Small Phosphomonoesters as Probes of Protein-Tyrosine Phosphatase Active Sites

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(ABSTRACT)

I evaluated the potential of isomers of the low molecular weight phosphomonoester naphthyl phosphate as general diagnostic substrates for differentiating between two families of protein phosphatases: the protein-tyrosine phosphatases [PTPs] and the dual-specificity protein phosphatases [DSPs]. Three PTPs, PTP-1B, Tc-PTPa, and PTP-H1, and three DSPs, Cdc-14, VHR, and IphP, were challenged in vitro with α-naphthyl phosphate and β-naphthyl phosphate. Both the DSPs and PTPs readily hydrolyzed β-naphthyl phosphate. As expected, the DSPs also hydrolyzed α-naphthyl phosphate at rates comparable to β-naphthyl phosphate and two of the PTPs, PTP-1B and Tc-PTPa, hydrolyzed α-naphthyl phosphate at a rate one-tenth that of β-naphthyl phosphate. However, PTP-H1 hydrolyzed both α- and β-naphthyl phosphate at nearly equal rates. Intriguingly, when challenged with radiolabeled phosphoproteins, PTP-H1 was markedly less stringent, by a factor of 40- to 200- fold, than PTP-1B or Tc-PTPa in its selectivity for [32P]phosphotyrosyl- over [32P]phosphoseryl-proteins in vitro.

The DSPs and PTPs listed above also were challenged in vitro with free phosphoserine. Each displayed little or no activity towards free phosphoserine. However, the addition of a hydrophobic “handle” to form N-(cyclohexane carboxyl)-O-phospho-L-serine produced a derivative that was hydrolyzed by IphP at rates comparable to that of the avid substrates ρ-nitrophenyl phosphate and β-naphthyl phosphate. VHR also hydrolyzed N-(cyclohexane carboxyl)-O-phospho-L-serine, though at a lower rate than IphP. Cdc14 displayed little activity towards N-(cyclohexane carboxyl)-O-phospho-L-serine.

The active site of VHR was mapped and amino acid residues potentially involved in binding N-(cyclohexane carboxyl)-O-phospho-L-serine were identified. The amino acid sequence of VHR was aligned with the amino acid sequences of IphP and Cdc14 to identify the nature of the corresponding residues in IphP and Cdc14.

Low molecular weight phosphomonoesters have proven to be effective in vitro indicators of protein phosphatase activity. They also have shown potential as diagnostic substrates for specific subclasses of protein phosphatases. However, neither α- and β-naphthyl phosphate nor N-(cyclohexane carboxyl)-O-phospho-L-serine proved to be universal discriminatory substrates for the functional subgroups within the family of protein-tyrosine phosphatases. Indeed, the probability of identifying such a substrate would appear to be relatively low.
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Set up the backgammon board, Crew -- I’m back in the rotation.
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LIST OF ABBREVIATIONS

ADP  adenosine diphosphate
ATP  adenosine triphosphate
BSA  bovine serum albumin
cAMP 3’, 5’-cyclic adenosine monophosphate
CPM  counts per minute
DSP  dual-specificity protein phosphatase
DTT  dithiothreitol
EDTA  ethylenediaminetetraacetic acid
EGTA  ethylenediaminoxydiethylenedinitrolo tetraacetic acid

\( g \)  acceleration due to gravity
GST  glutathione-S-transferase
HAT  His-Arg-Thiolate
HEPES  \( N \)-2-hydroxyethylpiperazine-\( N’ \)-2-ethanesulfonic acid
IPTG  isopropyl-\( \beta \)-D-thiogalactoside
kDa  kilodaltons
LB  Luria-Bertani medium
MBP  myelin basic protein
MOPS  4-morpholinepropanesulfonic acid

\( O \)-Ser, \( O \)-Thr, \( O \)-Tyr  \( O \)-phospho-L-serine, \( O \)-phospho-L-threonine, \( O \)-phospho-L-tyrosine

PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline

pH  log \( H^+ \) concentration

\( P_i \)  inorganic phosphate
PKA  protein kinase A
PMSF  phenylmethylsulfonyl fluoride

\( \rho \)-nitrophenyl phosphate

PSS-8105  \( N \)-(cyclohexane carboxyl)-\( O \)-phospho-L-serine
PSS-8108  \( N \)-benzoyl-\( O \)-phospho-L-serine
PTP  protein tyrosine phosphatase

\( [^{32}P] \)phosphoseryl protein

\( [^{32}P] \)phosphotyrosyl protein

RCML  reduced, carboxymethylated, and maleylated lysozyme
RCML-PS  \( [^{32}P] \)phosphoseryl RCM-lysozyme
RCML-PY  \( [^{32}P] \)phosphotyrosyl RCM-lysozyme
SDS  sodium dodecyl sulfate
TCA  trichloroacetic acid
Tris  tris (hydroxymethyl) aminomethane
CHAPTER I

INTRODUCTION

Protein Phosphorylation

The cell's environment is always changing. Therefore, the ability to sense information about the environment is vital to the cell's survival. However, it is not the information itself that is the key, but rather how it is used to enable the cell to adapt successfully to environmental changes. To survive, the cell must be able to take this information, sort it, evaluate it, determine a response, and then effect that response. In addition to numerous external stimuli, the cell must also respond to a multitude of internal signals as well.

The integration of a stimulus to an elicited response, signal transduction, is the relaying of a signal down an appropriate pathway to its final destination. Due to the vast quantities of signals and responses, nature cannot rely on linear pathways, that is, one pathway for each signal. Doing so would involve a waste of time and resources. Instead, nature has created a communication system of integrated pathways to form an information processing network. While this may seem overly complicated, it provides a means for vastly expanding the response options available to the cell. Instead of one signal, one pathway, one response, the cell can weigh numerous signals and selectively trigger the most appropriate pathways, leading to a quick and efficient adaptive response.

Protein phosphorylation is one of the essential mechanisms the cell uses for the regulation of its elaborate communications system. The phosphorylation state of a protein is
modulated by the balance between two opposing types of enzymes, protein kinases and protein phosphatases. Protein kinases transfer the γ-phosphate of ATP, and in some cases, any nucleoside triphosphate, to an amino acid side chain on a substrate protein. Examples of phosphoacceptor amino acids include serine, threonine, tyrosine, histidine, and aspartic acid. Protein phosphatases hydrolyze the protein-phosphate bond, restoring the protein to its original, dephosphorylated state (Fig. 1.1). The addition or removal of the highly charged phosphoryl group frequently perturbs the structure of the protein, leading to alterations in its function (31). Phosphorylation-dephosphorylation thus constitutes a very effective “on” and “off” switch for proteins. Since a protein population can have all, some, or none of its members phosphorylated, the cell is not restricted to absolutes of “on” and “off”, but can select intermediate degrees of phosphorylation as well.

Protein phosphorylation events regulate a variety of processes including cellular growth, proliferation, differentiation, metabolism, gene transcription, and immune response (7, 14). Disruptions in this intercellular communications system can play a role in many diseases, including cancer (38, 40).

**Protein Kinases**

In the 19th century the first phosphoproteins, casein from milk and phosvitin from egg yolk, were discovered. For almost a century, it was believed that protein phosphorylation was a gross metabolic reaction (38). The discovery of phosphorylase kinase by Krebs and Fischer in 1956 (34) demonstrated for the first time the potential of protein phosphorylation to function in a regulatory role. Over the past four decades, extensive studies have led to the
“Reversible” Reactions of Kinases and Phosphatases. The protein kinase catalyzes the transfer of the γ-phosphate from ATP to the phosphoacceptor protein. The protein phosphatase hydrolyzes the phospho-protein bond forming free inorganic phosphate and the phosphate-free acceptor protein. Whether the protein is active or inactive in its phosphorylated state depends on the individual protein.
identification of numerous protein kinases. These can be grouped into two superfamilies based upon their amino acid sequence similarity and enzymatic specificity (27).

The eukaryotic superfamily of protein kinases includes nearly every protein-serine/threonine and protein-tyrosine kinase found in eukaryotes (46). These kinases phosphorylate proteins by forming phosphomonoester bonds with seryl/threonyl and/or tyrosyl residues (27). On the other hand, members of the histidine kinase superfamily, mainly found in prokaryotes, undergo autophosphorylation on a nitrogen on a conserved histidine (1) to form a phosphoramidate intermediate. They subsequently phosphorylate substrate proteins by transferring the phosphoryl group to an aspartate residue.

**Protein Phosphatases**

The state of net phosphorylation of a phosphoprotein in the cell reflects the dynamic competition between the protein kinases and protein phosphatases for which it serves as substrate (7). Protein phosphatases are generally divided into two classes based upon their substrate specificity. These classes, in turn, can be divided into superfamilies based on sequence considerations. The protein serine/threonine phosphatases hydrolyze the phosphomonoester bond between a phosphoryl group and an oxygen of a seryl or threonyl residue, while the protein-tyrosine phosphatases dephosphorylate tyrosyl residues of phosphoproteins.

Protein serine/threonine phosphatases are metalloenzymes that hydrolyze the phosphate monoester by direct attack of an activated water molecule at the phosphorus atom of the phosphoprotein (2). The two major superfamilies of protein serine/threonine phosphatases are the PPP family, which includes PP1, PP2A, and PP2B; and the PPM family,
which includes PP2C (3). The protein Ser/Thr phosphatases of the PPP family share three conserved amino acid motifs contained in a catalytic domain approximately 220 amino acids in length. PPMs share eight highly conserved residues located within eleven generally conserved motifs that are contained in a catalytic domain approximately 290 amino acids in length (5, 11).

Protein tyrosine phosphatases hydrolyze phosphoryl groups on tyrosyl residues. However, a subclass of PTPs, referred to as dual-specificity protein phosphatases, can hydrolyze phosphoryl groups on seryl and threonyl residues as well. Low MW PTPs, like conventional PTPs, appear to be restricted to dephosphorylating tyrosine residues (57). Low MW PTPs have a catalytic domain of \(\approx 140\) amino acids that is much smaller than that of conventional PTPs/DSPs, which is estimated to span 250 amino acids (46). However, the catalytic domains of the PTPs/DSPs and low molecular weight PTPs share a conserved active site sequence: His/Val-Cys-Xaa<sub>5</sub>-Arg (Fig. 1.2) (17, 57). This sequence, His/Val-Arg-Thiolate (HAT), is located near the N-terminus of the catalytic domain of low MW PTPs. By contrast, the HAT motif of PTPs/DSPs is located near the center of the catalytic domain (46).

HAT phosphatases catalyze the removal of a phosphoryl group via a two step mechanism (Fig. 1.3) (22). In the first step, the thiol residue of the cysteine in the active site is deprotonated by the adjacent histidine, then performs a nucleophilic attack on the phosphoryl group of the phosphoprotein. In the second step, the dephosphorylated protein diffuses away and is replaced by a water molecule, which hydrolyzes the thiol-phosphate bond to generate free inorganic phosphate and free enzyme. A conserved aspartic acid residue acts as a general acid/base by protonating the leaving group alcohol on the displaced
**Figure 1.2**

**Conserved Active Site Sequence of Protein Tyrosine Phosphatases.**
Alignment of amino acid sequences of several protein tyrosine phosphatases: (A) Tyrosine-specific protein phosphatases, (B) Dual-specificity protein phosphatases, and (C) Low molecular weight protein phosphatases. Abbreviations used and literature references include: SptP, a PTP from *Salmonella typhimurium* (32); YopH, a PTP from *Yersinia pseudotuberculosis* (21); PTP-1B, PTP-H1 (54), and Tc-PTPa (25), protein tyrosine phosphatases from humans; IphP, a DSP from *Nostoc commune* UTEX 584 (43); Cdc14, a DSP from *Saccharomyces cerevisiae* (49); VHR (13) and Cdc25B (20), dual-specificity protein phosphatases from humans; slr0328, a putative low MW protein tyrosine phosphatase from *Synechocystis* sp. PCC 6803; YfkJ and YwlE, putative low MW protein tyrosine phosphatases from *Bacillus subtilis* (46).
Catalytic Mechanism of HAT Phosphatases. In the first step, the cysteine in the active site performs a nucleophilic attack on the phosphoryl group of the phosphoprotein. In the second step, the dephosphorylated protein diffuses away and is replaced by a water molecule, which hydrolyzes the thiol-phosphate bond to generate free inorganic phosphate and free enzyme. A conserved aspartic acid residue acts a general acid/base by protonating the leaving group alcohol on the displaced protein and then later abstracting a proton from the entering water molecule to activate it for nucleophile attack (57).
protein and then later abstracting a proton from the entering water molecule to activate it for nucleophile attack (57).

While the superfamily of HAT phosphatases shares a conserved active site sequence, they share little or no sequence similarity outside this discrete segment. This presumably reflects the emergence of several distinct families of PTPs by convergent evolution (44). Why do enzymes sharing identical catalytic mechanisms and active site functional groups exhibit such markedly different specificities?

The depth of the active site pocket has been suggested as the major factor in determining whether a HAT phosphatase acts as a tyrosine-specific PTP or a more broadly-specific DSP (30, 53). Prototypical PTPs, such as Yersinia PTP, have a deep active site pocket (53). Since the side chain of tyrosine is much longer than that of serine and threonine, those phosphatases possessing a “deep pocket” cannot dephosphorylate phosphoseryl/threonyl residues because they cannot reach the active site cysteine. In 1996, Dunn and coworkers confirmed this suggestion using a series of straight chain peptide-bound aliphatic phosphates as substrates for Yersinia PTP (14). PTPs had already been shown to greatly prefer aromatic phosphomonoesters over aliphatic phosphomonoesters (55). However, the experiment demonstrated that aliphatic phosphomonoesters, if long enough, could be dephosphorylated with reasonable efficacy by Yersinia PTP when compared to aromatic phosphomonoesters (14). The DSPs, on the other hand, possess a shallow active site pocket (16, 53), allowing phosphoseryl/threonyl residues, as well as phosphotyrosyl residues, ready access to the active site cysteine.

The determination of substrate specificity, and therefore classification, of a PTP has usually been accomplished by assaying with radiolabeled phosphoproteins in vitro (41, 54,
While $[^{32}\text{P}]-$labeled phosphotyrosyl, phosphoseryl, and phosphothreonyl proteins can readily determine whether a phosphatase is a PTP or DSP, many researchers are reluctant to use radioactive materials due to the expense of radioactive isotopes and to safety issues. Labs are also reluctant to engage in the enzymatic preparation of these substrates, which requires possession of a set of appropriate protein kinases.

Attempts to use simple, non-radioactive phosphoamino acids as alternative substrates have proved unsuccessful. While PTPs readily hydrolyze free $O$-phospho-$L$-tyrosine (55), DSPs show little activity toward $O$-phospho-$L$-serine and $O$-phospho-$L$-threonine (60). Thus, the free phosphoamino acids, P-$\text{Tyr}$, P-$\text{Ser}$, and P-$\text{Thr}$ cannot be used as discriminatory substrates. However, both PTPs and DSPs have been shown to hydrolyze other low molecular weight phosphomonoesters, in vitro (55, 56, 59). One of these, pNPP, has emerged as the standard substrate for assaying PTP/DSP activity (12, 22, 25, 29). However, because pNPP is readily hydrolyzed by both PTPs and DSPs, this compound is of little use in differentiating between them.

Two aromatic phosphoesters, the $\alpha$- and $\beta$- isomers of naphthyl phosphate (Fig. 1.4), have shown potential as possible substrates for differentiating PTPs from DSPs in vitro. In studies by Zhang and coworkers, PTP-H1 from *Yersinia pestis* (58) readily discriminated between the two isomers of naphthyl phosphate. The $k_{\text{cat}}$ for the hydrolysis of $\alpha$-naphthyl phosphate at pH 7.5 was $\approx 2$, while the $k_{\text{cat}}$ for $\beta$-naphthyl phosphate was $\approx 36$, yielding a ratio of $\alpha$- isomer to $\beta$- isomer activity of 1:18. Another PTP, PTP-1 (56), exhibited an even more dramatic $k_{\text{cat}}$ ratio of 1:44. In stark contrast, studies with IphP, a dual-specificity phosphatase from the cyanobacterium *Nostoc commune* UTEX 584 (43), and VHR, a DSP
**Figure 1.4**

*Isomers of Naphthyl Phosphate.* (A) $\alpha$-naphthyl phosphate and (B) $\beta$-naphthyl phosphate.
from humans (13), yielded a near equal $k_{\text{cat}}$ for the hydrolysis of $\alpha$- versus $\beta$-naphthyl phosphate for both DSPs (8, 26, 60). This marked difference in $k_{\text{cat}}$ ratios can be explained by steric hindrance (8). The phosphate ester of $\beta$-naphthyl phosphate can reach the cysteine in the shallow active site pocket of DSPs and, if inserted “lengthwise,” into the deep active site pocket of PTPs. Conversely, the phosphate ester on $\alpha$-naphthyl phosphate is sterically hindered from nucleophilic attack by the cysteine in the deep pocket of a PTP, but is not sterically hindered in the shallow pocket of a DSP (Fig. 1.5).

Work on IphP (26) suggested a second possibility for generating a DSP-specific substrate. A survey of nearly forty phosphoesters showed IphP to have a greater activity towards aryl phosphoesters than aliphatic phosphoesters in vitro, as expected. PTPs have also been shown to hydrolyze aryl phosphoesters much faster ($10^2$ to $10^3$ fold) than aliphatic phosphoesters (60). However, IphP exhibited relatively high activity towards aliphatic phosphoesters containing an adjacent aromatic ring, such as AMP (Fig. 1.6), suggesting that the presence of a aryl “handle” served as a potential recognition determinant. By adding such a handle, a poor substrate, such as $O$-phospho-L-serine, might potentially become a better substrate for IphP.
The Active Site Pockets of a PTP and DSP. The deep, narrow active site pocket of PTPs allows the entry of β-naphthyl phosphate but not α-naphthyl phosphate. However, the wide, shallow active site pocket of DSPs allow the entry of both naphthyl phosphate isomers.
Figure 1.6

Relative Activity of IphP Towards Different Substrate Classes. Bars represent the average activity ± S.D. of IphP toward different substrate classes relative to the standard substrate ρ-nitrophenyl phosphate (p-NPP). The number of phosphoesters assayed are shown in parantheses. The aryl phosphoesters assayed were phosphotyrosine, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), indoxyl 3-phosphate, α-naphthyl phosphate, β-naphthyl phosphate, 4-methylumbelliferyl phosphate, and pyridoxal phosphate. The aliphatic phosphoesters assayed were phosphoserine, phosphothreonine, glucose 6-phosphate, glucose 1-phosphate, sucrose 6′-phosphate, ribose 5-phosphate, inositol 2-phosphate, phytic acid, β-glycerol phosphate, hosphatidic acid, O-phosphorylethanolamine, and phosphocholine. The aliphatic phosphoesters with aryl “handles” assayed were 5′-AMP, 3′-AMP, 2′-AMP, 5′-dAMP, 2′-dAMP, 5′-GMP, 5′-dGMP, 5′-UMP, 5′-CMP, 5′-IMP, and thiamin phosphate (26).
Thesis Objectives:

Since the 1980’s, the study of PTPs has intensified as tyrosine phosphorylation has been implicated in the regulation of cellular growth, proliferation, differentiation, and the dysfunctions in these processes that occur in diseases such as cancer (7, 14, 28). Due to the wide variation in structure and amino acid sequence among the superfamily of PTPs, classification has relied primarily on substrate specificity. While $[^{32}\text{P}]$-labeled phosphotyrosyl, phosphoseryl, and phosphothreonyl proteins can readily determine whether a phosphatase is a PTP or DSP, many labs are reluctant to use radioactive materials or engage in the enzymatic preparation of these substrates, which requires possession of a set of appropriate protein kinases. Therefore, a need exists for non-radiolabeled diagnostic substrates that can be used “off the shelf” to differentiate DSPs from PTPs.

The first objective of this thesis was to examine the diagnostic potential of α-naphthyl phosphate and β-naphthyl phosphate as discriminatory substrates for PTPs and DSPs. Both reagents are commercially available and inexpensive. Moreover, their hydrolysis product, inorganic phosphate, can be assayed by a simple colorimetric assay. Our goal was to measure the ratio of α-naphthyl phosphatase activity to β-naphthyl phosphatase activity for several DSPs and PTPs to determine if a consistently large difference exists in the activity ratio between the two enzyme classes that then could be used as a reliable criterion for differentiating them.

The second objective was to test the validity of the aryl “handle” hypothesis for recognition of substrates by DSPs using two synthesized derivatives of O-phospho-L-serine (Fig. 1.7).
Synthesized *O*-Phospho-*L*-Serine Derivatives. *N*-benzoyl-*O*-phospho-*L*-serine, an aliphatic phosphoester with an aromatic “handle,” is synthesized by conjugating a benzyl ring to the α-amine of phosphoserine through an amide linkage. *N*-(cyclohexane carboxyl)-*O*-phospho-*L*-serine, an aliphatic phosphoester with a simple hydrophobic handle is synthesized by conjugating, then hydrogenating, a benzyl ring to the α-amine of phosphoserine through an amide linkage.
CHAPTER II

MATERIALS AND METHODS

Materials

Malachite green, ammonium molybdate, sodium citrate, $\rho$-nitrophenyl phosphate, $O$-phospho-$L$-serine, $O$-phospho-$L$-tyrosine, $\alpha$-naphthyl phosphate, $\beta$-naphthyl phosphate, cAMP-dependent protein kinase from bovine, BSA, glutathione-agarose beads were purchased from Sigma (St. Louis, MO). All radioisotopes were from Du Pont-New England Nuclear (Boston, MA). Chelating Sepharose Fast-Flo and Sephadex G-25 were from Pharmacia Biotech (Piscataway, NJ). Electrophoresis reagents, protein molecular weight standards, and mini-protean II apparatus were from Bio-Rad (Hercules, CA). Coomassie protein assay reagent was from Pierce (Rockford, IL). DE-52 cellulose was from Whatman (Clifton, NJ). Myelin Basic Protein was from Upstate Biotechnology (Lake Placid, NY). Scintisafe Plus 50% liquid scintillation fluid (Fisher Scientific, Pittsburgh, PA) as used for scintillation counting. All other buffers and routine lab chemicals, unless otherwise stated, were from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Spectrophotometric measurements were performed on a Hitachi UV-2000 Spectrophotometer. The pH of all solutions was measured on an Accumet Model 10 pH meter. Scintillation counting was performed using a Beckman LS5800 scintillation counter.
Procedures

Media Formulations

Luria Broth: 10 g tryptone, 5 g yeast extract, 10 g NaCl, water to 1 liter. pH adjusted to 7.0 with NaOH.

M9: 12.8 g Na$_2$HPO$_4$-7H$_2$O, 3 g KH$_2$PO$_4$, 0.5 g NaCl, 1 g NH$_4$Cl, 2 mM MgSO$_4$, 0.2 mM CaCl$_2$, 20% (w/v) glucose, water to 1 liter

2 x YT: 16 g tryptone, 10 g yeast extract, 5 g NaCl, water to 1 liter. pH adjusted to 7.0 with NaOH.

SDS-PAGE

SDS-polyacrylamide gels were prepared and electrophoresed as described by Laemmli (35). Electrophoresis was performed in a mini-protean II unit (Bio-Rad, Hercules, CA) at a constant 25 mA per gel. Samples, each, 5-20 µL were prepared by mixing the protein sample and SDS-PAGE gel sample buffer (62.5 mM Tris, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.00125% (w/v) bromophenol blue) so that protein was at concentration of about 1 mg/mL. The gels were stained with Coomassie Brilliant Blue as described by Fairbanks and coworkers (15).

Protein Phosphatases

A number of protein phosphatases used in these experiments were generously donated by the following persons:

VHR (13) was donated by Prof. Jack E. Dixon and coworkers from the Department of Biological Chemistry at the University of Michigan. Cdc14 (49) and the 45 kDa catalytic
domain of human Tc-PTPase, TC-PTPa (25), were donated by Prof. Harry Charbonneau and coworkers from the Department of Biochemistry at Purdue University. PTP-H1 (54) and pSK-PTP1B, a plasmid encoding PTP-1B (4), were donated by Dr. Nicholas K. Tonks and coworkers at Cold Spring Harbor Laboratories.

Expression and Isolation of IphP

*E. coli* sp. KM001 cells, containing the plasmid pMP005 bearing the *iphP* gene (51), were grown from a single ampicillin-resistant colony in 1 L of LB media supplemented with 0.05 mg/mL ampicillin, with vigorous shaking (~200 rpm), at 37°C until an absorbance of 0.7 at 600 nm was reached. Cells were harvested by centrifugation at 5,000 x g for 10 minutes at 4°C. The pellet was gently resuspended by swirling in 1 L of M9 media and prewarmed to 37°C. The cells were incubated at 37°C for 1 hour with vigorous shaking, after which 100 mM IPTG, in water, was added to a final concentration of 0.5 mM. The cells were incubated in presence of IPTG overnight at 25°C. Cells were harvested at 8,000 x g for 20 minutes at 4°C. The pellet was discarded. A solution of 100 mM PMSF in isopropanol, 10 mL, was added to the supernatant. The supernatant was concentrated to a final volume of ~20 mL by ultracentrifugation in an Amicon (Beverly, MA) concentrator with a YM10 membrane under nitrogen at 45 psi at 4°C. The supernatant was further concentrated by centrifugation at 2000 x g at 4°C in an Amicon (Beverly, MA) Centriprep 10 centricon to a volume of ~2 mL. The concentrate was then dialyzed against 25 mM sodium acetate, pH 5.0, 1 mM DTT, 0.5 mM EDTA and stored at 4°C.

Expression and Isolation of PTP-1B
Competent *E. coli* sp. DH5α cells were transformed with plasmid pSK-PTP-1B. Standard procedures were followed for all molecular biological manipulations (45). Cells were grown overnight at 37°C from a single ampicillin-resistant colony in 5 mL LB supplemented with 0.05 mg/mL ampicillin and the plasmid was purified using the supplied solutions of a Wizard Prep Kit (Promega, Madison, WI) according to the manufacturer’s protocols. The purified plasmid was cut with *Eco*RI and *Sal*I and the resulting fragment was subcloned into the expression vector pRSET-C (Invitrogen, Portland, OR) that had been cut with *Eco*RI and *Xho*I, to form plasmid pRSET-PTP-1B. Competent *E. coli* sp. DH5α cells were transformed with the plasmid pRSET-PTP-1B. Cells were grown for 12 hours at 37°C from a single ampicillin-resistant colony in 2 mL LB supplemented with 0.05 mg/mL ampicillin. The plasmid was purified using the supplied solutions of a Wizard Prep Kit (Promega, Madison, WI) according to the manufacturer’s protocols. Competent *E. coli* sp. BL21DE3/pCB424 cells (Novagen, Madison, WI) were transformed with the plasmid pRSET-PTP-1B. Cells were grown from a single ampicillin- and spectinomycin-resistant colony in 1 L of LB media supplemented with 0.05 mg/mL ampicillin and of 0.05 mg/mL spectinomycin, with vigorous shaking (~200 rpm), at 37°C until reaching an absorbance of 0.7 at 600 nm. IPTG, 100 mM in water, was added to a final concentration of 0.1 mM. The cells were incubated in presence of IPTG overnight at 25°C. Cells were harvested by centrifugation at 8,000 x g for 20 minutes at 4°C and resuspended in 10 mL of 20 mM Tris, pH 7.2, containing 750 mM NaCl, 5 mM imidazole, 1 mM DTT, 1 mM PMSF, and 10% (v/v) glycerol. Cells were lysed by passage through a French pressure cell three times at 12,000 psi. The lysate was centrifuged at 23,000 x g for 20 min. at 4°C. The supernatant was tested for hydrolase activity with pNPP and applied to a 1 x 11 cm column of Chelating Sepharose Fast-Flo (Pharmacia Biotech,
Piscataway, NJ) charged with 50 mM NiSO\textsubscript{4} and washed with 75 mL of 20 mM Tris, pH 7.2, containing 750 mM NaCl, 5 mM imidazole, and 1 mM DTT. The column-bound protein was eluted with 10 mL of 20 mM Tris, pH 7.2, containing 750 mM NaCl, and 250 mM imidazole. Fractions, 1 mL each, were collected and assayed for hydrolase activity with pNPP. The five most active fractions were pooled and concentrated by centrifugation at 2000 x \( g \) at 4°C in an Amicon (Beverly, MA) Centriprep 10 centricon. Glycerol was added to a final concentration of 40% (v/v), and the material was stored at –20°C.

**Expression and Purification of p56\textsuperscript{lyn} Kinase**

*E. coli* cells containing the plasmid, pGEX-KT, encoding a GST/lyn kinase fusion protein (52), were generously donated by Prof. Harry Charbonneau and coworkers from the Department of Biochemistry at Purdue University. The cells were grown at 37°C, with vigorous shaking, in 1 L of 2 x YT media supplemented with 0.05 mg/mL ampicillin, until an absorbance of 0.7 at 600 nm was reached. IPTG, 100 mM in water, was added to a final concentration of 0.2 mM and the cells incubated overnight at 25°C. The cells were harvested by centrifugation at 8000 x \( g \) for 20 minutes at 4°C. The cells were resuspended in 10 mL of PBS buffer, pH 7.4, containing 2 mM EDTA, 2 mM DTT, 2 mM PMSF, 1 mM benzamidine, and 1\( \mu \)g/mL leupeptin and lysed by passage through a French pressure cell three times at 12,000 psi. Triton-X-100, 25% (w/v) in water, was added to a final concentration of 1% (v/v). Cell debris was removed by centrifugation at 10,000 x \( g \) for 20 minutes at 4°C. A 1 mL suspension of 50 % (w/v) glutathione-agarose beads was added to the supernatant and incubated for 30 minutes at 4°C with rotation. The glutathione-agarose beads, with adherent protein, were washed four times in 10 mL of PBS buffer, pH 7.4, containing 2 mM EDTA, 2
mM DTT, 2 mM PMSF, 1 mM benamidine, and 1µg/mL leupeptin and collected by centrifugation at 1500 x g for 10 minutes at 4°C. The beads were then suspended as 50% slurry in PBS containing 10% (v/v) glycerol and stored at -20°C.

**Preparation of [³²P]Phosphotyrosyl-RCM-Lysozyme**

Lysozyme was reduced, carboxymethylated, and maleylated by the procedure of Crestfield et al. (10) as modified by Tonks et al. (50). Phosphorylation of the modified protein (RCM-lysozyme) on tyrosyl residues was performed in a volume of 1 mL of 50 mM HEPES, pH 7.5, containing 7.5 mM MgCl₂, 0.015% (w/v) Brij-35, 1 mM DTT, 0.5 mM [γ-³²P]ATP (0.5-2 x 10¹⁵ CPM/mol ATP), 1 mg RCM-lysozyme, and 200 µL of 20% (v/v) GST/lyn kinase bound to glutathione-agarose beads. The mixture was incubated overnight at 25°C with constant rotation. The kinase-bound beads were collected by micro-centrifugation at 12,000 rpm for 1 minute. The phosphotyrosyl-RCM-lysozyme was purified from the supernatant liquid by TCA precipitation as described by Tonks et al. (50) and stored at -20°C.

**Preparation of [³²P]Phosphoseryl-RCM-Lysozyme**

Lysozyme was reduced, carboxymethylated, and maleylated by the procedure of Crestfield et al. (10) as modified by Tonks et al. (50). Phosphorylation of the modified protein (RCM-lysozyme) on seryl residues using the catalytic subunit of cAMP-dependent protein kinase was performed as described by Kennelly and coworkers (33), with RCM-lysozyme substituted for casein. The reaction was performed in a volume of 1 mL of 50 mM HEPES, pH 7.5, containing 10.5 mM MgCl₂, 1 mM DTT, 0.1 mM EGTA, 0.5 mM [γ-³²P]ATP (0.5-2 x 10¹⁵ CPM/mol ATP), 1 mg RCM-lysozyme, and ~10 µg PKA catalytic
subunit and incubated overnight at 25°C. The phosphoseryl-RCM-lysozyme was purified from the supernatant liquid by TCA precipitation as described by Tonks et al. (50) and stored at -20°C.

**Preparation of [³²P]Phosphotyrosyl-Myelin Basic Protein**

Phosphorylation of the myelin basic protein on tyrosyl residues was under identical reaction conditions as those described above for RCM-lysozyme (³²P Tyr), with the exception that 1 mg MBP was substituted for RCM-lysozyme. After incubation, the glutathione-agarose beads were collected by centrifugation at 12,000 rpm for 1 minute and discarded. The radiolabeled phosphoprotein was purified by applying the supernatant to a 2 x 25 cm column of Sephadex G-25 (Pharmacia Biotech, Piscataway, NJ). The MBP was eluted with 500 mL of 50 mM Tris, pH 7.0, containing 1 mM DTT and 0.1 mM EGTA and 1 mL fractions were collected. Twenty microliters of each fraction were removed, dispersed into 1 mL of Scintisafe Plus 50% liquid scintillation fluid (Fisher Scientific, Pittsburgh, PA), and counted. Fractions from the first peak were collected, pooled, assayed for protein, and stored at -20°C.

**Preparation of [³²P]Phosphoseryl-Myelin Basic Protein**

Phosphorylation of the MBP on seryl residues was under identical reaction conditions as those described for RCM-lysozyme (³²P Ser), with the exception that 1 mg MBP was substituted for RCM-lysozyme. The radiolabeled phosphoprotein was purified as described for [³²P]phosphotyrosyl-MBP.

**Preparation of Malachite Green Reagent**
Malachite green reagent was prepared by adding 1 volume of 4.2% (w/v) ammonium molybdate in 4N HCl to 3 volumes 0.045% (w/v) malachite green. The mixture was let stand for at least 20 minutes at 25°C prior to use.

**Protein Assays**

Protein concentrations were determined by method of Bradford (6) using Coomassie protein assay reagent (Pierce, Rockford, IL) and a 2 mg/mL BSA solution. Protein purity was determined by SDS-PAGE (35).

**Phosphatase Assays**

Phosphatase activity towards low molecular weight phosphomonoesters was determined using malachite green to measure the release of inorganic phosphate as described by Lanzetta and coworkers (36), with the exception that Triton X-100 was omitted. Phosphatase assays were performed at 30°C in a volume of 50 µL of buffer containing 1 mM EDTA, 1 mM DTT, and 1 mM phosphomonoester substrate. The buffer used for assaying Cdc-14, Tc-PTPa, PTP-1B was 50 mM MOPS-HCl, pH 7.0. The buffer used for assaying VHR was 50 mM sodium succinate, pH 6.0. The buffer used for assaying IphP was 50 mM sodium acetate, pH 5.0. Reactions were terminated by adding 0.8 mL malachite green reagent, followed by 0.1 mL 34% (w/v) sodium citrate. The solution was vortexed briefly and allowed to stand at 25°C for ~20 minutes. The absorbance was measured at 660 nm. A solution of 0.5 mM K$_2$HPO$_4$ was used as standard. The limit of detection of the malachite green assay was 0.4 nmol PO$_4$. Phosphatase activity towards radiolabeled phosphoproteins was determined by measuring the liberated $[^{32}]$P inorganic phosphate using a scintillation counter.
Phosphatase assays were performed at 30°C in a volume of 25 µL of buffer containing 1 mM EDTA, 1 mM DTT, 0.08 mg/mL BSA, and radiolabeled phosphoprotein to a final concentration of 2 uM protein bound [³²P]phosphate. The buffer used for assaying Cdc-14, Tc-PTPa, PTP-1B was 50 mM MOPS-HCl, pH 7.0. The buffer used for assaying VHR was 50 mM sodium succinate, pH 6.0. The buffer used for assaying IphP was 50 mM sodium acetate, pH 5.0. The reaction was terminated by the addition of 0.125 mL of 20% (v/v) TCA. The sample was spun in a microcentrifuge at 12,000 rpm for 3 minutes to sediment the precipitate. Seventy-five microliters of the supernatant were removed, dispersed into 1 mL of Scintisafe Plus 50% liquid scintillation fluid (Fisher Scientific, Pittsburgh, PA), and counted for [³²P]phosphate present.

Molybdic acid extraction, performed as described by Martin and Doty (39), was used as an alternative method for the quantification of inorganic [³²P]phosphate. Protein phosphatase assays were performed as previously mentioned, with the exception that 74 µL of supernatant was removed to a 1.5 mL eppendorf tube and the following added: 0.2 mL of molybdate reagent (15 mg/ml (NH₄)₆Mo₇O₂₄·4H₂O in 25 mM H₂SO₄), 0.01 mL of 4mM K₂HPO₄, and 0.2 mL of isobutanol:toluene (1:1). The solution was vortexed for 30 seconds, then centrifuged at 12,000 rpm for 3 minutes in a microcentrifuge. A 50 µL aliquot from the organic upper layer was dispersed into 1 mL of Scintisafe Plus 50% liquid scintillation fluid (Fisher Scientific, Pittsburgh, PA) and counted for [³²P]inorganic phosphate.

All assay rates were determined from assays covering a minimum of five time points (Fig. 2.1-2.2).
Synthesis and Characterization of $^N$-Benzoyl-$^O$-Phospho-$^L$-Serine and $^N$-(Cyclohexane Carboxyl)-$^O$-Phospho-$^L$-Serine

In order to test the effectiveness of the addition of an aryl “handle” to a poorly hydrolyzed substrate, $^O$-phospho-$^L$-serine, Drs. Prashant Savle and Rich Gandour from the Department of Chemistry at Virginia Tech synthesized $^N$-Benzoyl-$^O$-phospho-$^L$-serine by conjugating a benzyl ring to the $\alpha$-amine of phosphoserine through an ester linkage, and as a control, the derivative, $^N$-(Cyclohexane carboxyl)-$^O$-phospho-$^L$-serine, by hydrogenating, then conjugating a benzyl ring to the $\alpha$-amine of phosphoserine through an ester linkage. A complete description of this procedure can be found in the Appendix.
The Dephosphorylation of \(N\)-Benzoyl-\(O\)-Phospho-L-Serine and \(N\)-(Cyclohexane Carboxyl)-\(O\)-Phospho-L-Serine by IphP Was Linear as a Function of Time. The protein phosphatase activity of IphP towards \(N\)-benzoyl-\(O\)-phospho-L-serine and \(N\)-(cyclohexane carboxyl)-\(O\)-phospho-L-serine was determined as described in Materials and Methods. Shown is the amount of liberated inorganic phosphate as a function of time. Substrates were present at a concentration of 1 mM. The concentration of IphP in assays was 0.02 \(\mu\)g/\(\mu\)L.
The Dephosphorylation of Phosphotyrosyl- and Phosphoseryl- RCM-Lysozyme by VHR and PTP-H1 Was Linear as a Function of Time. The protein phosphatase activity of VHR and PTP-H1 toward RCM-lysozyme phosphorylated on either tyrosine or serine residues was determined as described in Materials and Methods. Shown is the fraction of protein-bound $[^{32}\text{P}]$phosphate released as a function of time. Substrates were present at a concentration of 2 $\mu$M protein bound $[^{32}\text{P}]$phosphate. The concentration of VHR or PTP-H1 present in phosphotyrosine phosphatase assays was 0.04 or 0.0025 $\mu$g/$\mu$L, respectively, while the concentration present in phosphoserine phosphatase assays was 0.4 or 0.25 $\mu$g/$\mu$L, respectively.
CHAPTER III

RESULTS

PART A

Activity of PTPs and DSPs Towards Naphthyl Phosphate Isomers

The prediction that the ratio of $\alpha$- to $\beta$- naphthyl phosphate activity would be near unity for DSPs and high (e.g. 1 : 10+) for PTPs was examined. Phosphatases drawn from the two major classes of protein tyrosine phosphatases were assayed for activity toward $\alpha$-naphthyl phosphate and $\beta$-naphthyl phosphate under standard conditions as described in Materials and Methods. The PTPs assayed were Tc-PTPa (25), PTP-H1 (54), and PTP-1B (4). The DSPs assayed were VHR (13), Cdc14 (49), and IphP (43). The ratio of $\alpha$-to $\beta$-naphthyl phosphatase activity was determined for all six phosphatases (Table 3.1). The DSPs displayed ratios near the predicted value of ~1 : 1. Unexpectedly, only two of the PTPs, PTP-1B and Tc-PTPa, displayed a ratio of 1 : 10 or greater. The third, PTP-H1, displayed a very “DSP-like” ratio of 1 : 1.6. This suggested that either our hypothesis was incorrect, or that PTP-H1 might display DSP activity, at least in vitro.

A search of the literature showed PTP-H1 had been classified as a PTP by virtue of its ability to dephosphorylate tyrosine-phosphorylated MBP and RCML (54). However, these reports gave no indication that PTP-H1 activity towards serine-phosphorylated proteins had been examined. We therefore examined the activity of PTP-H1 towards $[^{32}\text{P}]$phosphoseryl
<table>
<thead>
<tr>
<th></th>
<th>Cdc14</th>
<th>IphP</th>
<th>VHR</th>
<th>PTP-1B</th>
<th>Tc-PTPa</th>
<th>PTP-H1</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthyl PO₄</td>
<td>22 ± 2  (3)</td>
<td>144 ± 10 (9)</td>
<td>1898 ± 228 (3)</td>
<td>n.d.</td>
<td>455 ± 12 (3)</td>
<td>137 (1)</td>
</tr>
<tr>
<td>β-Naphthyl PO₄</td>
<td>14 ± 2  (3)</td>
<td>147 ± 14 (9)</td>
<td>3185 ± 420 (3)</td>
<td>n.d.</td>
<td>8005 ± 637 (3)</td>
<td>221 (1)</td>
</tr>
<tr>
<td>α- : β-</td>
<td>1.5 : 1.0</td>
<td>1.0 : 1.0</td>
<td>1.0 : 1.7</td>
<td>1.0 : 18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.0 : 17.6</td>
<td>1.0 : 1.6</td>
</tr>
<tr>
<td>RCML-PY</td>
<td>0.053 ± 0.002 (3)</td>
<td>0.40 (1)</td>
<td>22.7 ± 0.9 (3)</td>
<td>577 (1)</td>
<td>22,900 ± 1000 (6)</td>
<td>111 (1)</td>
</tr>
<tr>
<td>RCML-PS</td>
<td>0.013 ± 0.002 (5)</td>
<td>0.15 (1)</td>
<td>0.90 ± 0.1 (3)</td>
<td>&lt; 0.005 (1)</td>
<td>0.02 ± 0.002 (5)</td>
<td>0.2 (1)</td>
</tr>
<tr>
<td>PY : PS (RCML)</td>
<td>4.1 : 1.0</td>
<td>2.7 : 1.0</td>
<td>24.6 : 1.0</td>
<td>1.15 × 10&lt;sup&gt;5&lt;/sup&gt; : 1.0</td>
<td>1.15 × 10&lt;sup&gt;6&lt;/sup&gt; : 1.0</td>
<td>553.5 : 1</td>
</tr>
<tr>
<td>MBP-PY</td>
<td>n.d.&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.d.</td>
<td>2.5 (1)</td>
<td>44.4 (1)</td>
<td>174.7 ± 7.2 (3)</td>
<td>205.8 (1)</td>
</tr>
<tr>
<td>MBP-PS</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.88 (1)</td>
<td>&lt; 0.003 (1)</td>
<td>&lt; 0.006 (3)</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td>PY : PS (MBP)</td>
<td>3.9 : 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7 : 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.8 : 1.0</td>
<td>1.5 × 10&lt;sup&gt;4&lt;/sup&gt; : 1.0</td>
<td>2.9 × 10&lt;sup&gt;4&lt;/sup&gt; : 1.0</td>
<td>343.0 : 1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>n.d. = not determined
<sup>b</sup>(49)
<sup>c</sup>(26)
<sup>d</sup>(54)

Table 3.1

Activity of PTPs and DSPs Toward α- and β-Naphthyl Phosphate and [³²P]Phosphotyrosyl- and [³²P]Phosphoseryl-RCML and MBP.

The rate of dephosphorylation of the indicated substrates by six protein phosphatases was determined under standard conditions as described in the Materials and Methods. Rates are reported in nmol/min/mg. Where sufficient materials were available, assays were performed in replicates, shown in parentheses, and reported as an average plus or minus standard error. The abbreviation n.d. indicates the rate was not measured experimentally during the study. The ratios of the activity of the phosphatases toward α-naphthyl phosphate versus β-naphthyl phosphate, reported as α- : β-; [³²P]phosphotyrosyl- versus [³²P]phosphoseryl-RCM-lysozyme, reported as PY : PS (RCML); and [³²P]phosphotyrosyl- versus [³²P]phosphoseryl-myelin basic protein, reported as PY : PS (MBP), are shown. Where indicated, ratios derived from literature values are shown.
proteins. Ideally, we would like to assay PTP-H1 against a protein substrate that can be phosphorylated on a single tyrosine or serine reside, however, no such substrate was available. Therefore, two proteins that could be phosphorylated on tyrosine or serine, albeit at different sites, RCM-lysozyme and myelin basic protein, were used as substrates. Both proteins are significantly different in sequence, isoelectric point, etc., so that the influence of any bias due to specific substrate-enzyme interactions can be minimized.

PTP-H1 was assayed against $^{32}$Pphosphoseryl-RCM-lysozyme and MBP. In order to provide a baseline measurement, PTP-H1 was also assayed against $^{32}$Pphosphotyrosyl-RCM-lysozyme and MBP. Prototypical PTPs, PTP-1B and Tc-PTPa, and DSPs, IphP, VHR, and Cdc14 were also assayed against $^{32}$Pphosphotyrosyl and $^{32}$Pphosphoseryl-RCM-lysozyme and MBP to provide a basis of comparison.

The six phosphatases were assayed under standard conditions as described in Materials and Methods in order to determine the ratio of activities toward RCML-PY versus RCML-PS, and MBP-PY versus MBP-PS (Table 3.1). The DSPs displayed ratios of PY:PS activity ranging from ≈ 3:1 to nearly 30:1. PTP-1B and Tc-PTPa, as predicted, exhibited an extremely high degree of selectivity for $^{32}$Pphosphotyrosyl proteins over the equivalent $^{32}$Pphosphoseryl proteins, displaying PY : PS ratios of greater than $1 \times 10^4$ :1. PTP-H1 exhibited a much different ratio of PY : PS activities than the other PTPs: 554:1 for RCML-PY versus RCML-PS and 343:1 for MBP-PY versus MBP-PS, respectively. When compared to the other PTPs, these ratios represent ≈ 40- to 200-fold decrease in the ability of PTP-H1 to discriminate $^{32}$Pphosphotyrosyl proteins from $^{32}$Pphosphoseryl proteins. Looking at this from another perspective, the hydrolysis rates of $^{32}$Pphosphoseryl proteins by PTP-H1 were greater than those of PTP-1B and Tc-PTPa, 0.2 nmol/min/mg versus 0.005 and 0.02
nmol/min/mg, respectively, for RCML-PS and 0.6 nmol/min/mg versus 0.003 and 0.006 nmol/min/mg, respectively, for MBP-PS. This represents ≈ 10 to 200 fold increase. To verify that PTP-H1 was the source of the phosphoserine phosphatase activity, assays were performed in the presence of 1.2 mM vanadate, a known inhibitor of PTPs (47, 48). Under these conditions, MBP-PS activity was abolished (Fig 3.1).
Inhibition of PTP-H1 Activity by Vanadate. Assays performed under standard conditions as described in the Materials and Methods with the exception that, where indicated, 1.2 mM vanadate was added to reaction mixture. MBP-PS was present at a concentration of 2 μM protein bound [\(^{32}\)P]phosphate. The concentration of PTP-H1 present in assays was 0.02 μg/μL.
CHAPTER III

RESULTS

PART B

Activity of PTPs and DSPs Toward N-Benzoyl-O-Phospho-L-Serine and N-(Cyclohexane Carboxyl)-O-Phospho-L-Serine

If an aryl “handle” provides a key determinant for substrate recognition by IphP, we reasoned that it should prove possible to transform a very poor substrate into a credible one by adding such a group to there. While PTPs readily dephosphorylate free O-phospho-L-tyrosine, O-phospho-L-serine is a poor substrate for DSPs (60). The activity of three DSPs, VHR (13), Cdc14 (49), and IphP (43), and two PTPs, Tc-PTPa(25) and PTP-1B (4), were assayed against β-naphthyl phosphate, N-benzyol-O-phospho-L-serine, N-(cyclohexane carboxyl)-O-phospho-L-serine, and free phosphoserine under standard conditions as described in Materials and Methods. The outcomes of these determinations are summarized in Table 3.2.

As expected, N-benzyol-O-phospho-L-serine, N-(cyclohexane carboxyl)-O-phospho-L-serine, and free phosphoserine proved to be poor substrates for PTP-1B (Fig. 3.2) and Tc-PTPa (Fig. 3.3). One likely possibility for this may be due to the steric hindrance of the deep active site pocket of PTPs.

When N-benzyol-O-phospho-L-serine was tested as an improved aryl “handle” version
### Table 3.2

<table>
<thead>
<tr>
<th></th>
<th>IphP</th>
<th>VHR</th>
<th>Cdc14</th>
<th>PTP-1B</th>
<th>Tc-PTPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSS-8105</td>
<td>65.2 ± 6.4</td>
<td>10.4 ± 1.1</td>
<td>2.0 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>PSS-8108</td>
<td>2.5 ± 0.2 (6)</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>P-Ser</td>
<td>0.6 ± 0.1 (6)</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

**Relative Activity of PTPs and DSPs Toward N-Benzoyl-O-Phospho-L-Serine, N-(Cyclohexane Carboxyl)-O-Phospho-L-Serine, and O-Phospho-L-Serine.** The activity of each of the protein phosphatases listed above towards the substrates listed was determined under standard conditions as described in Materials and Methods. The activities are reported as a percentage of the rate of dephosphorylation of β-naphthyl phosphate by each phosphatase. The concentration of each substrate was 1 mM. Each value represents the mean of nine replicates plus or minus standard error, with the exceptions listed in parentheses. The activity of each phosphatase towards β-naphthyl phosphate was as follows: IphP, 147 ± 13 nmols/min/mg; VHR, 2032 ± 78 nmols/min/mg; Cdc14, 829 ± 13 nmols/min/mg; PTP-1B, 118 ± 3 nmols/min/mg; Tc-PTPa, 4097 ± 266 nmols/min/mg.
Figure 3.2

Relative Activity of PTP-1B Towards N-Benzoyl-\textit{O}-Phospho-L-Serine, \textit{N}-(Cyclohexane Carboxyl)-\textit{O}-Phospho-L-Serine, and P-Ser. Assays were performed under standard condition as described in Materials and Methods. The values reported represent the mean of nine replicates plus or minus standard error. The activities are shown as a percentage of the total activity of PTP-1B towards β-naphthyl phosphate, 118 ± 3 nmols/min/mg.
Figure 3.3

Relative Activity of Tc-PTPa Towards \textit{N}-Benzoyl-\textit{O}-Phospho-L-Serine, \textit{N}-(Cyclohexane Carboxyl)-\textit{O}-Phospho-L-Serine, and \textit{P}-Ser. Assays were performed under standard condition as described in Materials and Methods. The values reported represent the mean of nine replicates plus or minus standard error. The activities are shown as a percentage of the total activity of Tc-PTPa towards β-naphthyl phosphate, 4097 ± 266 nmols/min/mg.
of P-Ser, it proved to be only a marginally better substrate for IphP than free phosphoserine. Similar results were obtained with the two other DSPs, VHR and Cdc14. However, surprising results were obtained with N-(cyclohexane carboxyl)-O-phospho-L-serine, which had been synthesized as a non-aromatic control. Whereas the aryl “handle” hypothesis predicts that N-(cyclohexane carboxyl)-O-phospho-L-serine should be a relatively poor substrate for IphP, the enzyme hydrolyzed the saturated cyclohexyl derivative at a rate almost 10²-fold greater than that of free phosphoserine and N-benzoyl-O-phospho-L-serine (Fig. 3.4). The rate of hydrolysis observed exceeded that of both free phosphotyrosine and pNPP, both regarded as good substrates, and approached that of β-naphthyl phosphate. VHR also hydrolyzed N-(cyclohexane carboxyl)-O-phospho-L-serine, though at a rate only ~30 fold greater than that of free phosphoserine (Fig. 3.5). Cdc14 exhibited very poor activity toward N-(cyclohexane carboxyl)-O-phospho-L-serine (Fig. 3.6), however, eliminating N-(cyclohexane carboxyl)-O-phospho-L-serine as a general diagnostic substrate for DSPs.

**Determination of Michaelis-Menten Constants for IphP Toward N-Benzoyl-O-Phospho-L-Serine and N-(Cyclohexane Carboxyl)-O-Phospho-L-Serine**

Kinetic analysis was performed to provide a more quantitative basis for the comparison of these substrates. The Kₘ and Vₘₐₓ values for the hydrolysis of N-benzoyl-O-phospho-L-serine, N-(cyclohexane carboxyl)-O-phospho-L-serine, and P-Tyr (as a control) by IphP were determined. IphP displayed a Kₘ of 4.4 mM for N-(cyclohexane carboxyl)-O-phospho-L-serine, while the Kₘ for P-Tyr, 10.1 mM, was roughly two times greater. The Vₘₐₓ for N-(cyclohexane carboxyl)-O-phospho-L-serine was 3380 nmols/min/mg, versus 1963 nmols/min/mg for phosphotyrosine. The Kₘ for N-benzoyl-O-phospho-L-serine was
**Figure 3.4**

**Relative Activity of IphP Towards** *N*-Benzoyl-*O*-Phospho-*L*-Serine, *N*-(*Cyclohexane Carboxyl)*-*O*-Phospho-*L*-Serine, and **P-Ser**. Assays were performed under standard condition as described in Materials and Methods. The values reported represent the mean of six replicates plus or minus standard error. The activities are shown as a percentage of the total activity of IphP towards β-naphthyl phosphate, 147 ± 13 nmols/min/mg.
Relative Activity of VHR Towards N-Benzoyl-O-Phospho-L-Serine, N-(Cyclohexane Carboxyl)-O-Phospho-L-Serine, and P-Ser. Assays were performed under standard condition as described in Materials and Methods. The values reported represent the mean of nine replicates plus or minus standard error. The activities are shown as a percentage of the total activity of VHR towards β-naphthyl phosphate, 2032 ± 78 nmols/min/mg.
Figure 3.6

Relative Activity of Cdc14 Towards N-Benzyol-O-Phospho-L-Serine, N-(Cyclohexane Carboxyl)-O-Phospho-L-Serine, and P-Ser. Assays were performed under standard condition as described in Materials and Methods. The values reported represent the mean of nine replicates plus or minus standard error. The activities are shown as a percentage of the total activity of Cdc14 towards β-naphthyl phosphate, 829 ± 13 nmols/min/mg.
estimated to be ~17.3 mM (Fig. 3.7), four times higher than the \( K_m \) for the cyclohexyl variant. A more accurate value could not be determined by virtue of our inability to achieve the high concentrations of substrate required.

**Visualization of The Active Site Pocket of VHR**

Since the \( V_{max} \) values for the benzoyl and cyclohexyl moieties were nearly equal, the large difference in their rates of hydrolysis by VHR and, especially, IphP can be most likely attributed to the differences in their \( K_m \) values. The aromatic ring of \( N\)-benzoyl-\( O\)-phospho-L-serine is rigid and planar, while the saturated ring of \( N\)-(cyclohexane carboxyl)-\( O\)-phospho-L-serine can adopt a more flexible conformation, perhaps allowing it greater access to hydrophobic binding regions in the active site pocket.

While IphP hydrolyzed \( N\)-(cyclohexane carboxyl)-\( O\)-phospho-L-serine with a greater efficacy than VHR, the crystal structure for the former has yet to be solved. The crystal structure of VHR is on file at the RCSB Protein Data Bank, PDB I.D. 1VHR (53). Through the use of modeling software, Quanta (Molecular Simulations, Inc., San Diego, CA), RasMol (Umass, Amherst, MA), and the CAST (37) server (Univ. of Minn., St. Paul, MN), we attempted to discern how \( N\)-(cyclohexane carboxyl)-\( O\)-phospho-L-serine might bind to the active site pocket of VHR. A molecule of HEPES buffer was in the active site of the VHR crystal structure. Because of its rough similarity in size and structure to \( N\)-(cyclohexane carboxyl)-\( O\)-phospho-L-serine (Fig. 3.8), it was used as the basis for mapping the region. We reasoned that the cyclohexyl moiety of \( O\)-phospho-L-serine requires a hydrophobic surface upon which to dock. One possible region of interaction is a hydrophobic groove consisting
Figure 3.7

Hanes-Woolf Plot of Hydrolysis Rates of N-Benzoyl-O-Phospho-L-Serine, N-(Cyclohexane Carboxyl)-O-Phospho-L-Serine, Free Phosphotyrosine, and β-Naphthyl Phosphate by IphP. Assays performed under standard conditions as described in Materials and Methods with the exception that substrates present in assays ranged in concentrations from 0.5 to 14 mM. IphP was present in these assays at a constant concentration of 0.02 µg/µL.
Comparison of $N$-(Cyclohexane Carboxyl)-$O$-Phospho-L-Serine and HEPES. A comparison of $N$-(cyclohexane carboxyl)-$O$-phospho-L-serine [LEFT] and a molecule of $N$-2-hydroxyethylpiperazine-$N'$-2-ethanesulfonic acid [RIGHT] showing their rough similarities in size and structure.
Figure 3.9

Rasmol Generated Side View of the Active Site Pocket of VHR (53). A side view showing the Leu 16, Tyr 23, Ile 160, Leu 25 and Gly 161 groove. The active site pocket is highlighted. HEPES is shaded gray. Carbon is shaded brown. Oxygen is shaded red. Nitrogen is shaded blue. Sulfur is shaded yellow.
of the residues, Leu 16, Tyr 23, Ile 160, Leu 25 and Gly 161 (Fig. 3.9). Another possible region found was a hydrophobic groove consisting of Met 69 and Phe 68 (Fig. 3.10).

**Comparison of VHR Active Site Residues With IphP and Cdc14 Residues**

With the amino acids of the two hydrophobic grooves mapped, we reasoned that the common ability of VHR and IphP to hydrolyze \(N\)-(cyclohexane carboxyl)-\(O\)-phospho-L-serine might be indicative of shared conserved amino acid residues in these regions. The amino acid sequence of IphP (Fig. 3.11) therefore was compared with that of VHR to determine whether similar residues might exist around the active site pocket. Using LaserGene computer software from DNASTAR (Madison, WI), the sequences were aligned using the Clustal method. As a control, the amino acid sequence of Cdc14 was also compared with VHR (Fig. 3.12). Although the sequence comparison revealed only two exact matches in the regions corresponding to the hydrophobic grooves on VHR, IphP did share some similar types of residues. Residue 60 (IphP) and residue 25 (VHR) have non-polar side chains. Residue 266 (IphP) and residue 160 (VHR) have uncharged side chains, while residue 270 (IphP) and residue 164 (VH) have charged side chains. Cdc14 and VHR, on the other hand, had five exact matches. In addition, Cdc14 and VHR shared similar residues, such as the uncharged, non-polar residues 103 and 25, respectively.
Figure 3.10

Rasmol Generated Top View of the Active Site Pocket of VHR (53). A top view showing the Met 69, Phe68 groove. The active site pocket is highlighted. HEPES is shaded gray. Carbon is shaded brown. Oxygen is shaded red. Nitrogen is shaded blue. Sulfur is shaded yellow.
Alignment Report of IphP and VHR. The sequences of VHR and IphP were compared using the Clustal method with PAM250 residue weight table. Residues around active site pocket of VHR are in bold. Corresponding IphP residues are in bold with exact matches in italics. The conserved active site sequence His-Cys-Xaa<sub>5</sub>-Arg is underlined.

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Figure 3.12

Alignment Report of Cdc14 and VHR. The sequences of VHR and Cdc14 were compared using the Clustal method with PAM250 residue weight table. Residues around active site pocket of VHR are in bold. Corresponding Cdc14 residues are in bold with exact matches in italics. The conserved active site sequence His-Cys-Xaa5-Arg is underlined.
CHAPTER IV

DISCUSSION

Three PTPs and three DSPs were challenged *in vitro* with the low molecular weight phosphomonoesters α-naphthyl phosphate and β-naphthyl phosphate. Our examination revealed that prototypical PTPs, such as PTP-1B, displayed a 10-fold or greater preference for β-naphthyl phosphate over α-naphthyl phosphate, while the DSPs displayed little ability to discriminate between the two phosphomonoesters. However, one PTP, PTP-H1, failed to discriminate between the two isomers. When challenged with $[^{32}\text{P}]$phosphoproteins, PTP-1B and Tc-PTPa displayed a marked preference, $10^4$-fold or more, for phosphotyrosyl- over phosphoseryl- proteins. Although PTP-H1 exhibited a clear preference for phosphotyrosyl proteins, it discriminated between the protein-bound phosphoamino acids to a much lesser degree, $3-5 \times 10^2$-fold, than did the other PTPs. This observation, considered in light of the naphthyl phosphate data, suggests that while classified as a PTP, PTP-H1 may possess a wider and perhaps shallower active site pocket than typical PTPs (Fig. 4.1) and, therefore, may be classified as an intermediate PTP.

The existence of intermediate PTPs, such as PTP-H1, eliminates α- and β-naphthyl phosphate as general diagnostic substrates for the differentiation of PTPs from DSPs. However, these compounds still may serve as effective diagnostic substrates for differentiating between prototypical PTPs and intermediate PTPs. Indeed, the identification
Figure 4.1

Schematic Representation of Possible Active Site Pocket Structure of PTP-H1. Shown is a schematic representation of the possible PTP-H1 active site, as well as representations of the active site pockets of prototypical PTPs and DSPs, with α-naphthyl phosphate. The data from experiments employing both naphthyl phosphate isomers and $[^{32}P]$phosphoproteins suggest that PTP-H1, while classified as a PTP, may possess a wider, shallower active site pocket than prototypical PTPs such as PTP-1B.
of naphthoquinone analogs as irreversible inactivators of a PTP and DSP (23, 24) reveals the potential usefulness of naphthyl derivatives as the foundation for a variety of substrates, inhibitors, and affinity-labeling reagents for PTPs and DSPs.

One possible interpretation of the laboratory behavior of PTP-H1 is that, rather than being an intermediate PTP, it may in fact be a misclassified DSP. Relatively little is known concerning the nature of the physiological substrates for PTP-H1. VCP (p97/CDC48), a cell cycle regulator (42), is the only such substrate identified to date. This protein was found to be phosphorylated on tyrosine residues. VCP was identified as a substrate by expressing a mutationally-altered form of PTP-H1 that was catalytically inactive. Immunoprecipitation with antibodies to the PTP also will bring down any substrate proteins bound to it in a dead-end E-S complex. Such substrate trapping mutants have been used to identify a number of tyrosine-phosphorylated protein substrates for various PTPs (9, 18, 19). However, no cases of the successful trapping of a serine or threonine phosphorylated protein by an inactive DSP have been reported in the literature. It therefore is unclear whether an E-S complex involving a phosphoseryl protein would be sufficiently stable to be successfully immunoprecipitated and, by extension, whether this technique would detect a serine or threonine phosphorylated substrate protein for PTP-H1 if one did exist. Until the crystal structure of PTP-H1 is solved, the question of its classification will remain. However, continued probing of the active site with various substrates could provide a preliminary answer. Challenging PTP-H1 with a series of increasing straight-chain peptide bound aliphatic phosphates, such as used by Dunn and coworkers (14), might reveal if PTP-H1 has shallower pocket than a prototypical PTP.

Two new phosphoserine derivatives were synthesized to evaluate the hypothesis that the addition of an aryl “handle” can transform a poor substrate into a credible one for IphP
and perhaps other DSPs. Three DSPs were challenged with the derivatives N-benzoyl-O-phospho-L-serine and N-(cyclohexane carboxyl)-O-phospho-L-serine. The activity of IphP and VHR towards N-benzoyl-O-phospho-L-serine was only marginally better than that towards free phosphoserine. Surprisingly, the cyclohexyl derivative proved to be a much more effective substrate for IphP and, to a lesser degree, VHR, than the benzoyl derivative. This suggests that it is not the aromaticity but, rather, the hydrophobicity of the adjacent ring that constitutes the determinant in substrate recognition. The aromatic ring of N-benzoyl-O-phospho-L-serine is rigid and planar, while the saturated ring of N-(cyclohexane carboxyl)-O-phospho-L-serine can adopt a more flexible conformation. The flexibility of the saturated ring may allow it to interact more effectively with one of the hydrophobic grooves adjacent to the active site pocket of VHR.

Sequence alignments indicate that IphP and VHR share only a few common residues around the active site pocket. Moreover, the degree of similarity appears to be no greater than that between Cdc14 and VHR. This suggests that while the hydrophobic “handle” may be important in substrate recognition, it is not the only determinant. The crystallization and solution of the three-dimensional structure of IphP will reveal possible binding regions directly. For now, further kinetic studies can be used to explore substrate recognition by IphP and VHR. Future experiments should involve challenging additional DSPs with both N-benzoyl-O-phospho-L-serine and N-(cyclohexane carboxyl)-O-phospho-L-serine in order to determine whether the low activity towards the substrates displayed by Cdc14 is shared by other DSPs.

Other experiments could explore new derivatives of phosphoserine in order to answer these questions:
1. **Is the substrate the right size?** What would be the result of increasing/decreasing the size of the ring or moving it farther away from C₁?

2. **Does a cyclic structure provide the best “handle”?** What would be the result of replacing the cyclohexyl ring with another R group such as a tertiary butyl group or naphthyl ring (Fig. 4.2a)?

3. **Would eliminating the potential charge of the carboxyl group result in a superior substrate?** What would be the result of replacing the charged carboxyl group with an uncharged, polar amide group (Fig. 4.2b)?

4. **Is a carboxyl “handle” better than an amino one?** What would be the result of linking the cyclohexyl moiety to the carboxyl end of phosphoserine (Fig. 4.2c)?

5. **Would a phosphorylated HEPES-like compound be a better substrate for VHR?** X-ray structures show HEPES bound within the active site of VHR. What would be the result of synthesizing a HEPES-like phosphomonoester and testing it as a substrate for VHR (Fig. 4.3a)?

6. **Would increasing the length of a HEPES-like phosphomonoester make it a better substrate?** What would be the result of increasing the number of straight chain carbons in a HEPES-like phosphomonoester (Fig. 4.3b)?
Derivatives of Phosphoserine for Future Experiments. Instead of challenging various DSPs with $N$-(cyclohexane carboxyl)-$O$-phospho-L-serine, other derivatives of phosphoserine could be synthesized and tested by replacing the cyclic ring with another hydrophobic group, (A), or replacing the charged carboxyl with an uncharged, polar amide, (B). In addition, a substrate with a carboxyl “handle” should be tested (C).
HEPES-Like Substrates for Challenging VHR. X-ray crystallography showed HEPES bound in the active site of VHR. Therefore, a phosphorylated HEPES-like compound might prove to be an effective substrate for VHR (A). If a phosphorylated HEPES-like compound is a substrate for VHR, it might be transformed into a better substrate by the addition of methyl groups to extend it more deeply into the active site (B).
Conclusion:

Our studies demonstrate the utility of small phosphomonoesters as probes of the substrate specificity and active site geometry of PTPs and DSPs. In many ways, modern organic synthetic techniques render such compounds malleable to a wider range of variations than is possible with phosphoprotein substrates.
REFERENCES

APPENDIX

The following description was provided by Dr. Prashant Savle from the Department of Chemistry at Virginia Tech.

Synthesis of $N$-Benzyol-$O$-Phospho-L-Serine

$N$-Benzyol-$O$-phospho-L-serine (Fig. B6) was envisaged from the reaction of the benzylester of L-serine (Fig. A1) and benzoyl chloride, followed by phosphorylation. Treatment of (Fig. A1) with benzoyl chloride in aqueous THF gave the $N$-benzyol derivative (Fig. A2) in 62% yield. Phosphate ester was prepared by reacting (Fig. A2) with diphenyloxyphosphonyl chloride in 90% yield. When compound (Fig. A3) was hydrogenated employing 5% PtO$_2$ as a catalyst, we observed the reduction of the phenyl ring (of $N$-benzyol) along with the cleavage of the phenyloxy and benzyl ester groups giving a $N$-cyclohexyoyl analogue of phosphoserine, (Fig. A4) in 84% yield. The reaction was over in 5 minutes and seemed to be associated with only PtO$_2$ catalyst. Since this was a new compound, we characterized it fully and assayed for its activity. The use of other catalysts could not cleave the phenyloxy protection groups from phosphate ester, (Fig. A3). We thus decided to prepare a dibenzyl phosphate derivative of (Fig. B2) employing $N,N$-diethyl dibenzylphosphoroamidate [Perich, 1987 #73]. The yield of dibenzyloxy phosphonate (Fig. B5) was 57%. Catalytic hydrogenation over 10% Pd-C gave us the required $N$-benzyol derivative (Fig. B6) of phosphoserine in 78% yield.
Experimental General Methods

$^1$H NMR spectra were recorded at 400 MHz with chemical shifts expressed in ppm downfield from internal TMS. $^{13}$C NMR spectra were recorded at 100 MHz with chemical shifts expressed in ppm relative to the solvent chemical shift. $^{31}$P NMR was recorded at 162 MHz with chemical shifts expressed in ppm relative to phosphoric acid chemical shift. Unless noted otherwise, all NMR spectra were recorded in CDCl$_3$. Coupling constants, (expressed as $J_{app}$ in Hz), were recorded as thin films on KBr cells. FAB MS samples were prepared as suspensions in glycerol. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA).

Unless otherwise noted, materials were obtained from commercial sources and used without further purification. THF was distilled from sodium-benzophenone ketyl. Diethyl ether was distilled from sodium. CH$_2$Cl$_2$ was distilled over CaH$_2$. Solutions were dried over Na$_2$SO$_4$ and concentrated by rotary evaporation, unless specified otherwise.

N-Benzoyl-L-Serine Benzy1 Ester (Fig. A2)

Benzoyl chloride (0.43 g, 3.6 mmol) was added to L-serine benzylesterHCl (Fig. A1) (0.76 g, 3.2 mmol) and K$_2$CO$_3$ (0.89 g, 6.5 mmol) in THF-water (1:1, 5 mL). The reaction mixture was extracted with EtOAc ($3 \times 30$ mL) and the organic layer was dried. Concentration of the extract gave a crude product, which was then chromatographed on silica (1” × 6” column). Elution with hexanes (500 mL), followed by 20% Et$_2$O-hexanes (400 mL), gave a colorless solid (0.48 g, 62%). $^1$H NMR 2.7 (1H, br, OH), 4.02-4.10 (2H, m, -CHCH$_2$OH), 4.88-4.92 (1H, m, -CHCH$_2$OH), 5.24 (2H, s, CO$_2$CH$_2$Ph), 7.16, 7.33-7.53 (8H, m, 2 × Ph), 7.82 (2H, d, $J_{app}$=10.4 Hz, COPh). $^{13}$C NMR 55.3, 63.5, 67.6, 127.2, 128.2,
**N-Benzoyl-O-Diphenoxyphosphoryl-L-Serine Benzyl Ester (Fig. A3)**

Diphenyloxyphosphonyl chloride (0.19 g, 0.71 mmol) was added to a solution of (Fig. A2) (0.17 g, 0.58 mmol), pyridine (0.06 g, 0.7 mmol) and DMAP (0.02 g) in methylene chloride (2 mL) under nitrogen atmosphere. The reaction mixture was stirred at 25°C overnight. The reaction mixture was diluted with water, extracted with EtOAc (3 × 10 mL) and the organic layer was dried. Concentration of the extract gave a crude product, which was chromatographed on silica (0.5” × 6” column). Elution with hexanes, (200 mL) followed by 1:1 Et₂O-hexanes (300 mL), gave a colorless solid (0.26 g, 90%). ¹H NMR 4.62-4.81 (2H, m, -CH₂OH), 5.01-5.09 (1H, m, -CH₂OH), 7.13-7.58 (18H, m, 4 × Ph), 7.73 (2H, d, J_app=10.4 Hz, COPh). ¹³C NMR 53.3 (d, J_C_P_app=7.6 Hz), 67.9, 68.5 (d, J_C_P_app=7.6 Hz), 119.9 (d, J_C_P_app=4 Hz), 125.6, 127.2, 128.3, 128.5, 128.6, 128.7, 129.5, 129.8, 131.9, 133.2, 134.8, 150.1 (d, J_C_P_app=9 Hz), 167.1, 168.6. ³¹P NMR 1.2. IR 1736, 1743, 3231.

**N-Cyclohexoyl-O-Phophoryl-L-Serine (Fig. A4)**

A solution of (Fig. A3) (0.21 g, 0.39 mmol) and PtO₂ (0.02 g) in EtOAc (5 mL) was stirred under hydrogen atmosphere for 5 minutes. The reaction mixture was filtered and concentrated to yield a crude product. Recrystallization from acetone and ether gave a colorless hygroscopic solid (0.096 g, 84%). ¹H NMR 0.97-1.31 (5H, m, cyclohexyl), 1.45
(5H, m, cyclohexyl), 2.11-2.32 (1H, m, cyclohexyl), 3.95-4.04 (1H, m, CHCH₃H₈OP), 4.07-4.14 (1H, m, CHCH₃H₈OP), 4.52-4.58 (1H, m, -CHCH₂OP). ¹³C NMR 25.2, 25.3, 25.5, 29.0, 29.1, 44.6, 52.8, 65.3, 171.1, 177.9. ³¹P NMR (CD₃OD) 1.43. The molecular mass, determined by FAB MS, was 296.0899 Da and closely corresponded to that calculated for the predicted product, C₁₀H₁₈NO₇P, of 296.0895 Da.

**N-Benzoyl-O-Dibenzyloxyphosphoryl-L-Serine Benzyl Ester (Fig. B5)**

1H-tetrazole (0.065 g, 0.93 mmol) was added to a solution of (Fig. B2) (0.09 g, 0.3 mmol) and N,N-dibenzyl diethyl phosphoroamidate[Perich, 1987 #73] (0.12 g, 0.31 mmol) (0.06 g, 0.7 mmol) in THF (1 mL), under a nitrogen atmosphere. The reaction mixture was stirred at 25°C for 30 minutes and then cooled to -40°C (dry ice-CH₃CN bath). m-Chloroperoxibenoic acid (m-CPBA) (0.1 g, 0.4 mmol) in methylene chloride (2 mL) was added at once. The reaction mixture was stirred at -40°C for 30 minutes, then allowed to warm up. The reaction mixture was diluted with water, extracted with Et₂O (2-10 mL) and the organic layer was washed with saturated NaHCO₃ (1-10 mL) and then dried. Concentration of the extract gave a crude product, which was chromatographed on silica (0.5” x 6” column). Elution with hexanes (100 mL), followed by 3:2 hexanes-EtOAc (200 mL), gave a colorless solid (0.096 g, 57%). ¹H NMR 4.33-4.53 (2H, m, -CHCH₂OP), 4.89-5.01 (5H, m, 2 × POCH₂Ph and CHCH₂OP), 5.16 (1H, d, J<sub>app</sub>=10.4 Hz, CO₂CH₃H₈Ph), 5.20 (1H, d, J<sub>app</sub>=10.4 Hz, CO₂CH₃H₈Ph), 7.13-7.58 (18H, m, 4 × Ph), 7.68 (1H, d, J<sub>app</sub>=6.8 Hz, NHCOPh), 7.84 (2H, d, J<sub>app</sub>=10.4 Hz, COPh). ¹³C NMR 53.7 (d, J<sub>CP</sub><sub>app</sub>=6.1 Hz), 67.5 (d, J<sub>CP</sub><sub>app</sub>=6.1 Hz), 67.9, 69.9 (d, J<sub>CP</sub><sub>app</sub>=6.1 Hz), 70.0 (d, J<sub>CP</sub><sub>app</sub>=6.1 Hz), 127.5, 128.2, 128.3, 128.4, 128.5, 128.7, 128.8, 128.9, 129.0, 129.1, 133.5, 135.3, 136.1 (d, J<sub>CP</sub><sub>app</sub>=9 Hz), 136.4
(d, $J_{CP_{app}}$=9 Hz), 167.3, 169.0. $^{31}$P NMR 0.52. IR 1728, 1743, 3307. Anal. Calcd. for C$_{31}$H$_{30}$NO$_7$P: C, 66.53 %; H, 5.41 %; N, 2.50 %. Found: C, 66.27 %; H, 5.36 %; N, 2.57 %.

**N-Benzoyl-O-Phosphoryl-L-Serine (Fig. B6)**

A solution of (Fig. B5) (0.35 g, 0.63 mmol) and 10% Pd-C (0.05 g) in EtOAc (5 mL) was stirred under hydrogen atmosphere in a parr apparatus at 40 psi overnight. The reaction mixture was filtered and concentrated to yield a crude product. Recrystallization from acetone and ether gave a colorless hygroscopic solid (0.14 g, 78%). $^1$H NMR 4.12-4.20 (1H, m, CH$_2$H$_2$OP), 4.24-4.32 (1H, m, CHCH$_3$H$_2$OP), 4.62-4.69 (1H, m, -CHCH$_2$OP), 7.32-7.41 (2H, m, COPh), 7.42-7.51 (1H, m, COPh), 7.74 (2H, d, $J_{app}$=10.4 Hz, COPh). $^{13}$C NMR 53.6 (d, $J_{CP_{app}}$=7.6 Hz), 64.8 (d, $J_{CP_{app}}$=7.6 Hz), 127.2, 128.6, 132.2, 132.5, 170.6, 174.9. $^{31}$P NMR (CD$_3$OD) 1.06. The molecular mass, determined by FAB MS, was 290.0443 Da and closely corresponded to that calculated for the predicted product, C$_{10}$H$_{12}$NO$_7$P, of 290.0445 Da.
Figure A

Synthesis of \( N-(\text{Cyclohexane Carboxyl})-O-\text{Phospho-L-Serine} \)
Figure B

Synthesis of N-Benzoyl-O-Phospho-L-Serine
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