 mmol) in dry THF (20 ml) was added dropwise (30 min) to a solution of aromatic alcohol (2.0 mmol) in dry THF (20 ml) and the resultant mixture was stirred at room temperature for 15 min (N2). The solvent was subsequently removed and the resultant oil residue phosphorylated as described below.

Procedure A (8)—The following procedure was employed for the preparation of compounds 10-15 and 17. POCl3 (2.1 mmol) in dry pyridine (20 ml) was added dropwise (30 min) to a solution of aromatic alcohol (2.0 mmol) in dry pyridine (30 ml) (N2). The reaction was stirred for 1 h at 0°C and then for 1 h at room temperature. The mixture was subsequently poured over ice (approximately 3 g) and then stirred for 15 min. Cyclohexylamine was added to precipitate out the desired aromatic phosphate. The solid was collected via filtration and then twice recrystallized as the cyclohexylammonium salt and were characterized as such. The structure of new compounds were confirmed by 1HNMR (400 MHz) and were characterized as phosphates. The solid was collected via filtration and then twice recrystallized as the cyclohexylammonium salt and were characterized as such. The structure of new compounds were confirmed by 1HNMR (400 MHz) and were characterized as phosphates.

Hydrolysis of 1,2-Phenylene Phosphorochloridite (Preparation of 24)—Water (0.25 ml) was added to a solution of 1,2-phenylene phosphorochloridite (2.0 mmol) in THF (10 ml) maintained at 20°C. The reaction was stirred and allowed to warm to room temperature over a period of 30 min. The solvent was subsequently removed in vacuo. Cyclohexylamine (6 ml) was added to the residue and the remaining solid was isolated via filtration and then twice recrystallized from ethanol.

PTPase Assay

All enzyme assays were performed at 30°C in 50 mM sucinate, 1 mM EDTA, pH 6.0 buffer with a constant ionic strength of 0.15 M (adjusted with NaCl). Initial rates for the enzyme-catalyzed hydrolysis of phosphate monoesters were measured by the production of inorganic phosphate using a colorimetric method described previously (11, 12). Michaelis-Menten kinetic parameters were determined from a direct fit of the velocity versus substrate concentration data to the Michaelis-Menten equation using the nonlinear regression program GraFit (Erlithicus Software).

Assay of Substrates 2, 13, and 17 as PTPase Inhibitors

The inhibition constants for 2, 13, and 17 were determined for the PTP1-catalyzed hydrolysis of p-nitrophenyl phosphate. At various fixed concentrations of inhibitors, the initial rates at various p-nitrophenyl phosphate concentrations were measured as described (6). The data were fit to equation 1 using KINETASYS (IntelliKinetics, State College, PA) to obtain the inhibition constant (K) as follows:

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

Results and Discussion

We have recently shown that PTPases will utilize a variety of non-naturally occurring aromatic and aliphatic phosphates as substrates (6, 7). For example, p-nitrophenyl phosphate, which is hydrolyzed by a variety of phosphatases, generally exhibits a Km in the mM range (13). Analogous aromatic and aliphatic phosphates display Km values that vary from 1 to 90 mM (7). Although we have found that the substrate efficacy of low molecular weight species can be dramatically improved by attacking them to active site-directed peptides (14), these "fusion" compounds still possess the principle disadvantage that inhibitor analogs are unlikely to serve as chemotherapeutic agents. With these features in mind, we investigated the active site substrate specificity of PTP1 with a structurally diverse array of low molecular weight aromatic phosphates. Several substrates examined in this study exhibit Km values in the low micromolar range and, as such, compare favorably with the very best peptide-based substrates ever reported for this enzyme (3).

p-Nitrophenyl phosphate 1 serves as a substrate for several PTPases. For example, it exhibits a Km of 1.7 mM with the Yersinia PTPase (3). With PTP1, we obtain a Km of 0.62 mM for the hydrolysis of 1 (Table I). For comparison, PTP1 hydrolyzes DADEpYLIPOQG with a Km of 2.6 μM (3). Although the Km values of these two substrates are dramatically different, the kcat values are nearly identical (63.7 s⁻¹ for 1 versus 75.7 s⁻¹ for DADEpYLIPOQG). In general, the enhancement in catalytic efficiency for the peptide-based (versus p-nitrophenyl phosphate) PTP1 substrates is primarily a consequence of differences in Km. In spite of the fact that the relationship between Km and Kj is often complex, it is tempting to ascribe the lower Km associated with peptide substrates to enhanced binding as a consequence of additional noncovalent interactions (outside of the active site) between enzyme and peptide.

Our initial survey of the active site substrate specificity of PTP1 focused on the relationship between aromatic substitution patterns and substrate efficacy. We prepared the ortho-, meta-, and para-ethyl-substituted phenyl phosphates 2, 3, and 4 (Table I). The ortho derivative is a dramatically poorer substrate than its meta and para counterparts. This is largely due to a precipitous drop in the kcat term, which is somewhat reminiscent of differences that we previously observed in the PTP1-catalyzed hydrolysis of aromatic versus aliphatic phosphates (7). We found that the former exhibits kcat values that are several orders of magnitude greater than those displayed by the latter. This appears to be a reflection of different rate-determining steps in the PTPase-catalyzed hydrolysis of these two different groups of substrates. In short, whereas the rate-
the carboxylate functionality is situated at the also characterized by a noteworthy strate, as it exhibits both an elevated turnover rate associated with the hydrolysis of 13 which has a deleterious impact on the enzyme and thereby render the k_cat step rate-limiting as well. This could account for the dramatically reduced k_cat value exhibited by this compound relative to those values displayed by compounds 3 and 4, where the ethyl moieties are positioned at a more distant site from the phosphate moiety. We also investigated the activity of the isopropyl derivatives 5 and 6. The k_cat associated with the meta-substituted derivative is an order of magnitude less than that obtained with its para-substituted counterpart. Furthermore, a similar pattern is evident with the corresponding phenyl substituted derivatives 7 and 8. One likely explanation for these observations is that relatively large substituents, even at the meta position, can interfere with phosphoryl transfer to the enzyme. Such a sensitivity to steric effect at the meta position has also been noted for the nitro- and chloro-substitutions (6). However, the results with compound 7 are notable for an additional reason. The alkyl and aryl substituted derivatives 2–6, and 8 all exhibit K_m values above 1 mM. In contrast, the K_m associated with 7 is 320 μM. Furthermore, the benzyl-containing derivative 9 exhibits a comparatively low K_m as well. However, unlike 7, which is hydrolyzed with a low k_cat, compound 9 enjoys a turnover rate that not only compares favorably with 1, but also with previously described peptide-based substrates (3). Due to the promising behavior of the para-benzyl derivative, we utilized this structural motif in several of the compounds described below.

We have previously shown that negatively charged residues, particularly on the NH2-terminal side of the phosphotyrosine moiety, substantially enhance the efficacy of PTP1 peptide-based substrates (3). Can appropriately positioned charged functionality on low molecular weight species likewise enhance substrate efficacy? Table II lists several derivatives of phenyl substituted phosphates appears to be formation (k_cat) of this intermediate (Scheme 1). In the case of compound 2 the ortho-substituent may sterically impede the ready formation of the phosphoenzyme and thereby render the k_cat step rate-limiting as well. This could account for the dramatically reduced k_cat value exhibited by this compound relative to those values displayed by compounds 3 and 4, where the ethyl moieties are positioned at a more distant site from the phosphate moiety. We also investigated the activity of the isopropyl derivatives 5 and 6. The k_cat associated with the meta-substituted derivative is an order of magnitude less than that obtained with its para-substituted counterpart. Furthermore, a similar pattern is evident with the corresponding phenyl substituted derivatives 7 and 8. One likely explanation for these observations is that relatively large substituents, even at the meta position, can interfere with phosphoryl transfer to the enzyme. Such a sensitivity to steric effect at the meta position has also been noted for the nitro- and chloro-substitutions (6). However, the results with compound 7 are notable for an additional reason. The alkyl and aryl substituted derivatives 2–6, and 8 all exhibit K_m values above 1 mM. In contrast, the K_m associated with 7 is 320 μM. Furthermore, the benzyl-containing derivative 9 exhibits a comparatively low K_m as well. However, unlike 7, which is hydrolyzed with a low k_cat, compound 9 enjoys a turnover rate that not only compares favorably with 1, but also with previously described peptide-based substrates (3). Due to the promising behavior of the para-benzyl derivative, we utilized this structural motif in several of the compounds described below.

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The relatively low k_cat values exhibited by compounds 13 and 16 may provide an unforeseen opportunity in the design of novel phosphatase inhibitors. For example, it may be possible to create phosphate-bearing compounds that bind well to specific phosphatases, yet are hydrolyzed slowly or not at all. Indeed, work by Greengard and his colleagues offers some enticing evidence that at least one naturally occurring inhibitor may utilize this mechanism as a basis for its inhibitory activity. These investigators have demonstrated that the active form of the protein phosphatase-1 inhibitor, DARPP-32 (the dopamine- and cAMP-regulated phosphoprotein) (15), is phosphorylated at an essential threonine residue. Synthetic peptides containing this phosphothreonine moiety are potent inhibitors of protein phosphatase-1 as well (16, 17). In contrast, the corresponding nonphosphorylated peptides are poor inhibitors. In short, although the precise fashion by which the phosphate moiety on DARPP-32 interacts with the target phosphatase remains to be established, it is tempting to ascribe the inhibitory potency of this species to the ability of a key phosphothreonine residue to occupy the active site in a fashion that promotes binding but precludes ready hydrolysis.

We have also examined the hydrolytic efficacy of several diphenylmethane-bearing compounds. The Michaelis constant (16 μM) associated with the hydrolysis of the diphenylmethane derivative 17 approaches those exhibited by the very best PTP1 peptide-based substrates (Table III). Indeed, 17 is the most potent low molecular weight substrate for PTPase that we have identified thus far. Nevertheless, the relatively low k_cat is surprising, particularly given the fact that the closely related
analog 9 exhibits a robust turnover rate. One possible explanation for these observations is that the additional phosphate moiety on 17 may enhance enzyme affinity, but at the expense of the ideal active site alignment required for rapid PTP1-catalyzed hydrolysis. Alternatively, 17 (as well as 13, 15, and others) may be engaged in a combination of productive and nonproductive binding modes. In the latter case, 17 may bind to the active site, but in a fashion that precludes phosphate hydrolysis. Such nonproductive binding would lower both the $k_{cat}$ and the $K_m$ (18). The $k_{cat}$ would be lowered since only a fraction of the substrate would be productively bound when the enzyme is saturated. The $K_m$ would be lowered because the existence of additional binding modes must lead to apparently tighter binding. Such a mechanism (nonproductive binding) has also been proposed to account for the decreased $k_{cat}$ and $K_m$ values by a series of substituted anilides substrates for chymotrypsin (19). Finally, the low $k_{cat}$ values associated with 13, 15, and 17 may be due to the presence of negatively charged substituents that serve to render the release of the phenol and phophates as PTP1 substrates. In general, the simple derivatives of low molecular weight, nonpeptidic, aromatic and aliphatic phosphates (6, 7, 21–24). However, these species are poor substrates relative to their peptidic counterparts, typically displaying $K_m$ values that are in excess of 1 mM. We have now established that low molecular weight aromatic phosphates can be hydrolyzed by PTP1 nearly as efficiently as full length peptide-based substrates. Indeed, compound 17 exhibits a very respectable $K_m$ of 16 mM. Is this Michaelis constant an accurate assessment of the affinity of 17 for PTP1? Fortunately, several of the substrates described in this study exhibit relatively low turnover rates, which provides us with an opportunity to estimate their PTP1 affinity. In particular, we investigated the ability of 13 and 17 to act as purely competitive inhibitors for PTP1. The $K_m$ values associated with these two substrates appear to slightly underestimate their affinity for the enzyme. Both exhibit $K_i$ values that are somewhat less than their corresponding $K_m$ values (13, $K_m = 80 \pm 18 \mu M$) and (5, 8, 0.1 $\mu M$); 17, $K_m = 16 \pm 3 \mu M$) and (4, 9, 0.7). As a control, we assessed the inhibitory activity of compound 2, which displays a $K_m$ of 4.2 mM. As expected, species 2 is a comparatively poor inhibitor ($K_i = 2.9 \pm 0.2 \mu M$). In short, the $K_m$ values for 2, 13, and 17, appear to provide a reasonable indication of how well these compounds bind to PTP1. Most importantly, the extraordinary inhibitory efficacy of 13 and 17 augurs well for the design of nonpeptidic protein phosphatase inhibitors.

In summary, we have examined the active site substrate specificity of rat brain PTP1 with a diverse structural array of aromatic phosphates. The active site of this enzyme is sensitive to steric bulk positioned orto to the phosphate moiety. Furthermore, the enzyme exhibits some sensitivity to certain sterically demanding residues at the meta site as well. In contrast, hydrophobic substituents, in conjunction with appropriately appended negatively charged residues, markedly enhance substrate efficacy. Although we have now established that low molecular weight compounds can serve as potent PTPase substrates, it still remains to be seen if all PTPases catalyze the hydrolysis of these simple aromatic phosphates with equal efficiency.

### Table III

| Phosphate | $k_{cat}$ (s$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$) |
|-----------|---------------------|------------|---------------------------------|
| 17        | 0.9 ± 0.4           | 0.016 ± 0.003 | 431 ± 85                      |
| 18        | 4.0 ± 0.1           | 0.13 ± 0.002  | 373 ± 58                      |
| 19        | 5.2 ± 0.2           | 0.036 ± 0.005 | 14 ± 2                        |
| 20        | 1.8 ± 0.02          | 2.2 ± 0.5    | 0.8 ± 0.2                     |

### Table IV

| Phosphate | $k_{cat}$ (s$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$) |
|-----------|---------------------|------------|---------------------------------|
| 21        | 7.1 ± 0.6           | 5.6 ± 1.0  | 1.1 ± 0.2                      |
| 22        | 5.3 ± 0.1           | 5.6 ± 0.6  | 0.9 ± 0.9                      |
| 23        | 7.0 ± 0.4           | 3.2 ± 0.4  | 22 ± 3                        |
| 24        | 3.6 ± 0.1           | 1.3 ± 0.1  | 27 ± 2                        |
| 25        | 60 ± 3              | 0.02 ± 0.06 | 97 ± 10                       |

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