CONVENTIONAL AND UNCONVENTIONAL APPROACHES TO
DRUG DESIGN AND DELIVERY

by

Arlette Nicole Dinaut

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Chemistry
University of Toronto

© Copyright by Arlette Nicole Dinaut 2001
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-63598-8
Abstract for:

Conventional and Unconventional Approaches to Drug Design and Delivery

Ph.D. Thesis by Arlette Nicole Dinart, 2001

Department of Chemistry, University of Toronto

Compounds bearing two difluoromethylenephosphonic acid (DFMP) groups, which are non-hydrolyzable phosphate mimetics, were examined as inhibitors of protein tyrosine phosphatase 1B (PTP1B), a therapeutically significant enzyme. These inhibitors were prepared by electrophilic fluorination of benzylic phosphonates or by CuCl-promoted coupling of (diethylphosphonyl)difluoromethyl cadmium reagent to aryl iodides. Several of these compounds exhibited low micromolar IC₅₀'s with PTP1B, and were considerably better inhibitors than α,α-difluorobenzylphosphonic acid. In collaboration with Prof. Zongchao Jia at Queen’s University, the crystal structures of two of these compounds complexed with PTP1B were obtained. These studies revealed that the additional DFMP group did not bind to a postulated second phosphate binding site in PTP1B. The selectivity of our best PTP1B inhibitors was also examined in collaboration with Merck-Frosst Canada. Generally, selectivity was modest to good when compared to receptor-like PTPases but poor when compared to cytosolic PTPases. The implication of these results concerning the utility of compounds bearing two phosphate mimetics as PTP1B inhibitors is discussed.

The use of catalytic antibodies as activators of N-methyl carbamate-based tripartate prodrugs was also examined. A tripartate prodrug was designed to spontaneously release a drug by a 1,6 elimination reaction after enzyme-catalyzed hydrolysis of the N-methyl carbamate moiety. Monoclonal antibodies were raised to a phosphoramidate transition state analogue resembling the transition state for the hydrolysis of the N-methyl carbamate portion.
of the prodrug system. Several antibodies were found to hydrolyze a model monocarbamate substrate that resembled the N-methyl carbamate portion of the prodrug. With this substrate, the most active of these antibodies, called ST51, exhibited a rate enhancement of 6500-fold and exhibited multiple turnover at pH 9.0. This is the first example of antibody-catalyzed hydrolysis of an N-methyl carbamate, one of the most difficult hydrolytic reactions ever catalyzed by an abzyme on a carbonyl derivative. ST51 could also activate a model N-methyl carbamate-based tripartate prodrug with a rate enhancement of 5000-fold with multiple turnover at pH 9.0. This is the first example of antibody-catalyzed substrate activation via a tandem hydrolysis/1,6-elimination process. The implication of these studies concerning the utility of N-methyl carbamate-based prodrugs for antibody-catalyzed prodrug activation is discussed.
Acknowledgements

First and foremost, I would like to extend my sincere gratitude to my supervisor, Professor Scott Taylor, for his guidance and support, both inside and outside of the laboratory. Professor Taylor has helped me define and achieve my research goals, and always took the time to listen to my ideas and provide feedback. His consistent advice, encouragement, and confidence were essential for the completion of this thesis, and his support is greatly appreciated.

I would like to thank the faculty and staff of the Departments of Chemistry at the University of Toronto and the University of Waterloo for the use of facilities, and for their support of my doctoral research. I would also like to extend my appreciation to Merck-Frosst Canada for the provision of certain chemicals, and for their collaboration with our group on select projects. Special thanks go to the members of the Taylor Group, both present and past, with whom I have had the pleasure of working, and the faculty and staff of the University of Toronto at Mississauga campus. I would like to thank Carmen Leung and Jenn Steere for providing me with accommodation in Waterloo, a sympathetic ear, and perspective. The University of Toronto, NSERC, OGSST, and the Canadian Federation of University Women are gratefully acknowledged for their generous financial support of my doctoral studies.

My parents, Dave and Janette Dinaut, have always been there to listen, to encourage, and support me through everything. This degree is as much theirs as it is mine! I would like to express my sincere appreciation to my husband Warren Lasiuk, my sisters Amy and Jennifer, and Jen's husband Randy, as well as Shannon (and Jack), and my other dear friends near and abroad, for their loving support. They have suffered through the setbacks and celebrated the successes every step of the way with me.
# Table of Contents

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>vii</td>
</tr>
</tbody>
</table>

**Chapter 1: Overview**

**Chapter 2: Design, Synthesis, and Characterization of Inhibitors of PTP1B**

2.1 Introduction

2.1.1 General Background on Protein Tyrosine Phosphatases 4
2.1.2 Catalytic Domain of PTPases 6
2.1.3 PTP1B 16
2.1.4 Early Inhibitors of PTP1B 22
2.1.5 Specific Objectives 26

2.2 Experimental

2.2.1 Materials and Methods 28
2.2.2 Synthesis of Inhibitors of PTP1B 30
2.2.3 Kinetic Studies with PTP1B 53

2.3 Results and Discussion

2.3.1 Naphthyl-based PTP1B Inhibitors 55
2.3.2 Biphenyl- and Triphenyl-based PTP1B Inhibitors 67
2.3.3 Crystallographic Studies 75
2.3.4 Bis-DFMP Inhibitors Bearing Alternative Linker Arms 83
2.3.5 The Second Phosphate Binding Site – Is It Real? 92
2.3.6 Selectivity and the Second Phosphate Binding Site 95
2.3.7 PTP1B Inhibitors Recently Reported by Other Groups 97
2.3.8 Future Directions 100

2.4 References 103

**Chapter 3: Antibody-Catalyzed Activation of a Tripartate Prodrug**

3.1 Introduction

3.1.1 Catalytic Antibodies 113
3.1.2 Antibody-Directed Abzyme Prodrug Therapy 124
3.1.3 Objectives 136

3.2 Experimental

3.2.1 Materials and Methods 140
3.2.2 Kinetic Studies with Carbamate Derivatives 3.42-3.46, and Carbamate 3.54 140
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.3</td>
<td>Synthesis of Transition State Analogue</td>
<td>141</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Conjugation of TSA to Bovine Serum Albumin and Keyhole Limpet Hemocyanin</td>
<td>145</td>
</tr>
<tr>
<td>3.2.5</td>
<td>Preparation of Standard Curves for Detection and Quantitation of PNP by HPLC</td>
<td>147</td>
</tr>
<tr>
<td>3.2.6</td>
<td>Initial Screening for Catalytic Activity of Antibodies with Substrate</td>
<td>148</td>
</tr>
<tr>
<td>3.2.7</td>
<td>Inhibition Studies with mAbs ST51, ST30, and ST24</td>
<td>149</td>
</tr>
<tr>
<td>3.2.8</td>
<td>Purification of ST51 from Ascites for Purpose of Detailed Kinetic Studies</td>
<td>149</td>
</tr>
<tr>
<td>3.2.9</td>
<td>Preparation of ST51 Fab</td>
<td>150</td>
</tr>
<tr>
<td>3.2.10</td>
<td>Testing for Catalytic Activity of BSA with Substrate</td>
<td>151</td>
</tr>
<tr>
<td>3.2.11</td>
<td>Screening for ST51 Catalytic Activity at Various pH</td>
<td>151</td>
</tr>
<tr>
<td>3.2.12</td>
<td>Stability tests of ST51 at pH 9.0 and 10.0</td>
<td>152</td>
</tr>
<tr>
<td>3.2.13</td>
<td>Detailed Kinetic Studies with ST51 and Substrate at pH 9.0 and 10.0</td>
<td>153</td>
</tr>
<tr>
<td>3.2.14</td>
<td>K_D Studies with ST51 and TSA using Indirect Competitive Enzyme-Linked Immunosorbant Assay</td>
<td>154</td>
</tr>
<tr>
<td>3.2.15</td>
<td>Synthesis of Ester, Amide, Carbamate and Carbonate Substrates</td>
<td>157</td>
</tr>
<tr>
<td>3.2.16</td>
<td>Rapid Screen for Catalytic Activity of ST51 with Various Carbamate, Ester, Amide and Carbonate Derivatives</td>
<td>172</td>
</tr>
<tr>
<td>3.2.17</td>
<td>Synthesis of Model Tripartate Prodrug Substrate</td>
<td>177</td>
</tr>
<tr>
<td>3.2.18</td>
<td>Preparation of Standard Curves for Detection and Quantitation of PNP and Trp by HPLC</td>
<td>182</td>
</tr>
<tr>
<td>3.2.19</td>
<td>Rapid Screen for Catalytic Activity with ST51 and Z-Tryptophan</td>
<td>183</td>
</tr>
<tr>
<td>3.2.20</td>
<td>Detailed Kinetic Studies with ST51 and Model Prodrug Substrate</td>
<td>183</td>
</tr>
<tr>
<td>3.3</td>
<td>Results and Discussion</td>
<td>185</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Prodrug Design</td>
<td>185</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Transition State Analogue Design and Synthesis</td>
<td>189</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Monoclonal Antibody Production and Screening</td>
<td>192</td>
</tr>
<tr>
<td>3.3.4</td>
<td>Is Enhanced Hydrolysis a Result of Abzyme Catalysis?</td>
<td>200</td>
</tr>
<tr>
<td>3.3.5</td>
<td>pH, Turnover, and Stability Studies</td>
<td>207</td>
</tr>
<tr>
<td>3.3.6</td>
<td>Determination of Michaelis-Menten Parameters with ST51 and Substrate</td>
<td>209</td>
</tr>
<tr>
<td>3.3.7</td>
<td>Determination of the K_D for the TSA and the Relationship Between k_cat/k_uncat and K_{m}K_D</td>
<td>213</td>
</tr>
<tr>
<td>3.3.8</td>
<td>Studies with Other Potential Substrates</td>
<td>223</td>
</tr>
<tr>
<td>3.3.9</td>
<td>Activation of a Model &quot;Prodrug&quot; Substrate by ST51</td>
<td>237</td>
</tr>
<tr>
<td>3.3.10</td>
<td>Conclusions, Perspectives, and Future Work</td>
<td>247</td>
</tr>
<tr>
<td>3.4</td>
<td>References</td>
<td>257</td>
</tr>
</tbody>
</table>

vi
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAPT</td>
<td>Antibody-directed abzyme prodrug therapy</td>
</tr>
<tr>
<td>ADEPT</td>
<td>Antibody-directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>c\text{H}</td>
<td>Constant region of the heavy chain of an antibody</td>
</tr>
<tr>
<td>c\text{L}</td>
<td>Constant region of the light chain of an antibody</td>
</tr>
<tr>
<td>CPG2</td>
<td>Carboxypeptidase G2</td>
</tr>
<tr>
<td>DAST</td>
<td>Dialkylaminosulfur trifluoride</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DFMP</td>
<td>(\alpha,\alpha)-difluoromethylene phosphonate</td>
</tr>
<tr>
<td>DIPC</td>
<td>Diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DIPEA</td>
<td>Diisopropylethylamine</td>
</tr>
<tr>
<td>DMAP</td>
<td>Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>EDC</td>
<td>1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EI</td>
<td>Electron impact</td>
</tr>
<tr>
<td>F\text{AB}</td>
<td>Fast atom bombardment</td>
</tr>
<tr>
<td>F\text{c}</td>
<td>Fragment of constant region of an antibody</td>
</tr>
<tr>
<td>FDP</td>
<td>Fluorescein diphosphate</td>
</tr>
<tr>
<td>HOBt</td>
<td>1-hydroxybenzotriazole</td>
</tr>
<tr>
<td>KDA</td>
<td>Potassium diisopropylamide</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>KPi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>m-CPBA</td>
<td>Meta-chloroperbenzoic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectra</td>
</tr>
<tr>
<td>NaHMDS</td>
<td>Sodium hexamethyldisilazane</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NFBS</td>
<td>N-fluorobenzenesulfonimide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>P-loop</td>
<td>Phosphate binding loop housing the signature motif of PTPases</td>
</tr>
<tr>
<td>Pmp</td>
<td>Phosphonomethyl phenylalanine</td>
</tr>
<tr>
<td>PNA</td>
<td>(p)-nitroaniline</td>
</tr>
<tr>
<td>PNP</td>
<td>(p)-nitrophenol</td>
</tr>
<tr>
<td>PNPP</td>
<td>(p)-nitrophenylphosphate</td>
</tr>
<tr>
<td>pSer</td>
<td>Phosphoserine</td>
</tr>
<tr>
<td>pThr</td>
<td>Phosphothreonine</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PTK's</td>
<td>Protein tyrosine kinases</td>
</tr>
<tr>
<td>PTP1B</td>
<td>Protein tyrosine phosphatase 1B</td>
</tr>
<tr>
<td>PTPases</td>
<td>Protein tyrosine phosphatases</td>
</tr>
<tr>
<td>pTyr</td>
<td>Phosphotyrosyl/phosphotyrosine</td>
</tr>
<tr>
<td>r.t.</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Sat'd</td>
<td>Saturated</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Ser/Thr phos</td>
<td>Serine/threonine phosphatases</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TMS</td>
<td>Trimethylsilyl</td>
</tr>
<tr>
<td>TNBS</td>
<td>Trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>TS</td>
<td>Transition state</td>
</tr>
<tr>
<td>TSA</td>
<td>Transition state analogue</td>
</tr>
<tr>
<td>vH</td>
<td>Variable region of the heavy chain of an antibody</td>
</tr>
<tr>
<td>vL</td>
<td>Variable region of the light chain of an antibody</td>
</tr>
<tr>
<td>WpD loop</td>
<td>Moveable loop in PTPases containing Trp and Asp</td>
</tr>
</tbody>
</table>
1. **OVERVIEW**

The design of new drugs, and new methods by which these drugs can be delivered to specific cells/tissues, is of central importance to modern medicine. This thesis deals with these two issues. One project outlined herein deals with the process of preparing and analyzing inhibitors of a therapeutically significant enzyme in the hope that information gleaned from such studies can be used for designing novel therapeutics. In this project, conventional medicinal chemistry tactics are employed, such as the synthesis and screening of substrate analogues, to obtain the desired inhibitors. The other project described in this thesis deals with the problem of site-specific drug delivery. Here we employ a rather unconventional approach to solving this problem. More specifically, we examine the possibility of using catalytic antibodies as vehicles for site-specific prodrug activation.

The project on inhibitor design involves an enzyme known as protein tyrosine phosphatase 1B (PTP1B). Proteins are phosphorylated on tyrosine residues in a reversible and dynamic process in vivo, and two classes of enzymes modulate the phosphorylation state of such proteins. Protein tyrosine kinases (PTK's) catalyze the formation of phosphotyrosyl residues in proteins and in peptides, and protein tyrosine phosphatases (PTPases) are responsible for dephosphorylation of phosphotyrosyl residues in these substrates. The process of protein tyrosine phosphorylation and dephosphorylation is known to play a crucial role in various cellular events, such as cell signaling, cell growth, tissue differentiation, and immune response. There is now considerable evidence that PTP1B is involved in the down-regulation of insulin signaling. Consequently, this enzyme is considered to be a primary target for therapeutic intervention for the treatment of non-insulin dependent diabetes mellitus, also known as type II diabetes. The goal of this project is to design inhibitors of
this enzyme in the hope that information arising from such studies can be used for designing new drugs for the treatment of diabetes. Based on our knowledge of the substrate specificity of PTP1B, its biological role, and previous inhibition studies that have been reported with this enzyme, we believed that compounds bearing two \( \alpha,\alpha \)-difluoromethylene phosphonic acid (DFMP) groups, which are non-hydrolyzable phosphate mimetics, would be potent and selective inhibitors of PTP1B. Thus, a series of naphthyl-, biphenyl-, and triphenyl-based inhibitors bearing two DFMP groups were constructed and examined as inhibitors of PTP1B. Our results indicated that such compounds are indeed relatively potent inhibitors of this enzyme, however, the selectivity of such compounds for PTP1B versus certain other PTPases is limited.

As mentioned above, the second project in this thesis involves using catalytic antibodies for site-specific activation of anticancer prodrugs. Our long-term goal is finding the “magic bullet” - a compound that will kill cancer cells without affecting healthy cells. One can envision an ideal drug delivery vehicle: a human, bispecific antibody possessing two completely different binding sites. One site would exhibit specificity for tumour-associated antigens, while the other would be a catalytic site, capable of activating the cytotoxic agent. Before such long-term goals can be realized, it was necessary to ascertain the feasibility of such an approach using simple model systems. The specific goal of this project was to determine if an abzyme can be obtained that is capable of activating an \( N \)-methyl carbamate-based tripartate prodrug. Towards this end, a tripartate prodrug was designed such that it would spontaneously release a drug by a 1,6-elimination reaction after abzyme-catalyzed hydrolysis of the \( N \)-methyl carbamate moiety. Monoclonal antibodies were raised to a phosphoramidate transition state analogue (TSA), which resembled the
transition state for the hydrolysis of the N-methyl carbamate portion of the prodrug system. From this pool of antibodies, an abzyme was obtained that was found to also be capable of activating a model N-methyl carbamate-based tripartate prodrug. These results are significant in that this is the first example of antibody-catalyzed hydrolysis of an N-methyl carbamate, one of the most difficult hydrolytic reactions ever catalyzed by an abzyme on a carbonyl derivative. Moreover, this is the first example of antibody-catalyzed activation of a substrate by a tandem hydrolysis/1,6-elimination process. However, although the abzyme was capable of catalyzing the desired reaction, it did not exhibit a sufficient rate enhancement for use as a prodrug activator under physiological conditions. Indeed, it appears that N-methyl carbamate hydrolysis is too challenging a reaction for abzyme catalysis. Nevertheless, these results provide a basis on which to design other tripartate prodrug systems that are more amenable to antibody catalysis.
2. DESIGN, SYNTHESIS, AND CHARACTERIZATION OF INHIBITORS OF PTP1B

2.1 INTRODUCTION

2.1.1 General Background on Protein Tyrosine Phosphatases (PTPases)

Protein phosphorylation on tyrosine (Tyr) residues is a reversible and dynamic process in vivo. The phosphorylation state of tyrosine residues in proteins is governed by the opposing actions of two families of enzymes: protein tyrosine kinases (PTK's), which catalyze the formation of phosphotyrosyl (pTyr) residues, and protein tyrosine phosphatases (PTPases), which are responsible for dephosphorylation of pTyr residues in these substrates (Scheme 2.1.1).

![Scheme 2.1.1. Opposing actions of PTPases and PTK's.](image)

Although tyrosine phosphorylation accounts for only 0.01 to 0.05% of all phosphorylated proteins, protein tyrosine phosphorylation and dephosphorylation is known to play a crucial role in various cellular events, such as cell signaling, cell growth, tissue differentiation, and immune response. The strength and duration of signals transmitted is dependent on the level of tyrosine phosphorylation, which is modulated by the action of PTK's and PTPases. Although both classes of enzymes are important, the present study is concerned with the
FIPases only. PTPases can effectively act as "on" and "off" switches during signal transduction. Based on genomic information to date, it is estimated that there are between 100 and 500 PTPases encoded by the human genome, and defective or overactive operation of these enzymes has been implicated in a number of disease states, including diabetes, cancer, and immune disorders. Thus, given the wide variety of PTPases and their important roles in cellular processes, much effort has been devoted to the elucidation of the form, function, and regulation of PTPases and their involvement in human diseases.

PTPases and Serine/Threonine (Ser/Thr) phosphatases represent the two classes of protein phosphatases, which are distinguished by their differences in substrate specificity. The Ser/Thr phosphatases hydrolyze phosphate groups from Ser/Thr residues, whereas PTPases, as aforementioned, dephosphorylate Tyr residues. Amino acid sequence alignments of the catalytic domains of PTPases with those of the Ser/Thr phosphatases indicate that, although these two classes of enzymes catalyze the same chemical reaction, there is no sequence similarity between the two families (<5% homology). Protein Ser/Thr phosphatases are two-metal-ion metalloenzymes that catalyze the hydrolysis of phosphate monoesters by direct attack of an activated water molecule at the phosphorus atom of a substrate. PTPases, however, are non-metalloenzymes that effect catalysis via the formation of a phosphocysteine intermediate. Thus, these two classes of enzymes use quite different approaches to catalyze the hydrolysis of phosphate monoesters.

Based on their cellular localization, PTPases have been classified as either receptor-like or intracellular. The receptor-like PTPases, such as CD45, consist of a single transmembrane region, highly conserved tandem intracellular catalytic domains, and
extracellular domains that are thought to be involved in cell signaling. The first intracellular domain accounts for the majority of catalytic activity, whereas the function of other catalytic domains remains unknown.1.5.21 Intracellular, or cytosolic, PTPases, such as Yersinia PTPase22 and protein tyrosine phosphatase 1B (PTP1B),23 contain a single catalytic domain, with various noncatalytic amino- and carboxy-terminal extensions that are thought to target the enzymes or regulate their catalytic activity.5.21

2.1.2 Catalytic Domain of PTPases

Although PTPases can be composed of more than 400 amino acids, their catalytic domains are generally contained within a region consisting of about 250 amino acids.3 This is the only structural element within these enzymes that exhibits amino acid sequence identity from bacteria to mammals.3.24 An important feature of the catalytic domain is what is commonly referred to as the PTPase active site “signature motif”,1.3.5.21 which is the sequence (His/Val)-Cys-(X)5-Arg-(Ser/Thr), or (H/V)C(X)5R(S/T), where X can be any amino acid. This signature motif was also found in two additional groups of phosphatases that can bring about pTyr hydrolysis, thus a more recent classification of PTPases includes the VH1-like dual specificity phosphatases25 and the low-molecular weight phosphatases.36.37 The dual specificity phosphatases accept phosphoserine (pSer) and phosphothreonine (pThr) protein substrates, in addition to pTyr-containing proteins,28.29 and have recently been shown to dephosphorylate the critical pThr and pTyr residues within MAP kinases.30 The low-molecular weight phosphatases accept primarily pTyr-containing substrates, with weaker activities associated with pSer/pThr proteins.5.26.31 There is little amino acid sequence homology between the classic PTPases (receptor-like and intracellular) and these two recent additions to the PTPase superfamily.3.5 The only similarities among these three groups of
phosphatases are the relative locations of the Cys and Arg residues in the active site signature motif and the common mechanism they use catalyze the hydrolysis of phosphate monoesters.\textsuperscript{3,5}

To date, several three-dimensional structures of active PTPase domains have been determined, including PTP1B,\textsuperscript{32,33} the Yersinia PTPase,\textsuperscript{34-36} PTP\textalpha,\textsuperscript{37} PTP\mu,\textsuperscript{38} LAR,\textsuperscript{39} SHP-1,\textsuperscript{40,41} SHP-2,\textsuperscript{42} and the dual specificity phosphatase VHR.\textsuperscript{43} PTPases are $\alpha + \beta$ proteins containing tertiary folds composed of a highly twisted mixed $\beta$-sheet surrounded by $\alpha$-helices on both sides.\textsuperscript{3,5} The PTPases share common secondary structure scaffolds and close similarity in tertiary structure within their catalytic domains, despite the fact that their amino acid sequences may be quite different (e.g. Yersinia exhibits only $\approx$20\% sequence homology with mammalian PTPases).\textsuperscript{3,5} Detailed biochemical studies and the crystal structure data currently in hand have greatly enhanced the general knowledge of PTPase structure and mechanism. The core active site structural elements common to the superfamily of PTPases are the phosphate-binding loop, or P-loop,\textsuperscript{3} and an adjacent moveable loop on the surface of the enzyme that contains an amino acid residue essential for catalysis.

**The P-loop.** Residues forming the PTPase signature motif (\((H/V)C(X)_2R(S/T)\)) are located in the P-loop, the phosphate-binding loop,\textsuperscript{3} which is located between the $\beta$-turn at the carboxy-terminus of a $\beta$-strand and the first turn of an $\alpha$-helix.\textsuperscript{5} The P-loop forms the base of the phosphatase active site, which is located in a crevice $\approx 9$ Å deep for Tyr-specific phosphatases, and $\approx 6$ Å deep for VHR, on the molecular surface of the enzyme.\textsuperscript{3,5} The relatively shallow pocket of VHR can thus accommodate both \(p\text{Tyr}\) and \(p\text{Thr}/p\text{Ser}\) substrates, whereas the deeper crevice found in the classical PTPases can only accommodate the longer \(p\text{Tyr}\) residue.\textsuperscript{43} The crystal structures of PTPases complexed with various
oxanions, such as transient or phosphate, provide insight into the interactions that take place between the enzymes and their phosphorylated substrates.

The invariant Arg residue of the P-loop, is observed to form hydrogen-bonds between its guanidinium group and two of the oxygen atoms of oxanions bound in the active site of PTPases in the crystal structures mentioned above. The oxygen atoms of the oxanion are also stabilized through hydrogen-bonding to NH amides of the peptide backbone comprising the P-loop.\(^5\) Zhang and coworkers,\(^{44,45}\) as well as other groups,\(^{27,46-48}\) have used site-directed mutagenesis to ascertain the important role of the invariant Arg residue in substrate binding and particularly transition state stabilization of PTPase-catalyzed reactions. Mutations in the invariant Arg residue in the PTPase signature motif have significant effects on the catalytic activity of these enzymes. Replacing this Arg residue with Lys or Ala in \emph{Yersinia} PTPase dramatically decreased \(k_{\text{cat}}\) values, while only modestly increasing the \(K_m\) values for the substrate \(p\)-nitrophenylphosphate.\(^{44}\) The same trend was observed for PTP1B.\(^{48}\) These results suggest that while the invariant Arg plays a role in substrate binding, it serves a more important function in stabilizing the transition state of the catalyzed reaction.\(^5\)

The guanidinium group of this essential Arg is a planar moiety, and is capable of forming multiple hydrogen-bonds, thus it is ideally suited for phosphate group binding.\(^{3,5,49}\) In crystal structures of PTPase-oxanion complexes, the ability of the Arg to form hydrogen-bonds between its guanidinium group and two of the oxanion oxygens illustrates why this residue is important in substrate binding. However, it is most likely that the conserved Arg residue is ideally positioned to interact most favorably with two of the equatorial oxygen atoms present on the phosphate in the trigonal bipyramidal transition state.\(^{3,5}\) This would
explain why mutations of this essential Arg residue affect the $k_{cat}$ of PTPases more adversely than the $K_m$ values exhibited by the enzymes.

Site-directed mutagenesis studies clearly indicate that the invariant Cys residue in the signature motif of PTPases is absolutely required for phosphatase activity.\textsuperscript{3,5,22,27,28,46,50} and there is formation of a covalent phosphoenzyme intermediate when catalysis of pTyr hydrolysis occurs. CysteinyI phosphoenzyme intermediates have been observed by \textsuperscript{31}P NMR,\textsuperscript{51} through trapping experiments,\textsuperscript{27,50,52-54} and in crystal structures.\textsuperscript{55} In PTPase-tungstate complexes, the active site Cys $\gamma$ atoms of \textit{Yersinia} PTPase\textsuperscript{35} and PTP1B\textsuperscript{32} are within a few Å of the W atom, which is consistent with the Cys $\gamma$ atom acting as a nucleophile during catalysis.\textsuperscript{3,5} The apparent thiol pK$_a$ of the invariant active site Cys residue present in the PTPase signature motif is 5.4 in rat PTP1\textsuperscript{56} and 4.7 in \textit{Yersinia} PTPase.\textsuperscript{57} Thus, the invariant Cys residue exists in anionic form at physiological pH, in contrast to free cysteine, which has a pK$_a$ of 8.5. The Cys $\gamma$ is within a few Å of every amide nitrogen in the P-loop, by virtue of its position at the very center of this loop. Its orientation therein is crucial for phosphate binding and thiolate stabilization.\textsuperscript{5} The guanidinium group of the essential Arg residue within the P-loop,\textsuperscript{32} as well as the hydroxyl groups of the invariant Ser/Thr residue in the signature motif\textsuperscript{58,59} may also help to lower the pK$_a$ of the Cys residue, by stabilizing the anionic form of its side chain.\textsuperscript{3,5}

A conserved Ser or Thr residue is located in the PTPase signature motif, C-terminal to the invariant Arg residue.\textsuperscript{58} In several PTPase crystal structures, the hydroxyl group of the conserved Ser/Thr is within hydrogen-bonding distance of the $\gamma$ of the invariant Cys residue.\textsuperscript{3,5} Site-directed mutagenesis has been used to investigate the role of this conserved amino acid in stabilizing the Cys thiolate anion and facilitating the phosphatase-catalyzed
reaction. Results from mutagenesis studies of the *Yersinia* PTPase,\(^5^8\) PTP1,\(^5^6\) the dual specificity phosphatase VHR,\(^6^0\) and the low-molecular weight phosphatase Stp\(^1^6^1\) indicate that this conserved Ser/Thr residue facilitates the breakdown of the phospho-enzyme intermediate.

**The Moveable Loop (WpD Loop).** An invariant Asp residue has been established as a general acid in the catalytic mechanism of the superfamily of PTPases.\(^3^5^\)\(^2^4^\)\(^5^6^\)\(^6^2^\)\(^6^6^\) Crystal structures of PTPases complexed with oxyanions (such as tungstate) indicate that this essential Asp residue is located on a moveable loop adjacent to the P-loop, but opposite from the nucleophilic Cys residue, and points toward the bound oxyanion.\(^3^5^\)\(^3^5^\)\(^4^3^\)\(^6^7^\)\(^6^8^\) The carboxylate of the Asp residue is located within hydrogen-bonding distance of the negatively charged oxygen that represents the scissile oxygen of pTyr. It would seem that the carboxyl group of this Asp residue is perfectly positioned to act as a proton donor to the Tyr leaving group during catalysis. It has also been suggested that this Asp residue acts as a general base in the breakdown of the phospho-enzyme intermediate.\(^5^1^\)\(^5^6^\)\(^6^4^\)

A movement in the surface loop harboring this Asp residue is the only significant conformational difference between the ligand-free and ligand-bound structures of PTPases.\(^5^\) For example, in *Yersinia* PTPase, this Asp residue (Asp356) is more than 10 Å away from the phosphate binding site. When tungstate binds to this site, the moveable loop closes like a flap, and covers the active site – bringing the Asp residue 6 Å closer to the active site in the process.\(^3^5^\)\(^3^6^\) Substrate binding in PTP1B induces a similar loop closure.\(^3^3^\) This moveable surface loop has been dubbed the WpD loop, since there is little amino acid sequence homology amongst PTPases apart from the catalytic Asp residue and a Trp residue, which is located near the hinge portion of the loop.\(^5^\) The closing of this WpD loop upon substrate
binding, bringing the Asp residue close to the active site and, consequently, the scissile oxygen of the substrate, is thought to be part of the catalytic mechanism. This "closed, active" conformation of the WpD loop is stabilized through interactions with the substrate. Following catalysis, this loop would have to assume its "open, inactive" form in order to allow for the release of the phosphate product. Because it has been observed in so many members of the PTPase family, this mechanism of ligand-induced loop closure is likely to be common to the PTPase superfamily. However, this has not yet been confirmed for the dual specificity phosphatases and the low-molecular weight phosphatases, since only the ligand-bound crystal structures have been resolved.\(^5\)

It has been observed that the dynamic behavior of the WpD loop in *Yersinia* PTPase is ligand-dependent.\(^5\) Whereas in the absence of ligand, the wild-type *Yersinia* PTPase alternates between its closed and open forms of the moveable loop over five orders of magnitude faster than its \(k_{\text{cat}}\) value, ligand binding dramatically reduces the rate of loop opening.\(^5\) It has been suggested that the dynamics of the moveable loop may be directly related to the catalytic power of PTPases,\(^5,36\) which varies by over six orders of magnitude.\(^69\) WpD loop dynamics may be in part dependent on the amino acid sequence of the \(\beta\)-turn within this loop, which is quite divergent amongst PTPases.\(^5\)

**Mechanism of Catalysis.** Numerous biochemical studies have led to the conclusion that hydrolysis of phosphate monoesters catalyzed by classical PTPases, as well as dual-specificity and low-molecular weight phosphatases, proceeds by a common mechanism, shown in Scheme 2.1.2.\(^3,5\) The PTPase-catalyzed reaction proceeds *via* a double displacement mechanism. In the first step, the phosphoryl group of the substrate is
transferred to the active site Cys residue, forming a thiophosphoenzyme intermediate, which is then hydrolyzed by water in the second step of the catalytic mechanism.\textsuperscript{3,5,51,53,54,70}

\[ \text{Scheme 2.1.2. General catalytic mechanism of all PTPases.}^{3,5} \]

The basic kinetic mechanism of PTPases can be described as follows:\textsuperscript{3,5} substrate (ROPO\textsubscript{3}\textsuperscript{2−}) binding leads to the formation of an enzyme-substrate complex (E*ROPO\textsubscript{3}\textsuperscript{2−}). The WpD loop then undergoes a conformational change to bring the general acid Asp close to the scissile oxygen of the substrate (E*ROPO\textsubscript{3}\textsuperscript{2−}), which, after attack of the Cys thiolate, releases its leaving group (ROH). The covalent intermediate, resulting from phosphoryl transfer to the nucleophilic Cys residue in the active site (E-PO\textsubscript{3}\textsuperscript{2−}), is then hydrolyzed by water, to produce the non-covalent enzyme phosphate complex (E*PO\textsubscript{3}\textsuperscript{2−}). Inorganic phosphate then leaves the active site, completing the cycle, and the PTPase is ready to act on another substrate molecule. Scheme 2.1.3 illustrates the catalytic mechanism of PTPases, with specific reference to residues directly involved in catalysis of phosphate monoester hydrolysis.\textsuperscript{5}
Scheme 2.1.3. Common chemical mechanism for reaction catalyzed by superfamily of PTPases.

Both the structure of the P-loop (containing the invariant Cys and Arg residues within the PTPase signature motif, as well as the essential Ser/Thr residue) and the moveable loop, which houses the general acid Asp, are conserved within the superfamily of PTPases. Thus, from these common structures emerges a common catalytic mechanism, shown in Scheme 2.1.3. The invariant Cys residue acts as a nucleophile to attack the phosphate monoester,
forming a thiophosphate intermediate. The invariant Arg present in the signature motif is responsible for both substrate and transition state stabilization. Protonation of the ester oxygen atom in the leaving group by the conserved Asp residue in the moveable loop stabilizes the transition state and facilitates formation of the cysteinyl-phosphate intermediate. Once formed, the thiophosphoryl linkage is then hydrolyzed by a water molecule, which approaches from the same side on which the leaving group departed. The Asp residue that functioned as a general acid in the formation of the phosphoenzyme intermediate then acts as a general base to activate a water molecule for the dephosphorylation reaction. The conserved Ser/Thr present in the signature motif facilitates hydrolysis of the thiophosphate moiety through its stabilization of the developing negative charge on the Cys 5.

The proposed transition state structures for the double displacement mechanism by which PTPases catalyze phosphate monoester hydrolysis are shown in Scheme 2.1.4. Site-directed mutagenesis, in combination with isotope effect studies and Brønsted correlations, suggests that these transition states are highly dissociative in nature, both for the formation and hydrolysis of the phosphoenzyme intermediate. In the formation of the thiophosphoryl linkage, the P-O bond to the leaving group is largely broken, proton transfer to the leaving group oxygen is quite advanced (the departing phenol is without charge), and the central phosphoryl group resembles metaphosphate in structure. The hydrolysis of the phosphoenzyme intermediate proceeds through a similarly dissociative transition state.5
Scheme 2.1.4. Proposed transition state structures for formation and hydrolysis of the phosphoenzyme intermediate during PTPase-catalyzed phosphate monoester hydrolysis.\(^{5}\)

The dissociative transition state in the formation of the P-S linkage is stabilized by the Asp residue in the WpD loop, which acts as a proton donor to the leaving group.\(^{5,64,66,71-73}\) Site-directed mutagenesis studies indicate that the role of Asp as a general acid in the first step of the double-displacement reaction catalyzed by PTPases is actually more important than its role as a general base in the hydrolysis of the phosphoenzyme intermediate.\(^{64}\) In the transition state of the dephosphorylation step, however, P-S bond breakage is advanced,\(^{58,61}\) and there is a detrimental effect on the rate of P-S hydrolysis in the absence of the active site hydroxyl group,\(^{58,60,61}\) since the Ser/Thr located in the signature motif stabilizes the developing charge on the Cys \(\text{S'}\). The decrease in the rate of P-S hydrolysis is greater when
the hydroxyl group is eliminated than the corresponding rate decrease in P-S formation. The essential Arg residue in the signature motif is ideally positioned to stabilize the $sp^2$ hybridization of the PO$_3$ oxygens in the metaphosphate-like transition states. Thus, all of these stabilizing factors lower the energy of the transition states and contribute to the rate enhancements in the PTPase-catalyzed hydrolysis of phosphate monoesters.

PTPases catalyze their reactions in two chemical steps, and there is some debate as to which of these steps is rate-limiting for classical PTPases and dual-specificity phosphatase-catalyzed reactions. While hydrolysis of the phosphoenzyme intermediate is thought to be the rate-determining step for low-molecular weight phosphatases, the rate-limiting step in reactions catalyzed by the other members of the superfamily of PTPases may vary with the form of the enzyme used in the experiment, the substrate, as well as other experimental variables.

2.1.3 PTP1B

This thesis is concerned mainly with a PTPase known as PTP1B. PTP1B is an intracellular PTPase consisting of a single catalytic domain. This enzyme is expressed in a wide variety of human tissues, and is one of the most studied of all the PTPases. PTP1B was the first PTPase to be isolated in homogenous form, and its three dimensional structure has been determined in complex with various ligands. Although this enzyme has been purified from human placenta as a protein of 321 amino acid residues, the cDNA would indicate that this protein is the amino-terminal portion of a larger molecule consisting of 435 residues. The conserved catalytic domain that is found in all PTPases is located within residues 30 to 278 of this protein, and the carboxy-terminal, non-catalytic portion of PTP1B
serves a regulatory function. The last 35 carboxy-terminal amino acid residues target PTP1B to the cytoplasmic face of the endoplasmic reticulum. The three dimensional structure of the 37-kD form of PTP1B consists of a single domain, with the polypeptide chain organized into eight α-helices and twelve β-strands. The catalytic Cys residue is located within the PTPase signature motif (His214 to Ser222) on loop 15, the P-loop, connecting the carboxy-terminus of β-12 to the amino-terminus of α-4. PTP1B catalyzes the hydrolysis of pTyr-containing peptides and proteins using the same general mechanism as previously described.

**PTP1B and Peptide Substrates.** Numerous studies have been carried out to determine the substrate specificity of PTP1B, both with PTP1B and with the structural homologue of human PTP1B, rat PTP1. Substrate recognition studies with rat PTP1 are equivalent to those with PTP1B, as their catalytic domains are 97% identical. PTP1B is unable to dephosphorylate proteins that are phosphorylated on Ser and Thr residues, and phosphotyrosine alone is a relatively poor substrate for this enzyme, with a \( K_m \) of 5 mM. PTP1B exhibits a wide range of \( k_{cat}/K_m \) values for relatively short peptide substrates, and these values can approach the diffusion-controlled limit for some pTyr-containing molecules. As the values for \( k_{cat}/K_m \) are 3 to 4 orders of magnitude greater than for pTyr alone, this suggests that residues adjacent to the pTyr moiety in substrates can contribute to binding and catalysis by PTP1B. Using synthetic pTyr-containing peptides that correspond to natural phosphorylation sites in proteins, the undecapeptide DADE(pY)LIPQQG, which was modeled on the autophosphorylation site (residues 988-998) of the epidermal growth factor receptor (EGFR), was found to be an optimal peptide substrate for PTP1B. The \( k_{cat}/K_m \) value for this peptide and PTP1B approaches the diffusional limit. PTP1B displays a
preference for acidic residues N-terminal to the pTyr, and requires a minimum of six amino acid residues (DADE(pY)L) for efficient substrate binding and catalysis. As well, the presence of a pTyr residue is necessary for binding; PTPases will not bind dephosphorylated peptides.

Professor Zongchao Jia and coworkers have obtained the crystal structure of (C21S-PTP1B) (catalytically inactive mutant) in complex with pTyr and with the high-affinity peptides DADE(pY)L-NH₂ [EGFR-(988-993)] and Ac-DE(pY)L-NH₂ [EGFR(990-993)] (Km = 3.2 and 20 μM). Unlike the PTP1B three-dimensional structure with and without tungstate present, binding of either phosphopeptide or pTyr to PTP1B caused a conformational change in the WpD loop, bringing the general acid Asp residue 8 Å closer to the pTyr in the active site. The closed conformation of the loop was stabilized by hydrophobic interactions between the side chain of Phe182 on the moveable loop and the phenyl ring of the pTyr, and other nonpolar residues also line the recognition site for the phenyl ring of the pTyr moiety. These hydrophobic interactions were not possible in the PTP1B-tungstate complex, which could be the reason for the open-loop conformation being adopted by the enzyme in this structure. As well, hydrogen-bonds and salt bridges provide stabilization for the closed-loop conformation of the PTP1B pTyr/phosphopeptide complex. The binding pocket, amphipathic in character due to the nonpolar residues lining the site for the pTyr aromatic ring and invariant Arg221 lining the base of the pocket, is exactly the depth of a pTyr residue (= 9 Å), which explains why PTP1B cannot hydrolyze pSer- and pThr-containing substrates. These phosphorylated residues cannot reach the catalytic amino acids at the base of the cleft.
Residues at the P-4 and P-2 through P+1 sites of the peptide substrates form interactions with PTP1B, and offer clues as to why this enzyme prefers acidic residues N-terminal to the pTyr. Peptide binding causes the guanidinium group of Arg47 to move by 3.5 Å and form salt bridges with the P-2 and P-1 positions of the peptide. Arg47 also forms a long hydrogen-bond with the main-chain carbonyl at P-4. The interactions of Arg47 with acidic side chains would explain why PTP1B exhibits a preference for negatively charged residues N-terminal to the pTyr group.

PTP1B and Non-peptidyl Substrates. PTPases, including PTP1B, will catalyze the hydrolysis of aliphatic and aromatic phosphorylated species, even if the phosphorylated moieties are not contained within a peptide-based molecule. In general, aromatic substrates are better substrates for PTP1B than aliphatic molecules, with alkyl phosphates exhibiting \( k_{\text{cat}} \) values orders of magnitude lower than aryl phosphates.\(^8\) While \( \beta \)-naphthyl phosphate has a lower \( K_m \) than phenyl phosphate for rat PTP1, it has been noted that substitution of the phenyl phosphate ring with certain substituents can result in a lowering of the \( K_m \) to levels less than that of \( \beta \)-naphthyl phosphate.\(^8\) The general trend has been that \textit{ortho} substituents on the phenyl ring, and, to a lesser extent, \textit{meta}-substituted groups exhibit higher \( K_m \) values than their \textit{para}-substituted counterparts, which has been attributed to steric hindrance.\(^8\) PTP1 seems to bind substrates possessing negatively charged groups distal to the phosphate group with higher affinities.

Early studies by Merck-Frosst with PTP1B have demonstrated that fluoresceindiphosphate (2.3) has a significantly lower \( K_m \), and hydrolysis of the first phosphate group occurs much faster, than fluoresceinmonophosphate (2.4).\(^8\) Zhang and coworkers have also observed a similar phenomenon with phenolphthalein di- and
monophosphate (2.5 and 2.6, respectively). Thus, bisphosphorylated substrates display lower $K_m$ values with PTP1B than their monophosphorylated counterparts. This observation could be due to the favorable binding interactions between PTP1B and acidic residues N-terminal to the pTyr moiety, or there could be other as-of-yet undiscovered interactions between PTP1B and bisphosphorylated substrates that might explain why they are bound with higher affinity.

**PTP1B and Disease States.** At the time we began this work, several reports had appeared indicating that overexpression of PTP1B occurs in certain cancers and in insulin-resistant states. In fact, considerable evidence had accumulated that implicated PTP1B in the down-regulation of insulin signaling by dephosphorylating specific pTyr residues on the insulin receptor kinase.

A pictorial description of the proposed role of PTP1B in insulin signaling is shown in Scheme 2.1.5. The insulin receptor is a tyrosine kinase composed of two extracellular ligand-binding α subunits linked by disulfide bonds to two transmembrane β subunits containing tyrosine kinase domains. When insulin binds to the α subunits, this activates the intrinsic tyrosine kinase activity of the β subunits, which results in autophosphorylation of critical tyrosine residues (Tyr1146, Tyr1150, and Tyr1151) contained within the regulatory domain. This completely activates the tyrosine kinase activity of the insulin receptor, which
then goes on to phosphorylate its various substrates, such as the insulin receptor substrate 1, in order to propagate the insulin signal. To terminate insulin action, even after insulin has left the receptor, dephosphorylation of the insulin receptor, as well as insulin receptor substrates, is required.

![Diagram of insulin signaling cascade](image)

**Scheme 2.1.5.** Proposed role of PTP1B in insulin signaling.

Earlier studies by Kusari and coworkers indicated that PTP1B complexed with the insulin receptor in vivo, and that the association of PTP1B with the insulin receptor was
absolutely dependent on receptor autophosphorylation. The binding of PTP1B to the receptor was also inhibited by phosphopeptides modeled after the kinase domain of the insulin receptor: DIPYETDPYPYRK. Note that in the sequence reported for the insulin receptor kinase domain there are three pTyr residues, two of which are side-by-side (pTyr 1150 and 1151). Since evidence points to the involvement of PTP1B in dephosphorylation and inactivation of the insulin receptor, any changes in the expression levels of PTP1B relative to the insulin receptor could affect insulin signaling. As aforesaid, PTP1B has been shown to be overexpressed in insulin-resistant states. Type 2 diabetes mellitus is a very serious disease state that affects 80% of people with diabetes. This form of diabetes is characterized by a resistance of normally insulin-sensitive tissues such as muscle, liver, and fat, to insulin action. Thus, overexpression of PTP1B could represent a causal effect in such insulin-resistant states. Therefore, we became specifically interested in designing inhibitors of PTP1B, anticipating that these inhibitors might have therapeutic potential for the treatment of diabetes. As well, PTP1B-specific inhibitors could be utilized to further study the role of this important enzyme in complex signal transduction pathways.

2.1.4 Early Inhibitors of PTP1B

The first attempts to design PTPase-specific inhibitors were based on peptide substrate structure and substrate-enzyme interactions. PTPases exhibit a wide range of \( k_{cat}/K_m \) values for relatively short peptide substrates. Thus, once a high-affinity substrate is found for a specific PTPase, replacement of the pTyr residue in the PTPase peptide substrate with a non-hydrolyzable pTyr mimetic could result in a very potent and specific PTPase inhibitor. This strategy was used by Terrence Burke and coworkers to develop an exceptionally potent peptide-based inhibitor of PTP1B. Burke's strategy was to incorporate
phosphonomethyl phenylalanine ((D/L)-Pmp, 2.7, see below) and difluorophosphonomethyl phenylalanine ((L)-F$_2$-Pmp, 2.8) into a high-affinity peptide sequence for PTP1B, AcDADE(M)L-amide, where M indicates the position of the pTyr mimic in the peptide and all other amino acids therein are of the L-configuration. Pmp is a phosphonic acid that is isosteric with its parent compound pTyr, yet is chemically and enzymatically resistant to P-C bond cleavage. The peptide sequence containing the (D/L)-Pmp residue, where the scissile oxygen was replaced with a methylene moiety, had an IC$_{50}$ of 200 μM for the PTP1B-catalyzed dephosphorylation of the autophosphorylated insulin receptor. The estimated IC$_{50}$ of the enantiomerically pure (L)-Pmp-containing peptide was 100 μM, due to the preference of PTP1B for L-amino acid peptides. It has been suggested that an α,α-difluoromethylenephosphonate (DFMP) group is an effective phosphate mimic because it is both isosteric and isopolar to the phosphate group. When a difluoromethylene group was put in place of the methylene group, as in the case of the (L)-F$_2$-Pmp-containing residue, the IC$_{50}$ was decreased dramatically to 100 nM. This significant enhancement in binding affinity was initially thought to be due to a two-fold effect of the fluorine atoms. Fluorine substitution lowers the ionization constants of the phosphonate hydroxyls relative to Pmp, and provides hydrogen-bonding interactions similar to the parent oxygen-containing phosphate that are absent in the Pmp-containing peptide. However, inhibition was found to be independent of pH in the pH region spanning the second ionization constant of the phosphonates. This suggests that the monoanionic and dianionic forms bind equally well and that the enhanced potency of the F$_2$-Pmp-containing peptide over the analogous Pmp-containing molecule is not due to pK$_a$ effects, but is rather due to the ability of the fluorine atoms to interact with specific residues in the active site of PTP1B.
Not only were F₂-Pmp-containing peptides found to be good inhibitors of PTPases, these inhibitors also displayed some selectivity with certain PTPases. For example, the peptide AcDADE(F₂-Pmp)LNH₂ was a potent inhibitor of PTP1 (Kᵢ = 0.18 μM), but a much poorer inhibitor for the receptor-like PTPase LAR (Kᵢ = 376 μM). Thus, the significant differences in active site specificity for peptide substrates of PTPases can be exploited in this way to design potent and PTPase-specific inhibitors.

While peptide-based inhibitors are useful in determining important properties for substrate-PTPase interactions, there are problems associated with such molecules. Due to their proteolytic susceptibility and poor bioavailability, peptide-based inhibitors are not ideal for use as probes for cellular studies, or for the development of medicinally effective drugs. At the time we began these studies, very little work on the synthesis and evaluation of small molecule inhibitors of PTP1B had been reported. Burke and coworkers examined a series of nonpeptidyl benzylic phosphonates (2.9a-d) and naphthylphosphonates (2.10, 2.11a) as reversible inhibitors of PTP1B, PP1, and PP2A, the latter two of which are Ser/Thr phosphatases. In general, the phenylphosphonates were found to be very poor inhibitors of the PTPases. Little or no inhibition was observed with PTP1B or PP1, regardless of the substituent at the α position of the phosphonate, however, there was an increase in inhibitory
activity as the methylene unit (2.9a) was replaced with -CHOH- (2.9b), -CHF- (2.9c), and -CF₂- (2.9d) for the Ser/Thr phosphatase PP2A. The naphthyl DFMP-bearing compounds, on the other hand, were much more potent. Upon introducing the second aryl ring, both 2.10 and 2.11a inhibited PTP1B and PP2A effectively and equally well. The Kᵢ values for 2.10 and 2.11a with PTP1B were determined to be 255 and 179 μM, respectively.

![Chemical structures](image)

In order to understand the structural basis for inhibition, and to provide a springboard for the design of more potent inhibitors of PTP1B, Burke et al. obtained the crystal structure of (C215S-PTP1B) complexed with the β-substituted naphthyl DFMP derivative 2.11a. This three-dimensional structure revealed that the enhanced inhibitory activity of this naphthyl derivative over the phenyl-based inhibitors (2.9a-d) was due to extensive hydrophobic interactions formed with the naphthalene ring which were not possible with derivatives bearing a single phenyl ring. A number of X-ray crystallographic water molecules were found adjacent to the inhibitor 2.11a, bound tightly to the enzyme. It was reasoned through molecular modeling studies that the affinity of this inhibitor for PTP1B could potentially be increased by placing a hydroxyl group at the C-4 position of the naphthalene ring, in order to replace and mimic two water molecules found in this region of
the three-dimensional structure. The newly designed inhibitor, 2.11b, exhibited a $K_i$ that was 2-fold lower ($K_i = 93$ µM) in comparison to the parent molecule 2.11a.

2.1.5 Specific Objectives

While the naphthyl-based DFMP-containing derivatives (2.10 – 2.11a,b) are more potent inhibitors of PTPases than the phenyl DFMP derivative 2.9d, these competitive inhibitors are not selective for PTP1B, and their $K_i$ values are much higher than the peptide-based PTP1B inhibitors. It was clear to us that there was much room for improvement in this area. Our specific objective was to develop potent and selective inhibitors of PTP1B. Such compounds could be used as lead compounds for the development of drugs for the treatment of type II diabetes and as tools for studying signal transduction pathways.

Our approach to meeting the above objective was as follows. Burke’s studies with the naphthalene derivatives $^{102,103}$ and the observation that bisphosphorylated substrates are better substrates for PTP1B than their monophosphorylated counterparts $^{83,84}$ suggested to us that incorporation of two DFMP groups into an aryl scaffold might provide good inhibitors of PTP1B. While the preference of PTPases for acidic residues adjacent (N-terminal) to the pTyr moiety in peptide substrates $^{78,79}$ bodes well for this supposition, at least as far as potency was concerned, we also believed that potent and selective PTP1B inhibitors could be obtained with this approach. As previously mentioned, the insulin receptor kinase is believed to be an in vivo substrate for PTP1B $^{87-84}$. The autophosphorylation site of the insulin receptor acted upon by PTP1B contains several pTyr residues, two of which are side by side (pTyr 1150 and 1151). We hypothesized that PTP1B might contain two phosphate binding sites, one of which is the catalytically active site, and another, non-catalytic site, that functions to bind a second pTyr residue. This structural feature could provide a basis for the design of
inhibitors specific for PTP1B. To test this hypothesis, we constructed naphthyl- (2.12), biphenyl- (2.13), and triphenyl-based (2.14) compounds that bear two DFMP groups and examined these compounds as inhibitors of PTP1B as well as with other PTPases.
2.2 EXPERIMENTAL

2.2.1 Materials and Methods

General. Unless otherwise noted, all reagents for syntheses and biological assays were obtained from commercial suppliers (Sigma-Aldrich, Lancaster Synthesis Inc.) and were used without further purification. Certain solvents for chromatography (chloroform, dichloromethane, hexanes) were distilled before use. Tetrahydrofuran (THF) and diethyl ether (ether) were distilled from sodium/benzophenone ketyl under argon. Dichloromethane was distilled from calcium hydride under argon. Dimethylformamide (DMF) was distilled under reduced pressure from calcium hydride and stored over 4-Å molecular sieves under argon. Reactions involving moisture-sensitive reagents were executed under an atmosphere of dry argon or dry nitrogen. Liquid transfers were made using oven-dried syringes and needles. Chromatographic purifications were performed with silica gel from Silicycle (230-400 mesh, 60-Å). Melting points were obtained on an Electrothermal capillary melting point apparatus and are uncorrected. \(^1\)H, \(^{19}\)F, \(^{13}\)C, and \(^{31}\)P NMR spectra were recorded on various Varian and Bruker instruments, and the frequencies at which the spectra were obtained have been noted accordingly in the experimental details. For \(^1\)H NMR spectra run in deuterated chloroform, chemical shifts (\(\delta\)) are reported in parts per million (ppm) downfield relative to the internal standard tetramethylsilane (TMS). For \(^1\)H NMR spectra run in deuterated water (D\(_2\)O), DMSO-d6, acetone-d6, and deuterated methanol (CD\(_3\)OD), chemical shifts are reported in parts per million relative to the solvent's residual protons (D\(_2\)O, \(\delta\) 4.79; DMSO-d6, \(\delta\) 2.50 for the central peak of the quintet; acetone-d6, \(\delta\) 2.05 for the central peak of the quintet; and CD\(_3\)OD, \(\delta\) 3.31 for the central peak of the quintet). For \(^{19}\)F NMR, chemical shifts are reported in parts per million relative to trifluoroacetic acid (external), except for
those compounds that are specifically noted as being referenced to fluorobenzene (external). For $^{13}$C NMR spectra run in CDCl$_3$, chemical shifts are reported in parts per million relative to the CDCl$_3$ residual carbons ($\delta$ 77.0 for the central peak). For $^{13}$C spectra run in D$_2$O, chemical shifts are reported in parts per million relative to the CH$_3$ peak of 3-(trimethylsilyl)-1-propanesulfonic acid (external). For $^{13}$C spectra run in DMSO-$d_6$, acetone-$d_6$, and CD$_3$OD, chemical shifts are reported in parts per million relative to the solvent signal ($\delta$ 39.5, 29.8, 49.0 for central peaks, respectively). For $^{31}$P NMR spectra, chemical shifts are reported in parts per million relative to 85% phosphoric acid (external). Abbreviations s, d, t, q, qt, m, and br are used for singlet, doublet, triplet, quadruplet, quintuplet, multiplet, and broad, respectively. All NMR couplings are given in Hz. Electron impact (EI) and fast atom bombardment (FAB) mass spectra (MS) were obtained on a Micromass 70-S-250 mass spectrometer. Electrospray mass spectra were obtained using a Micromass Platform mass spectrometer. Analytical and preparative HPLC was performed using reverse-phase HPLC (Waters 600 Controller, Waters 2487 Dual Wavelength (\(\lambda\)) detector), with a Vydac #218TPS4 reverse-phase analytical column and Vydac #218TP1022 reverse-phase preparative column, respectively. A Varian Cary 1 spectrophotometer was used for enzyme kinetics studies. Ultra-pure water (Millipore®) was used for the preparation of all biological samples. Fluorescein diphosphate (FDP), PTP1B and PP2A were kindly provided by Merck-Frosst.
2.2.2 Synthesis of Inhibitors of PTP1B

**Preparation of Benzyl Bromides.** Literature procedures were used to prepare the benzyl bromide precursors 2.23b and 2.28,\textsuperscript{104} 2.41a-e and 2.42.\textsuperscript{105} Bromides 2.23a, 2.35, and \( p \)-iodobenzyl bromide are commercially available.

**Preparation of Phosphonates.** Phosphonates were prepared via an Arbuzov reaction with trimethyl or triethyl phosphite, using the following general procedure. The benzyl bromides were added to a solution of trimethyl or triethyl phosphite (5 - 15 eq.) in benzene (= 5 mL benzene per gram of benzyl bromide), and the solution was heated to reflux for 6 to 18 hours. The reaction was then cooled and benzene and unreacted phosphite were removed by vacuum distillation. The phosphonates were then obtained in pure form by subjecting the residue to silica gel chromatography or by recrystallization.

**Dimethylbenzylphosphonate (2.24a).** Purified using silica gel chromatography (methanol:chloroform = 2:98, \( R_f = 0.4 \)), clear, colorless oil, 89% yield. \( ^1 \)H NMR(200 MHz, CDCl\(_3\)): \( \delta \) 3.17 (d, \( J = 21.6 \) Hz, 2H), 3.69 (d, \( J = 10.6 \) Hz, 6H), 7.30 (m, 5H, aryl); \( ^31 \)P NMR(81 MHz, CDCl\(_3\)): \( \delta \) 26.65; \( ^{13} \)C NMR(100 MHz, CDCl\(_3\)): \( \delta \) 32.6 (d, \( J = 137.6 \) Hz), 52.5 (d, \( J = 6.6 \) Hz), 126.7 (d, \( J = 3.7 \) Hz), 128.3 (d, \( J = 2.2 \) Hz), 129.4 (d, \( J = 6.6 \) ), 131.1 (d, \( J = 9.5 \) Hz); MS (EI): \( m/z \) 200 (M\(^+\), 45%), 91 (M\(^+\) - PO(OMe)_2, 100%); HRMS(EI) calcd for C\(_9\)H\(_{13}\)O\(_3\)P 200.0602, found 200.0607.

**Dimethyl 2-naphthylmethylphosphonate (2.24b).** Purified via silica gel chromatography (ethyl acetate, \( R_f = 0.4 \)), white solid, 92% yield. \( \text{mp} = 77-78 \) °C; \( ^1 \)H NMR(200 MHz, CDCl\(_3\)): \( \delta \) 3.34 (d, \( J = 21.6 \) Hz, 2H), 3.68 (d, \( J = 11.0 \) Hz, 6H), 7.47 (m, 3H), 7.79 (m, 4H); \( ^31 \)P NMR(81 MHz, CDCl\(_3\)): \( \delta \) 26.50; \( ^{13} \)C NMR(125 MHz, CDCl\(_3\)): \( \delta \) 33.1 (d, \( J = 138.2 \) Hz), 52.9 (d, \( J = 6.8 \) Hz), 125.8 (d, \( J = 1.9 \) Hz), 126.2, 127.6 (d, \( J = 2.0 \) Hz)
Hz), 127.7 (d, J = 1.9 Hz), 127.8 (d, J = 4.9 Hz), 128.3 (d, J = 1.5 Hz), 128.5 (d, J = 8.6 Hz), 128.8 (d, J = 9.6 Hz), 132.4 (d, J = 2.8 Hz), 133.5 (d, J = 2.5 Hz); MS (EI): m/z 250 (M⁺, 68%), 141 (M⁺ - PO(OMe)₂, 100%); HRMS (EI) calcd for C₁₃H₁₅O₃P 250.0759, found 250.0757.

1,3-Bis[(dimethylphosphono)methyl]naphthalene (2.29a). Purified via silica gel chromatography (chloroform:methanol = 95:5, R_f = 0.4), white solid, quantitative yield. mp = 42-44 °C; ¹H NMR (500 MHz, CDCl₃): δ 3.31 (d, J = 21.8 Hz, 2H), 3.64 (m, 1H), 7.42 (m, 1H), 7.50 (m, 2H), 7.69 (bs, 1H), 7.81 (d, J = 7.9 Hz, 1H), 8.04 (d, J = 8.3 Hz, 1H); ³¹P NMR (81 MHz, CDCl₃): δ 26.18; ¹³C NMR (125 MHz, CDCl₃): δ 29.7 (d, J = 139.0 Hz), 32.6 (d, J = 139.0 Hz), 52.8 (m), 124.0 (bt, J = 2.2 Hz), 126.2, 128.1 (d, J = 1.8 Hz), 128.2 (t, J = 3.7 Hz), 128.3, 128.3 (td, J = 8.6 Hz, J = 3.8 Hz), 130.1 (d, J = 5.4 Hz), 130.2 (d, J = 4.4 Hz), 130.8 (m), 133.9 (t, J = 3.3 Hz); MS (EI): m/z 372 (M⁺, 98%), 263 (M⁺ - PO(OMe)₂, 100%); HRMS (EI) calcd for C₁₈H₂₂O₆P₂ 372.0892, found 372.0898.

1,5-Bis[(diethylphosphono)methyl]naphthalene (2.29b). Purified via silica gel chromatography (ethyl acetate, R_f = 0.4), white solid, 68% yield. mp = 105-107 °C; ¹H NMR (200 MHz, CDCl₃): δ 1.15 (t, J = 7.0 Hz, 12H), 3.65 (d, J = 22.0 Hz, 4H), 3.85-4.05 (m, 8H), 7.45-7.55 (m, 4H), 8.02-8.11 (m, 2H); ³¹P NMR (202.5 MHz, CDCl₃): δ 23.71; ¹³C NMR (50 MHz, CDCl₃): δ 16.2 (d, J = 5.5 Hz), 31.3 (d, J = 139.1 Hz), 62.0 (d, J = 6.9 Hz), 124.1, 125.3, 128.4 (d, J = 6.8 Hz), 128.7 (m), 132.6 (bs); MS (EI): m/z 428 (M⁺, 100%), 291 (M⁺ - PO(OEt)₂, 27%); HRMS (EI) calcd for C₂₀H₂₀O₆P₂ 428.1518, found 428.1503.

1,6-Bis[(dimethylphosphono)methyl]naphthalene (2.29c). Purified via silica gel chromatography (chloroform:methanol = 95:5, R_f = 0.5), white solid, 82% yield. mp = 76-78 °C; ¹H NMR (300 MHz, CDCl₃): δ 3.31 (d, J = 21.9 Hz, 2H), 3.56-3.70 (m, 14H), 7.38-7.50
(m, 3H), 7.69-7.76 (m, 2H), 8.02 (d, J = 8.7 Hz, 1H); $^{31}$P NMR (202.5 MHz, CDCl$_3$): $\delta$
26.10, 26.18; $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 29.8 (d, J = 139.1 Hz), 32.7 (d, J = 138.2 Hz),
52.7-52.9 (m), 124.6 (t, J = 2.3 Hz), 125.7-125.8 (m), 127.4-127.6 (m), 128.1 (d, J = 3.9 Hz),
128.3 (d, J = 7.5 Hz), 128.7 (d, J = 10.7 Hz), 129.1 (d, J = 8.7 Hz), 130.8 (m), 133.9 (t, J =
2.8 Hz); MS (EI): m/z 372 (M+, 98%), 263 (M+ - PO(OMe)$_2$, 100%); HRMS (EI) calcd for
C$_{16}$H$_{22}$O$_6$P$_2$ 372.0892, found 372.0898.

1,7-Bis[(diethylphosphono)methyl]naphthalene (2.29d). Purified via silica gel chromatography (chloroform:methanol = 95:5, R$_f$ = 0.4), white solid, quantitative yield. mp =
70-72 °C; $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 1.05-1.25 (m, 12H), 3.33 (d, J = 21.6 Hz, 2H), 3.59
(d, J = 22.0 Hz, 2H), 3.80-4.10 (m, 8H), 7.30-7.50 (m, 3H), 7.60-7.70 (m, 2H), 7.94 (s, 1H);
$^{31}$P (81 MHz, CDCl$_3$): $\delta$ 23.99, 24.13; $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 16.2 (d, J = 5.3 Hz),
16.3 (d, J = 5.3 Hz), 30.6 (d, J = 138.9 Hz), 34.2 (d, J = 138.1 Hz), 62.0 (d, J = 6.9 Hz),
124.9-125.2 (m), 127.3-127.8 (m), 128.5-128.9 (m), 129.5 (d, J = 9.2 Hz), 132.0, 132.7
(bt); MS (EI): m/z 428 (M+, 100%); HRMS (EI) calcd for C$_{26}$H$_{30}$O$_6$P$_2$ 428.1518, found
428.1517.

2,6-Bis[(diethylphosphono)methyl]naphthalene (2.29e). Purified using silica gel chromatography (methanol:chloroform = 2:98, R$_f$ = 0.3), white solid, 81% yield. mp = 123-
125 °C; $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 1.24 (t, J = 7.1 Hz, 12H), 3.30 (d, J = 21.2 Hz, 4H),
3.95-4.10 (m, 8H), 7.38-7.48 (m, 2H), 7.70-7.79 (m, 4H); $^{31}$P NMR (81 MHz, CDCl$_3$): $\delta$
24.03; $^{13}$C NMR (50 MHz, CDCl$_3$): $\delta$ 16.3 (d, J = 2.3 Hz), 35.4 (d, J = 138.6 Hz), 62.1 (d, J
= 3.2 Hz), 127.3-128.7 (m), 129.1, 132.4; MS (EI): m/z 428 (M+, 100%), 291 (M+ - PO(OEt)$_2$, 48%); HRMS (EI) calcd for C$_{26}$H$_{30}$O$_6$P$_2$ 428.1518, found 428.1507.
**2,7-Bis[(dimethylphosphono)methyl]naphthalene (2.29f).** Purified via silica gel chromatography (chloroform:methanol = 95:5, Rf = 0.4), white solid, 63% yield. mp = 98-100 °C; $^1$H NMR(200 MHz, CDCl$_3$): $\delta$ 3.30 (d, $J$ = 22.0 Hz, 4H), 3.67 (d, $J$ = 10.6 Hz, 12H), 7.40 (d, $J$ = 8.0 Hz, 2H), 7.65-7.85 (m, 4H); $^{31}$P NMR(81 MHz, CDCl$_3$): $\delta$ 26.43; $^{13}$C NMR(125 MHz, CDCl$_3$): $\delta$ 34.0 (d, $J$ = 138.2 Hz), 52.9 (d, $J$ = 6.8 Hz), 127.7 -127.8 (m), 128.0 (t, $J$ = 1.8 Hz), 128.2 (d, $J$ = 9.5 Hz), 129.2 (d, $J$ = 9.7 Hz), 131.3 (t, $J$ = 2.9 Hz), 133.5 (t, $J$ = 3.3 Hz); MS (El): m/z 372 (M$^+$, 100%), 263 (M$^+$ - PO(OMe)$_2$, 94%); HRMS(EI) calcd for C$_{16}$H$_{12}$O$_5$P$_2$ 372.0892, found 372.0903.

**2,7-Bis[(diethylphosphono)methyl]naphthalene (2.29g).** Purified via silica gel chromatography (chloroform:methanol = 95:5, Rf = 0.3), white solid, 90% yield. mp = 67-69 °C; $^1$H NMR(200 MHz, CDCl$_3$): $\delta$ 1.23 (t, $J$ = 7.0 Hz, 12H), 3.30 (d, $J$ = 21.9 Hz, 4H), 3.95-4.10 (m, 8H), 7.41 (d, $J$ = 8.0 Hz, 2H), 7.65-7.80 (m, 4H); $^{31}$P NMR(81 MHz, CDCl$_3$): $\delta$ 23.90; $^{13}$C NMR(50 MHz, CDCl$_3$): $\delta$ 16.2 (d, $J$ = 5.9 Hz), 34.0 (d, $J$ = 138.2 Hz), 62.1 (d, $J$ = 6.9 Hz), 127.4-128.3 (m), 129.6 (d, $J$ = 9.6 Hz), 131.2 (bt), 133.5 (bt); MS (El): m/z 428 (M$^+$, 100%), 291 (M$^+$ - PO(OEt)$_2$, 68%); HRMS(EI) calcd for C$_{20}$H$_{30}$O$_6$P$_2$ 428.1518, found 428.1515.

**Dimethyl 4-phenylbenzylphosphonate (2.36).** Purified using silica gel chromatography (methanol:chloroform = 1:99, Rf = 0.3), white solid, 90% yield. mp = 68-70 °C; $^1$H NMR(200 MHz, CDCl$_3$): $\delta$ 3.22 (d, $J$ = 21.7 Hz, 2H), 3.71 (d, $J$ = 10.6 Hz, 6H), 7.50 (m, 9H); $^{31}$P NMR(81 MHz, CDCl$_3$): $\delta$ 26.60; $^{13}$C NMR(125 MHz, CDCl$_3$): $\delta$ 32.44 (d, $J$ = 138.6 Hz), 52.9 (d, $J$ = 6.9 Hz), 126.9, 127.3 (m), 128.7, 130.0 (d, $J$ = 6.8 Hz), 130.1 (d, $J$ = 9.8 Hz), 139.8 (d, $J$ = 3.9 Hz), 140.5; MS (El): m/z 276 (M$^+$, 44%), 167 (M$^+$ - PO(OMe)$_2$, 100%); HRMS(EI) calcd for C$_{15}$H$_{17}$O$_3$P 276.0915, found 276.0922.
4,4'-Bis[(dimethylphosphono)methyl]biphenyl (2.43a). Purified using silica gel chromatography (methanol:chloroform = 2:98, Rf = 0.3), white solid, 47% yield. mp = 128-130 °C; 1H NMR(200 MHz, CDCl3): δ 3.21 (d, J = 21.6 Hz, 4H), 3.71 (d, J = 10.6 Hz, 12H), 7.37 (dd, J = 8.0 Hz, 2.4 Hz, 4H), 7.54 (d, J = 7.7 Hz, 4H); 31P NMR(81 MHz, CDCl3): δ 26.56; 13C NMR(125 MHz, CDCl3): δ 32.5 (d, J = 138.6 Hz), 52.9 (d, J = 6.9 Hz), 127.1, 130.0 (d, J = 9.8 Hz), 139.2; MS (EI): m/z 398 (M+, 76%), 289 (M+ - PO(OMe)2, 100%); HRMS(EI) calcd for C18H24O6P2 398.1058, found 398.1048.

1,1'-Bis[4-((dimethylphosphono)methyl)benzene)methane (2.43b). Purified using silica gel chromatography (methanol:chloroform = 5:95, Rf = 0.3), yellow oil, 76% yield. 1H NMR(200 MHz, CDCl3): δ 3.13 (d, J = 21.6 Hz, 4H), 3.67 (d, J = 11.0 Hz, 12H), 3.93 (s, 2H), 7.20 (m, 8H); 13C NMR(125 MHz, CDCl3): δ 26.79; 31P NMR(81 MHz, CDCl3): δ 26.79; 13C NMR(125 MHz, CDCl3): δ 32.3 (d, J = 138.6 Hz), 41.0, 52.8 (d, J = 6.8 Hz), 128.8 (d, J = 9.8 Hz), 129.1, 129.7 (d, J = 6.8 Hz), 139.6; MS (EI): m/z 412 (M+, 100%), 303 (M+ - PO(OMe)2, 38%), 194 (M+ - 2(PO(OMe)2), 16%); HRMS(EI) calcd for C19H26O6P2 412.1205, found 412.1216.

1,2'-Bis[4-((dimethylphosphono)methyl)benzene]ethane (2.43c). Purified via silica gel chromatography (methanol:chloroform = 4:96, Rf = 0.4), white solid, 75% yield. 1H NMR(200 MHz, CDCl3): δ 2.88 (s, 4H), 3.13 (d, J = 21.6 Hz, 4H), 3.66 (d, J = 10.6 Hz, 12H), 7.15 (m, 8H); 13C NMR(125 MHz, CDCl3): δ 32.5 (d, J = 138.4 Hz), 37.3, 52.8 (d, J = 7.3 Hz), 128.6, 128.7 (d, J = 2.9 Hz), 129.6 (d, J = 6.6 Hz), 140.35 (d, J = 3.6 Hz); MS (EI): m/z 426 (M+, 36%), 213 (100%); HRMS(EI) calcd for C26H26O6P2 426.1361, found 426.1366.

1,3'-Bis[4-((dimethylphosphono)methyl)benzene]propane (2.43d). Purified using silica gel chromatography (methanol:chloroform = 4:96, Rf = 0.3), 35% yield. Obtained as
an unresolvable mix of isomers. $^1$H NMR(200 MHz, CDCl$_3$): $\delta$ 1.80 – 2.00 (m, 2H), 2.55 – 2.75 (m, 4H), 3.05 – 3.25 (m, 4H), 3.55 – 3.75 (m, 12H), 7.05 – 7.30 (m, 8H).

1,4'-Bis[4-((dimethylphosphono)methyl)benzene]butane (2.43e). Purified using silica gel chromatography (methanol:chloroform = 2:98, $R_f$ = 0.3), white solid, 69% yield. mp = 88-90 °C; $^1$H NMR(200 MHz, CDCl$_3$): $\delta$ 1.63 (m, 4H), 2.60 (bt, 4H), 3.13 (d, $J$ = 21.2 Hz, 4H), 3.66 (d, $J$ = 11.0 Hz, 12 H), 7.15 (m, 8H); $^{31}$P NMR(81 MHz, CDCl$_3$): $\delta$ 26.90; $^{13}$C NMR(125 MHz, CDCl$_3$): $\delta$ 30.9, 31.9 (d, $J$ = 138.6 Hz), 35.3, 52.8 (d, $J$ = 6.8 Hz), 128.3 (d, $J$ = 8.8 Hz), 128.7 (d, $J$ = 2.9 Hz), 129.5 (d, $J$ = 6.9 Hz), 141.2, (d, $J$ = 3.9 Hz); MS (EI): m/z 454 (M$^+$, 100%), 227 (M$^+$ - (CH$_2$)$_2$PhCH$_2$PO(OMe)$_2$, 66%); HRMS(EI) calcd for C$_{22}$H$_{32}$O$_6$P$_2$ 454.1674, found 454.1684.

3,3'-Bis[(dimethylphosphono)methyl]biphenyl (2.44). Purified using silica gel chromatography (methanol:chloroform = 5:95, $R_f$ = 0.3), clear, colorless oil, 85% yield. $^1$H NMR(200 MHz, CDCl$_3$): $\delta$ 3.19 (d, $J$ = 20.5 Hz, 4H), 3.66 (d, $J$ = 11.7 Hz, 12H), 7.35 (m, 8H), $^{31}$P NMR(81 MHz, CDCl$_3$): $\delta$ 26.75; $^{13}$C NMR(50 MHz, CDCl$_3$): $\delta$ 32.8 (d, $J$ = 138.2 Hz), 52.6 (d, $J$ = 6.4 Hz), 125.6 (d, $J$ = 3.7 Hz), 128.6 (m), 131.9 (d, $J$ = 9.1 Hz), 141.0; MS (EI): m/z 398 (M$^+$, 100%), 289 (M$^+$ - PO(OMe)$_2$, 81%); HRMS(EI) calcd for C$_{18}$H$_{24}$O$_6$P$_2$ 398.1048, found 398.1044.

**Preparation of α,α-difluoromethylene phosphonates.** Unless otherwise noted, all fluorination reactions were performed as per the following general procedure. To prepare monodifluoromethylene phosphonate derivatives (mono-DFMP compounds), to a solution of sodium hexamethyldisilazane (NaHMDS) (Aldrich, 1.0 M in THF, 2.2 eq.) in dry THF (approximately 0.4 mL NaHMDS/mL THF) at -78 °C was added a solution of the benzylic phosphonates (1.0 eq.) in dry THF (approximately 15-20 mL THF/mmol phosphonate) over a
period of 2 min. The resulting orange to dark red solution (sometimes a suspension forms) was stirred for 1 h at -78 °C. A solution of N-fluorobenzenesulfonamide (NFBS) (Aldrich, 2.5 eq.) in dry THF (approximately 2-4 mL THF/mmol NFBS) was added over a period of two minutes, during which time the solution (suspension becomes a solution) turned from dark red or orange to yellow-brown. After addition, the solution was stirred for 1-2 hours and then allowed to warm to -30 °C during which time a precipitate formed. The reaction was quenched with 0.01 N HCl and the resulting solution (precipitate dissolves) was extracted with ethyl acetate. The organics were combined and washed with a solution of NaHCO₃ (5% aqueous), brine, and dried (MgSO₄) and concentrated by rotary evaporation to give a yellow oil which was purified by flash chromatography. For the formation of bis-difluoromethylenephosphonate derivatives (bis-DFMP compounds), 5.5 eq. NaHMDS and 7.3 eq. NFBS were used.

[(Dimethylphosphono)difluoromethyl]benzene (2.25a). Purified via silica gel chromatography (hexane:ethyl acetate = 70:30, Rf = 0.3), colorless oil, 63% yield. ¹H NMR(200 MHz, CDCl₃): δ 3.78 (d, J = 10.6 Hz, 6H), 7.50 (m, 5H); ³¹P NMR(81 MHz, CDCl₃): δ 6.52 (t, J = 116.7 Hz); ¹⁹F NMR(188 MHz, CDCl₃): δ -31.6 (d, J = 116.7 Hz); ¹³C NMR(125 MHz, CDCl₃): δ 54.9 (m), 118.3 (td, J = 263.4 Hz, J = 218.8 Hz), 126.1 (td, J = 6.8 Hz, J = 2.4 Hz), 128.5, 130.9 (d, J = 1.4 Hz), 132.3 (td, J = 22.0 Hz, J = 13.9 Hz); MS (EI): m/z 236 (M⁺, 14%), 127 (M⁺ - PO(OMe)₂, 100%); HRMS(EI) calcd for C₉H₁₁F₂O₃P 236.0414, found 236.0424.

Dimethyl difluoro(2-naphthyl)methylphosphonate (2.25b). Purified via silica gel chromatography (hexanes:ethyl acetate = 60:40, Rf = 0.5), white solid, 70% yield. mp = 52-54 °C; ¹H NMR(200 MHz, CDCl₃): δ 3.84 (d, J = 10.6 Hz, 6H), 7.56 (m, 2H), 7.69 (d, J =
8.8 Hz, 1H), 7.92 (m, 3H), 8.15 (s, 1H); $^{31}$P NMR(81 MHz, CDCl$_3$): $\delta$ 6.67 (t, $J$ = 116.7 Hz);
$^{19}$F NMR(188 MHz, CDCl$_3$): $\delta$ 5.96 (d, $J$ = 116.7 Hz) {referenced to fluorobenzene, external}; $^{13}$C NMR(100 MHz, CDCl$_3$): $\delta$ 54.9 (d, $J$ = 7.4 Hz), 118.3 (td, $J$ = 262.1 Hz, $J$ = 217.7 Hz), 122.5 (t, $J$ = 6.2 Hz), 126.4 (td, $J$ = 8.1 Hz, $J$ = 2.9 Hz), 126.8, 129.6 (td, $J$ = 22.0 Hz, $J$ = 13.9 Hz), 132.4, 134.1 (d, $J$ = 1.4 Hz); MS (EI): m/z 286 (M$^+$, 28%), 177 (M$^+$ - PO(OMe)$_2$, 100%); HRMS(EI) calcd for C$_{13}$H$_{13}$F$_2$O$_3$P 286.0570, found 286.0570.

1,3-Bis[(dimethylphosphono)difluoromethyl]naphthalene (2.18a). Purified via silica gel chromatography (hexanes:ethyl acetate = 20:80, $R_f$ = 0.3), yellow oil, 55% yield.
$^1$H NMR(200 MHz, CDCl$_3$): $\delta$ 3.84 (m, 12H), 7.65 (m, 2H), 7.97 (m, 2H), 8.27 (bs, 1H).
8.48 (d, $J$ = 8.4 Hz, 1H); $^{11}$P NMR(81 MHz, CDCl$_3$): $\delta$ 6.20 (t, $J$ = 116.7 Hz);
$^{19}$F NMR(188 MHz, CDCl$_3$): $\delta$ 5.23 (d, $J$ = 115.2 Hz), 11.18 (d, $J$ = 112.9 Hz) {referenced to fluorobenzene, external}; $^{13}$C NMR(100 MHz, CDCl$_3$): $\delta$ 55.0 (d, $J$ = 7.4 Hz), 117.8 (td, $J$ = 263.7 Hz, 219.0 Hz), 119.4 (td, $J$ = 265.0 Hz, 216.7 Hz), 122.8 (m), 125.8 (t, $J$ = 5.1 Hz), 127.2, 128.5 (weak td), 128.7, 129.3 (weak td), 129.5, 130.3-130.6 (m), 133.2; MS (EI): m/z 444 (M$^+$, 33%), 335 (M$^+$ - PO(OMe)$_2$, 100%); HRMS(EI) calcd for C$_{13}$H$_{13}$F$_2$O$_3$P 444.0515, found 444.0518.

1,5-Bis[(diethylphosphono)difluoromethyl]naphthalene (2.18b). Purified using silica gel chromatography (hexanes:ethyl acetate = 20:80, $R_f$ = 0.4), white solid, 48% yield.
mp = 102-103 °C; $^1$H NMR(200 MHz, CDCl$_3$): $\delta$ 1.28 (t, $J$ = 7.0 Hz, 12H), 4.00-4.30 (m, 8H), 7.62 (t, $J$ = 7.9 Hz, 2H), 7.87 (d, $J$ = 7.0 Hz, 2H), 8.65 (d, $J$ = 8.8 Hz, 2H); $^{31}$P NMR(81 MHz, CDCl$_3$): $\delta$ 4.47 (t, $J$ = 113.7 Hz); $^{19}$F NMR(188 MHz, CDCl$_3$): $\delta$ 11.88 (d, $J$ = 113.7 Hz) {referenced to fluorobenzene, external}; $^{13}$C NMR(100 MHz, CDCl$_3$): $\delta$ 16.3 (d, $J$ = 5.9 Hz), 64.8 (d, $J$ = 6.6 Hz), 119.6 (td, $J$ = 265.1 Hz, $J$ = 216.7 Hz), 125.1, 126.6 (td, $J$ = 11.0 Hz).
Hz, J = 3.6 Hz), 128.9 (td, J = 19.8 Hz, J = 13.2 Hz), 129.8 (bt), 130.6; MS (EI): m/z 500 (M+, 40%), 363 (M+ - PO(OEt)2, 100%); HRMS(EI) calcd for C20H28F2O8P2 500.1141, found 500.1125.

1,6-Bis[(dimethylphosphono)difluoromethyl]naphthalene (2.18c). Purified using silica gel chromatography (hexanes:ethyl acetate = 20:80, Rf = 0.2), yellow oil, 51% yield. 

$^1$H NMR (200 MHz, CDCl3): $\delta$ 3.75-3.90 (m, 12H), 7.59 (t, J = 7.9 Hz, 1H), 7.76 (d, J = 9.1 Hz, 1H), 7.90 (d, J = 7.3 Hz, 1H), 8.06 (d, J = 8.1 Hz, 1H), 8.17 (s, 1H), 8.52 (d, J = 9.1 Hz, 1H); $^{31}$P NMR (81 MHz, CDCl3): $\delta$ 6.33 (t, J = 115.2 Hz), 6.54 (t, $J$ = 113.7 Hz); $^{19}$F NMR (188 MHz, CDCl3): $\delta$ 5.44 (d, $J$ = 115.9 Hz), 11.57 (d, $J$ = 113.7 Hz) (referenced to fluorobenzene, external); $^{13}$C NMR (100 MHz, CDCl3): $\delta$ 54.9 (d, $J$ = 2.9 Hz), 55.0 (d, $J$ = 3.6 Hz), 118.1 (td, $J$ = 262.9 Hz, $J$ = 218.9 Hz); 119.7 (td, $J$ = 265.1 Hz, $J$ = 218.2 Hz), 123.5 (t, $J$ = 5.8 Hz), 125.5, 126.5 (t, $J$ = 5.4 Hz), 127.1 (td, $J$ = 10.2 Hz, $J$ = 3.6 Hz), 128.0 (td, $J$ = 10.2 Hz, $J$ = 2.9 Hz), 128.4 (m), 130.1 (td, $J$ = 22.0 Hz, $J$ = 13.9 Hz), 130.6, 133.0, 133.2; MS (EI): m/z 444 (M+, 30%), 335 (M+ - PO(OME)2, 100%); HRMS(EI) calcd for C16H18F2O8P2 444.0515, found 444.0504.

1,7-Bis[(diethylphosphono)difluoromethyl]naphthalene (2.18d). Purified using silica gel chromatography (hexanes:ethyl acetate = 20:80, Rf = 0.5), white solid, 46% yield.

$^1$H NMR (200 MHz, CDCl3): $\delta$ 1.20-1.40 (m, 12H), 4.05-4.35 (m, 8H), 7.61 (t, $J$ = 7.7 Hz, 1H), 7.76 (d, $J$ = 8.4 Hz, 1H), 7.85-8.05 (m, 3H), 8.66 (s, 1H); $^{31}$P (81 MHz, CDCl3): $\delta$ 4.34 (t, $J$ = 114.4 Hz), 3.98 (t, $J$ = 114.4 Hz); $^{19}$F NMR (188 MHz, CDCl3): $\delta$ 4.89 (d, $J$ = 116.0 Hz), 11.35 (d, $J$ = 114.5 Hz) (referenced to fluorobenzene, external); $^{13}$C NMR (100 MHz, CDCl3): $\delta$ 16.1-16.3 (m), 64.2 (d, $J$ = 2.2 Hz), 64.9 (d, $J$ = 2.2 Hz), 118.2 (td, $J$ = 263.6 Hz, $J$ = 217.5 Hz), 119.7 (td, $J$ = 264.3 Hz, $J$ = 217.5 Hz), 123.3 (t, $J$ = 5.5 Hz), 124.1-124.6 (m),
126.1, 127.4 (td, J = 9.6 Hz, J = 3.0 Hz), 128.8, 129.1, 129.4 (weak td), 130.8 (weak td), 131.7, 134.7; MS (EI): m/z 500 (M⁺, 31%), 363 (M⁺ - PO(OEt)₂, 100%); HRMS(EI) calcd for C₂₀H₂₆F₄O₆P₂ 500.1141, found 500.1119.

2,6-Bis[(diethylphosphono)difluoromethyl]naphthalene (2.18e). Purified using silica gel chromatography (hexanes:ethyl acetate = 20:80, Rf = 0.4), white solid, 23%. mp = 77-79 °C; ¹H NMR(200 MHz, CDCl₃): δ 1.32 (t, J = 7.0 Hz, 12H), 4.05-4.35 (m, 8H), 7.76 (d, J = 8.4 Hz, 2H), 8.00 (d, J = 8.4 Hz, 2H), 8.17 (s, 2H); ³¹P NMR(81 MHz, CDCl₃): δ 4.07 (t, J = 115.2 Hz); ¹⁹F NMR(188 MHz, CDCl₃): δ 4.78 (d, J = 115.2 Hz) (referenced to fluorobenzene, external); ¹³C NMR(100 MHz, CDCl₃): δ 16.3 (d, J = 5.8 Hz), 64.9 (d, J = 7.3 Hz), 118.0 (td, J = 263.7 Hz, J = 218.2 Hz), 123.7 (t, J = 5.1 Hz), 126.3 (btd), 129.2, 131.7 (td, J = 22.0 Hz, J = 13.9 Hz), 133.1; MS (EI): m/z 500 (M⁺, 33%), 363 (M⁺ - PO(OEt)₂, 100%); HRMS(EI) calcd for C₂₀H₂₆F₄O₆P₂ 500.1141, found 500.1147.

2,7-Bis[(diethylphosphono)difluoromethyl]naphthalene (2.18f). Purified using silica gel chromatography (hexanes:ethyl acetate = 20:80, Rf = 0.3), white solid, 57% yield. mp = 71-72 °C; ¹H NMR(200 MHz, CDCl₃): δ 3.81 (d, J = 10.7 Hz, 12H), 7.74 (d, J = 8.8 Hz, 2H), 7.94 (d, J = 8.7 Hz, 2H), 8.19 (s, 2H); ³¹P NMR(81 MHz, CDCl₃): δ 6.24 (t, J = 115.2 Hz); ¹⁹F NMR(188 MHz, CDCl₃): δ 5.61 (d, J = 115.2 Hz) (referenced to fluorobenzene, external); ¹³C NMR(100 MHz, CDCl₃): δ 55.0 (d, J = 6.6 Hz), 118.1 (td, J = 263.7 Hz, J = 219.0 Hz), 124.4 (t, J = 5.5 Hz), 127.2 (td, J = 8.0 Hz, J = 3.6 Hz), 128.4, 130.8 (td, J = 21.9 Hz, J = 13.1 Hz), 131.4, 134.9; MS (EI): m/z 444 (M⁺, 20%), 335 (M⁺ - PO(OMe)₂, 100%); HRMS(EI) calcd for C₁₆H₁₄F₄O₆P₂ 444.0515, found 444.0507.

2,7-Bis[(diethylphosphono)difluoromethyl]naphthalene (2.18g). Purified using silica gel chromatography (hexanes:ethyl acetate = 35:65, Rf = 0.4), pale yellow oil, 46%. ¹H
NMR(200 MHz, CDCl₃): δ 1.31 (t, J = 7.0 Hz, 12H), 4.05-4.30 (m, 8H), 7.77 (d, J = 8.8 Hz, 2H), 8.00 (d, J = 8.4 Hz, 2H), 8.20 (s, 2H); ³¹P NMR(81 MHz, CDCl₃): δ 4.05 (t, J = 114.5 Hz); ¹⁹F NMR(188 MHz, CDCl₃): δ 4.93 (t, J = 115.1 Hz); ¹³C NMR(100 MHz, CDCl₃): δ 16.3 (d, J = 5.9 Hz), 64.9 (d, J = 6.6 Hz), 117.9 (td, J = 263.7 Hz, J = 218.3 Hz), 124.5 (t, J = 5.5 Hz), 127.2 (td, J = 7.3 Hz, J = 2.9 Hz), 128.3, 131.0 (td, J = 22.7 Hz, J = 13.9 Hz), 131.4, 134.8; MS (EI): m/z 500 (M⁺, 41%), 363 (M⁺ - PO(OEt)₂, 100%); HRMS(EI) calcd for C₂₀H₇₆F₄O₈P₂ 500.1141, found 500.1124.

4-[4-((Dimethylphosphono)difluoromethyl)phenyl]benzene (2.37). Purified using silica gel chromatography (hexanes:ethyl acetate = 70:30, Rf = 0.3), white solid, 59% yield. mp = 75-78 °C; ¹H NMR(200 MHz, CDCl₃): δ 3.87 (d, J = 10.2 Hz, 6H), 7.60 (m, 9H); ³¹P NMR(81 MHz, CDCl₃): δ 6.64 (t, J = 117.0 Hz); ¹⁹F NMR(188 MHz, CDCl₃): δ -31.3 (d, J = 117.0 Hz); ¹³C NMR(100 MHz, CDCl₃): δ 54.9 (d, J = 6.6 Hz), 118.3 (td, J = 263.4 Hz, J = 219.4 Hz), 126.6 (td, J = 6.6 Hz, J = 2.2 Hz), 127.3 (b dd), 128.0, 128.9, 131.2 (td), 140.0, 143.8 (d, J = 2.2 Hz); MS (EI): m/z 312 (M⁺, 19%), 203 (M⁺ - PO(OMe)₂, 100%); HRMS(EI) calcd for C₁₅H₁₅F₂O₃P 312.0727, found 312.0730.

4,4'-Bis[(dimethylphosphono)difluoromethyl]biphenyl (2.45a). Purified using silica gel chromatography (hexanes:ethyl acetate = 20:80, Rf = 0.45), white crystalline solid, 21% yield. mp = 92-94 °C; ¹H NMR(200 MHz, CDCl₃): δ 3.85 (d, J = 10.6 Hz, 12H), 7.69 (m, 8H); ³¹P NMR(81 MHz, CDCl₃): δ 6.41 (t, J = 116.7 Hz); ¹⁹F NMR(188 MHz, CDCl₃): δ -31.8 (d, J = 116.7 Hz); ¹³C NMR(125 MHz, CDCl₃): δ 55.0 (d, J = 6.8 Hz), 118.2 (td, J = 262.8 Hz, J = 218.9 Hz), 126.8 (td, J = 3.9 Hz, J = 2.0 Hz), 127.4, 132.0 2 (td, J = 22.4 Hz, J = 13.7 Hz), 142.5; MS (EI): m/z 470 (M⁺, 14%), 361 (M⁺ - PO(OMe)₂, 100%), 252 (M⁺ - 2(PO(OMe)₂), 39%); HRMS(EI) calcd for C₁₈H₂₆F₄O₃P₂ 470.0671, found 470.0678.
1,1'-Bis[4-((dimethylphosphono)difluoromethyl)benzene]methane (2.45b). Purified using silica gel chromatography (hexanes:ethyl acetate = 20:80, Rf = 0.3), yellow oil, 16.5%. \(^1\)H NMR(200 MHz, CDCl\(_3\)): \(\delta\) 3.83 (d, \(J = 10.3\) Hz, 12H), 4.06 (s, 2H), 7.27 (d, \(J = 8.1\) Hz, 4H), 7.55 (d, \(J = 8.1\) Hz, 4H); \(^31\)P NMR(81 MHz, CDCl\(_3\)): \(\delta\) 6.58 (t, \(J = 117.0\) Hz); \(^19\)F NMR(188 MHz, CDCl\(_3\)): \(\delta\) -31.4 (d, \(J = 117.0\) Hz); \(^13\)C NMR(125 MHz, CDCl\(_3\)): \(\delta\) 41.4, 54.9, 118.1, 126.3, 129.1, 130.4, 130.1, 130.4, 126.3, 126.3, 129.1, 130.4, 130.4; MS (EI): \(m/z\) 484 (M\(^+\), 14%), 375 (M\(^+\) - PO(OMe)\(_2\), 100%).

1,2'-Bis[4-((dimethylphosphono)difluoromethyl)benzene]ethane (2.45c). Purified via silica gel chromatography (hexanes:ethyl acetate = 20:80, Rf = 0.3), white solid, 37% yield. \(^1\)H NMR(200 MHz, CDCl\(_3\)): \(\delta\) 2.97 (s, 4H), 3.79 (d, \(J = 10.6\) Hz, 12H), 7.23 (d, \(J = 8.0\) Hz, 4H), 7.50 (d, \(J = 8.0\) Hz, 4H); \(^31\)P NMR(81 MHz, CDCl\(_3\)): \(\delta\) 6.64 (t, \(J = 117.5\) Hz); \(^19\)F NMR(188 MHz, CDCl\(_3\)): \(\delta\) -31.5 (d, \(J = 117.5\) Hz); \(^13\)C NMR(125 MHz, CDCl\(_3\)): \(\delta\) 37.2, 54.9, 118.2, 126.1, 128.6, 130.1, 144.2; MS (EI): \(m/z\) 489 (M\(^+\), 24%), 389 (M\(^+\) - PO(OMe)\(_2\), 35%), 249 (M\(^+\) - 249, 100%); HRMS(EI) calcd for C\(_{20}\)H\(_{20}\)F\(_4\)O\(_6\)P\(_2\) 498.0984, found 498.0973.

1,4'-Bis[4-((dimethylphosphono)difluoromethyl)benzene]propane (2.45d). Purified using silica gel chromatography (hexanes:ethyl acetate = 20:80, Rf = 0.35), 21% yield. Obtained as an unresolvable mixture of isomers. \(^1\)H NMR(200 MHz, CDCl\(_3\)): \(\delta\) 1.80 - 2.05 (m, 2H), 2.60 - 2.95 (m, 4H), 3.50 - 4.00 (m, 12H), 7.20 - 7.60 (m, 8H).

1,4'-Bis[4-((dimethylphosphono)difluoromethyl)benzene]butane (2.45e). Purified using silica gel chromatography (hexanes:ethyl acetate = 20:80, Rf = 0.35), white solid, 64%
yield. mp = 99-101 °C; $^1$H NMR(200 MHz, CDCl$_3$): $\delta$ 1.66 (bs, 4H), 2.67 (bt, 4H), 3.82 (d, $J$ = 10.2 Hz, 12H), 7.25 (d, $J$ = 7.4 Hz, 4H), 7.52 (d, $J$ = 7.3 Hz, 4H); $^{31}$P NMR(81 MHz, CDCl$_3$): $\delta$ 6.75 (t, $J$ = 118.3 Hz); $^{19}$F NMR(188 MHz, CDCl$_3$): $\delta$ 31.0 (d, $J$ = 118.3 Hz); $^{13}$C NMR(100 MHz, CDCl$_3$): $\delta$ 30.7, 35.5, 54.8 (d, $J$ = 7.4 Hz), 118.3 (td, $J$ = 262.9 Hz, $J$ = 219.7 Hz), 126.1 (td, $J$ = 7.3 Hz, $J$ = 2.2 Hz), 128.6, 129.9 (td, $J$ = 21.9 Hz, $J$ = 13.1 Hz), 145.5; MS (EI): $m/z$ 526 (M+, 12%), 417 (M$^+$ - PO(OOMe)$_2$, 13%), 93 (M$^+$ - 433, 100%); HRMS(EI) calcd for C$_{22}$H$_{29}$F$_7$O$_4$P$_2$: 526.1297, found 526.1319.

3,3'-Bis[(dimethylphosphono)difluoromethyl]biphenyl (2.46). Purified via silica gel chromatography (ethyl acetate:hexanes = 80:20, R$_f$ = 0.3), pale yellow oil, 42% yield. $^1$H NMR(200 MHz, CDCl$_3$): $\delta$ 3.87 (d, $J$ = 10.3 Hz, 12H), 7.30 (m, 8H); $^{31}$P NMR(81 MHz, CDCl$_3$): $\delta$ 6.60 (t, $J$ = 116 Hz); $^{19}$F NMR(188 MHz, CDCl$_3$): $\delta$ 31.5 (d, $J$ = 116 Hz); $^{13}$C NMR(50 MHz, CDCl$_3$): $\delta$ 54.6 (d, $J$ = 7.3 Hz), 118.13 (td, $J$ = 263.5 Hz, $J$ = 215.9 Hz), 124.9 (bt), 125.5 (bt), 129.1, 129.7, 136.0 (m), 140.6; MS (EI): $m/z$ 470(M$^+$, 37%), 361 (M$^+$ - PO(OOMe)$_2$, 100%); HRMS(EI) calcd for C$_{18}$H$_{26}$F$_7$O$_4$P$_2$: 470.0671, found 470.0671.

Preparation of Ammonium Salts or Free Acids of Phosphonic Acids. Deprotection of the phosphonate esters was accomplished using the following general procedure. To a solution of the phosphonate ester in dry dichloromethane (approximately 1 mL dichloromethane per 0.1 mmol phosphonate) was added TMSBr (approximately 1.5 eq. TMSBr per methyl or ethyl group). The solution was stirred at room temperature for 12 h (for methyl esters) or at reflux for 36 hours (for ethyl esters). The solution was concentrated and the residue was subjected to high vacuum for several hours. The residue was then dissolved in dichloromethane (approximately 1 mL dichloromethane per 0.1 mmol phosphonate) and a solution of NH$_4$HCO$_3$ (2 eq. per silyl ester moiety) in water.
(approximately 15 mL water per gram of NH₄HCO₃) was added, or an equal volume of water alone was added. The biphasic mixture was stirred vigorously for 30-60 minutes and the organic layer was removed by rotary evaporation. The aqueous layer was then lyophilized, several times where NH₄HCO₃ was used, leaving the desired phosphonic acids in essentially quantitative yield as their ammonium salts (fluffy white powders) or free acids (relatively dense white powders).

**α,α-Difluorobenzylphosphonic acid, ammonium salt (2.9d).** Prepared using the general procedure from 2.25a. ¹H NMR(200 MHz, D₂O): δ 7.49 (m, 3H), 7.62 (M, 2H); ³¹P NMR(81 MHz, D₂O): δ 5.83 (t, J = 91.6 Hz); ¹⁹F NMR(188 MHz, D₂O): δ -27.5 (d, J = 93.8 Hz); ¹³C NMR(100 MHz, D₂O): δ 122.8 (weak td), 126.7 (bt), 129.0, 130.4, 137.2 (btd); FABMS: m/z 207 (M²⁺ + 1H⁺, 100%).

**α,α-Difluoro(2-naphthyl)methylphosphonic acid (2.11a).** Prepared using the general procedure from 2.25b. ¹H NMR(200 MHz, D₂O): δ 7.75 (bm, 3H), 8.15 (bm, 3H), 8.21 (bs, 1H); ³¹P NMR(81 MHz, DMSO-d6): δ 5.22 (t, J = 107.6 Hz); ¹⁹F NMR(188 MHz, DMSO-d6): δ 10.85 (d, J = 109.9 Hz) {referenced to fluorobenzene, external}; ¹³C NMR(100 MHz, DMSO-d6): δ 120.2 (weak td), 124.0 (bt), 126.7 (bt), 127.9, 128.5, 128.6, 128.9, 129.4, 132.4 (td, J = 21.9 Hz, J = 12.4 Hz), 132.9, 134.4; FABMS: m/z 257 (M²⁺ + 1H⁺, 100%).

**1,3-[(phosphono)difluoromethyl]naphthalene, ammonium salt (2.12a).** Prepared using the general procedure from 2.18a. ¹H NMR(200 MHz, D₂O): δ 7.45-7.55 (m, 2H), 7.90-7.98 (m, 2H), 8.14 (bs, 1H), 8.42-8.52 (m, 1H); ³¹P NMR(81 MHz, D₂O): δ 5.58 (t, J = 92.3 Hz); ¹⁹F NMR(188 MHz, D₂O): δ 9.60 (d, J = 97.7 Hz), 15.87 (d, J = 94.6 Hz) {referenced to fluorobenzene, external}; ¹³C NMR(125 MHz, D₂O): δ 120-122 (2xweak td).
122.8, 126.6, 126.7, 127.4, 128.5, 129.2, 129.9, 131.8-132.6 (m), 133.1; FABMS: m/z 387 (M⁺ + 3H⁺, 82%), 91 (100%).

1,5-[(phosphonomethyl)naphthalene, free acid (2.12b). Prepared using the general procedure from 2.18b. ¹H NMR(500 MHz, D₂O): δ 7.41 (t, J = 7.6 Hz, 2H), 7.65 (t, J = 6.6 Hz, 2H), 8.35 (d, J = 8.1 Hz, 2H); ³¹P NMR(81 MHz, D₂O): δ 4.90 (t, J = 102.3 Hz); ¹⁹F NMR(188 MHz, D₂O): δ 15.19 (d, J = 100.7 Hz) [referenced to fluorobenzene, external]; ¹³C NMR(125 MHz, D₂O): δ 121.4 (td, J = 262.0 Hz, J = 198.6 Hz), 125.2, 126.3 (t, J = 9.6 Hz), 129.0, 130.0 (m), 130.1; FABMS: m/z 387 (M⁺ - 1H⁺, 42%), 91 (100%).

2,7-[(phosphono)difluoromethyl]naphthalene, ammonium salt (2.12f). Prepared using the general procedure from 2.18f. ¹H NMR(200 MHz, D₂O): δ 7.89 (m, 2H), 8.17 (m, 2H), 8.35 (m, 2H); ³¹P NMR(81 MHz, D₂O): δ 5.07 (t, J = 98.5 Hz); ¹⁹F NMR(188 MHz, D₂O): δ 8.54 (d, J = 100.7) [referenced to fluorobenzene, external]; ¹³C NMR(100 MHz, D₂O): δ 121.9 (weak td), 125.4, 127.3, 129.0, 132.4, 134.5 (m), 134.9; FABMS: m/z 387 (M⁺ + 3H⁺, 92%), 367 (100%).

2,7-[(phosphonomethyl)naphthalene, free acid (2.31). Prepared using the general procedure from 2.29f. ¹H NMR(200 MHz, CD₃OD): δ 3.28 (d, J = 21.6 Hz, 4H), 7.43 (d, J = 8.5 Hz, 2H), 7.70-7.85 (m, 4H); ³¹P NMR(81 MHz, DMSO-d₆): δ 23.73; ¹³C NMR(100 MHz, DMSO-d₆): δ 35.9 (d, J = 131.0 Hz), 128.2, 128.4 (d, J = 8.8 Hz), 128.9 (weak td), 131.2, 132.4 (d, J = 10.3 Hz), 133.9 (bt); FABMS: m/z 315 (M⁺ - 1H⁺, 25%), 91 (100%).

2,7-[(phosphono)monofluoromethyl]naphthalene, ammonium salt (2.32). Prepared using the general procedure from 2.33. ¹³C NMR(200 MHz, D₂O): δ 5.62 (d, J = 7.3 Hz, 1H), 5.85 (d, J = 7.4 Hz, 1H), 7.67 (d, J = 8.7 Hz, 2H), 7.90-8.05 (m, 4H); ³¹P
NMR(81 MHz, D₂O): δ 11.86 (bs); ¹⁹F NMR(188 MHz, D₂O): δ -77.51 (d, J = 45.8 Hz), -77.90 (d, J = 45.8 Hz); ¹³C (125 MHz, D₂O): δ 92.2 (d, J = 157.3 Hz), 93.6 (d, J = 156.4 Hz), 125.5 (bs), 126.6 (bt), 128.4, 128.7, 133.3 (d, J = 50.5 Hz), 134.6 (d, J = 18.0 Hz); FABMS: m/z 351 (M⁺ + 3H⁺, 4%).

4-[4-((Phosphono)difluoromethyl)phenyl]benzene, ammonium salt (2.38). Prepared using the general procedure from 2.37. ¹H NMR(200 MHz, D₂O): δ 7.50 (m, 3H), 7.72 (m, 6H); ³¹P NMR(81 MHz, D₂O): δ 5.84 (bt, J = 92.0 Hz); ¹⁹F NMR(188 MHz, D₂O): δ -27.2 (d, J = 92.0 Hz); ¹³C NMR(100 MHz, D₂O): δ 123.1 (td), 127.5 (bs), 127.6 (bs), 128.1, 129.0, 130.1, 136.9 (bt), 141.0, 142.4; FABMS: m/z 283 (M⁺ + 1H⁺, 100%).

4,4'-Bis[(phosphono)difluoromethyl]biphenyl, ammonium salt (2.13a). Prepared using the general procedure from 2.45a. ¹H NMR(200 MHz, D₂O): δ 7.70 (d, J = 8.8 Hz, 4H), 7.78 (d, J = 8.4 Hz, 4H); ³¹P NMR(81 MHz, D₂O): δ 6.18 (bt); ¹⁹F NMR (188 MHz, D₂O): δ -27.5 (d, J = 93.9 Hz); ¹³C NMR(100 MHz, D₂O): δ 123.1 (weak td), 127.6 (t, J = 6.6 Hz), 137.2 (bt), 141.8; FABMS: m/z 413 (M⁺ + 3H⁺, 51%).

1,1'-Bis[4-((phosphono)difluoromethyl)benzene]methane, ammonium salt (2.13b). Prepared using the general procedure from 2.45b. ¹H NMR(200 MHz, D₂O): δ 4.02 (s, 2H), 7.32 (d, J = 7.3 Hz, 4H), 7.50 (d, J = 6.9 Hz, 4H); ³¹P NMR(81 MHz, D₂O): δ 5.81 (bt); ¹⁹F NMR(188 MHz, D₂O): δ -27.5 (d, J = 94.7 Hz); ¹³C NMR(125 MHz, D₂O): δ 41.5, 122.8 (td, J = 262.2 Hz, J = 184.5 Hz), 127.1 (bt, J = 6.8 Hz), 129.4, 135.3 (td, J = 22.7 Hz, J = 11.7 Hz), 143.8; FABMS: m/z 427 (M⁺ + 3H⁺, 100%).

1,2'-Bis[4-((phosphono)difluoromethyl)benzene]ethane, ammonium salt (2.13c). Prepared using the general procedure from 2.45c. ¹H NMR(200 MHz, D₂O): δ 2.99 (s, 4H), 7.31 (d, J = 8.1 Hz, 4H), 7.50 (d, J = 8.0 Hz, 4H); ³¹P NMR(81 MHz, D₂O): δ 5.84 (t, J =
93.9 Hz); $^{19}$F NMR(188 MHz, D$_2$O): $\delta$ -27.41 (d, $J = 96.1$ Hz); $^{13}$C NMR(125 MHz, D$_2$O): $\delta$ 37.0, 122.7 (weak td), 126.8 (bt, $J = 6.4$ Hz), 129.2, 134.9 (btd), 144.1; FABMS: $m/z$ 441 (M$^+$ + 3H$^+$, 100%).

1,3'-Bis[4-((phosphono)difluoromethyl)benzeno]propane, ammonium salt (2.13d). Prepared using the general procedure from 2.45d. Preparative HPLC purification was necessary to obtain the desired product in pure form: 100 µL injection loop, $\lambda_{det} = 265$ nm; mobile phase = 84/16 water (0.1% TFA)/acetonitrile for 40 min, ramp to 100% acetonitrile over 10 min, holding 100% acetonitrile for 15 min, ramp back to 84/16 water (0.1% TFA)/acetonitrile over 3 min, holding this solvent system for 15 min, flowrate = 5 mL/min throughout. The retention time for the desired product was 41 min. $^1$H NMR(200 MHz, D$_2$O): $\delta$ 1.94 (bt, $J = 7.3$ Hz, 2H), 2.67 (bt, $J = 7.3$ Hz, 4H), 7.30 (d, $J = 8.8$ Hz, 4H), 7.52 (d, $J = 8.7$ Hz, 4H); $^{31}$P NMR(81 MHz, D$_2$O): $\delta$ 4.98 (t, $J = 106.8$ Hz); $^{19}$F NMR(188 MHz, D$_2$O): $\delta$ 10.42 (d, $J = 94.6$ Hz) [referenced to fluorobenzene, external]; $^{13}$C NMR(125 MHz, D$_2$O): $\delta$ 33.2, 35.1, 126.8 (bt, $J = 6.4$ Hz), 129.1, 134.2 (weak td), 145.2; FABMS: $m/z$ 456 (M$^+$ + 3H$^+$, 100%).

1,4'-Bis[4-((phosphono)difluoromethyl)benzeno]butane, ammonium salt (2.13e). Prepared using the general procedure from 2.45e. $^1$H NMR(200 MHz, D$_2$O): $\delta$ 1.63 (bt, 4H), 2.67 (bt, 4H), 7.29 (d, $J = 8.0$ Hz, 4H), 7.51 (d, $J = 8.0$ Hz, 4H); $^{31}$P NMR(81 MHz, D$_2$O): $\delta$ 6.11 (t, $J = 93.5$ Hz); $^{19}$F NMR(188 MHz, D$_2$O): $\delta$ -27.2 (d, $J = 93.5$ Hz); $^{13}$C NMR(100 MHz, D$_2$O): $\delta$ 31.1, 35.6, 123.0 (weak td), 127.0 (bt, $J = 6.9$ Hz), 129.1, 135.0 (btd), 145.6; FABMS: $m/z$ 469 (M$^+$ + 3H$^+$, 100%).

3,3'-[Bis((phosphono)difluoromethyl)]biphenyl, ammonium salt (2.47). Prepared using the general procedure from 2.46. $^1$H NMR(200 MHz, D$_2$O): $\delta$ 7.58 (m, 4H), 7.77 (s,
2H), 7.91 (s, 2H); $^{31}$P NMR(81 MHz, D$_2$O): $\delta$ 6.90 (bt); $^{19}$F NMR(188 MHz, D$_2$O): $\delta$-27.3 (d, $J$ = 94.6 Hz); $^{13}$C NMR(50 MHz, D$_2$O): $\delta$ 125.3 (bt), 126.2 (bt), 129.2, 129.8, 137.4 (bt); 140.8; FABMS: m/z 413 (M$^+$ + 3H', 100%).

4,4'-Bis[phosphonomethyl]biphenyl, ammonium salt (2.49). Prepared using the general procedure from 2.43a. $^1$H NMR(200 MHz, D$_2$O): $\delta$ 3.10 (d, $J$ = 20.8 Hz, 4H), 7.45 (d, $J$ = 8.0 Hz, 4H), 7.69 (d, $J$ = 7.7 Hz, 4H); $^{31}$P NMR(81 MHz, D$_2$O): $\delta$ 21.00 (s); $^{13}$C NMR(125 MHz, D$_2$O): $\delta$ 49.1 (d, $J$ = 128.6 Hz), 140.2, 143.7 (d, $J$ = 5.7 Hz), 148.2 (d, $J$ = 8.6 Hz), 151.6 (d, $J$ = 2.9 Hz); FABMS: m/z 341 (M$^+$ + 3H', 100%).

**Synthesis of Inhibitor 2.57**

**Synthesis of 2.53.** Diethylbromodifluoromethane phosphonate (14.5 mL, 82 mmol, 4.6 eq.) was added to a stirring suspension of cadmium (10.0 g, 89 mmol, 5.0 eq.) in anhydrous DMF (50 mL) and the mixture was stirred for 4 h in a round-bottom flask fitted with a condenser (exothermic reaction). The mixture was then filtered quickly under an inert atmosphere through oven-dried Celite into a round-bottom flask containing p-iodotoluene (3.90 g, 18 mmol, 1.0 eq.) and copper (I) chloride (4.39 g, 44 mmol, 2.5 eq.) and the mixture was stirred for 12 h at room temperature. The mixture was filtered and the filtrate was transferred to a separatory funnel, to which was added CH$_2$Cl$_2$ (100 mL). The solution was washed with water (2×50 mL), a solution of NaHCO$_3$ (5% aqueous, 1×50 mL), and brine (1×50 mL), dried (anhyd. MgSO$_4$), filtered, and concentrated by rotary evaporation. The product was purified via silica gel chromatography (ethyl acetate:hexane = 30:70, Rf = 0.5) and was obtained as a colorless oil (4.05 g, 82%). $^1$H NMR (200 MHz, CDCl$_3$) $\delta$ 1.31 (t, $J$ = 7.3 Hz, 6H), 2.40 (s, 3H), 4.20 (m, 4H), 7.25 (d, $J$ = 8.8 Hz, 2H), 7.51 (d, $J$ = 7.3 Hz, 2H); $^{31}$P NMR (81 MHz, CDCl$_3$) $\delta$ 4.35 (t, $J$ = 117.5 Hz); $^{19}$F NMR (188 MHz, CDCl$_3$) $\delta$ -32.09
(d, J = 119.0 Hz); $^{13}$C NMR (50 MHz, CDCl$_3$) δ 16.1 (d, J = 3.7 Hz), 21.1, 64.5 (d, J = 6.4 Hz), 118.2 (weak td, CF$_2$), 126.1 (br t), 129.0, 129.6 (m), 140.9; MS (EI): $m/z$ 278 (M$^+$, 16%), 141 (M$^+$ - P(O)(OEt)$_2$, 100%); HRMS(EI) calcd for C$_{13}$H$_{17}$O$_3$F$_2$P 278.0883, found 278.0889.

Synthesis of 2.54. A solution of 2.53 (1.0 eq.) and N-bromosuccinimide (1.1 eq.) in benzene (60 mL per gram of 15) was irradiated using a Reflector IR Heat lamp (250 W, 120 v) for 1.5 hours. The solution was washed with water, a solution of NaHCO$_3$ (5% aqueous), brine (60 mL aqueous solution per gram of 15), dried (MgSO$_4$), and concentrated by rotary evaporation. The desired product was partially purified by silica gel chromatography (ethyl acetate:toluene = 30:70, R$_f$ = 0.4) to yield a clear, colorless oil. $^1$H NMR analysis of the crude product revealed both the desired product 2.54 as well as the undesired dibrominated material and unreacted 2.53 in a 68: 6.5 : 25.5 ratio. This compound was used without further purification for subsequent syntheses.

Synthesis of 2.56. To a solution of p-iodobenzyl bromide (2.1 g, 7.07 mmol, 3.0 eq.) in anhydrous CH$_2$Cl$_2$ (5 mL) was added ethylene glycol (0.13 mL, 2.33 mmol, 1.0 eq.) and silver (I) oxide (1.64 g, 7.08 mmol, 3.0 eq.). The reaction was stirred for 48 h at room temperature, filtered, and the filtrate was concentrated by rotary evaporation. The product was obtained as a white solid after purification via flash chromatography (ethyl acetate:hexane = 20:80, R$_f$ = 0.3) and recrystallization in dichloromethane/hexane (0.60 g, 52%). mp 75-77°C; $^1$H NMR (200 MHz, CDCl$_3$) δ 3.64 (s, 4H), 4.51 (s, 4H), 7.09 (d, J = 7.3 Hz, 4H), 7.67 (d, J = 8.7 Hz, 4H); $^{13}$C NMR (50 MHz, CDCl$_3$) δ 69.9, 72.7, 92.9, 129.5, 137.5, 138.2; MS (EI): $m/z$ 494 (M$^+$, trace), 277 (loss of CH$_2$ArI, 100%); HRMS(EI) calcd for C$_{16}$H$_{16}$O$_2$I$_2$ 493.9239, found 493.9229.
Synthesis of 2.55. Diethylbromodifluoromethane phosphonate (2.7 mL, 15.2 mmol, 9.5 eq.) was added to a stirring suspension of cadmium (1.8 g, 16.0 mmol, 10.0 eq.) in anhydrous DMF (10 mL) and the mixture was stirred for 4 h in a round-bottom flask fitted with a condenser (exothermic reaction). The mixture was then filtered quickly under an inert atmosphere through oven-dried Celite into a round-bottom flask containing 2.56 (0.8 g, 1.6 mmol, 1.0 eq.) and copper (I) chloride (0.8 g, 8.1 mmol, 5.1 eq.) and was stirred for 12 h. The mixture was filtered and the filtrate was transferred to a separatory funnel, to which was added CH₂Cl₂ (30 mL). The organic layer was washed with water (2×25 mL), a solution of NaHCO₃ (5% aqueous, 1×25 mL), and brine (1×25 mL), dried (MgSO₄), filtered, and concentrated by rotary evaporation. The product was purified via silica gel chromatography (ethyl acetate:hexane = 55:45, Rf = 0.4) and was obtained in pure form as a colorless oil (0.78 g, 80%). ¹H NMR (200 MHz, CDCl₃) δ 1.30 (t, J = 7.0 Hz, 12H), 3.69 (s, 4H), 4.17 (m, 8H), 4.62 (s, 4H), 7.43 (d, J = 8.1 Hz, 4H), 7.59 (d, J = 8.0 Hz, 4H); ³¹P NMR (81 MHz, CDCl₃) δ 4.13 (t, J = 117.5 Hz); ¹⁹F NMR (188 MHz, CDCl₃) δ -32.25 (d, J = 117.5 Hz); ¹³C NMR (50 MHz, CDCl₃) δ 16.2 (d, J = 5.5 Hz), 64.6 (d, J = 6.4 Hz), 70.0, 72.7, 118.1 (td, J = 263.5 Hz, 217.8 Hz), 126.4, 127.3, 132.0, 141.4; MS (EI): m/z 616 (M⁺ + H⁺, trace), 278 (CH₂ArCF₂P(O)(OEt)₂ + H⁺, 77%), 140 (100%).

Synthesis of 2.57. To a solution of ester 2.55 (0.100 g, 0.16 mmol, 1.0 eq.) in dry CH₂Cl₂ (4 mL) was added TMSBr (0.17 mL, 1.3 mmol, 8.0 eq.) and the solution was refluxed for 2 d under an argon atmosphere. The solvent was removed by rotary evaporation and the resulting oil was dissolved in dry dichloromethane and the solution concentrated again by rotary evaporation. This process was repeated two more times. The resulting colorless oil was then placed under high vacuum for 16 h. The oil was redissolved in
benzene (3 mL) and an aqueous solution (3 mL) of NH₄HCO₃ (0.077 g, 0.97 mmol, 6.0 eq.) was added. After vigorous stirring for 30 min, the organic layer was evaporated (rotary evaporation) and the aqueous solution was lyophilized repeatedly to yield 2.57 as a pure, white solid in near quantitative yield (0.093 g). ^1H NMR (200 MHz, D₂O) δ 3.74 (s, 4H), 4.65 (s, 4H), 7.49 (d, J = 8.0 Hz, 4H), 7.63 (d, J = 7.7 Hz, 4H); ^31P NMR (202.5 MHz, D₂O) δ 4.65 (t, J = 96.4 Hz); ^19F NMR (188 MHz, D₂O) δ -28.36 (d, J = 96.1 Hz); ^13C NMR (50 MHz, D₂O) δ 68.9, 72.0, 119.8 (weak td), 126.0 (t, J = 6.6 Hz), 127.9, 133.7 (m), 139.8; MS (Electrospray): m/z 501.1 (M^+ + 3H^+), 250.1 (M^+ + 2H^+).

Synthesis of Inhibitors 2.14a-c.

Synthesis of 2.58. To a solution of resorcinol (0.100 g, 0.91 mmol, 1.0 eq.) in dry DMF (3 mL) was added K₂CO₃ (0.370 g, 2.69 mmol, 3.0 eq.) and p-iodobenzyl bromide (0.800 g, 2.69 mmol, 3.0 eq.). The reaction was stirred at room temperature for 48 h, and then dichloromethane (10 mL) was added to the flask. The solution was washed with 0.1 M NaOH (10 mL), 0.1 M HCl (10 mL), water (10 mL) then brine (10 mL). After drying (MgSO₄), the organic layer was concentrated via rotary evaporation. The product was purified via silica gel chromatography (hexane:ethyl acetate = 95:5, Rf = 0.3), and was obtained as a white, crystalline solid (86%). mp = 105-107 °C; ^1H NMR(200 MHz, CDCl₃): δ 4.99 (s, 4H), 6.50-6.60 (m, 3H), 7.05-7.25 (m, 5H), 7.72 (d, J = 8.4 Hz, 4H); ^13C(50 MHz, CDCl₃): δ 69.6, 93.3, 102.8, 107.9, 129.2, 130.0, 136.9, 137.7, 160.0; MS (EI): m/z 542 (M^+ , 39%), 217 (CH₂ArI, 100%); HREIMS calcd for C₃₀H₁₆O₂I₂ 541.9240, found 541.9247.

Synthesis of 2.59a-c. To a solution of the diol (hydroquinone, resorcinol, or catechol, 1.0 eq.) was added K₂CO₃ (3.0 eq.) and anhydrous DMF (12 mL/mmol diol), followed by a solution of crude 2.54 (approximately 3.0 eq. 2.54) in anhydrous DMF (5 mL DMF/g crude
After 12 h, ethyl acetate (90 mL/mmol diol) was added and the organic layer was washed twice with water and once with brine (90 mL/mmol diol of each aqueous solution). The organic layer was dried (MgSO$_4$) filtered and concentrated by rotary evaporation. The product was purified via silica gel chromatography and was obtained as a white, crystalline solid or colorless oil.

**Compound 2.59a:** Colorless oil, 76% yield; TLC: $R_f = 0.5$ (ethyl acetate:hexane = 70:30); $^1$H NMR (200 MHz, CDCl$_3$) $\delta$ 1.30 (t, $J = 7.3$ Hz, 12H), 4.20 (m, 8H), 5.08 (s, 4H), 6.58 (m, 3H), 7.18 (t, $J = 8.8$ Hz, 1H), 7.51 (d, $J = 7.3$ Hz, 4H), 7.64 (d, $J = 7.3$ Hz, 4H); $^{31}$P NMR (81 MHz, CDCl$_3$) $\delta$ 4.13 (t, $J = 117.5$ Hz); $^{19}$F NMR (188 MHz, CDCl$_3$) $\delta$ -32.30 (d, $J = 116.7$ Hz); $^{13}$C NMR (50 MHz, CDCl$_3$) $\delta$ 16.2 (d, $J = 5.5$ Hz), 64.6 (d, $J = 6.4$ Hz), 69.6, 102.8, 107.9, 118.1 (td, $J = 263.5$ Hz, $J = 218.0$ Hz), 126.6 (br t), 127.1, 130.0, 133.1 (m), 140.1, 160.0; MS (EI): $m/z$ 662 (M$^+$ trace), 642 (M$^+$/HF, 100%), 277 (CH$_2$ArCF$_2$P(O)(OE$_2$)$_2$, 50%); HREIMS calcd for C$_{30}$H$_{36}$O$_8$F$_4$P$_2$ 662.1822, found 662.1806.

**Compound 2.59b:** White solid, 44% yield; mp: 91-93 °C; TLC: $R_f = 0.3$ (ethyl acetate:dichloromethane, 12:88); $^1$H NMR (200 MHz, CDCl$_3$) $\delta$ 1.32 (t, $J = 7.0$ Hz, 12H), 4.20 (m, 8H), 5.07 (s, 4H), 6.90 (s, 4H), 7.51 (d, $J = 8.4$ Hz, 4H), 7.64 (d, $J = 7.6$ Hz, 4H); $^{31}$P NMR (81 MHz, CDCl$_3$) $\delta$ 4.07 (t, $J = 116.0$ Hz); $^{19}$F NMR (188 MHz, CDCl$_3$) $\delta$ -32.30 (d, $J = 116.0$ Hz); $^{13}$C NMR (50 MHz, CDCl$_3$) $\delta$ 16.3 (d, $J = 4.6$ Hz), 64.7 (d, $J = 6.4$ Hz), 70.3, 116.1, 118.0 (weak td), 126.5, 127.1, 132.3 (m), 140.3, 153.3; MS (EI): $m/z$ 662 (M$^+$, 40%), 277 (CH$_2$ArCF$_2$P(O)(OE)$_2$, 100%); HREIMS calcd for C$_{30}$H$_{36}$O$_8$F$_4$P$_2$ 662.1822, found 662.1788.

**Compound 2.59c:** Colorless oil, 69% yield; TLC: $R_f = 0.3$ (ethyl acetate:dichloromethane = 12:88); $^1$H NMR (200 MHz, CDCl$_3$) $\delta$ 1.31 (t, $J = 7.1$ Hz,
12H), 4.18 (m, 8H), 5.21 (s, 4H), 6.90 (m, 4H), 7.59 (m, 8H); $^{31}$P NMR (81 MHz, CDCl$_3$) δ 4.05 (t, $J = 116.0$ Hz); $^{19}$F NMR (188 MHz, CDCl$_3$) δ -32.31 (d, $J = 116.0$ Hz); $^{3}$C NMR (50 MHz, CDCl$_3$) δ 16.2 (t, $J = 4.6$ Hz), 46.6 (d, $J = 6.4$ Hz), 71.0, 115.9, 118.0 (weak t), 122.1, 126.5 (t, $J = 6.0$ Hz), 127.1, 132.4 (m), 140.4, 149.1; MS (EI): $m/z$ 662 (M$^+$, 7%), 642 (M$^+$ - HF, 50%), 277 (CH$_2$ArCF$_2$P(O)(OEt)$_2$, 100%); HREIMS calcd for C$_{30}$H$_{36}$O$_8$F$_2$P$_2$ 662.1822, found 662.1799.

Synthesis of 2.14a-c. General procedure: To a solution of the ethyl esters 2.59a-c (1.0 eq.) in dry CH$_2$Cl$_2$ (9 mL/0.1 mmol ester) was added TMSBr (8.0 eq.) and the solution was refluxed for 2 d under an argon atmosphere. The solvent was removed by rotary evaporation and the resulting oil was dissolved in dry dichloromethane and the solution was concentrated again by rotary evaporation. This process was repeated two more times. The resulting colorless oil was then placed under high vacuum for 16 h. The oil was redissolved in benzene (3 mL) and water (3 mL) was added. After vigorous stirring for 30 min, the organic layer was removed by rotary evaporation and the aqueous solution was lyophilized to yield pure 2.14a-c as white solids in near quantitative yield.

Compound 2.14a: Prepared according to the general procedure from 2.59a. $^1$H NMR (200 MHz, D$_2$O) δ 4.92 (s, 4H), 6.50 (m, 3H), 7.06 (t, $J = 8.0$ Hz, 1H), 7.37 (d, $J = 8.8$ Hz, 4H), 7.52 (d, $J = 7.3$ Hz, 4H); $^{31}$P NMR (81 MHz, D$_2$O) δ 4.56 (t, $J = 106.0$ Hz); $^{19}$F NMR (188 MHz, D$_2$O) δ -30.00 (d, $J = 105.3$ Hz); $^{13}$C NMR (75 MHz, D$_2$O) δ 70.1, 102.8, 108.6, 126.5 (t, $J = 6.8$ Hz), 127.8, 130.7, 136.1 (m), 137.9, 159.2; MS (Electrospray): $m/z$ 549 (M$^+$ + 3H$^+$), 274 (M$^+$ + 2H$^+$).

Compound 2.14b: Prepared according to the general procedure from 2.59b. $^1$H NMR (200 MHz, CD$_3$OD) δ 5.10 (s, 4H), 6.93 (s, 4H), 7.53 (d, $J = 8.0$ Hz, 4H), 7.63 (d, $J =$
8.0 Hz, 4H); $^{31}$P NMR (81 MHz, D$_2$O) $\delta$ 4.47 (t, $J = 105.2$ Hz); $^{19}$F NMR (188 MHz, D$_2$O) $\delta$ -29.82 (d, $J = 105.2$ Hz); $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 70.9, 116.9, 127.5 (t, $J = 6.8$ Hz), 128.1, 134.7 (m), 141.5, 154.4; MS (Electrospray): $m/z$ 549 ($M^+ + 3H^+$), 274 ($M^+ + 2H^+$).

**Compound 2.14c**: Prepared according to the general procedure from 2.59c. $^1$H NMR (200 MHz, D$_2$O) $\delta$ 4.87 (s, 4H), 6.70 (bdt, 4H), 7.29 (d, $J = 7.4$ Hz, 4H), 7.47 (d, $J = 8.8$ Hz, 4H); $^{31}$P NMR (81 MHz, D$_2$O) $\delta$ 4.64 (t, $J = 106.1$ Hz); $^{19}$F NMR (188 MHz, D$_2$O) $\delta$ -29.98 (d, $J = 106.9$ Hz); $^{13}$C NMR (75 MHz, D$_2$O) $\delta$ 70.6, 115.4, 119.9 (td, $J = 261.0$ Hz, $J = 201.0$ Hz), 122.3, 126.2 (t, $J = 6.8$ Hz), 127.7, 134.0 (m), 139.0, 147.7; MS (Electrospray): $m/z$ 549 ($M^+ + 3H^+$), 274 ($M^+ + 2H^+$).

### 2.2.3 Kinetic Studies with PTP1B

The majority of the inhibition studies reported in this chapter were performed by Dr. Chris Kotoris, a former graduate student in the Taylor group, or by Qingping Wang and Deena Waddleton, who are employees of Merck-Frosst Canada Inc. The exception to this are the inhibition studies with compounds 2.57 and 2.14a-c and PTP1B, which were performed by the author in the Taylor group. These studies were performed as follows. Rates of PTP1B-catalyzed dephosphorylation in the presence or absence of inhibitors were determined using fluorescein diphosphate (FDP) as substrate$^{84}$ in assay buffer containing 50 mM BIS-TRIS, 2 mM EDTA, 5 mM DTT, and 0.2 mg/mL BSA, pH 6.2, at 25 °C. Stock solutions of inhibitors were prepared in assay buffer and were found to be stable under these conditions indefinitely. Assays were carried out in 1 mL cuvettes with total volumes of 700 µL. Reactions were initiated by the addition of PTP1B (final concentration of 0.15 µg/mL). Rates were obtained by continuously monitoring the production of fluorescein monophosphate (FMP) at 450 nm using a Varian Cary 1 spectrophotometer. IC$_{50}$
Determinations were determined in duplicate at six different inhibitor concentrations with FDP at $K_m$ concentration (20 μM). IC$_{50}$'s were obtained from plots of % inhibition vs. log[inhibitor].
2.3 RESULTS AND DISCUSSION

2.3.1 Naphthyl-based PTP1B Inhibitors

In order to construct our bis-DFMP-substituted naphthyl compounds (General Structure 2.12, Section 2.1.5), we wished to develop a methodology that would allow for the synthesis of these inhibitors from isomerically pure, commercially available naphthyl starting materials. We first attempted to synthesize DFMP-containing naphthyl compounds via fluorination of bis-(α-ketophosphonates) using dialkylaminosulfur trifluoride, or DAST. This procedure had been previously used with success in the synthesis of DFMP-based inhibitors, and, as well, the diacid precursors 2.15 could be prepared in high yields through oxidation of commercially available, isomerically pure dimethylnaphthalenes (Scheme 2.3.1).

![Chemical Reaction Diagram]

**Scheme 2.3.1.** First attempted synthesis of naphthyl-based DFMP-containing inhibitors.
Unfortunately, many attempts to synthesize the bis-(α-ketophosphonates) by reacting the bis-(acid chloride), with trimethyl or triethyl phosphite were unsuccessful. We therefore turned to alternative procedures. In 1991, Differding and coworkers reported that α,α-difluorophosphonates could be prepared by electrophilic fluorination using the electrophilic fluorinating agent N-fluorobenzenesulfonimide (NFBS) (Scheme 2.3.2). This was accomplished using a two-step procedure in which 1.3 – 2.5 equivalents of potassium diisopropylamine (KDA) was used to remove an α-proton from the starting material 2.19 at -78 °C in tetrahydrofuran (THF), followed by the addition of 1.3 – 2.5 equivalents of NFBS (2.22) to form the α-monofluorophosphonates (2.20). The α-monofluorophosphonates were then purified and subjected to the same procedure at -85 °C in THF to yield the desired α,α-difluorophosphonates (2.21) in 29 – 36% yield overall (yields were based on non-fluorinated starting material).

**Scheme 2.3.2.** Synthetic procedure developed by Differding for the synthesis of α,α-difluorophosphonates.
Although Differding did not attempt this reaction on benzylic phosphonates, we reasoned that we could synthesize our aryl DFMP compounds using this methodology. Aromatic rings are fluorinated by this reagent, but only under relatively harsh conditions (heated without solvent to 100-150 °C, or to reflux for low boiling compounds). This commercially available electrophilic fluorinating agent therefore has intermediate reactivity, situated somewhere between the more powerful perfluorosulfonimides and less reactive alkylsulfonimides.\textsuperscript{111}

We first attempted the synthesis of benzylic mono-DFMP compounds (Scheme 2.3.3).\textsuperscript{112} Although a previous report seemed to indicate that benzylic DFMP-containing compounds could not be obtained through electrophilic fluorination,\textsuperscript{107} we found that we could readily obtain our benzylic DFMP molecules in relatively good yields, in a single-step reaction. The benzylic phosphonates 2.24a and 2.24b were synthesized in high yields by reacting the benzyl bromide precursors (2.23a and 2.23b,\textsuperscript{104} respectively) with trimethyl phosphite (Arbusov reaction). The benzylic DFMP compounds 2.25a and 2.25b were obtained in 63% and 70% yields respectively by reacting their corresponding phosphonates with 2.2 equivalents of a non-nucleophilic base, sodium hexamethyldisilazane (NaHMDS), at -78°C in THF, followed by the addition of 2.5 equivalents of NFBS (Scheme 2.3.3). The DFMP esters 2.25a and 2.25b were then deprotected and obtained as a free acid (2.11a) or ammonium salt (2.9d), in near quantitative yields, by reaction with trimethylsilylbromide (TMSBr) in dichloromethane, followed by hydrolysis of the resulting TMS esters with water or aqueous ammonium bicarbonate. In contrast to Differding’s original report on the fluorination of non-benzylic phosphonates,\textsuperscript{110} we found that yields were not improved by isolation of monofluoro derivatives (using half the equivalents of base and NFBS), followed by another fluorination.
Studies in our lab\textsuperscript{113} indicated that our method of preparing these mono-substituted phenyl- and naphthyl-DFMP derivatives is superior to the DAST procedure. The synthesis of \textit{2.25a} and \textit{2.25b} using DAST, from the corresponding \(\alpha\)-ketophosphonates, resulted in yields of less than 50\%, and the compounds were quite difficult to purify. As well, fluorination using the DAST procedure requires a large excess of expensive DAST (5 - 15 equivalents), and, when performed on a multigram scale, can sometimes react explosively.\textsuperscript{114} While these studies were in progress, Burton and Qui reported the synthesis of benzylic \(\alpha,\alpha\)-difluorophosphonates (Compound \textit{2.27}, Scheme 2.3.4) by CuCl-promoted coupling of (diethylphosphonyl)difluoromethyl cadmium reagent with aryl iodides (\textit{2.26}).\textsuperscript{115} The yields that we obtained using the electrophilic fluorination procedure are comparable to those obtained by Burton and Qui.
Scheme 2.3.4. Synthesis of benzylic α,α-difluorophosphonates by Burton and Qui; CuCl-promoted coupling of aryl iodides with a cadmium reagent.

Having worked out a facile synthesis for mono-substituted naphthyl-based DFMP derivatives, we then turned our attention to obtaining our bis-substituted compounds (General Structure 2.12, Section 2.1.5), using a similar approach.\(^{112}\) The phosphonate precursors 2.29a-g were readily obtained in excellent yields by reacting trimethyl or triethyl phosphite with their corresponding bis(bromomethyl)-naphthalene starting materials (2.28), which were prepared according to literature procedures.\(^{104}\) We found that a variety of benzylic bis(DFMP)phosphonates could be obtained in a single fluorination reaction by reacting bisphosphonates 2.29a-g with 5.5 equivalents of NaHMDS at -78 °C followed by addition of 7.3 equivalents of NFBS in THF at -78 °C (Scheme 2.3.5). The results of these fluorination reactions are summarized in Table 2.3.1. With the exception of the 2,6-naphthyl isomer (2.18e, 23% yield), the fluorination reactions gave 46% yield or higher, and both methyl and ethyl ester protecting groups could be tolerated. We believe that this difference in percent yield may be due to a competing elimination reaction during the fluorination of 2.29e (Scheme 2.3.6).

By reducing the number of equivalents of NaHMDS (2.5 eq.) and NFBS (3.0 eq.), benzylic bis-(α-monofluoro-phosphonates) could be isolated as the major product from a mixture of mono- and bis-substituted products. We were unable to detect any benzylic mono(DFMP) product, which suggests that the reaction proceeds via initial formation of the
dianion and the benzylic bis-(α-monofluorophosphonate) when 5.5 equivalents of NaHMDS and 7.3 equivalents of NFBS are used.

\[
\begin{align*}
\text{R} = \text{Me or Et} \\
(63\% - \text{quantitative})
\end{align*}
\]

**Scheme 2.3.5.** Synthesis of bis-DFMP naphthyl derivatives 2.12a-f.

Intermediate in fluorination reaction of 2.29e

**Scheme 2.3.6.** Proposed elimination reaction to account for low yield of 2.18e.
Table 2.3.1. Preparation of Benzylic Bis-DFMP compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Naphthyl Derivative</th>
<th>R</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.18a</td>
<td>1,3-</td>
<td>Me</td>
<td>55</td>
</tr>
<tr>
<td>2.18b</td>
<td>1,5-</td>
<td>Et</td>
<td>48</td>
</tr>
<tr>
<td>2.18c</td>
<td>1,6-</td>
<td>Me</td>
<td>51</td>
</tr>
<tr>
<td>2.18d</td>
<td>1,7-</td>
<td>Et</td>
<td>46</td>
</tr>
<tr>
<td>2.18e</td>
<td>2,6-</td>
<td>Et</td>
<td>23</td>
</tr>
<tr>
<td>2.18f</td>
<td>2,7-</td>
<td>Me</td>
<td>57</td>
</tr>
<tr>
<td>2.18g</td>
<td>2,7-</td>
<td>Et</td>
<td>46</td>
</tr>
</tbody>
</table>

The methyl or ethyl protecting groups of our bis-DFMP compounds were removed using TMSBr in dichloromethane, as with the mono-DFMP compounds 2.25a and 2.25b, to give the silyl esters. Upon treatment with water or aqueous ammonium bicarbonate, followed by repeated lyophilizations, the desired bis-DFMP inhibitors 2.12a-f were obtained, as either free acids or ammonium salts. All of the compounds were found to be stable in neutral aqueous solution, and exhibited no detectable decomposition during storage as solids or in neutral aqueous solution.

While we were most interested in designing potent inhibitors of PTP1B, we also wished to screen these inhibitors against other phosphatases, in order to determine their selectivity. Thus, the naphthyl bis-DFMP compounds were examined for inhibition of CD45 and PP2A, in addition to PTP1B. CD45 is a transmembrane or receptor-like PTPase.
expressed on the surface of hematopoietic cells. This enzyme is thought to dephosphorylate Src PTK's, resulting in the up-regulation of their catalytic activity, leading to cell activation.\(^{117}\) PP2A is a serine/threonine phosphatase, and has been implicated in regulating many cellular functions.\(^{118}\) An initial assessment of the inhibitory potency of these inhibitors was performed by Qingping Wang at Merck-Frosst using a fluorogenic assay.\(^{119}\) This involved using fluorescein diphosphate (FDP) as substrate at \(K_m\) concentration and 500 \(\mu\)M inhibitor. The results, in terms of percent inhibition, are summarized in Table 2.3.2. Consistent with studies by Terrence Burke and coworkers,\(^{102}\) the phenyl-DFMP derivative 2.9d is a very poor inhibitor of PTP1B, resulting in only 41\% inhibition at 500 \(\mu\)M. The mono-substituted naphthyl inhibitors 2.10 and 2.11a, however, were better inhibitors of PTP1B, showing 83\% and 84\% inhibition at 500 \(\mu\)M, respectively. While these results are in keeping with those reported by Burke \textit{et al.},\(^{102}\) we found that 2.10 and 2.11a were less potent with PP2A, whereas Burke reported that these compounds inhibited PTP1B and PP2A equally well. Inhibitors 2.10 and 2.11a did not significantly inhibit CD45. In general, addition of a second DFMP group did not enhance inhibitory activity of the bis-DFMP compounds with PP2A. Conversely, incorporation of two DFMP groups at certain positions on the naphthyl ring had a profound effect on PTP1B and CD45 inhibition. For example, 500 \(\mu\)M of the 2,7- (2.12f) and 2,6- (2.12e) naphthyl derivatives completely inhibited PTP1B activity, and 500 \(\mu\)M of the 1,7-derivative (2.12d) resulted in 98\% and 99\% inhibition of PTP1B and CD45, respectively.
Table 2.3.2. Percent Inhibition of PTP1B, CD45 and PP2A with Naphthyl- and Phenyl-DFMP Compounds.\textsuperscript{119}

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>CD45\textsuperscript{a}</th>
<th>PTP1B\textsuperscript{b}</th>
<th>PP2A\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Inhibitor 2.9d" /></td>
<td>2.9d</td>
<td>n.d.\textsuperscript{d}</td>
<td>41 \textsuperscript{e,f}</td>
</tr>
<tr>
<td><img src="image" alt="Inhibitor 2.10" /></td>
<td>2.10</td>
<td>20</td>
<td>83</td>
</tr>
<tr>
<td><img src="image" alt="Inhibitor 2.11a" /></td>
<td>2.11a</td>
<td>5</td>
<td>84</td>
</tr>
<tr>
<td><img src="image" alt="Inhibitor 2.12a" /></td>
<td>2.12a</td>
<td>78</td>
<td>81</td>
</tr>
<tr>
<td><img src="image" alt="Inhibitor 2.12b" /></td>
<td>2.12b</td>
<td>26</td>
<td>51</td>
</tr>
<tr>
<td><img src="image" alt="Inhibitor 2.12c" /></td>
<td>2.12c</td>
<td>70</td>
<td>89</td>
</tr>
<tr>
<td><img src="image" alt="Inhibitor 2.12d" /></td>
<td>2.12d</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td><img src="image" alt="Inhibitor 2.12e" /></td>
<td>2.12e</td>
<td>62</td>
<td>100</td>
</tr>
<tr>
<td><img src="image" alt="Inhibitor 2.12f" /></td>
<td>2.12f</td>
<td>64</td>
<td>100</td>
</tr>
</tbody>
</table>

\textsuperscript{a}[FDP] = 20 \mu M; \textsuperscript{b}[FDP] = 20 \mu M; \textsuperscript{c}[FDP] = 35 \mu M; \textsuperscript{d}n.d. = not determined; \textsuperscript{e}value obtained by C. Kotoris, Taylor Group; \textsuperscript{f}IC\textsubscript{50} for this inhibitor was determined to be 610 \mu M.\textsuperscript{120}
The monosubstituted inhibitors 2.10 and 2.11a, and compounds 2.12d-f were examined in further detail with PTP1B and CD45. IC₅₀ determinations were performed by Qingping Wang at Merck-Frosst with FDP at Kᵣ concentrations, and these results are summarized in Table 2.3.3.

Table 2.3.3. IC₅₀'s of Selected Naphthyl-DFMP Derivatives.¹¹⁹

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>CD45°</th>
<th>PTP1B°</th>
<th>PP2A°</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.11a</td>
<td>&gt;1000</td>
<td>95</td>
<td>753</td>
</tr>
<tr>
<td>2.10</td>
<td>&gt;1000</td>
<td>112</td>
<td>636</td>
</tr>
<tr>
<td>2.12d</td>
<td>27</td>
<td>118</td>
<td>481</td>
</tr>
<tr>
<td>2.12e</td>
<td>&gt;100</td>
<td>29</td>
<td>354</td>
</tr>
<tr>
<td>2.12f</td>
<td>180</td>
<td>26</td>
<td>306</td>
</tr>
</tbody>
</table>

°FDP = 20 µM; ²[FDP] = 20 µM; ³[FDP] = 35 µM.

Compounds 2.12d, 2.12e, and 2.12f exhibited IC₅₀'s of 118 µM, 29 µM, and 26 µM, respectively, with PTP1B. Thus, 2.12e and 2.12f have IC₅₀ values approximately four- to five-fold lower than those of the mono-DFMP-substituted naphthyl compounds, 2.10 and 2.11a, with PTP1B. The 1,7-derivative, 2.12d, is about five times more potent with CD45 (IC₅₀ = 27 µM) than with PTP1B (IC₅₀ = 118 µM), and roughly eighteen times more potent with CD45 relative to PP2A (IC₅₀ = 481 µM). As with inhibitors 2.12e and 2.12f,
incorporation of an additional DFMP group into the naphthyl scaffold greatly increased the inhibition of CD45 relative to the monosubstituted naphthyl derivatives. Inhibitor 2.12f appears to be the most potent and selective inhibitor of PTP1B, at an IC$_{50}$ of 26 µM, and exhibiting approximately seven- and twelve-times greater inhibitory potency with PTP1B relative to CD45 and PP2A, respectively. Therefore, the $K_i$ values were determined for compound 2.12f with PTP1B, and for 2.12d and CD45. The $K_i$ for 2.12f with PTP1B was 16 µM, and the $K_i$ for 2.12d with CD45 was 9 µM. At the time we first published these findings in early 1998, these inhibitors were among the most potent, reversible, small molecule inhibitors of PTP1B and CD45 reported to date.$^{119}$

To determine whether the fluorine atoms were necessary for potency and selectivity of inhibition, we prepared the nonfluorinated 2,7-derivative 2.31 and the monofluorinated 2,7-derivative 2.32, and examined these compounds for inhibition of PTP1B and CD45 (see below). These derivatives were prepared using the same synthetic approach as the other naphthyl inhibitors. Compound 2.31 was quantitatively deprotected from its phosphonate precursor 2.29f, and compound 2.32 was obtained via quantitative deprotection of its monofluorinated phosphonate precursor 2.33.$^{106}$

The non-fluorinated compound, 2.31, was a very poor inhibitor of both PTP1B and CD45. The decrease in potency was relatively small for CD45 (three-fold) but was much more significant for PTP1B (at least twenty-fold). The bis-monofluorophosphonate, 2.32, was a five-fold poorer inhibitor of PTP1B compared to its difluoro analogue 2.12f. However, little difference in inhibition between these two compounds was found for CD45. Thus, it appears that for PTP1B, inhibition decreases as the number of fluorines decreases.$^{119}$
As mentioned in Section 2.1.4, fluorine substitution lowers the ionization constants of the phosphonate moiety relative to the unsubstituted compound, and can provide hydrogen-bonding interactions similar to the parent oxygen-containing phosphate that are absent in the non-fluorinated derivative. Studies by Burke suggested that the effect of the fluorines is not due to a pK\textsubscript{a} effect. Shortly after we began work in this area, Burke and coworkers published the crystal structure of (C215S-PTP1B) complexed with the \( \beta \)-substituted naphthyl DFMP derivative 2.11a. This inhibitor binds to the active site of PTP1B in a manner similar to that of pTyr. The phosphate group of pTyr in the PTP1B-DADE(pY)L crystal structure superimposes on the DFMP group of the former, and the same holds true for the phenyl ring of the pTyr and the proximal naphthyl ring (bearing the DFMP group) of 2.11a.

Binding of 2.11a to the active site induces the motion of a loop consisting of Trp179 to Ser187, which was also observed in the binding of pTyr to PTP1B. A water molecule located between the main chain NH of Phe182 and the leaving group oxygen of pTyr is absent in the PTP1B-2.11a complex. In the PTP1B-DADE(pY)L crystal structure, the side chain of Asp181 forms a hydrogen-bond with the buried water molecule and a long contact (3.5 Å) with the scissile oxygen of PTP1B. However, in Burke's crystal structure the two
fluorine atoms of 2.11a partly occupy the site where the water binds, which causes displacement of the water molecule and a shift in the position of the side chain of Asp181. Concerted with this shift is a rotation of the side chain of Phe182 to avoid close contact with the fluorine atoms of 2.11a, and the phenyl ring of Phe182 forms van der Waals contacts with these fluorine atoms. The oxygen atoms of the DFMP group form hydrogen-bonds to the main chain nitrogens of residues Ser215 to Arg221, and there are also two salt bridges formed between the phosphonate oxygens and the guanidinium side chain of Arg221. The naphthalene ring in 2.11a forms hydrophobic interactions with the side chains of nonpolar residues in the binding pocket, namely Tyr46, Phe182, Ala217, Ile219 and the nonpolar groups of Gln262. Most interestingly, it was noted that the pro-S fluorine was in hydrogen-bonding distance (2.2 Å) from the backbone N-H of Phe182 while the pro-R fluorine did not interact with any residues beyond simple van der Waals contacts. On the basis of this crystal structure and molecular dynamics calculations, it was suggested that the pro-S fluorine is involved in an unusually strong hydrogen-bond with the backbone N-H of Phe182 and contributes 4.6 kcal/mol more in binding energy than the pro-R fluorine. This 4.6 kcal/mol can account for the >1000-fold difference in affinity of the fluorinated versus the non-fluorinated peptides discussed in Section 2.1.4. Thus, it appears the fluorines enhance binding affinity through hydrogen-bonding interactions.

2.3.2 Biphenyl- and Triphenyl-based PTP1B Inhibitors

Although the bis-DFMP naphthyl compounds were more potent than their mono-DFMP analogues we wished to design and synthesize more potent inhibitors of PTP1B. We therefore extended our methodology to produce biphenyl bis-DFMP compounds (General Structure 2.13, Section 2.1.5). We decided to focus on para-substituted biphenyl
derivatives, due to the fact that studies with rat PTP1 and phenyl phosphate substrates indicated that ortho and, to a lesser extent, meta substitution generally interferes with substrate binding (Section 2.1.3). Also, substrate 2.34 for rat PTP1 is the most potent low-molecular weight substrate ever reported for a PTPase, and exhibits a $K_m$ of 16 μM (which is 19 times lower than its mono-phosphorylated counterpart, and nearly as low as the $K_m$ values for the best peptidyl substrates). Thus, we had reason to believe that para-DFMP substituted biphenyl inhibitors might prove to be potent inhibitors of PTP1B.

We first attempted the synthesis of a mono-DFMP biphenyl inhibitor, compound 2.38 (see below, Table 2.3.4). This inhibitor was synthesized in the usual manner, from its commercially available benzylic bromide precursor (2.35). The methyl-protected phosphonate (2.36) was obtained in 90% yield, while the protected DFMP derivative (2.37) was recovered in 59% yield. Quantitative deprotection yielded the desired product, compound 2.38.

Compound 2.38 was screened for inhibition with PTP1B, by Dr. Chris Kotoris (formerly of the Taylor Group). As well, we had in hand compounds 2.39 and 2.40, the ortho- and meta-phenyl (phenyl-DFMP) derivatives, respectively, so these compounds were also screened for inhibition with PTP1B. This initial assessment was performed using 500 μM inhibitor with PTP1B and FDP as substrate, at a concentration equal to its $K_m$ value (20 μM) at pH 6.2. The results of these studies are shown in Table 2.3.4.
Table 2.3.4. Percent inhibition of PTP1B and IC₅₀'s for inhibitors 2.38, 2.39 and 2.40.¹²³¹²⁴

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% Inhibition</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.38, X = p-phenyl</td>
<td>67</td>
<td>210</td>
</tr>
<tr>
<td>2.39, X = m-phenyl</td>
<td>94</td>
<td>35</td>
</tr>
<tr>
<td>2.40, X = o-phenyl</td>
<td>25</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

¹500 µM inhibitor, 20 µM FDP, pH 6.2; ²various inhibitor, 20 µM FDP, pH 6.2; ³n.d. = not determined.

The ortho-substituted phenyl-DFMP derivative, 2.40, was a poorer inhibitor than phenyl-DFMP (2.9d, Table 2.3.2), exhibiting 25% inhibition at 500 µM as opposed to 41% inhibition for the unsubstituted analogue 2.9d. The para-phenyl (phenyl-DFMP) derivative 2.38 inhibited the activity of PTP1B by 67% at 500 µM, however, the most potent inhibition, was exhibited by the meta-phenyl derivative, compound 2.39, at 94%. The IC₅₀ value for 2.9d with PTP1B was determined to be 610 µM. The IC₅₀ value for 2.39 (35 µM) is six times lower than that obtained for 2.38 (210 µM). This is consistent with the finding of Montserat et al. in that the Kₘ reported for meta-phenyl phenylphosphate (0.32 mM) is seven times lower than that reported for the para-phenyl phenylphosphate derivative (2.4 mM). This trend was not observed by Montserat et al. for non-peptidyl substrates that were meta-substituted with aliphatic groups (such as ethyl or isopropyl), as these substrates exhibited higher Kₘ values than meta-phenyl phenylphosphate with rat PTP1. Therefore, the effect observed with meta-phenyl substituted substrates and inhibitors is likely due to more than merely hydrophobic interactions, and may involve pi-stacking interactions with aromatic residues proximal to the active site.
The para- and meta-substituted bis-DFMP biphenyl derivatives (2.13a and 2.47 in Scheme 2.3.7) were prepared in the usual manner. Both the para- and meta-substituted bis-DFMP biphenyl derivatives were prepared because we had previously demonstrated (see above, Table 2.3.4) that the meta-phenyl (phenyl-DFMP) compound 2.39 was a considerably better PTP1B inhibitor than the para-phenyl derivative 2.38. We wished to see if a similar effect would occur with the bis-DFMP compounds. It is interesting to note that the fluorination reaction of 2.43a to form 2.45a proceeded in only a 21% yield while the analogous reaction for the meta-substituted compound (fluorination of 2.44) proceeds in a 42% yield. We believe that this difference may be due a competing elimination reaction which can only occur in the para-substituted compounds (Scheme 2.3.8).

Scheme 2.3.7. Synthesis of bis-DFMP biphenyl inhibitors 2.13a and 2.47.
Intermediate in fluorination reaction of 2.43a

Elimination Product

Scheme 2.3.8. Proposed elimination reaction during the fluorination of 2.43a.

The IC₅₀’s of the bis-DFMP substituted meta and para biphenyl compounds, 2.47 and 2.13a, are shown below. The symmetrical meta-substituted bis-DFMP derivative 2.47 was a slightly poorer inhibitor of PTP1B than the mono-DFMP analogue (2.39, IC₅₀ = 35 μM), exhibiting an IC₅₀ value of 45 μM. However, the bis-DFMP derivative 2.13a exhibited an IC₅₀ value of 15 μM, which is 14-fold lower than that of its mono-DFMP analogue 2.38. This compound was determined to be a competitive inhibitor of PTP1B with a Kᵢ value of 4.5 μM.

To determine whether the fluorines at the α-positions of each DFMP group were necessary for potent inhibition, the non-fluorinated analogue of 2.13a, compound 2.49 (shown below), was prepared through quantitative deprotection of phosphonate 2.43a. Compound 2.50 (also shown below), a bis-phosphonate in which only one of the benzylic phosphonates was α-fluorinated, was also examined for inhibition with PTP1B. The completely non-fluorinated analogue, 2.49, was a very poor inhibitor of PTP1B, exhibiting an IC₅₀ of about 2 mM, over 150-fold greater than that of 2.13a. The singly α-fluorinated bis-phosphonate 2.50, was a much better inhibitor of PTP1B. This inhibitor exhibited an
IC₅₀ value of 51 μM, which, although three-fold greater than that of the fully fluorinated 2.13a, is about 40-fold less than the IC₅₀ value obtained for the completely non-fluorinated derivative, 2.49.¹²³,¹²⁴

While the enhanced binding effect of increased fluorine substitution at the α-position of para-substituted biphenyl phosphonates is less dramatic than the >1000-fold decrease in Kᵢ of F₂-Pmp-containing peptides relative to Pmp-containing peptides observed by Burke and coworkers with PTP1B,⁹⁹ the effect is nonetheless significant. The inhibition constant of compound 2.13a with PTP1B was found to be independent of pH between pH 5.5 and 7.2.¹²⁴,¹²⁶ This is in agreement with earlier studies that have also revealed that monoanionic and dianionic forms of phosphonates bind PTP1B with equal affinity.¹⁰⁰ Thus, the increased potency of inhibition of the increasingly fluorinated para-substituted biphenyl derivatives is probably due to the capability of the fluorines to form hydrogen-bonds with specific residues in PTP1B.

The incorporation of a second DFMP group into a biphenyl scaffold offered improved inhibition of PTP1B relative to the mono-substituted derivative, but only in the case of para substitution. We therefore synthesized a series of bis-DFMP para-substituted biphenyl
derivatives, 2.13b-e (Scheme 2.3.9). Percent yields obtained for the synthesis of inhibitor 2.13a are also included in the synthetic scheme for comparison. As was the case with 2.43a, the yield of the fluorination reaction for 2.43b, where the phenyl rings are separated by a single methylene unit, was very low (16%). It is possible that for 2.43b, an elimination reaction is also occurring by removal of a proton on the methylene unit separating the two rings. However, no product arising from the fluorination of the methylene bridge was isolated. The low yield for compound 2.45d (21%) is attributed to failed attempts to separate a mixture of unresolvable isomers. (The final deprotected product 2.13d was HPLC-purified.) The yield for compound 2.45c was 37%, while compound 2.45e was obtained in a respectable 64% yield. Thus, it seems that, in general, increasing the distance between the two phenyl rings increases the yield of the fluorination reaction, although we are uncertain as to why this might occur.

Our para-substituted bis-DFMP biphenyl derivatives were then screened by Dr. Chris Kotoris, formerly of the Taylor group, for inhibition with PTP1B, and the results are shown in Table 2.3.5. The bis-DFMP diphenylmethyl derivative, 2.13b, exhibited an IC$_{50}$ value of 23 µM, slightly higher than that of 2.13a. The diphenylethane and diphenylpropane derivatives 2.13c and 2.13d were better inhibitors, with IC$_{50}$ values of 6.8 µM and 5.8 µM, respectively. The best biphenyl inhibitor was compound 2.13e, which exhibited an IC$_{50}$ value of 4.4 µM, and competitively inhibited PTP1B with a K$_i$ of 1.5 µM. This inhibitor was also screened for inhibition of the phosphatase PP2A, and was found to exhibit an IC$_{50}$ value of roughly 200 µM, which is about 45-fold greater than the IC$_{50}$ of 2.13e with PTP1B.
Scheme 2.3.9. Synthesis of bis-DFMP biphenyl derivatives 2.13a-e.

Thus, not only is 2.13e a potent inhibitor of PTP1B, it also exceeds the potency and selectivity of inhibition of our best naphthyl inhibitor 2.12f (see Table 2.3.3). Moreover, it exhibits an affinity for PTP1B that is two orders of magnitude greater than the simple phenyl DFMP compound 2.9d.
Table 2.3.5. IC$_{50}$ values for bis-phosphonates 2.13a-e.$^{123,124}$

| 2.13a: n=0 | 15 |
| 2.13b: n=1 | 23 |
| 2.13c: n=2 | 6.8 |
| 2.13d: n=3 | 5.8 |
| 2.13e: n=4 | 4.4 (K$_i$ = 1.5 µM) |

*various [inhibitor], 20 µM FDP, pH 6.2.

2.3.3 Crystallographic Studies

While the simple phenyl-DFMP derivative 2.9d is a poor inhibitor of PTP1B, it is interesting that joining two such derivatives together results in much more potent inhibitors of this enzyme. Why does the presence of an additional phenylphosphonate or phenylphosphate mimetic enhance the binding affinity of both inhibitors and substrates for PTP1B? The fact that PTP1B prefers to bind peptides with acidic residues N-terminal to the pTyr residue is one explanation. However, our hypothesis from the onset of our work with this enzyme was that a second, non-catalytic phosphate binding site might exist in PTP1B, and that this structural feature could be exploited for the design of potent and selective PTP1B inhibitors. Our results with our biphenyl-based DFMP inhibitors suggested that this hypothesis might be correct.

Shortly before the publication of our work, a report appeared by Puius et al. describing a second non-catalytic aryl phosphate-binding site with lower binding affinity in PTP1B.$^{127}$ The second site was discovered by crystallizing PTP1B in the presence of the high affinity bis-phosphonate substrate 2.34 or saturating (53 mM) amounts of pTyr. The pTyr molecule was found to bind in two different modes. In one mode (pTyr A), the
phosphate group of pTyr binds in the catalytic site in the usual manner. In the other mode (pTyr B), the phosphate group binds by forming ionic interactions with Arg254 and Arg24 and water-mediated hydrogen-bonds with Met258 and Gly259. Although, with saturating pTyr, both sites could be occupied simultaneously, only 50% of the pTyr was bound as pTyrB. These results suggest that PTP1B contains one high affinity, catalytic aryl phosphate binding site and, adjacent to it, one low affinity non-catalytic, aryl phosphate binding site in which Arg254 and Arg24 are key residues. Compound 2.34 was found to bind to PTP1B in two mutually exclusive modes. In one mode, the one phosphate group binds in the catalytic site, while the other phosphate makes a water-mediated hydrogen-bond with Gln262. In the other mode, one phosphate again makes a water-mediated hydrogen-bond with Gln262. However, the other phosphate group binds in the second aryl phosphate binding site. Each site was approximately 50% occupied. Two molecules of 2.34 are incapable of binding simultaneously to the enzyme as a result of steric repulsion between the two distal phosphate groups. On the basis of these studies, it was suggested that a compound that simultaneously occupies both aryl phosphate binding sites may be a potent inhibitor of PTP1B. The possibility that some of the residues involved in the second non-catalytic site may be less conserved amongst PTPases than those found in the catalytic site suggests that such compounds could also be selective for PTP1B.

The presence of a second aryl phosphate binding site seems to have offered an explanation as to why compounds bearing two DFMP groups are more effective inhibitors than the analogous compounds bearing only a single DFMP group. To learn more about how these inhibitors interact with PTP1B we collaborated with Professor Zongchao Jia, a protein crystallographer in the Department of Biochemistry at Queen's University. Modeling studies
performed in Professor Jia's laboratory suggested that the distance separating the two DFMP groups in the naphthyl inhibitor in 2.12f was too short and the naphthyl ring too rigid to allow both DFMP groups to interact with both phosphate binding sites simultaneously. This suggests that the second DFMP group on 2.12f enhances inhibitory potency by interacting with residues that are not part of the second phosphate binding site. However, modeling studies on 2.13e suggested that the linker arm separating the two DFMP groups was of sufficient length and flexibility to allow them to interact with the two phosphate binding sites simultaneously. Does the second DFMP group on inhibitor 2.13e occupy the second phosphate binding site? Why is compound 2.13e a better inhibitor than 2.12f? Is the second site an important motif for the development of potent and selective PTP1B inhibitors? In an attempt to answer these questions, and to learn more about those features that are important for the rational design of small molecule PTP1B inhibitors, the x-ray crystal structures of PTP1B complexed with compounds 2.12f and 2.13e were obtained

The PTP1B-2.13e complex. The PTP1B-2.13e complex, refined to 2.35 Å, clearly indicates that one of the phenyl-DFMP groups (which we call the proximal DFMP group) occupies the active site of the enzyme (Figures 2.3.1-4). The binding of this phenyl-DFMP group to PTP1B is similar to previously reported PTP1B-phosphonate crystal structures. The three oxygen atoms of the DFMP group participate in hydrogen-bonds with main-chain amide groups of the P-loop, as well as electrostatic interactions with the guanidinium group of Arg221. A water molecule occupies a cavity and forms hydrogen-bonds with the pro-R fluorine, the N-H of Phe182, and one of the oxygens of the phosphonate group. The WpD loop is in its closed (catalytically active) conformation, and
**Figure 23.1.** Ribbon diagram of PTP1B-2.13e complex. Compound 2.13e is shown in ball and stick model. The extended conformation of compound 2.13e (shown in green) is evident. Side chains of some of the contact residues of PTP1B are also shown (yellow).

**Figure 23.2.** Electron density map of inhibitor 2.13e and PTP1B contact residues.
Figure 2.3.3. Spacefilling model of 2.13e bound to PTP1B. The carbons of the 2.13e are shown in light green, the distal phosphate group in yellow and the fluorines of the distal phosphate group in dark green. The proximal phosphate group is buried in a cleft in the active site and cannot be seen. Arg47 is in red while Arg24 and Arg254, which are part of the second phosphate binding site, are in blue.

Figure 2.3.4. Schematic of inhibitor 2.13e-PTP1B interactions. A distance cutoff of 3.2 Å was used, except for those involving certain aromatic rings.
residues such as Phe182 and Tyr46 may form hydrophobic interactions with the proximal phenyl ring and adjacent portions of the hydrocarbon linker chain. Due to the exposed position of the protein region in the vicinity of the second aryl phosphate group (which we call the distal DFMP group), the number of contacts between the enzyme and 2.13e is limited. Higher temperature factors of both the inhibitor and enzyme are observed, as evident by the weak and non-continuous density in the solvent exposed region (Figure 2.3.2). However, even with the weak density, the extended conformation of the inhibitor, which only binds to the primary or active site, is beyond any doubt. We therefore conclude that this inhibitor does not bind to the second aryl phosphate-binding site. The hydrocarbon linker chain does not bend as is required to direct the distal phenyl DFMP group towards the second phosphate binding site. This is especially well-illustrated in the space-filling model shown in Figure 2.3.3. Instead, the distal DFMP group extends out into the solvent and is involved in water mediated hydrogen-bonding interactions with Arg47 (Figure 2.3.4). One of the fluorines may be involved in a long hydrogen-bond with Arg47 and both fluorines appear to be involved in long hydrogen-bonds with the bound water molecule.

**The PTP1B-2.12f complex.** The crystal structure of our 2,7-bis(DFMP) substituted naphthyl inhibitor, compound 2.12f, with PTP1B was refined to 2.5 Å resolution. The WpD loop is in its closed conformation, and Phe182 and Tyr46 may form hydrophobic interactions with the naphthalene ring. Besides this similarity, 2.12f binds quite differently from the mono-DFMP naphthyl inhibitor 2.11a reported by Burke. A schematic representation outlining the important interactions is shown in Figure 2.3.5. First, the naphthalene ring is rotated 180° relative to that in the 2.11a-PTP1B complex. Second, the proximal (active site bound) phosphate does not interact with the P-loop backbone to the same extent as that
found for the phosphate group of 2.11a. However, it does form electrostatic interactions with Arg221. Third, the pro-R fluorine forms a hydrogen-bond with the side chain of Lys120. As expected, the distal DFMP group does not interact with the second phosphate binding site but instead one of the oxygens forms hydrogen-bonds with the N-H backbone amides of Arg47 and Asp48. The fluorines do not appear to interact with any residues on the enzyme. Thus, both the proximal and distal DFMP groups in 2.13e and 2.12f interact with the enzyme in a very different ways and these differences are the most probable cause for their differences in affinity for the enzyme.

Figure 2.35. Schematic of inhibitor 2.12f-PTP1B interactions. A distance cutoff of 3.2 Å was used, except for those involving certain aromatic rings.

Quite recently, Groves et al.128 have reported the crystal structures of PTP1B complexed with naphthalene-2-difluorophosphonic acid derivatives 2.51 and 2.52. The $K_i$ values of these inhibitors103,129 are only slightly greater than that obtained for 2.12f.
Since the structure of 2.51 is quite similar to 2.12f in that it bears an additional acidic moiety directly attached to the naphthalene ring (except it is located at the 6-position instead of the 7-position) it would be of interest to compare the two structures. The overall structure of the PTP1B-2.51 complex is virtually identical to the previously described PTP1B-2.11a complex (discussed above). Unlike the distal DFMP group in 2.12f, the carboxylate group in 2.51 is involved in interactions, via a bound water molecule, with the side chains of Arg47 and Asp48. Thus, although 2.12f and 2.51 have similar $K_i$'s, the additional acidic moieties interact with the enzyme in different ways. These additional interactions are most likely responsible for the increased potency of 2.51 and 2.12f relative to 2.11a.

Structure 2.52 contains a carboxamidoglutamic acid group designed to mimic a peptide moiety. The complex of compound 2.52 with PTP1B also indicates that the WpD loop is in the closed conformation, similar to the structures described above and other PTP1B-peptide complexes. The phosphonate group interacts with the P-loop in the usual way. The carboxamidoglutamic acid group of 2.52 participates in several main-chain hydrogen-bond interactions with PTP1B, which are similar to those exhibited by the PTP1B-DADE(pY)L peptide complex. The side chain of the glutamate moiety in 2.52 forms a water-mediated hydrogen-bond with Arg47 similar to that found in the 2.13e-PTP1B complex discussed above. However, 2.13e is an approximately 8-fold better inhibitor than
suggesting that the water-mediated hydrogen-bonds between the distal phosphonate in 2.13e and Arg47 are superior to that found for 2.52.

The fact that compound 2.13e does not occupy both phosphate binding sites is actually consistent with our inhibition studies which indicate that increasing the length of the hydrocarbon linker chain from one methylene unit to four does not result in a dramatic increase in inhibitory potency. If the second site is indeed designed to interact with phenylphosphate groups, it would be reasonable to expect that bis-(phenyl-DFMP) inhibitors that occupy both binding sites would be more potent than those that only interact by hydrogen-bonding or electrostatic interactions with an Arg residue found outside the phosphate binding sites. Previously mentioned studies with synthetic peptide substrates have indicated that certain PTPases, including PTP1B, display a strong affinity for acidic residues N-terminal to pTyr. The crystal structure of the high-affinity peptide substrate DADE(pY)L revealed that the preference for acidic residues at positions P-1 and P-2 is largely due to the formation of salt bridges between Arg47 and the side chains of Glu and Asp at the P-1 and P-2 positions. There may be other residues besides Arg47 that are capable of interacting with anionic side chains, since acidic residues at positions P-3 and P-4 are also known to enhance the affinity of peptides for PTP1B. Thus, as is the case with inhibitor 2.13e, the enhanced affinity of inhibitors 2.13a-d may well be due to interactions of the second DFMP group with residues that are not found in either of the two phosphate binding sites.

2.3.4 Bis-DFMP Inhibitors Bearing Alternative Linker Arms

Although the modeling studies suggested that a linker arm consisting of four CH₂ groups would be sufficient for allowing both DFMP groups to interact with the two

83
phosphate binding sites, dual occupancy may require a longer linker and, more importantly, afford more variation in torsion angle freedom. Thus, compound 2.57 (Scheme 2.3.10-11) was prepared in which the two aryl DFMP groups were joined by a longer, yet still flexible, linker. As well, compounds 2.14a-c were prepared, in which the linker arm joining the two DFMP groups was longer, yet was more rigid than that in inhibitors 2.13e and 2.57 (Scheme 2.3.12-13).

![Chemical structure](image)

Our approach to the synthesis of 2.57 involved preparing the phosphonate 2.55 as an intermediate (Scheme 2.3.10). Initially, it was thought that the desired DFMP compound 2.55 could be obtained by reaction of compound 2.54 and ethylene glycol. A test reaction was attempted with benzyl bromide (1.0 eq.) and ethylene glycol (0.3 eq.), in dichloromethane in the presence of silver(I) oxide (1.0 eq.), and after a 12 hour reaction at room temperature the coupled product was obtained in 57% yield. Encouraged by these results, compound 2.54 was prepared in two steps by a CuCl-promoted coupling of (diethylphosphonyl)difluoromethyl cadmium reagent (Burton’s procedure – see Scheme 2.3.4, Section 2.3.1)\(^\text{115}\) with p-iodotoluene followed by bromination of the resulting phosphonate, 2.53, with NBS. We chose to prepare 2.53 via this route instead of electrophilic fluorination since the aryl iodide starting material was commercially available,
and the yields from this procedure are similar to ours for the electrophilic fluorination reaction with NFBS. This also cut the number of synthetic steps down from two to one, as an Arbusov reaction followed by electrophilic fluorination would have to be performed with the commercially available brominated precursor to obtain compound 2.53 by our method. Although the coupling reaction proceeded well (82% yield) we found that in order to get a good yield an excess (4.6 equiv) of the cadmium reagent had to be used. This is in contrast to the original report by Burton and Qui who reported that these couplings should proceed well using just 1.7 equiv of cadmium reagent.\textsuperscript{115} The bromination via NBS yielded the desired monobrominated compound 2.54 in 68% yield, which was contaminated with unreacted starting material 2.53 and a trace amount of dibrominated material. This compound was used without further purification in a reaction with ethylene glycol and silver(I) oxide in dichloromethane. However, after 48 hours, we were surprised to find that the starting material remained, and no desired product, 2.55, was obtained.

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {\includegraphics[width=\textwidth]{scheme.png}};
\node at (4.5, -3) {\textbf{Scheme 2.3.10.} Failed attempt to synthesize compound 2.55.};
\end{tikzpicture}
\end{center}
It was then decided to reverse the order of reaction, and instead link the two phenyl groups first and then perform the coupling reaction (Scheme 2.3.11). The linking of commercially available p-iodobenzyl bromide to ethylene glycol in the presence of 1.0 equiv. of Ag₂O to give 2.56 proceeded reasonably well (52% yield). This was followed by a CuCl-promoted coupling of (diethylphosphonyl)difluoromethyl cadmium reagent with 2.56 to give the bis-phosphonate 2.55 in 80% yield. Subsequent deprotection using TMSBr gave the desired compound, 2.57 as the ammonium salt.

Scheme 2.3.11. Synthesis of inhibitor 2.57.

The synthesis of the triphenyl-type inhibitors 2.14a-c was first attempted in somewhat the same manner as with inhibitor 2.57 (Scheme 2.3.12). The 1,3-benzene diol derivative was reacted with p-iodobenzyl bromide in the presence of K₂CO₃ in DMF, to yield the
iodinated compound 2.58 in 86% yield. However, the product 2.59a of the subsequent coupling reaction with (diethylphosphonyl)difluoromethyl cadmium reagent could not be obtained in pure form.

\[
\text{Scheme 2.3.12. Failed attempt to synthesize compound 2.59a.}
\]

A synthesis was then attempted whereby compound 2.54 was reacted with 1,2-, 1,3-, and 1,4-benzene diols in the presence of K₂CO₃ in DMF (Scheme 2.3.13). The desired triphenyl ethyl-protected esters 2.59a-c were obtained in 44-69% yield by this method. The free acids 2.14a-c were isolated in essentially quantitative yields following deprotection using TMSBr.
Compounds 2.57 and 2.14a-c exhibited IC₅₀ values of 2 - 5 µM with PTP1B (see below), which are similar to the IC₅₀ value of 4.4 µM obtained for the biphenyl inhibitor 2.13e. Thus, increasing the length and altering the flexibility of the linker chain between the two phenyl DFMP groups made little difference in inhibitory potency.
Very recently, Taing et al. also reported PTP1B inhibition studies with a wide variety of compounds bearing two DFMP groups.\textsuperscript{130} The best inhibitor from Taing et al.'s series, compound 2.60 (shown below), exhibited a $K_i$ of 0.93 \textmu M with PTP1B.

Although it was suggested that the two DFMP groups in 2.60 interacted with the two phosphate binding sites in PTP1B, the possibility of less specific interactions with residues
outside the second site was also raised. It is of interest to compare the results obtained by Taing et al. with our results.

Among the inhibitors reported by Taing et al. was compound 2.14c, which they obtained via a 5-step synthesis, and in their hands this compound exhibited a $K_i$ of 2.7 $\mu$M with PTP1B. This is very close to the IC$_{50}$ value of 2 $\mu$M we obtained for compound 2.14c with PTP1B. This result indicates that a comparison of our IC$_{50}$ values with those obtained by Taing et al. is valid.

It is of significance that compound 2.13e ($K_i = 1.5 \mu$M), which exhibits an affinity for PTP1B that is only slightly less than compound 2.60, interacts with Arg47 and not the second phosphate binding site. It is also of significance that compounds 2.57 and 2.14a-c exhibit little difference in inhibitory potency with PTP1B compared to compound 2.13e, yet the length and flexibility of the units linking the two phenyl DFMP groups in compounds 2.57 and 2.14a-c differs significantly from that found in inhibitor 2.13e. Indeed, the potency of compounds 2.57 and 2.14a-c with PTP1B differs little from compound 2.60, which has an even longer spacer separating the two phenyl DFMP groups. There are two possible explanations for these results. One possibility is that, in spite of the differences in the length and flexibility of the spacers separating the two phenyl DFMP groups in both our and Taing et al.’s studies, no none of the compounds reported here or by Taing et al. interact with both phosphate binding sites simultaneously. The second phenyl DFMP increases inhibitory potency by interacting with other positively charged residues that are within the vicinity of the active site, of which there are several (Arg43, Arg45, Arg47, Lys36, Lys116, Lys120). The other possibility is that some of the compounds reported here and by Taing et al. do interact with the second site. However, if this is the case, then bis-DFMP inhibitors that
interact with both phosphate binding sites may not be significantly more potent than bis-DFMP inhibitors that interact with both the catalytic site and other residues that are not part of the second site, such as Arg47.

Compounds 2.13e, 2.57, and 2.14a-c were screened for selectivity by Qingping Wang and Deena Wadeton at Merck-Frosst by comparing their IC$_{50}$ values obtained for PTP1B with those obtained with seven other PTPases (Table 2.3.6).

**Table 2.3.6.** IC$_{50}$ values of inhibitors 2.13e, 2.57, and 2.14a-c.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PTP1B</th>
<th>TCPTP</th>
<th>SHP-1</th>
<th>SHP-2</th>
<th>CD45</th>
<th>Cdc25a</th>
<th>PTPβ</th>
<th>PTPmeg-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.13e</td>
<td>4.4</td>
<td>6.4</td>
<td>31.4</td>
<td>90.6</td>
<td>197.8</td>
<td>16% at</td>
<td>342</td>
<td>9% at</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>250 μM</td>
<td></td>
<td>250 μM</td>
</tr>
<tr>
<td>2.57</td>
<td>3.9</td>
<td>6.1</td>
<td>60.6</td>
<td>151.9</td>
<td>236.1</td>
<td>13% at</td>
<td>300</td>
<td>2% at</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>250 μM</td>
<td></td>
<td>250 μM</td>
</tr>
<tr>
<td>2.14a</td>
<td>5.3</td>
<td>7.9</td>
<td>16.0</td>
<td>31.9</td>
<td>108.1</td>
<td>163.0</td>
<td>77.2</td>
<td>30% at</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>380 μM</td>
</tr>
<tr>
<td>2.14b</td>
<td>4.2</td>
<td>4.6</td>
<td>6.3</td>
<td>15.9</td>
<td>14.2</td>
<td>29.9</td>
<td>15.2</td>
<td>145.7</td>
</tr>
<tr>
<td>2.14c</td>
<td>1.7</td>
<td>3.2</td>
<td>14.5</td>
<td>59.5</td>
<td>41.6</td>
<td>162.8</td>
<td>76.9</td>
<td>30% at</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 μM</td>
</tr>
</tbody>
</table>

The values obtained for the IC$_{50}$'s of compounds 2.13e, 2.57, and 2.14a-c with PTP1B by researchers at Merck-Frosst, shown in Table 2.3.6, are nearly identical to the IC$_{50}$'s we obtained in our lab for these PTP1B inhibitors. Similar to PTP1B, the PTPases TCPTP, SHP-1, SHP-2, and PTPmeg-1 are intracellular PTPases. CD45 and PTPβ are receptor-like PTPases, and Cdc25a is a dual specificity PTPase. Inhibitor 2.14b exhibits a 35-fold preference for PTP1B over PTPmeg-1, but demonstrated poor selectivity with the other PTPases studied. With the exception of 2.14b, the inhibitors in Table 2.3.6 exhibited good selectivity for PTP1B in comparison with Cdc25a and PTPmeg-1. For the other four compounds, selectivity for PTP1B vs. PTPβ ranged from good (45- to 77-fold for compounds 2.13e, 2.57, and 2.14a-c) to poor (2- to 3-fold for compounds 2.14b and 2.14c).
2.13e, 2.57 and 2.14c) to modest (15-fold for inhibitor 2.14a). A similar trend is apparent for the selectivity of these inhibitors for PTP1B vs. CD45. However, the selectivity of all of the inhibitors in Table 2.3.6 for PTP1B vs. TCPTP, SHP-1 and SHP-2 was modest to very poor. The exception to this generalization is compound 2.57, which exhibits a 39-fold preference for PTP1B over SHP-2. Thus, although we have designed fairly potent inhibitors of PTP1B, further work needs to be done in order to render these compounds more selective for our target enzyme.

2.3.5 The Second Phosphate Binding Site – Is it Real?

Given the above results, one might be tempted to disregard the proposed second phosphate binding site as unimportant, or question its very existence. However, a very recent report has further confirmed the existence of this second phosphate binding site in PTP1B, as well as the important role of this enzyme in type II, or insulin-resistant, diabetes. Not surprisingly, the latter is inherently related to the former. Early studies by Kusari and coworkers97 indicated that PTP1B complexed with the insulin receptor in vivo, and that the association of PTP1B with the insulin receptor was absolutely dependent on receptor autophosphorylation. The binding of PTP1B to the receptor was also inhibited by phosphopeptides modeled after the kinase domain of the insulin receptor: D1pY1146ETDPY1150pY1151RK.98 However, Kusari et al.97 indicated that “the structural features of PTP1B that enable it to associate with the autophosphorylated insulin receptor are...unclear.” However, very recently, Barford and coworkers131 determined the molecular basis for the association of PTP1B with the activation segment of the insulin receptor by a combination of crystallographic, kinetic and binding studies utilizing a series of mono-, bis-, and tris-phosphorylated peptides corresponding to the phosphorylated sequence of the
activation segment of the insulin receptor kinase (RD[pY\textsuperscript{1146}ETDpY\textsuperscript{1150}pY\textsuperscript{1151}]RKGGKOLL). Their findings are as follows. Native PTP\textsubscript{1B} exhibits a striking preference for the bis-phosphorylated ((pY)\textsubscript{1150,1151}) and tris-phosphorylated ((pY)\textsubscript{1146,1150,1151}) substrates (K\textsubscript{m} of 14 and 8 \(\mu\)M, respectively) over the mono-phosphorylated substrates (K\textsubscript{m} > 100 \(\mu\)M). The presence of tandem pTyr residues resulted in a 70-fold increase in affinity for a substrate trapping mutant (D181A-PTP\textsubscript{1B}) compared to the monophosphorylated peptides. Crystal structures of the complexes between (C215A-PTP\textsubscript{1B}) (catalytically inactive) and mono-(pTyr\textsubscript{1151}), bis-(pTyr\textsubscript{1150,1151}) and tris-phosphopeptides revealed the following. In the complex of the bis-phosphorylated peptide with PTP\textsubscript{1B}, pTyr\textsubscript{1150} is located in the active site, which indicates substrate specificity for pTyr\textsubscript{1150} relative to pTyr\textsubscript{1151}. The pTyr residue in the catalytic site (1150) interacts with PTP\textsubscript{1B} in a manner identical to that observed for the pTyr residue in the EGFR peptide-PTP\textsubscript{1B} crystal structure obtained by Jia et al.\textsuperscript{33} The carboxylate group of Asp\textsubscript{1149} accepts a hydrogen-bond from the guanidinium group of Arg47, which is not surprising given the preference of PTP\textsubscript{1B} for acidic residues N-terminal to the substrate pTyr. Similar interactions between acidic residues in the EGFR peptide (Glu(P-1) and Asp(P-2)) in complex with PTP\textsubscript{1B} were apparent.\textsuperscript{33} However, the conformation of the Arg47 side chain differs somewhat between the two complexes, indicating that conformational flexibility of this side chain allows Arg47 to interact with a variety of acidic groups N-terminal to the pTyr moiety, or a pTyr-mimetic (as was observed in the case of our compound 2.13e). Residues C-terminal to the active-site-bound pTyr also contribute to substrate-protein interactions. The second pTyr residue (pTyr\textsubscript{1151}) is located within a shallow groove on the protein surface, connected via a channel to the active site cleft. \textit{The location of this second pTyr is in}
a similar position to that which was observed by Puius and coworkers\textsuperscript{127} for high to saturating concentrations of aryl phosphates bound to PTP1B. In other words, pTyr1151 is bound in the postulated second phosphate binding site of PTP1B. Residue pTyr1151 forms salt bridges between its phosphate group and the side chains of Arg24 and Arg254, which are components of the second phosphate binding site in PTP1B. However, it is significant to note that mutagenesis studies revealed that Arg254 is important for good binding while Arg24 contributes very little to the enhanced affinity. The phenyl ring of pTyr1151 participates in long contacts with the side chain of Met258 and the amide side chain of Gln262. Thus, as pointed out by the authors, the pocket for this phenyl ring is not well-defined, and the ring may therefore adopt numerous conformations when bound at this site. Nevertheless, it seems that the purported second phosphate binding site of PTP1B is real, and there is a relevant biological purpose to its existence. However, this binding site is clearly very poorly defined and the main factor that contributes to the enhanced affinity of the tandem pTyr peptides is an electrostatic interaction of the phosphate group of pTyr 1151 with Arg 254.

The tris-phosphorylated substrate was bound in essentially the same manner as the bis-phosphorylated peptide, and there appeared to be no defined binding pocket for pTyr1146, which is consistent with the similar $K_m$ values exhibited by these substrates. The mono-phosphorylated insulin receptor peptide formed strong interactions with PTP1B only with the catalytic site pTyr side chain, and nonspecific peptide-enzyme main-chain interactions were also observed. All of the other peptide side chains were poorly ordered and did not form interactions with PTP1B. This is consistent with the fact that mono-
phosphorylated peptide substrates exhibited $K_m$ values more than 10-fold higher than the multiply-phosphorylated peptides.

The above studies by Barford and coworkers strongly support a supposition in a recent report by Desmarais et al. that the high affinity of E-(F$_2$-Pmp)-(F$_2$-Pmp) for PTP1B ($K_i = 40 \text{ nM}$) is a result of the additional F$_2$-Pmp residue interacting with the second phosphate binding site. However, this does not necessarily mean that inhibitors that interact with both phosphate binding sites will be significantly more potent than those that interact with other positively charged residues that are close to the active site but not part of the second site. Indeed, we now know that 2.13e interacts with Arg47 and not the second phosphate binding site yet is over two orders of magnitude more potent than the mono-DFMP compound 2.9d. The distance between the two phosphate groups in peptides bearing tandem pTyr residues, such as the ones examined by Barford, is greater than in compound 2.13e which may explain why 2.13e does not exhibit dual binding. However, an array of non-peptidyl compounds bearing two DFMP groups attached by linker arms of varying length and/or flexibility, reported here and by Taing et al., did not differ significantly from 2.13e in terms of inhibitory potency. This suggests that either none of these compounds occupy both sites or bis-DFMP inhibitors that interact with both phosphate binding sites may not be significantly more potent than those that interact with other positively charged residues that are close to the active site but not part of the second site. We are unable to distinguish between these two possibilities.

2.3.6 Selectivity and the Second Phosphate Binding Site

It is significant to note that our inhibitors 2.13e, 2.57, and 2.14a-c exhibit very poor selectivity for PTP1B vs. TCPTP (Table 2.3.6). It is also interesting that none of the other
research groups reporting seemingly selective inhibitors of PTP1B examined their inhibitors with TCPTP.\textsuperscript{130,132-136} TCPTP is an intracellular PTPase, and is abundantly and widely expressed.\textsuperscript{137} TCPTP has recently been shown to dephosphorylate the insulin receptor kinase, as well as a tris-phosphorylated phosphopeptide corresponding to the insulin receptor kinase activation (autophosphorylation) site, and a substrate-trapping mutant of TCPTP was capable of trapping the insulin receptor kinase.\textsuperscript{138} Thus, these results extend earlier findings that TCPTP may be a physiological enzyme for the insulin receptor kinase. Unlike mice that lack the gene for PTP1B,\textsuperscript{139} mice that completely lack the gene for TCPTP die soon after birth.\textsuperscript{140} The function of this PTPase has yet to be completely resolved. These results do, however, underscore the need for selective inhibitors of PTP1B vs. TCPTP. Obtaining potent inhibitors that are selective for PTP1B over TCPTP will be a formidable challenge, as these enzymes exhibit a high degree of sequence homology.\textsuperscript{8} Barford and coworkers have compared PTP1B to other PTPase structures, and have suggested that the Asp-pTyr-pTyr-Arg motif that is strongly recognized by PTP1B will be unique to only to this enzyme, and to its close relative, TCPTP.\textsuperscript{131} In particular, the mode of recognition of pTyr1151 in the insulin receptor activation segment, by the second phosphate binding site of PTP1B, is thought to be unique to PTP1B and TCPTP, based on phylogenetic analysis.\textsuperscript{131} As well, like PTP1B, TCPTP has an arginine residue at a position equivalent to Arg47 in PTP1B.\textsuperscript{8} Thus, targeting inhibitor moieties to Arg47 or to the second phosphate binding site is unlikely to result in selectivity between PTP1B and TCPTP. These motifs can potentially be utilized, however, to obtain selective inhibitors of PTP1B relative to other PTPases. For example, PTPases SHP-1 and SHP-2 possess a Lys at the position corresponding to Arg47 in PTP1B,\textsuperscript{8,40} which may not exhibit the same flexibility of binding relative to the arginine residue. This could
explain why the IC\textsubscript{50} value we obtained for compound 2.13e with PTP1B, which is observed to interact with Arg47 in the PTP1B-2.13e complex, was 7- and 21-fold lower than the IC\textsubscript{50}'s exhibited by SHP-1 and SHP-2, respectively, with this inhibitor. As well, certain, but not all, structural elements of the second phosphate binding site are present in SHP-1 and SHP-2.\textsuperscript{8,40} Barford's studies\textsuperscript{131} indicate that the presence of a glycine residue at position 259 is absolutely essential in order for the bulky pTyr moiety to enter into this second phosphate binding site. Any PTPases possessing amino acids with larger side chains at this location would not be able to accommodate a pTyr residue in this pocket. Interestingly, all receptor-like PTPases fall in this category. Also, other residues of the second site are much less highly conserved in the receptor-like PTPases such as PTP\beta and CD45.\textsuperscript{8} As well, PTP\beta and CD45 possess Asn and Val residues, respectively, at the position corresponding to Arg47 in PTP1B,\textsuperscript{8} which could explain why 2.13e was better inhibitor of PTP1B than CD45 or PTP\beta.

2.3.7 PTP1B Inhibitors Recently Reported by other Groups

As mentioned in Section 2.1.3, at the time that we began these studies, there was already a considerable amount of evidence implicating PTP1B as a negative regulator of insulin signaling.\textsuperscript{88-94,97} While our studies were in progress, a seminal paper was published by researchers at McGill University and Merck-Frosst which described the deletion of the PTP1B gene in mice and the resulting physiological effects.\textsuperscript{139} Mice lacking the PTP1B gene had enhanced insulin sensitivity and exhibited increased phosphorylation of the insulin receptor in liver and muscle tissue after insulin injection compared with PTP1B +/- mice. When fed a high fat diet, these mice maintained insulin sensitivity and were resistant to weight gain. Whereas earlier studies were provocative, these results established an unequivocal, functional link between PTP1B and the insulin receptor and strongly suggest
that inhibitors of PTP1B can indeed be useful for the treatment of type II diabetes and perhaps even obesity. Thus, it is not surprising that the area of PTP1B inhibitors has grown tremendously since we began work in this area. Having outlined the potency and selectivity of our inhibitors of PTP1B, perhaps it would be prudent to highlight some of the more relevant developments in the area of small, organic, non-peptidyl, reversible PTP1B inhibitors that have arisen since the onset of our work.

Rice et al. have created a library of small-molecule tyrosine and dual-specificity phosphatase inhibitors, derived from a pharmacophore modeled on natural product inhibitors of phosphothreonine phosphatases. Their inhibitors are based on an oxazole ring, substituted with nonpolar groups, incorporated into a triamide structure, with further nonpolar substituents, as well as a carboxyl group. One of their inhibitors, compound 2.61 shown below, exhibited a $K_i$ of 850 nM with PTP1B (noncompetitive) and $K_i$ of approximately 10 μM (competitive inhibition) for the dual-specificity phosphatases Cdc25A, -B, and -C (a modest 12-fold preference for PTP1B). Inhibitor 2.61 did not inhibit PP1 or PP2A, and its selectivity with other PTPases was not examined.

![Image of compound 2.61]
Researchers at Wyeth-Ayerst have reported azolidinedione derivatives as inhibitors of PTP1B, with submicromolar IC\textsubscript{50} values, however, the selectivity of these inhibitors with other PTPases has not been examined.\textsuperscript{134} A series of benzofuran and benzothiophene derivatives has also been examined by this group for inhibitory activity with PTP1B, and two of these inhibitors, 2.62\textsuperscript{141} and 2.63,\textsuperscript{135} are shown below.

Based on modeling studies, inhibitors such as 2.62 are thought to bind to the active site of PTP1B, with their carboxylic acid moiety interacting with the active site amino acid Arg221. The benzyl moiety was incorporated near this position in the molecule in order to occupy a large pocket that was not being taken advantage of by analogues lacking a large hydrophobic residue at this position. Compound 2.62 was a potent inhibitor of PTP1B, exhibiting an IC\textsubscript{50} value of 83 nM, and showed some selectivity between PTPases, ranging from 5- to 163-fold.\textsuperscript{141} Its selectivity with receptor-like PTPases such as LAR and PTP\textbeta was relatively poor (5- to 6-fold preference for PTP1B, respectively). Compound 2.63 was found to exhibit an IC\textsubscript{50} of 322 nM with PTP1B, and demonstrated from 10- to 100-fold selectivity against the
tested PTPases (LAR, PTPα, VH-R, and He-PTP). The lowest selectivity was observed with LAR (10-fold preference for PTP1B).

Also recently reported was the inhibition of PTP1B with a series of oxalylaminothiophene derivatives. The most potent of these, compound 2.64, exhibits an IC₅₀ value of 5 μM with PTP1B at physiological pH. This inhibitor binds in the phosphate binding loop of PTP1B, and by introducing a basic nitrogen in the core structure of the molecule, interactions are possible with Asp48 in PTP1B. Other PTPases contain an asparagine residue in the equivalent position, which is thought to result in the observed selectivity (>300-fold) demonstrated for 2.64 for PTP1B relative to the PTPases SHP-1, PTPα, PTPε, PTPβ, and LAR. The selectivity of inhibitor 2.64 for PTP1B drops off to a mere 170-fold for the PTPase CD45. This is most likely due to the fact that CD45 also contains an Asp residue at the position equivalent to Asp48 in PTP1B that can interact in a similar manner with the basic nitrogen moiety in the inhibitor.

![Chemical Structure](image)

### 2.3.8 Future Directions

As can be seen from the above discussion, the development of both highly potent and selective inhibitors of PTP1B has yet to be achieved. Our results indicate that using inhibitors bearing two DFMP groups can provide fairly potent inhibitors of PTP1B. However, it is clear that even if such compounds can be designed to interact with both
phosphate binding sites, such compounds will not be selective for PTP1B versus TCPTP unless additional "selectivity" elements are incorporated. The crystal structure of TCPTP has been obtained by Barford and coworkers\textsuperscript{142} but it has not yet been reported in the literature and the coordinates have not been made available to the public. Thus, we are unable to use a rational approach to the design of PTP1B selective inhibitors. However, another means by which selective and potent potential inhibitors can be obtained is through combinatorial chemistry methods. Combinatorial chemistry has been described as a "brute force alternative to the reasoned, intellectual efforts of chemists to design, in a rational manner, compounds for specific purposes."\textsuperscript{143} Combinatorial synthesis assembles building blocks to build new, larger molecules. Large libraries of compounds of interest can be made by assembling all combinations of a set of building blocks. For example, if 20 compounds are reacted with 50 reagents, and the 1000 products of this reaction are reacted with 40 other reagents, the end result is a library of 40,000 members. For reasons of purification and record keeping, combinatorial chemistry is usually performed on a polymer support. Ongoing work in our lab in this area involves the synthesis of small molecule libraries of DFMP-bearing compounds (Scheme 2.3.14). It is hoped that PTP1B-specific inhibitors will be obtained from these libraries. It has also not escaped our attention that inhibitors bearing the DFMP group may not exhibit sufficient cellular penetration to be useful as therapeutics. Consequently, current work in the Taylor group is also focussed upon finding alternative phosphate mimetics that are not dianionic. Recently, the Taylor group has shown that the difluoromethanesulfonic acid (DFMS) moiety is an effective monoanionic phosphate mimic for obtaining PTP1B inhibitors.\textsuperscript{144} A combinatorial approach to the synthesis of DFMS-bearing inhibitors is also being explored in the Taylor group.
Scheme 2.3.14. Combinatorial synthesis of derivatives of aryl-DFMP and aryl DFMS compounds on a soluble solid support.
2.4 REFERENCES


(4) Hunter, T. Cell 1995, 80, 225.


(10) Kennedy, B. P.; Ramachandran, C. Biochem. Pharma. 2000, 60, 877.


(14) Zenner, G.; Zurhausan, J. D.; Burn, P.; Mustelin, T. Bioessays 1995, 17, 967.


(22) Guan, K.; Dixon, J. E. *Science* 1990, 249, 553.


(88) Goldstein, B. J. Receptor 1993, 2, 1.

293, 1156.


19810.


(94) Ramachandran, C.; Aebersold, R.; Tonks, N. K.; Pot, D. A. Biochemistry 1992, 31,
4232.


(98) Seely, B. L.; Staubs, P. A.; Reichart, D. R.; Berhanu, P.; Milarski, K. L.; Saltiel, A.


(106) Compound 2.33 was received from Avinash Thadani, a former undergraduate in the Taylor Group.


(108) These studies were performed by Avinash Thadani, a former undergraduate in the Taylor Group.


Comparison of the synthesis of aryl DFMP compounds via fluorination by DAST vs. NFBS was carried out by Dr. Chris Kotoris, a former graduate student in the Taylor Group.


Deprotections of compounds 2.18c-e were performed by Avinash Thadani, a former undergraduate in the Taylor Group.


Synthesized by Chris Kotoris, a former graduate student in the Taylor Group.

Compounds 2.38-2.40, 2.47, 2.49 and 2.13a-e were screened for inhibitory activity with PTP1B by Dr. Chris Kotoris, a former graduate student in the Taylor Group.


Compound 2.50 was synthesized by Dr. Chris Kotoris, a former graduate student in the Taylor Group.

The Kᵢ values of compound 2.13a with PTP1B at various pHs were determined by Dr. Chris Kotoris, a former graduate student in the Taylor Group.
(127) Puius, Y. A.; Zhao, Y.; Sullivan, M.; Lawrence, D. S.; Almo, S. C.; Zhang, Z.-Y.


(130) Taing, M.; Keng, Y.-F.; Shen, K.; Wu, L.; Lawrence, D. S.; Zhang, Z.-Y.

*Biochemistry* 1999, 38, 3793.


(137) Ibarra-Sanchez, M. d. J.; Simoncic, P. D.; Nestel, F. R.; Duplay, P.; Lapp, W. S.;
Tremblay, M. Seminars in Immunology 2000, 12, 379.

J. Biol. Chem. 2000, 275, 9792.

(139) Elchebyl, M.; Payette, P.; Michaliszyn, E.; Cromlish, W.; Collins, S.; Loy, A. L.;

(140) You-Ten, K. E.; Muise, E. S.; Itie, A.; Michaliszyn, E.; Wagner, J.; Jothy, S.; Lapp,


(142) Personal communication from Barford to S. Taylor.


3. ANTIBODY-CATALYZED ACTIVATION OF A TRIPARTATE PRODRUG

3.1 INTRODUCTION

3.1.1 Catalytic Antibodies

Catalytic antibodies, or “abzymes” were first envisioned thirty-two years ago when Professor William Jencks proposed that one could “synthesize an enzyme” through the use of antibodies. This proposal arose from Linus Pauling’s theory of enzyme catalysis.

Scheme 3.1.1 illustrates a chemical reaction in terms of the free energy changes of the reactants as a function of a reaction coordinate. The transition state (TS) of the reaction occurs at the highest point on the reaction coordinate. The rate of the chemical transformation is proportional to the free energy of activation, which corresponds to the difference in free energy between the reactants and TS. The TS of a molecule is a short-lived, high-energy species containing fractional bond orders, stretched bond lengths, distorted bond...
angles, partial charges and expanded valences.\(^3\) TS theory indicates that an enzyme acts as a flexible molecular template that stabilizes and binds in a complementary fashion to the activated TS of its substrate, with the binding of the enzyme to the reactant and product of the catalyzed reaction being much weaker in comparison. Catalysis of a reaction is therefore due to the enzyme’s ability to stabilize the transition-state structure of the substrate relative to the ground state.\(^4\) This provides a pathway of lower free energy by which the reaction can proceed.

The immune system, specifically the humoral immune system, is capable of generating extremely large numbers of antibodies, or immunoglobulins, in response to a foreign molecule. These protein molecules bind virtually any naturally-occurring or synthetic molecule, or antigen, with high affinity and selectivity. Antibodies are large proteins. Although there are several different classes of antibodies, this thesis will be concerned with only one class, the IgG class. IgG class antibodies are \(\alpha\beta\) dimers with molecular weights in the range of 150 kDa (Scheme 3.1.2).

The two larger peptides are called the heavy chains and have a molecular weight of about 50 kDa, while the two smaller proteins are called the light chains and have molecular weights of about 25kDa. The heavy and light chains are held together by disulfide bonds. The light chains are composed of two domains, \(V_L\) (variable) and \(C_L\) (constant). The heavy chains are divided into \(V_H\), \(C_H1\), \(C_H2\), and \(C_H3\) domains. The variable portions of the heavy and light chains, \(V_H\) and \(V_L\), respectively, are highly polymorphic and vary according to each antigen. These regions are not completely variable, rather most of the variation in amino acid sequence is concentrated into three short hypervariable sequences. It is known that the hypervariable sequences line the antibody’s antigen-binding site and determine the binding
Scheme 3.1.2. A diagram of an IgG molecule.

specificity of the immunoglobulin. Proteolysis by papain cleaves the antibody molecule at the hinge portion of the molecule (shown in Scheme 3.1.2), producing two Fab fragments (antigen binding fragments) and one Fc fragment (named in this way because it is easily crystallized). The Fab portion of the antibody molecule retains all the binding specificity of the entire antibody molecule, since this segment of the immunoglobulin contains the hypervariable sequences.

Conventional enzymes and antibodies share certain similarities in structure and function. Both molecules are proteinaceous, and both participate in binding interactions with their target molecules. Both molecules contain binding pockets that allow them to interact with their targets. The fundamental difference between enzymes and antibodies lies in the events that occur once binding has been achieved. Enzymes have developed to catalyze reactions, whereas antibodies have evolved to form tight binding interactions with antigens
or antigenic determinants. In essence, the former selectively bind transition states, while the latter bind ground states. While enzymes are capable of making and breaking covalent bonds, antibodies use weaker forces for noncovalent binding such as hydrogen bonds, electrostatic interactions, and Van der Waals forces.

Professor William Jencks proposed that the immune system could be exploited for the “synthesis” of new catalysts by raising antibodies to haptenic (immunogenic) groups that resemble the TS of a given reaction. Theoretically, antibodies that bind the TS of a substrate with higher affinity than the substrate or product(s) of the desired reaction could exhibit catalytic activity and facilitate the conversion of substrate to product(s). This idea stems logically from Jencks’ argument that molecules which mimic the TS of an enzyme-catalyzed reaction should be potent inhibitors of the enzyme. While no stable molecule can completely mimic the unstable TS of a reacting species, it has been well-established that transition state analogues (TSAs) can bind tightly as enzyme inhibitors. It was thought that the principles used to design these potent enzyme inhibitors could be applied to the generation of catalytic antibodies for the catalysis of standard reactions in new ways, and especially for the generation of completely novel catalysts. These abzymes could have enormous potential for biological, chemical, and medical applications.

Due to difficulties in the isolation and purification of single-species antibodies, it was not until eighteen years after Jencks’ initial proposal that the notion of catalytic antibodies could be productively pursued. Homogenous immunoglobulins cannot be obtained in quantity by simply cloning a single lymphocyte (antibody-producing cell) and harvesting the immunoglobulin the clone produces, since lymphocytes do not grow continuously in cell culture. The advent of monoclonal antibody technology made it possible to continuously
produce a single molecular species (monoclonal) of antibody by immortalizing antibody-producing clones. This discovery was an important prerequisite for the development of the catalytic antibody field.

Scheme 3.1.3. Schematic overview of monoclonal antibody technology.

In monoclonal antibody technology, as outlined in Scheme 3.1.3, mice are immunized with a haptenic molecule, for example, a transition state analogue (TSA)-carrier protein conjugate. Carrier proteins must be conjugated to the TSA in order to elicit an immune response, since small molecules are not otherwise immunogenic. Following a period of weeks to months, antibody-producing mouse lymphocytes from the spleen are harvested, and are fused with myeloma cells, which are essentially cancer cells and are easily grown in cell culture. The resulting hybridoma cells can be grown in cell culture and produce the antibodies specific for the hapten (TSA-carrier protein conjugate). Thus, sufficient quantities of monoclonal antibodies of a desired specificity can be produced and purified.

Another means of obtaining catalytic antibodies that has developed more recently is through the use of combinatorial libraries of monoclonal antibodies. Nature itself takes a
combinatorial approach to antibody production, so it is only natural that researchers have followed suit. With a repertoire of approximately 100 million antibody molecules of differing specificity, the body cannot carry the genetic information for all of these molecules in every cell. To circumvent this problem, a much smaller set of gene fragments is carried in each cell, and these are randomly recombined in antibody-producing cells to generate the full set of antibody molecules.\textsuperscript{12} In a similar approach, scientists have succeeded in constructing highly diverse combinatorial libraries of Fabs from human origin through a phage vector system.\textsuperscript{12,13} The number of members in these libraries can rival the diversity exhibited by the humoral immune system.\textsuperscript{14}

The first abzyme-catalyzed reactions were reported in 1986, when Peter Schultz\textsuperscript{15} and Richard Lerner\textsuperscript{16} independently published reports of antibody catalysis of the hydrolysis of carbonates and aryl ester molecules, respectively. Schultz and coworkers\textsuperscript{15} characterized abzyme MOTC167, which was capable of catalyzing the hydrolysis of the \( p \)-nitrophenyl carbonate substrate 3.1 shown in Scheme 3.1.4.

![Scheme 3.1.4. Reaction catalyzed by catalytic antibody MOTC167.](image)
This abzyme was obtained by raising antibodies to TSA 3.3 using hybridoma technology. There are similarities between the structure of TSA 3.3 and the TS of the reaction which was believed to resemble intermediate 3.2. Both contained \( p \)-nitrophenyl and \( N \)-trimethylammonioethyl groups, but most importantly, the tetrahedral phosphate group in the TSA mimics the negatively charged tetrahedral carbon center in the TS. These features of the TSA increase the likelihood that the antibody will bind the TS of the desired reaction with higher affinity than the substrate or product(s) of the reaction. MOTC167 antibody was found to exhibit Michaelis-Menten kinetics. It exhibited a \( k_{\text{cat}} \) value of 0.007 s\(^{-1} \), \( K_m \) of 208 \( \mu \)M, and rate enhancement of 770-fold \((k_{\text{cat}}/k_{\text{uncat}})\) for substrate 3.1 in Scheme 3.1.4.\(^\text{15}\) This abzyme bound the TSA with high affinity \((K_d = 1.4\times10^{-6} \text{ M})\) and the TSA inhibited the catalytic activity of MOTC167.\(^\text{15}\)

The research group led by Lerner elicited antibodies to a tetrahedral phosphorus species as well, an anionic phosphonate hapten\(^\text{16}\) (Compound 3.6, Scheme 3.1.5), in order to raise abzymes capable of ester hydrolysis. Lerner's abzyme, antibody 6D4, catalyzed the hydrolysis of the ester substrate 3.4 with a \( k_{\text{cat}} \) of 0.027 s\(^{-1} \), \( K_m \) of 1.9 \( \mu \)M, \( K_i(3.6) \) of 1.6\times10^{-7} \text{ M} \) and rate enhancement of 960-fold. In the case of the TSA 3.6 used to generate abzyme 6D4, it is apparent that the antibody was not raised against the ester substrate 3.4 or against the alcohol or acid hydrolytic products. Rather, the abzyme was raised against the antigenic determinants that appeared in the proposed TS of the reaction, which is thought to resemble intermediate 3.5 in Scheme 3.1.5, namely the charge and geometry of the phosphonate group. It should be noted that the pyridine-2,6-dicarboxylate functionality in the hapten was incorporated to include a metal ion coordination site, with the notion that metalloenzymes employ metal ions to effect hydrolysis.\(^\text{16}\) TSA 3.6, however, was first used as a non-chelated
hapten, and catalytic antibodies were nonetheless elicited to this molecule. Lerner and coworkers were somewhat uncertain as to the role of the picolinyl appendage in catalysis, as preliminary experiments seemed to indicate that metal ions were not necessary for the catalytic reaction.\textsuperscript{16}

![Scheme 3.1.5. Reaction catalyzed by catalytic antibody 6D4.](image)

Great care must be taken in designing an appropriate TSA for inducing a catalytic antibody. If the hapten resembles the starting material too closely, the activation energy may be increased for the reaction, due to substrate stabilization, which would decrease the rate of reaction. If the antibody binds the products of the reaction with high affinity, this may lead to tight complexes and product inhibition. For any chemical reaction for which a catalytic antibody is desired it is important to have a detailed understanding of the reaction mechanism. With such knowledge in hand, features that are unique to the proposed TS of
this reaction (e.g. charges, geometry) may be incorporated into the hapten, and the likelihood of obtaining a catalytic antibody for the reaction will be increased.

Since these initial reports, a number of different strategies have been implemented for hapten design. Catalytic antibody generation is not limited strictly to the use of haptens that function as TSAs (see Reference 3 for a review of this subject) – other approaches to generating abzymes can be used, and often more than one approach is utilized to obtain the abzyme of interest. Catalytic antibodies can function as entropic traps to lower the translational and rotational entropy of a reaction.\textsuperscript{17,18} For example, several abzymes have been reported to catalyze the Diels-Alder [4π+2π] cycloaddition reaction.\textsuperscript{19-25} As the TS of a Diels-Alder reaction resembles the product of the reaction more strongly than the reactants, it is difficult to choose an appropriate hapten for generating antibodies that will not result in the abzyme becoming severely product-inhibited. Hilvert and coworkers\textsuperscript{19} overcame this problem by choosing a Diels-Alder reaction for antibody catalysis where the reaction product does not resemble the TSA for the reaction. The Diels-Alder reaction between tetrachlorothiophene and N-ethylmaleimide (Compounds 3.7 and 3.8, respectively, Scheme 3.1.6) results in the formation of an unstable, tricyclic adduct, 3.9, that spontaneously releases sulfur dioxide to yield a planar bicyclic adduct, 3.10, which does not resemble the TSA, 3.11, to which the antibody was raised. Catalytic antibody 1E9 promoted the target reaction with multiple turnover and a rate enhancement of over 110-fold.
Scheme 3.1.6. Reaction catalyzed by Diels-Alderase antibody 1E9.

Catalytic antibodies can be elicited by “bait and switch” strategies,\textsuperscript{36-35} whereby groups in the hapten are used to generate complementary charges in the antibody binding site. In this approach, the hapten acts as a “bait” for the antibody. The bait contains ionic functional groups that represent charge distributions that are thought to be present in the TS of the reaction. These charged groups then elicit oppositely charged counterparts in the antibody combining site during the immunization process. This counter-charged residue may then result in catalytic activity when the hapten is “switched” to the substrate for the desired reaction. The “bait and switch” approach was first utilized by Schultz\textsuperscript{26} in 1989, in an effort to raise abzymes to catalyze β-elimination reactions. By raising antibodies to an alkylammonium ion, hapten 3.14 in Scheme 3.1.7, Schultz and coworkers succeeded in isolating abzyme 43D4-3D12, which was able to catalyze the β-elimination of a β-fluoroketone substrate (3.12) with a rate enhancement ($k_{\text{cat}}/k_{\text{uncat}}$) of approximately $10^5$. 

\[ [3.7] = 0.61 \text{ mM}; k_{\text{cat}} = 0.07 \text{ s}^{-1}, K_m(3.8) = 21 \text{ mM}, \]
\[ k_{\text{cat}}/k_{\text{uncat}} = 110, K_D(3.11) = 126 \text{ nM}. \]
Catalytic antibody 43D4-3D12 contained a carboxylate group to complement the positive charge in the hapten, which was responsible for the catalytic activity of this abzyme.\textsuperscript{36}

\begin{equation*}
\text{3.12} \xrightarrow{43D4-3D12} \text{3.13}
\end{equation*}

\begin{center}
\begin{tabular}{|c|}
\hline
\text{Scherne 3.1.7.}\hspace{1cm} \text{P-dimination reaction catalyzed by abzyme 43D4-3D12.}\hspace{1cm} \text{TSA to which abzyme 43D4-3D12 was raised} \\
\hline
\end{tabular}
\end{center}

\textbf{Scheme 3.1.7.} \(\beta\)-elimination reaction catalyzed by abzyme 43D4-3D12.

Catalysis by desolvation is another means by which abzymes can catalyze reactions. One example of this phenomenon is the abzyme-catalyzed decarboxylation of nitro-3-carboxybenzisoxazole derivatives.\textsuperscript{37} Kemp\textsuperscript{38} has demonstrated that the rate of decarboxylation of such compounds is largely due to the properties of the surrounding solvent. The rate of decarboxylation increases dramatically by transferring the reagent from an aqueous media to a dipolar aprotic solvent. This is thought to be due to substrate destabilization through loss of hydrogen bonding to a protic solvent and stabilization of the charge-delocalized TS in the dipolar aprotic solvent. Jencks\textsuperscript{1} has noted that enzymes are able to create hydrophobic environments in order to accelerate the rates of reactions, therefore Hilvert\textsuperscript{37,39-41} sought to create a TSA (3.17, Scheme 3.1.8) that could elicit hydrophobic binding pockets with appropriate size and charge distribution postulated for the TS (3.16) for

\[ k_{\text{cat}} = 0.193 \text{ min}^{-1}, \quad K_m = 182 \mu M, \]
\[ k_{\text{cat}}/k_{\text{uncat}} = 10^6, \quad K_i (3.14) = 290 \text{ nM} \]
the decarboxylation reaction. One of Hilvert's best antibody catalysts,\textsuperscript{37} abzyme 21D8, exhibited a rate acceleration of 19,000 for the decarboxylation of 5-nitro-3-carboxybenzisoxazole, which was comparable to rate enhancements observed in mixed solvent systems.\textsuperscript{17}

![Scheme 3.1.8. Decarboxylation reaction catalyzed by abzyme 21D8.](image)

The abzyme-catalyzed reactions described above are merely a small sampling of the capabilities of catalytic antibodies. Over 70 different chemical reactions have been catalyzed by antibodies, including carbamate, amide, phosphate ester, and ether hydrolysis reactions, cycloreversions, isomerizations, cationic cyclizations, and acyl transfer reactions, to name a few.\textsuperscript{17,18,42} Both Thomas\textsuperscript{18,42} and Blackburn\textsuperscript{17} have published excellent recent reviews of the catalytic antibody literature.

3.1.2 Antibody-Directed Abzyme Prodrug Therapy (ADAPT)

One of the most exciting prospects for catalytic antibodies is their potential for therapeutic use in place of enzymes: for example, an abzyme could be used to replace the
activity of an enzyme that is defective in certain disease states. Antibody molecules exhibit an unlimited number of specificities, and methods exist to screen libraries of upwards of 100 million of these potential catalysts. However, there are, in contrast, perhaps only 4000 known enzymes. As well, enzymes may function properly only in certain conditions, whereas catalytic antibodies can be raised that function in a variety of different reaction conditions. Initial work on catalytic antibodies arose from detailed studies of existing enzymes, however, abzyme-catalyzed reactions are not limited to those for which an enzymatic counterpart already exists. As long as a reasonable mechanism for a reaction can be formulated, and an appropriate hapten can be synthesized, one can attempt to generate an abzyme for this reaction.

Antibody-Directed Enzyme Prodrug Therapy, or ADEPT, is a therapeutic strategy aimed at improving the selectivity of anticancer drugs. In the search for the “magic bullet”, a compound that will kill cancer cells without affecting healthy cells, researchers have created antibody-enzyme constructs for the selective delivery of cytotoxic drugs to tumour cells (Scheme 3.1.9). The antibody exhibits specificity for antigens preferentially expressed on the surface of cancerous cells, while the attached enzyme catalyzes the transformation of a prodrug to an active drug. In the first step, the antibody-enzyme conjugate is administered and accumulates where cancer cells are located, and unbound conjugate is given time to clear from the bloodstream. In the second stage of ADEPT, an inactive prodrug is injected. Ideally, a functional group on the free drug known to be critical for cytotoxicity is sequestered through an attachment to the enzyme’s substrate moiety. The prodrug is then converted to the active cytotoxic agent by the enzyme in the conjugate. The advantage of this system is that a single antibody-enzyme conjugate is able to activate
many prodrug molecules, thus leading to an accumulation of the cytotoxic agent in the vicinity of the cancer cells expressing the antigen, while minimizing its presence at healthy cells.

Napier and coworkers$^{46}$ have recently reported the use of ADEPT in the treatment of colorectal carcinoma in cancer patients. Ten patients with colorectal carcinoma expressing carcinoembryonic antigen (CEA) received ADEPT treatment with A5B7 Fab antibody to CEA conjugated to carboxypeptidase G2 (CPG2). This research group has focused on developing ADEPT systems that incorporate the bacterial enzyme CPG2 because this enzyme has no known mammalian equivalent, and catalyzes the hydrolytic cleavage of the prodrug $3.18$, shown in Scheme 3.1.10. After administering a galactosylated antibody directed to the active site of CPG2, to clear and inactivate circulating enzyme, when plasma enzyme levels had fallen to a safe level ($=48$ h), the benzoic acid mustard-glutamate prodrug $3.18$ was administered. This prodrug was converted by CPG2 in the tumour to its cytotoxic
form (3.19), and prodrug conversion to drug was confirmed by finding detectable levels of 3.19 in the plasma, with no detectable levels of circulating antibody-enzyme conjugate.

Scheme 3.1.10. ADEPT system used for treatment of colorectal carcinoma in cancer patients.

Interestingly, the two patients with the highest plasma drug levels had large tumours. Given that no active enzyme was found in the plasma, the drug in the circulation was likely the result of “leakback” from the tumour. Prodrug 3.18 administered alone (in a previous study) did not cause myelosuppression (bone marrow suppression), which was observed to occur in the ADEPT trial due to the circulating free drug. Effective enzyme levels persisted in the tumour for at least up to 85 h. There was evidence of a tumour response – one patient had a partial response, and six of the ten patients had stable disease for an average of four months after previous tumour progression. However, human anti-CPG2 antibodies were found in all patients after 2 weeks, preventing any further therapy.

Preliminary in vivo studies such as these highlight the potential of the ADEPT approach. One of the limitations of the ADEPT approach, however, is the immunogenicity of the antibody-enzyme conjugate. To avoid non-specific activation of the prodrug, a non-endogenous enzyme, such as CPG2, is generally used in the construct, and this renders the
conjugate highly immunogenic. For effective therapy, repeated doses of the cytotoxic agent are often required for the treatment of cancer, so immunogenicity of the conjugate is a severe drawback to ADEPT. The antibody-enzyme construct is also a very large protein molecule, and there may be limitations on dosing patients with this conjugate.

One way to avoid the problems associated with immunogenicity and, to a certain extent, the size of the construct, is to replace the enzyme in the conjugate with a catalytic antibody. One can envision a human, bispecific antibody possessing two completely different binding sites (Scheme 3.1.11). One site would exhibit specificity for tumour-associated antigens, while the other would be a catalytic site, capable of activating the cytotoxic agent. The use of human antibodies would make the conjugate much less immunogenic, and the use of Fab fragments of the antibodies would significantly decrease the size of the construct. This modified version of ADEPT has been referred to as Antibody-Directed Abzyme Prodrug Therapy (ADAPT).

![Scheme 3.1.11. Antibody-Directed Abzyme Prodrug Therapy (ADAPT).](image)

The first group to succeed in generating an antibody capable of prodrug activation was that of Ikou Fujii in 1993. Fujii\(^{47}\) isolated a catalytic antibody, 6D9, that could
hydrolyze an acyl prodrug (Scheme 3.1.12, compound 3.20) of the antibiotic chloramphenicol (3.21) with a rate enhancement ($k_{cat}/k_{uncat}$) of 1800. Prodrug activation was also demonstrated by 6D9 through the inhibition of growth of Bacillus subtilis cells. The chloramphenicol monoester 3.20 inhibited growth of the bacterial cells only when 6D9 was also present. Product inhibition of 6D9 might have been expected, given that the chloramphenicol product (3.21) is incorporated into the TSA (3.22). However, a conformational change between the monoester substrate (and TSA geometry) and the chloramphenicol product minimized product inhibition of 6D9.


Shortly following Fujii's report, Schultz and coworkers reported abzymes possessing similar activity for activating a prodrug (Scheme 3.1.13, compound 3.23) of the
anticancer agent 5-fluorodeoxyuridine (3.24). Catalytic antibody 49.AG.659.12 was raised to TSA 3.25 and catalyzed the hydrolysis of the ester prodrug (3.23) with a rate enhancement of approximately 1000-fold. *In vitro* activity of this abzyme was evidenced by its ability to completely inhibit the growth of *Escherichia coli* HB101 in the presence of the prodrug 3.23, whereas the antibody alone or prodrug alone did not affect the growth of the bacteria at the concentrations used in the activity assay.

Scheme 3.1.13. Conversion of 5-fluorodeoxyuridine prodrug 3.23 to active drug 3.24 by catalytic antibody 49.AG.659.12

Wentworth and coworkers\(^4^9\) have reported the production of a series of catalytic antibodies that can cleave the carbamate prodrug 3.26, shown in Scheme 3.1.14, to effect human tumour cell kill *ex vivo* through the generation of the anti-cancer nitrogen mustard drug 3.27. These abzymes were raised to the phosphonamidate hapten 3.28, based on 5-
aminoisophthalic acid, which was designed to create a more stable, entropically-limited mimic of one rotamer of the glutamic acid prodrug 3.26. Abzyme EA11-D7 was the most effective at reducing the viability of a human tumour cell line in vitro when incubated with prodrug 3.26. This catalytic antibody exhibited a $K_m$ value of 201 $\mu$M with prodrug 3.26, a $k_{cat}$ of 0.03 s$^{-1}$, and the $K_d$ for the hapten 3.28 was approximately 5 nM. Abzyme EA11-D7 turned over roughly 3 equivalents of substrate per antibody site in one hour.


Wentworth and coworkers$^{50}$ also performed detailed kinetic studies with abzyme DF8-D5, which was also raised to hapten 3.28 and was capable of activating prodrug 3.26 (although not quite as well as abzyme EA11-D7). Catalytic antibody DF8-D5 was able to hydrolyze carbamates of type 3.29, permitting a mechanistic analysis of the DF8-D5-catalyzed reaction.
The significantly smaller Hammett $\rho$ value ($\rho = +0.53$) obtained for the antibody-catalyzed reaction compared to that obtained for the uncatalyzed reaction ($\rho = +2.68$) suggested that the antibody reaction proceeded via the highly disfavoured $B_{AC2}$ mechanism rather than the more favoured $E1cB$ process found for the uncatalyzed reaction.\textsuperscript{50} However, abzyme DF8-D5 was not able to hydrolyze $N$-methyl carbamate substrates.\textsuperscript{50} Thus, antibodies raised to TSA 3.28 could be applied antibody-catalyzed prodrug activation, and also were capable of catalyzing a highly disfavoured chemical transformation.

While these early reports demonstrated that antibodies could be used to activate drugs in a specific manner, they all had certain drawbacks. First, all three systems are bipartate prodrugs. In a bipartate prodrug, a specifier is used to target the drug by making it a substrate for a certain enzyme or abzyme, and catalysis at the specifier (e.g. hydrolysis reaction) releases the active drug. \textit{The specifier moiety is linked directly to the drug} (Scheme 3.1.15). As a result, in the above three examples of antibody-catalyzed prodrug activation, the drug had to be incorporated into the TSA. This limits the use of the abzymes to the activation of one particular drug and no other. Ideally, it would be better if a variety of drugs could be activated by a single catalytic antibody. This feature would allow for treatment regimes involving cocktails of prodrugs. Second, since the drug is incorporated into the TSA, the risk of product inhibition by the released drug could be a significant problem.
Finally, prodrugs based on ester linkages (of the type used by Fujii and Schultz) are fairly labile and hydrolyze spontaneously in solution, and there are many endogenous enzymes that hydrolyze esters. Alternate functionalities would be preferable, in order to avoid nonspecific activation of prodrugs in an in vivo setting.¹⁴

Katzenellenbogen and coworkers⁵¹ have described certain problems associated with prodrug design, with specific reference to bipartate and tripartate prodrug systems. As mentioned above, in a bipartate system the specifier is used to target the drug by making it a substrate for a certain enzyme or abzyme, and catalysis at the specifier releases the active drug. Thus, electronic or steric features of the drug may inhibit the activity of the catalytic agent. One way of getting around this problem is to use a tripartate prodrug, in which the specifier and drug are connected by a special linker (Scheme 3.1.15).⁵¹

**Bipartate prodrug system:**

\[ \text{DRUG} \rightarrow \text{SPECIFIER} \rightarrow \text{activation} \rightarrow \text{DRUG} \rightarrow + \text{SPECIFIER} \]

**Tripartate prodrug system:**

\[ \text{DRUG} \rightarrow \text{LINKER} \rightarrow \text{SPECIFIER} \rightarrow \text{activation} \rightarrow \text{SPECIFIER} \rightarrow + \text{LINKER} \rightarrow \text{DRUG} \rightarrow \text{LINKER} \rightarrow \text{spontaneous} \rightarrow \text{DRUG} \rightarrow \text{LINKER} \]

**Scheme 3.1.15.** Bipartate and tripartate prodrug design.

This arrangement serves to distance the drug portion of the molecule from the site of activation, thus the drug moiety would be expected to play a minimal role in enzyme or
abzyme recognition of the specifer. The linker must be designed in such a way that, following activation of the prodrug, there is a spontaneous cascade reaction that releases free drug.

One example of a tripartate prodrug system is that reported by Katzenellenbogen and coworkers,\textsuperscript{51} outlined in Scheme 3.1.16. In this model system, which is activated by the enzyme trypsin, the $N$-Boc Lys group is the specifer moiety, the $p$-amidobenzylxoycarbonyl group is the link, and the drug portion of the system is represented by $p$-nitroaniline. This tripartate prodrug (3.30) was quite stable in aqueous buffer (0.05 M BIS-TRIS, pH 6.9) in the absence of trypsin ($t_{1/2} = 40$ h at 25 °C). However, in the presence of trypsin prodrug 3.30 underwent rapid hydrolysis to release the chromophore $p$-nitroaniline (3.36), which was used to represent the “drug” portion of the prodrug. The half-life of this compound in the presence of trypsin was approximately 11 minutes at 25 °C.

The rapid release of $p$-nitroaniline (3.36) following activation of prodrug 3.30 was thought to be due to the conversion of the weakly electron-donating acyl-amido group in 3.30 to a strongly electron-donating amine group (compound 3.31). This additional release of electron density into the $\pi$ system promotes the spontaneous decomposition of 3.31 to produce the iminoquinone methide 3.33 and the carbamic acid derivative 3.34. This latter molecule is unstable and rapidly loses CO$_2$ to yield $p$-nitroaniline (3.36). The former reacts with water to yield $p$-aminobenzy1 alcohol (3.35). As well, in the absence of the specificier 3.32 incorporated into the prodrug molecule, no activation was observed in the presence of trypsin, thus the carbamate group itself is not susceptible to hydrolysis by trypsin. Katzenellenbogun and coworkers\textsuperscript{51} also indicate that the lability of a benzylic carbamate similar to compound 3.31 should be relatively unaffected by changes in the nature of the
amine-containing drug moiety, thus such a tripartate prodrug system could be used to activate a wide variety of drugs.

Scheme 3.1.16. Tripartate prodrug system developed by Katzenellenbogen et al.\textsuperscript{51}
3.1.3 Objectives

Our specific objective is to determine whether it is possible to obtain an abzyme that is capable of activating a tripartate prodrug similar to that reported by Katzenellenbogen and coworkers.\textsuperscript{51} Tripartate prodrug 3.37 was utilized as a model system (Scheme 3.1.17) to examine this possibility. The idea is to raise an antibody that catalyzes the hydrolysis of the N-methyl carbamate moiety in 3.37. The initial reaction, which would proceed through intermediate 3.38, would produce p-nitrophenol and amine 3.39. Amine 3.39 is unstable and would then undergo a spontaneous and rapid fragmentation \textit{via} an electron relay mechanism resulting in the release of the free drug (3.40).\textsuperscript{51-53}

Several features of our model system warrant mention. We chose p-nitrophenol (PNP) as the specifier since this would allow us to readily monitor the reaction by spectrophotometry or HPLC, and it was anticipated that the synthesis of a prodrug bearing this group would not be overly complex. Carbamate moieties were chosen for attaching the drug to the linker and the linker to the specifier for several reasons. First, carbamate derivatives have been used extensively in the development of prodrugs for ADEPT.\textsuperscript{44,45,54} These compounds are known to be relatively stable under physiological conditions and can be designed to be resistant to hydrolysis by esterases and proteases. Second, in order for an abzyme to be truly catalytic, it must exhibit multiple turnover. Negatively charged products found with ester or amide hydrolysis often experience significant charge interactions with positively charged residues in the antibody active site generated in response to the negative TSAs to which they are generally raised. For this reason, certain hydrolase abzymes can be strongly product inhibited.\textsuperscript{55} Since carbamate hydrolysis yields neutral CO\textsubscript{2}, as opposed to
Scheme 3.1.17. General structure of tripartate prodrug 3.37 for our ADAPT system.
negatively charged carboxylic acid groups, it is expected that this feature will result in an abzyme capable of activating prodrugs with multiple turnover. Third, there are a number of anti-cancer agents (e.g., doxorubicin, melphalan, vinblastine-3-carboxyhydrazide) bearing amino groups, which, when functionalized as carbamates, yield prodrugs with dramatically reduced cytotoxicity. We chose to target an $N$-methyl carbamate instead of an $N$-H carbamate substrate since $N$-H carbamates bearing good leaving groups, such as PNP, hydrolyze very rapidly, making it difficult to perform accurate kinetics studies. On the other hand, $N$-methyl carbamates, even those bearing good leaving groups, are much more stable and hydrolyze approximately $10^8$ times slower. Although $N$-methyl carbamate hydrolysis is a highly challenging reaction for an abzyme, and has never before been reported, Wentworth and coworkers, as previously mentioned, have shown that it is possible to obtain antibodies that catalyze the hydrolysis of $N$-H carbamates by the $B_{AC2}$ mechanism. This suggests that it is possible to obtain antibodies that hydrolyze $N$-methyl carbamates.

In order to obtain an abzyme that will catalyze the hydrolysis of 3.37, antibodies would be raised to a compound (TSA) that mimics the rate-determining TS for the reaction which would resemble intermediate 3.38 (Scheme 3.1.17). Some of the most efficient hydrolytic antibodies have been obtained using phosphorus-based TSA's. Consequently, phosphoramidate 3.41 (Scheme 3.1.17) was chosen as a mimic of the rate determining TS for hydrolysis of the carbamate substrate. It is important to note that the drug component of the prodrug is not incorporated into the TSA. Instead, a carrier protein (necessary for antibody production and screening) is attached to a position on the TSA that is equivalent to the point of attachment of the drug to the prodrug carbamate substrate. Since abzymes are insensitive to changes in the region where the TSA is attached to the carrier
protein antibodies raised to 3.41 should only bind the "non-drug" portion of the prodrug substrate 3.37. By not incorporating the drug into the TSA, the drug should be free to enter the cell rather than bind to the abzyme. This approach also has the advantage that a variety of prodrugs can be activated by a single catalytic antibody.

Here we report the first example of antibody-catalyzed activation of a model tripartate prodrug of type 3.37. We also report here the first example of antibody-catalyzed N-methyl carbamate hydrolysis, one of the most difficult hydrolytic reactions of a carbonyl derivative ever reported for an abzyme.
3.2 EXPERIMENTAL

3.2.1 Materials and Methods

General. Please refer to Section 2.2.1 for a general description of materials and methods.

Instrumentation for HPLC and Spectrophotometry. Analytical and preparative HPLC was performed using reverse-phase HPLC (Waters 600 Controller, Waters 2487 Dual Wavelength (λ) detector), with a Vydam #218TP54 reverse-phase analytical column and Vydam #218TP1022 reverse-phase preparative column, respectively. Kinetic studies employing spectrophotometry were performed using a Varian Cary 1 spectrophotometer except for small-scale kinetic or end-point spectrophotometric assays where a Spectramax190 platereader (Molecular Devices) was used.

3.2.2 Kinetic Studies with Carbamate Derivatives 3.42–3.46, and Carbamate 3.54

Kinetic Studies with Carbamate Derivatives 3.42–3.46. Rates of hydrolyses of 50 μM solutions of the carbamates 3.42–3.46 in 1 M NaOH and/or 50 mM KPi, pH 8.0, 1% DMSO were determined by continuously monitoring the formation of their breakdown products with time using spectrophotometry. The hydrolysis of compounds 3.42, 3.44, and 3.45 were monitored by following the formation of N-methyl-4-nitroaniline at 409 nm. Compounds 3.43 and 3.46, were monitored by following the formation of p-hydroxybenzoic acid at 280 nm. The pseudo-first-order rate constants (k) were obtained by fitting the data to a first-order rate equation using GraphIt, and half-lives were determined from these rate constants (t½ = 0.693/k).

Determination of the pseudo-first-order rate constant for hydrolysis of compound 3.54 at pH 9.0 and 10.0. Due to the stability of compound 3.54, the rate of hydrolysis of 3.54
at pH 9.0 and 10.0 could not be directly measured. Instead, a stock solution of 3.54 was prepared (1 mM) in DMSO, and an aliquot of this solution (50 µL) was added to basic solutions containing 0.10 M NaOH - 1.0 M NaOH (μ = 1.0 [NaCl]) to a final volume of 1.000 mL. The hydrolysis of 3.54 was followed by repetitively scanning the UV/VIS spectrum of the solution at 25°C. The change in absorbance at 400 nm with time was recorded, and pseudo-first-order rate constants were determined using GraFit, after following the hydrolysis to several half lives. The pseudo-first-order rate constants were then plotted vs. hydroxide ion concentration, and from this data the second-order rate constant for the hydrolysis reaction was obtained. The uncatalyzed rates (kUNCAT) at pH 9.0 and 10.0 were obtained through multiplication of the second-order rate constant by 1×10⁻⁵ M and 1×10⁻⁴ M, respectively.

3.2.3 Synthesis of Transition State Analogue (TSA) (3.41)

**O-Methyl-O'-[4-nitrophenyl]-N-methyl-N-[4-(2-alloxycarbonyl)ethyl]phenyl-phosphoramidate (3.49).** Dry ether (20 mL) was added to a 100 mL 3-neck round bottom flask containing methyldichlorophosphite (0.850 mL, 8.95 mmol, 1.0 eq.), and the solution was then cooled to -78°C (dry ice/acetone). A solution of diisopropylethylamine (DIPEA) (3.45 mL, 19.8 mmol, 2.2 eq.) in dry ether (15 mL) was added via syringe over a period of 3 min to the stirred phosphite solution. Stirring was continued for an additional 10 min. A solution of p-nitrophenol (PNP) (1.27 g, 9.13 mmol, 1.02 eq.) in dry ether (15 mL) was added over a period of 60 min, and the solution was stirred for 1 h. Allyl 3-[4-(methylamino)phenyl]propanoate (3.47)59 (2.00 g, 9.13 mmol, 1.02 eq.) was dissolved in dry ether (15 mL) and was added to the reaction mixture over a period of 30 min, at which time the reaction was left to warm to room temperature, stirring for an additional 2.5 h. The
reaction mixture was then filtered quickly through a sintered glass funnel, and the filtrate was concentrated via rotary evaporation. The yellow residue was then placed under high vacuum for 15 min, redissolved in dry dichloromethane (30 mL) and cooled to -40°C (dry ice/acetonitrile). A solution of *meta*-chloroperbenzoic acid (*m*-CPBA) (2.64 g, 15.3 mmol, 1.7 eq.) in dry dichloromethane (30 mL) was added over a period of 10 min, during which time a precipitate formed. The solution was maintained at -40°C for 2 h, and was then filtered quickly through a sintered glass funnel. The filtrate was transferred to an erlenmeyer flask, and a solution of Na$_2$SO$_3$ (5% aqueous, 20 mL) was added, with vigorous stirring. The organic layer was separated, washed with a solution of NaHCO$_3$ (5% aqueous, 20 mL), dried (MgSO$_4$), and concentrated by rotary evaporation. Following purification via silica gel chromatography (hexanes:ethyl acetate = 60:40, $R_f = 0.2$; then repurified in dichloromethane:ethyl acetate = 90:10, $R_f = 0.4$), the desired product was obtained as a light yellow oil (0.600 g, 15%). $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 2.65 (t, $J = 7.7$ Hz, 2H), 2.94 (t, $J = 7.7$ Hz, 2H), 3.21 (d, $J = 9.5$ Hz, 3H), 3.89 (d, $J = 11.7$ Hz, 3H), 4.59 (m, 2H), 5.15 – 5.35 (m, 2H), 5.75 – 6.00 (m, 1H), 7.12 – 7.18 (m, 4H), 7.28 – 7.35 (m, 2H), 8.20 (d, $J = 8.8$ Hz, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 30.2, 35.6, 37.8, 53.9, 65.1, 118.2, 120.6, 123.7, 125.5, 129.1, 132.0, 137.6, 141.1, 144.5, 155.7 (d, $J = 6.5$ Hz), 172.3; $^{31}$P NMR (202.5 MHz, (CD$_3$)$_2$C(O)): $\delta$ 8.45; MS (EI): m/z 434 (M$^+$, 6%), 120 (100%); HRMS(EI) calcd for C$_{30}$H$_{22}$N$_2$O$_7$P 434.1243, found 434.1246.

**Lithium salt O-(4-nitrophenyl) N-methyl-N-[4-(2-allyxycarbonyl)ethyl]phenylphosphoramic acid (3.50).** Compound 3.49 (0.150 g, 0.346 mmol, 1.0 eq.) was dissolved in dry butanone (5 mL), and to this solution was added lithium bromide (0.0294 g, 0.339 mmol, 0.98 eq.). The solution was then refluxed for 1.5 h. The solvent was removed via
rotary evaporation, and the residue was placed under high vacuum for 15 min. The residue was then redissolved in water (50 mL) and washed with ether (2×50 mL) in order to remove any remains of the methyl ester. The aqueous layer was then lyophilized, and the product was recovered as a yellow powder in pure form (0.100 g, 69%). \(^1\)H NMR (200 MHz, (CD\(_3\))\(_2\)C(O)): \(\delta\) 2.59 (t, \(J = 7.5\) Hz, 2H), 2.82 (t, \(J = 7.5\) Hz, 2H), 3.09 (d, \(J = 8.0\) Hz, 3H), 4.54 (dt, \(J = 5.8\) Hz, 1.5 Hz, 2H), 5.10 – 5.35 (m, 2H), 5.80 – 6.05 (m, 1H), 7.01 (d, \(J = 8.4\) Hz, 2H), 7.20 – 7.30 (m, 4H), 7.94 (d, \(J = 9.1\) Hz, 2H); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)C(O)): \(\delta\) 30.6, 36.4, 37.1, (d, \(J = 3.4\) Hz), 65.2, 117.8, 120.2 (d, \(J = 5.1\) Hz), 121.8 (d, \(J = 5.1\) Hz), 125.4, 128.7, 133.3, 133.7, 143.5, 145.1 (d, \(J = 5.1\) Hz), 159.6 (d, \(J = 6.8\) Hz), 172.7 (d, \(J = 1.7\) Hz); \(^{31}\)P NMR (202.5 MHz, (CD\(_3\))\(_2\)C(O)): \(\delta\) 4.13; MS (Electrospray): 419 (M\(^+\) - Li\(^+\), trace), 138 (PNP, 100%).

Dilithium salt \(O-\)-(4-nitrophenyl) \(N\)-methyl-\(N\)-[4-(2-hydroxycarbonyl)ethyl]-phenylphosphoramidic acid (3.41). Compound 3.50 (0.119 g, 0.280 mmol, 1.0 eq.) was dissolved in dry THF (4 mL), and a solution of lithium hydroxide (0.0118 g, 0.280 mmol, 1.0 eq.) in water (2 mL) was added dropwise with stirring. The pale yellow, monophasic solution was stirred at room temperature for 24 h, at which point \(^{31}\)P NMR (202.5 MHz) indicated that the reaction had reached 97% completion. The THF was removed by rotary evaporation, and the water was removed by lyophilization, yielding a yellow solid. The solid was washed extensively with THF to remove all traces of starting material, and the compound was placed on a high-vacuum rotary evaporator to remove the remaining solvent. Trace amounts of water were removed from the product by dissolving the residue in methanol and adding toluene, and once again placing the flask on the high-vacuum rotary evaporator to remove solvent. This procedure yielded a crystalline yellow solid (0.070 g,
64%). $^1$H NMR (200 MHz, CD$_3$OD): $\delta$ 2.39 (t, $J = 8.2$ Hz, 2H), 2.84 (t, $J = 8.2$ Hz, 2H), 3.13 (d, $J = 8.5$ Hz, 3H), 7.09 (d, $J = 8.4$ Hz, 2H), 7.22 (d, $J = 8.5$ Hz, 2H), 7.27 (d, $J = 9.3$ Hz, 2H), 8.12 (d, $J = 9.2$ Hz, 2H); $^{13}$C NMR (125 MHz, CD$_3$OD): $\delta$ 32.1, 36.6 (d, $J = 4.1$ Hz), 40.2, 120.6 (d, $J = 5.1$ Hz), 121.4, 124.9, 128.0, 136.0, 143.1, 144.4 (d, $J = 4.6$ Hz), 159.3 (d, $J = 6.9$ Hz), 180.9; $^{31}$P NMR (202.5 MHz, CD$_3$OD): $\delta$ 0.05; MS (Electrospray): 379 (M$^+ - 2Li^+ + H^+$, 100%).

**O-**Allyl-$^O'$-(4-nitrophenyl) $N$-methyl-$^N'$-[4-(2-alkoxycarbonyl)ethyl]phenyl phosphoramidate (3.52). Dry ether (10 mL) was added to a 100 mL 3-neck round bottom flask containing allyl phosphoryl dichloride$^{60}$ (0.420 g, 2.66 mmol, 1.0 eq.) and the solution was then cooled to -78°C (dry ice/acetonite). A solution of DIPEA (0.920 mL, 5.26 mmol, 2.0 eq.) in dry ether (7 mL) was added via syringe over a period of 15 min to the stirred phosphite solution. Stirring was continued for an additional 15 min. A solution of PNP (0.340 g, 2.44 mmol, 0.9 eq.) in dry ether (7 mL) was added over a period of 60 min, and the solution was stirred for 3.5 h. Allyl 3-[4-(methylamino)phenyl]propanoate (3.47)$^{59}$ (0.500 g, 2.44 mmol, 0.9 eq.) was dissolved in dry ether (7 mL) and was added to the reaction mixture over a period of 30 min, and the reaction was stirred at -78°C for an additional 3 h. The reaction mixture was then allowed to warm to room temperature, was filtered quickly through a sintered glass funnel, and the filtrate was concentrated via rotary evaporation. The brown residue was then placed under high vacuum for 15 min, redissolved in dry dichloromethane (10 mL) and cooled to -40°C (dry ice/acetonitrile). A solution of m-CPBA (0.700 g, 4.06 mmol, 1.5 eq.) in dry dichloromethane (10 mL) was added over a period of 10-15 min, during which time a precipitate gradually formed. The solution was maintained at -40°C for 1 h, and was then filtered quickly through a sintered glass funnel. The filtrate was transferred
to an erlenmeyer flask, and a solution of Na$_2$SO$_3$ (5% aqueous, 20 mL) was added, with vigorous stirring. The organic layer was separated, washed with a solution of NaHCO$_3$ (5% aqueous, 20 mL), dried (MgSO$_4$), and concentrated by rotary evaporation. Following purification via silica gel chromatography (dichloromethane:ethyl acetate = 96:4, R$_f$ = 0.25) the product was obtained as a light brown oil (0.100 g, 10%). $^1$H NMR (300 MHz, CDCl$_3$): δ 2.65 (t, $J = 7.5$ Hz, 2H), 2.94 (t, $J = 7.5$ Hz, 2H), 3.21 (d, $J = 9.3$ Hz, 3H), 4.50 - 4.70 (m, 4H), 5.20 - 5.40 (m, 4H), 5.80 - 6.00 (m, 2H), 7.10 - 7.20 (m, 4H), 7.30 (d, $J = 8.5$ Hz, 2H), 8.20 (d, $J = 8.7$ Hz, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 30.3, 35.8, 37.9, 65.3, 68.2 (d, $J = 5.1$ Hz), 118.4, 119.0, 120.8 (d, $J = 5.8$ Hz), 123.9 (d, $J = 3.4$ Hz), 125.2, 125.6, 129.2, 132.1, 137.6, 141.8 (d, $J = 4.1$ Hz), 144.6, 155.8 (d, $J = 6.7$ Hz), 172.4; $^{31}$P NMR (202.5 Hz, (CD$_3$)$_2$C(O)): δ 6.87; MS (EI): $m/z$ 460 (M$^+$, 100%); HRMS(EI) calcd for C$_{22}$H$_{25}$N$_2$O$_7$P 460.1399, found 460.1394.

3.2.4 Conjugation of TSA 3.41 to Bovine Serum Albumin (BSA) and Keyhole Limpet Hemocyanin (KLH)

To a solution of 3.41 (0.0064 g, 0.0163 mmol, 1.0 eq.) in 60% DMF/H$_2$O (3.2 mL) was added N-hydroxysulfosuccinimide (sulfo NHS) (0.0039 g, 0.0180 mmol, 1.1 eq.) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (0.0034 g, 0.0180 mmol, 1.1 eq.). This solution was stirred at room temperature for 3h. Half of this crude succinimide ester solution (=1.5 mL) was then slowly added to a solution of BSA (0.0060 g, approx. 9×10$^{-5}$ mmol, 0.0055 eq. BSA = 0.18 eq. free lysine residues) in phosphate buffer, pH 7.45 (50 mM KPi, 3 mL), and this clear solution was shaken gently for 12 h at 4°C. A solution of KLH in phosphate buffer, pH 7.45 (50 mM KPi, 2.5 mL) was prepared by dissolving KLH in water (2 mL), and slowly adding a solution of 400 mM KPi (0.3 mL) with
gentle shaking. To this solution was added the remaining crude succinimide ester solution (=1.5 mL) with gentle shaking, and this somewhat cloudy solution was then gently mixed for 12 h at 4°C.

The solution containing the KLH conjugate was dialyzed extensively against phosphate buffer, pH 7.45 (50 mM KPi, 6×1 L) in order to remove unconjugated 3.41 from the sample. The BSA-3.41 conjugate was concentrated via centrifugation using Centricon-10’s™ (Amicon Inc.), and washed extensively with phosphate-buffered saline (PBS: 10 mM NaPi, 150 mM NaCl, 0.1% NaN₃, pH 7.5), in order to remove unconjugated 3.41 from the sample. The BSA-3.41 conjugate was then stored in PBS.

The degree of conjugation to the carrier proteins was determined using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay of Habeeb61, described below. This procedure was carried out with the BSA-3.41 solution only, as KLH is known to precipitate easily from solution, and it is therefore difficult to obtain quantitative results with this protein.

First, the concentration of BSA in the conjugate solution was determined via the bicinchoninic acid (BCA) protein assay, using a kit designed for this purpose (Pierce). A BSA solution was prepared in PBS, with a concentration of BSA identical to the BSA-3.41 conjugate solution. To determine the number of free amino groups, a TNBS assay was used.61 TNBS reacts readily with the primary amino groups of amino acids in basic aqueous solutions, forming brightly colored yellow adducts, and there are roughly 33 free lysine residues per molecule of BSA. Samples were prepared consisting of Millipore water (150 µL), a solution of NaHCO₃ (4% aqueous, 250 µL), TNBS (0.1%, 250 µL), and either BSA alone, BSA-3.41 conjugate, or PBS (blank) solutions (150 µL). These samples were incubated at 40°C for 2 h. Once the samples had cooled to room temperature, aliquots of 650
μL were transferred to test tubes, to which solutions of sodium dodecylsulfate (SDS) (10% aqueous, 250 μL) and HCl (1.0 M, 125 μL) were added. The absorbances of these solutions were read at 335 nm, and from this data the number of transition state analogues (3.41) attached to BSA in the BSA-3.41 conjugate was determined.

3.2.5 Preparation of Standard Curves for Detection and Quantitation of PNP by HPLC

Smaller-range standard curve: A series of stock solutions of PNP were prepared (0 μM to 10 μM, in 5/95 DMSO/bicine [50 mM, pH 9.0]), and to an aliquot of each of these solutions (60 μL) was added a solution of p-nitroaniline (PNA) (70 μM, 10 μL, in 5/95 DMSO/bicine [50 mM, pH 9.0]; internal standard) and concentrated perchloric acid (3.5 μL). Using a 5 μL injection loop, these samples were analyzed via reverse-phase analytical HPLC: λ_{det} = 325 nm; mobile phase = 20/80 acetonitrile/water (0.1 % TFA) for 20 min, ramp to 100% acetonitrile over 5 min, holding 100% acetonitrile for 15 min, ramp back to 20/80 acetonitrile/water (0.1 % TFA) over 5 min, holding this solvent system for 15 min, flowrate = 1 mL/min throughout. Retention times for the analytes were as follows: PNP 13 - 14 min; PNA 10 - 11 min. The plot of concentration of PNP/PNA vs. peak area ratio of PNP/PNA was found to be linear. This standard curve was used for the detection of PNP in the initial screening for catalytic activity of antibodies with substrate 3.54 (Section 3.2.6).

Longer-range standard curve: A longer-range standard curve was prepared, with CHES (50 mM, 100 mM NaCl, and 0.01% NaN₃, pH 10.0) as the buffer system. A series of stock solutions of PNP were prepared (0 μM to 50 μM, in 5/95 DMSO/CHES [50 mM CHES, 100 mM NaCl, and 0.01% NaN₃, pH 10.0]), and to an aliquot of each of these solutions (40 μL) was added a solution of 5/95 DMSO/CHES (as above, 10 μL), PNA (70
µM, 10 µL, in 5/95 DMSO/CHES (as above); internal standard) and concentrated perchloric acid (3.5 µL). Using a 5 µL injection loop, these samples were analyzed via reverse phase analytical HPLC, using the same conditions as with the smaller-range standard curve. The plot of concentration of PNP/PNA vs. peak area ratio of PNP/PNA was found to be linear, and this linear relationship was essentially exactly the same as with the smaller range standard curve. This standard curve was used for the detection of PNP in the detailed kinetic studies of ST51 with substrate 3.54 (Section 3.2.13) at pH 9.0 and 10.0.

3.2.6 Initial Screening for Catalytic Activity of Antibodies with Substrate 3.54

An aliquot (10 µL) of a stock solution of substrate 3.54 (20 mM) in DMSO was added to bicine buffer (100 mM bicine, 100 mM NaN₃, pH 9.0) and the solution was mixed well. A solution of the antibody in bicine buffer (100 mM bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0) was added, so that the final volume was 200 µL and the final concentration of antibody was approximately 4 µM. The final concentration of DMSO was 5%, and the final concentration of 3.54 was 1 mM in the antibody-containing reactions. The control reaction consisted of a solution of 1 mM 3.54 in 5/95 DMSO/bicine buffer (as above), with no antibody present. These solutions were mixed gently and were maintained at 25°C in a water bath for a period of 40 h. At this point, the solutions were gently mixed, and an aliquot (40 µL) of each reaction mixture was withdrawn. To this was added a solution of 5/95 DMSO/bicine buffer (as above) (10 µL), a solution of PNA (10 µL of 70 µM in 5/95 DMSO/bicine buffer; internal standard), and concentrated perchloric acid (3.5 µL). This solution was mixed well, precipitated antibody was removed via centrifugation (microcentrifuge), and the concentration of PNP in the solution was determined by reverse-phase analytical HPLC (as outlined in Section 3.2.5, using the smaller-range standard curve data).
3.2.7 Inhibition Studies with mAbs ST51, ST30, and ST24

An aliquot (10 μL) of a stock solution of substrate 3.54 (20 mM) in DMSO was added to bicine buffer (100 mM bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0) and the solution was mixed well. A solution of the antibody (ST51, ST30, ST24) in bicine buffer (100 mM bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0) was added, so that the final volume was 200 μL and the final concentration of antibody was 4 μM. The final concentration of DMSO was 5%, and the final concentration of 3.54 was 1 mM in the antibody-containing reactions. Duplicates of each antibody solution were prepared, and to one of the duplicates was added a solution of 3.41 (2 μL of 1 mM) in DMSO, to yield a final concentration of 3.41 of 10 μM in these reaction mixtures. The control reaction consisted of a solution of 1 mM 3.54 in 5/95 DMSO/bicine buffer (as above), 10 μM 3.41, with no antibody present. These solutions were mixed gently and were maintained at 25°C in a water bath for a period of 46 h. At this point, after gentle mixing of the solutions, an aliquot (40 μL) of each reaction mixture was withdrawn. To this was added a solution of 5/95 DMSO/bicine buffer (as above) (10 μL), a solution of PNA (10 μL of 70 μM in 5/95 DMSO/bicine buffer; internal standard), and concentrated perchloric acid (3.5 μL). This solution was mixed well, precipitated antibody was removed via centrifugation (micro-centrifuge), and the concentration of PNP in the solution was determined by reverse-phase analytical HPLC (as outlined in Section 3.2.5, using the smaller-range standard curve data).

3.2.8 Purification of ST51 from Ascites for Purpose of Detailed Kinetic Studies

Ascites fluid (received from Banting and Best) containing ST51 was diluted 1:1 with Immunopure® Binding Buffer (Pierce), to a total volume of 2 to 8 mL. This solution was filtered through glass wool if precipitates were present, and was then applied to an
Immunopure® Plus Immobilized Protein A column (Pierce; capacity = 17 mg mouse IgG), which had been equilibrated with the Binding Buffer (5 mL = 5 column volumes). The column was then washed with Binding Buffer (10 to 20 mL), and the absorbance of the fractions, collected in small aliquots (= 1 mL), was monitored at 280 nm. Once all nonspecific protein had been eluted, the column was then washed with Immunopure® Mouse IgG1 Mild Elution Buffer (Pierce), the eluate was collected in small fractions (= 1 mL; generally 5 to 8 mL were collected), and the absorbance of the fractions was again monitored at 280 nm. The fractions containing antibody were pooled and dialyzed against an appropriate buffer system.

The concentration of ST51 in solution after dialysis was determined using the Bradford assay. The samples of ST51 were then subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in a 7.5% acrylamide gel according to the method developed by Laemmli. The gels were stained with Coomassie Brilliant Blue R, destained in 7/3/6 methanol/acetic acid/water, and vacuum-dried at 80 °C.

Regardless of the buffer used for kinetic studies with ST51, a portion of the purified antibody was always dialyzed against bicine, pH 9.0 (50 - 100 mM bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0). A rapid screen was then performed with substrate 3.54 to verify the catalytic activity of the purified ST51 (as described in Section 3.2.6).

3.2.9 Preparation of ST51 Fab

ST51 Fab was prepared using protocols and reagents from a Pierce Immunopure® Fab Preparation Kit that has been specially designed to generate and isolate mouse Fab fragments. Pure ST51 mAb was dialyzed extensively against a 20 mM phosphate/10 mM
EDTA buffer at pH 7.0. This solution was concentrated and was subjected to papain digestion for 12 h at 37°C. The papain used for proteolysis was immobilized on a support of cross-linked 6% beaded agarose, and was easily separated from the digested antibody sample. The Fab fragments were purified by application of the digested mAb to a Protein A-agarose column (Pierce). The solution containing the pure Fab fragments was concentrated and dialyzed against bicine buffer (50 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0). The concentration of ST51 Fab was determined via the Bradford assay. ST51 Fab was analyzed by SDS-PAGE (as previously described in Section 3.2.8), and a rapid screen was performed to verify the catalytic activity of the purified Fab (as described in Section 3.2.6).

3.2.10 Testing for Catalytic Activity of BSA with Substrate 3.54

BSA was tested for catalytic activity with compound 3.54 in the same manner as described in Section 3.2.6, except that no antibodies were present in the reaction mixture and the final concentration of BSA was approximately 4 μM.

3.2.11 Screening for ST51 Catalytic Activity at Various pH

An aliquot (10 μL) of a stock solution of substrate 3.54 (20 mM) in DMSO was added to each of three buffered solutions (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 7.4; 100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0; 50 mM CHES, pH 10.0) and the solutions were mixed well. A solution of ST51 in an appropriate buffer was added to each solution, so that the final volume was 200 μL and the final concentration of antibody was 4 μM. The final concentration of DMSO was 5%, and the final concentration of 3.54 was 1 mM in the antibody-containing reactions. The control reaction consisted of a solution of 1 mM 3.54 in 5/95 DMSO/buffer, with no antibody present, for each of the three buffer systems. These solutions were mixed gently and were maintained at 25°C in a water bath for
a period of 91 h. At various intervals, after gentle mixing of the solutions, an aliquot (40 μL) of each reaction mixture was withdrawn. To this was added a solution of 5/95 DMSO/appropriate buffer (10 μL), a solution of PNA (10 μL of 70 μM in 5/95 DMSO/appropriate buffer; internal standard), and concentrated perchloric acid (3.5 μL). This solution was mixed well, precipitated antibody was removed via centrifugation (microcentrifuge), and the concentration of PNP in the solution was determined by reverse-phase analytical HPLC (as outlined in Section 3.2.5, using longer-range standard curve).

3.2.12 Stability tests of ST51 at pH 9.0 and 10.0

Stock solutions of ST51 in bicine (100 mM bicine, 100 mM NaCl, 0.01 % NaN3, pH 9.0) and CHES (50 mM CHES, 100 mM NaCl, 0.01 % NaN3, pH 10.0) were incubated at 25°C (water bath) over a period of several days. These stock solutions were used to initiate ST51-catalyzed reactions roughly every 24 h, and each reaction was analyzed for PNP after a 24 h time period. The ST51-catalyzed reactions consisted of 5 μM ST51, 1 mM 3.54, in 5/95 DMSO/buffer (described above), to a total volume of 55 μL. After 24 h, the solutions were mixed gently, and an aliquot (40 μL) of each reaction mixture was withdrawn. To this was added a solution of 5/95 DMSO/appropriate buffer (10 μL), a solution of PNA (10 μL of 70 μM in 5/95 DMSO/appropriate buffer; internal standard), and concentrated perchloric acid (3.5 μL). This solution was mixed well, precipitated antibody was removed via centrifugation (microcentrifuge), and the concentration of PNP in the solution was determined by reverse-phase analytical HPLC (as outlined in Section 3.2.5, using longer-range standard curve).
3.2.13 Detailed Kinetic Studies with ST51 and Substrate 3.54 at pH 9.0 and 10.0

An aliquot (12.5 μL) of a stock solution of substrate 3.54 (3 mM - 40 mM) in DMSO was added to bicine buffer (100 mM bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0) and the solution was mixed well. A solution of the antibody in bicine buffer (100 mM bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0) was added, so that the final volume was 250 μL and the final concentration of antibody was 5 μM. The final concentration of DMSO was 5%, and the final concentration of 3.54 ranged from 0.150 mM to 2 mM in the antibody-containing reactions. The control reactions consisted of a solution of 0.150 mM to 2 mM 3.54 in 5/95 DMSO/bicine buffer (as above), with no antibody present. These solutions were mixed gently and were maintained at 25°C in a water bath. At various time intervals, the solutions were gently mixed, and an aliquot (40 μL) of each reaction mixture was withdrawn. To this was added a solution of 5/95 DMSO/bicine buffer (as above) (10 μL), a solution PNA (10 μL of 70 μM in 5/95 DMSO/bicine buffer; internal standard), and concentrated perchloric acid (3.5 μL). This solution was mixed well, precipitated antibody was removed via centrifugation (micro-centrifuge), and the concentration of PNP in the solution was determined by reverse-phase analytical HPLC (as outlined in Section 3.2.5, using longer-range standard curve). The reactions were followed to approximately 6% completion, and plots of the concentration of PNP over time exhibited excellent linearity. The initial velocities of the reactions at the various substrate concentrations were determined by taking the slopes of these plots. This procedure was repeated in duplicate for each substrate concentration. Studies carried out at pH 10.0 (50 mM CHES, 100 mM NaCl, 0.01 % NaN₃, pH 10.0) were performed in a similar manner. Lineweaver-Burk plots were generated by plotting \((V_{\text{init}})^{-1}\) as a function of \([S]^{-1}\), using Grafit.
3.2.14 Kinetic Studies with ST51 and TSA 3.41 using Indirect Competitive Enzyme-Linked Immunosorbent Assay (ELISA)⁶⁵

**Test for linearity of relationship between [ST51] and absorbance of final assay:**

*Coating the microplate with BSA-3.41:* A 96-well microplate (Nunc Maxisorp) was coated with BSA-3.41 conjugate (80 µL, 1.25 µg/mL in 50 mM potassium phosphate buffer, pH 7.45) for 15 h (covered, 4°C), washed (150 mM NaCl; 4×50 mL), and blocked with BSA.

*Blocking unbound sites on microplate wells:* The microplate wells were filled with blocking solution (10 mg/mL BSA, in 20 mM Tris, 150 mM NaCl, pH 7.4 (TBS)) and the plate was incubated at room temperature for 5 h. After blocking, the plate was washed (TBS: 4×50 mL), and was dried by shaking onto a pad of paper towel on the laboratory bench.

*Incubation of ST51 in microplate wells:* Solutions of ST51 (0 to 0.5 nM, in 100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0, supplemented with 10 mg/mL BSA) were incubated at room temperature for 1 h, and were transferred to the microtitre plate (150 µL per well) directly following the blocking step. Each ST51 sample was analyzed in quadruplicate. The microplate was covered, incubated at room temperature for 1 h, washed with Millipore water (4×50 mL), and dried as described above.

*Final assay for detecting ST51 that bound to BSA-3.41 in microplate wells:* Goat-anti-mouse (Fc) alkaline phosphatase conjugate (Sigma) was added to the microplate (100 µL per well, 0.25 µg/mL in blocking solution (10 mg/mL BSA in TBS)), and the microplate was covered and incubated at room temperature for 1.7 h. The plate was then washed (150 mM NaCl, 50 mM Tris, 0.05 % Tween-20, pH 7.4 (TBS/Tween); 4×50 mL), rinsed with assay buffer (10 mM diethanolamine, 0.5 mM MgCl₂, pH 9.5; 2×25 mL), and dried. A solution of p-nitrophenyl phosphate (PNPP) (disodium salt hexahydrate) (0.030 g) in assay buffer was added, and the plate was covered and incubated for 1 h at room temperature. The plate was then washed (150 mM NaCl, 50 mM Tris, 0.05 % Tween-20, pH 7.4 (TBS/Tween); 4×50 mL), and the absorbance of the wells was measured.
buffer (10 mM diethanolamine, 0.5 mM MgCl₂, pH 9.5; 15 mL) was prepared immediately before use. This solution was transferred to the microplate (100 μL per well) and the development of yellow color was monitored over time using a microplate reader, detecting at 405 nm. The resulting absorbance values were corrected for background absorbance of blanks/controls (see below) and were plotted vs. [ST51].

**Controls/Blanks used:** Nonspecific binding of ST51 to the wells was tested by adding solutions of various concentrations of ST51 to microplate wells that had been blocked with BSA, but not coated with BSA-3.41, and no nonspecific binding was detected. The absorbance of the final assay was small and constant for these wells. As well, bicine (100 mM bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0) containing 10 mg/mL BSA was added to the coated and blocked microplate wells, in place of ST51-containing solutions, to determine the background absorbance of the final assay. Again, the absorbance of the final assay was small and constant, and was the same as the wells that had been tested for nonspecific binding of ST51.

**Test for <10% binding:** The same experimental conditions were used as described above, except that following incubation of the various concentrations of ST51 solutions in the wells of the microplate (previously coated with BSA-3.41 and blocked with BSA), the contents of each well were transferred to the wells of another coated and blocked microplate, and were incubated in the new plate for the same amount of time, concurrently with wells containing solutions of the same concentration of ST51 that had not been previously incubated in the first microplate. In all wells, the antibody trapped in the ELISA was detected as described above, and the percentage of antibody retained in the first well was calculated. Each ST51 sample was analyzed in quadruplicate.
Determination of $K_D$ for TSA 3.41 and ST51. *Coating the microplate with BSA-3.41; blocking unbound sites on microplate wells:* same as described above.

*Preincubation of ST51 and TSA 3.41, followed by incubation of ST51/TSA solutions in microplate wells:* TSA 3.41 at various concentrations (0.3125 nM to 100 nM) was mixed with a constant amount of ST51 (0.5 nM, in 100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0, supplemented with 10 mg/mL BSA). These solutions were incubated at room temperature for 1 h, then each solution was transferred to the microtitre plate (150 µL per well) directly following the blocking step. Each ST51/TSA sample was analyzed in quadruplicate. After covering the microplate and incubating at room temperature for 1 h, the microplate was then washed with Millipore water (4×50 mL), and dried.

*Final assay for detecting ST51 bound to BSA-3.41 in microplate wells:* same as described above. The resulting absorbance values were corrected for the background absorbance of the blanks/controls (see below) and were used to create a Scatchard plot for measuring the affinity of ST51 for TSA 3.41. See Section 3.3.7 for actual equations used for this analysis.

*Controls/Blanks used:* Nonspecific binding of ST51 and TSA 3.41 to the wells was tested by adding solutions of 0.5 nM ST51, and solutions of TSA (0.3125 nM to 100 nM) and 0.5 nM ST51 to microplate wells that had been blocked with BSA, but *not* coated with BSA-3.41, and no nonspecific binding was detected of ST51 or the TSA 3.41. The absorbance of the final assay was small and constant for these wells. As well, bicine (100 mM bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0) containing 10 mg/mL BSA was added to the coated and blocked microplate wells, in place of ST51/TSA-containing solutions, to determine the background absorbance of the final assay. Again, the absorbance of the final assay was small.
and constant, and was the same as the wells that had been tested for nonspecific binding of ST51 and the TSA 3.41.

3.2.15 Synthesis of Ester, Amide, Carbamate and Carbonate Substrates 3.62-3.89

3-(4-[(4-Nitrophenoxy)carbonyl]amino)phenyl)propanoic acid (3.62). To a cooled and stirred solution of tert-butyl 3-(4-aminophenyl)propanoate (3.60) (0.200 g, 0.904 mmol, 1.0 eq.) and N-methylmorpholine (0.109 mL, 0.991 mmol, 1.1 eq.) in dry dichloromethane (10 mL) was slowly added p-nitrophenyl chloroformate (0.200 g, 0.994 mmol, 1.1 eq.). Within 15 min, a fine precipitate had formed (N-methylmorpholine hydrochloride salt). After 2 h, none of the amine derivative remained (observed by TLC). Dichloromethane (15 mL) was added to the flask, and the organic layer was washed with water (2×20 mL), 1.0 M HCl (2×20 mL), brine (2×20 mL), dried (MgSO4) and concentrated under reduced pressure to yield a pale yellow oil. The crude material 3.61 was then redissolved in a solution of trifluoroacetic acid (TFA) in dry dichloromethane (30 % TFA, 5 mL), and the solution was stirred at room temperature for 2 h, at which time the reaction had reached completion (TLC). The solvent was removed by rotary evaporation, the product was redissolved/resuspended in dichloromethane, and the flask was again placed on the rotary evaporator. After repeating this procedure several times to remove most of the TFA, the product was placed under high vacuum for 6 h. The pale yellow solid was recrystallized in dichloromethane/ethyl acetate, and pure 3.62 was obtained as a white solid (0.085 g, 29%). mp = 148-150 ºC; 1H NMR (200 MHz, (CD3)2C(O)): δ 2.62 (t, J = 7.7 Hz, 2H), 2.91 (t, J = 7.7 Hz, 2H), 7.28 (d, J = 8.8 Hz, 2H), 7.54 (d, J = 8.8 Hz, 4H), 8.33 (d, J = 9.5 Hz, 2H), 9.12 (bs, = 1H), 10.57 (bs, = 1H); MS (EI): m/z 330 (M+, trace), 191 (M+ - HOArNO2, 48%), 132 (100%); HRMS(EI) calcd for C16H14N2O6 330.0852, found 330.0844.
**Tert-butyl 3-[[4-(4-nitrobenzyl)oxy]carbonyl]amino]phenyl]propanoate (3.63).**

To a cooled and stirred solution of tert-butyl 3-(4-aminophenyl)propanoate (3.60) (0.200 g, 0.904 mmol, 1.0 eq.) and *N*-methylmorpholine (0.109 mL, 0.991 mmol, 1.1 eq.) in dry dichloromethane (10 mL) was slowly added *p*-nitrobenzyl chloroformate (0.214 g, 0.994 mmol, 1.1 eq.). Within 5 min, a fine precipitate had formed (*N*-methylmorpholine hydrochloride salt). After 20 min, none of the amine derivative remained (observed by TLC). Dichloromethane (15 mL) was added to the flask, and the organic layer was washed with water (2×20 mL), 1.0 M HCl (2×20 mL), brine (2×20 mL), dried (MgSO₄) and concentrated under reduced pressure to yield a white solid. Compound 3.63 was obtained in pure form following recrystallization in hexanes/dichloromethane (0.255 g, 71%). mp = 118-119 °C; $^1$H NMR (300 MHz, CDCl₃): δ 1.42 (s, 9H), 2.51 (t, $J$ = 7.7 Hz, 2H), 2.87 (t, $J$ = 7.7 Hz, 2H), 5.29 (s, 2H), 6.67 (s, 1H), 7.16 (d, $J$ = 8.0 Hz, 2H), 7.31 (d, $J$ = 8.0 Hz, 2H), 7.56 (d, $J$ = 8.0 Hz, 2H), 8.24 (d, $J$ = 8.0 Hz, 2H); $^{13}$C NMR (50 MHz, CDCl₃): δ 28.1, 30.5, 37.1, 65.3, 80.4, 119.3, 123.7, 128.3, 128.9, 135.8, 136.4, 143.7, 147.8, 153.1, 172.2; MS (EI): m/z 400 (M$^+$, 8%), 344 (M$^+$ + H$^+$ - C(CH₃)₃, 58%), 136 (CH$_3$ArNO$_2$, 62%), 57 (C(CH$_3$)$_3$, 100%); HRMS (EI) calcd for C$_{21}$H$_{24}$N$_2$O$_6$ 400.1634, found 400.1631.

**3-[[4-(4-Nitrobenzyl)oxy]carbonyl]amino]phenyl]propanoic acid (3.64).** The tert-butyl ester 3.63 (0.150 g, 0.375 mmol) was dissolved in a solution of TFA in dry dichloromethane (30 % TFA, 5 mL), and the solution was stirred at room temperature for 2 h, at which time the reaction had reached completion (TLC). The solvent was removed by rotary evaporation, the product was redissolved/resuspended in dichloromethane, and the flask was again placed on the rotary evaporator. After repeating this procedure several times to remove most of the TFA, the product was placed under high vacuum for 12 h.
Recrystallization in ethyl acetate yielded pure product (0.107 g, 83%) as a white solid. mp = 195-196 °C; $^1$H NMR (200 MHz, (CD$_3$)$_2$C(O)): $\delta$ 2.60 (t, $J = 7.5$ Hz, 2H), 2.88 (t, $J = 7.5$ Hz, 2H), 5.34 (s, 2H), 7.22 (d, $J = 7.9$ Hz, 2H), 7.50 (d, $J = 7.9$ Hz, 2H), 7.72 (d, $J = 8.1$ Hz, 2H), 8.28 (d, $J = 8.1$ Hz, 2H), 8.86 (bs, < 1H); $^{13}$C NMR (50 MHz, (CD$_3$)$_2$C(O)): $\delta$ 31.0, 36.1 65.7, 119.8, 124.4, 129.3, 129.6, 136.8, 138.1, 145.7, 148.8, 154.2, 173.9; MS (EI): $m/z$ 344 (M$^+$, 5%), 153 (HOCH$_2$ArNO$_2$, 15%), 191 (M$^+$ - HOCH$_2$ArNO$_2$, 50%), 132 (100%); HRMS(EI) calcd for C$_{17}$H$_{16}$N$_2$O$_6$ 344.1008, found 344.1009.

*Tert*-butyl 3-[(phenoxy carbonyl)amino]phenyl]propanoate (3.65). To a cooled and stirred solution of *tert*-butyl 3-(4-aminophenyl)propanoate (3.60) (0.200 g, 0.904 mmol, 1.0 eq.) and N-methylmorpholine (0.109 mL, 0.991 mmol, 1.1 eq.) in dry dichloromethane (10 mL) was slowly added phenyl chloroformate (0.125 mL, 0.994 mmol, 1.1 eq.). Within 5 min, a fine precipitate had formed (N-methylmorpholine hydrochloride salt) and none of the amine derivative remained (observed by TLC). Dichloromethane (25 mL) was added to the flask, and the organic layer was washed with water (2 x 25 mL), brine (25 mL), dried (MgSO$_4$) and concentrated under reduced pressure to yield an off-white solid. Compound 3.65 was obtained in pure form following recrystallization in hexanes/ethyl acetate (0.230 g, 75%). mp = 80 – 81 °C; $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 1.42 (s, 9H), 2.52 (t, $J = 7.7$ Hz, 2H), 2.89 (t, $J = 7.7$ Hz, 2H), 6.86 (bs, 1H), 7.1 – 7.5 (m, 9H); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 28.1, 30.4, 37.1, 80.4, 118.8, 121.6, 125.6, 129.0, 129.3, 135.4, 136.4, 150.6, 151.6, 172.2; MS (EI): $m/z$ 341 (M$^+$, 8%), 191 (M$^+$ - OAr - C(CH$_3$)$_3$, 96%), 57 (C(CH$_3$)$_3$, 100%); HRMS(EI) calcd for C$_{20}$H$_{23}$NO$_4$ 341.1627, found 341.1618.

3-[(phenoxy carbonyl)amino]phenyl]propanoic acid (3.66). The *t*-butyl ester 3.65 (0.150 g, 0.440 mmol) was dissolved in a solution of TFA in dichloromethane (30 %
TFA, 5 mL), and the solution was stirred at room temperature for 2 h, at which time the reaction had reached completion (TLC). The solvent was removed by rotary evaporation, the product was redissolved/resuspended in dichloromethane, and the flask was again placed on the rotary evaporator. After repeating this procedure several times to remove most of the TFA, the product was placed under high vacuum for 12 h. The resulting white solid was recrystallized in hexanes/ethyl acetate, and was obtained in pure form (0.107 g, 86%). mp = 154-155°C; $^1$H NMR (200 MHz, (CD)$_3$C(0)): δ 2.61 (t, J = 7.5 Hz, 2H), 2.90 (t, J = 7.5 Hz, 2H), 7.1 - 7.6 (m, 9H), 9.09 (bs, 1H); $^{13}$C NMR (75 MHz, (CD)$_3$C(0)): δ 31.9, 37.0, 120.6, 123.7, 127.1, 130.6, 131.1, 137.8, 138.7, 153.0, 153.6, 175.1; MS (EI): m/z 285 (M$^+$, trace), 191 (M$^+$ - HOAr, 53%), 132 (100%); HRMS(EI) calcd for C$_{16}$H$_{15}$NO$_4$ 285.1001, found 285.0994.

Tert-butyl 3-[(4-methoxyphenoxy)carbonyl]amino]phenyl]propanoate (3.67).

To a cooled and stirred solution of tert-butyl 3-(4-aminophenyl)propanoate (3.60) (0.216 g, 0.976 mmol, 1.0 eq.) and N-methylmorpholine (0.120 mL, 1.09 mmol, 1.1 eq.) in dry dichloromethane (10 mL) was slowly added p-methoxyphenyl chloroformate (0.160 mL, 1.08 mmol, 1.1 eq.). Within 5 min, a fine precipitate had formed (N-methylmorpholine hydrochloride salt), and after 1 h, none of the amine derivative remained (observed by TLC). Dichloromethane (20 mL) was added to the flask, and the organic layer was washed with 0.1 M HCl (25 mL), water (25 mL), brine (25 mL), dried (MgSO$_4$) and concentrated under reduced pressure to yield a white solid. Compound 3.67 was obtained in pure form (white solid) following recrystallization in dichloromethane (0.31 g, 86%). mp = 144-146 °C; $^1$H NMR (300 MHz, CDCl$_3$): δ 1.42 (s, 9H), 2.52 (t, J = 7.7 Hz, 2H), 2.89 (t, J = 7.7 Hz, 2H), 3.81 (s, 3H), 6.84 (bs, 1H), 6.90 (d, J = 8.7 Hz, 2H), 7.10 (d, J = 8.7 Hz, 2H), 7.17 (d, J = 8.6
Hz, 2H), 7.35 (d, J = 8.6 Hz, 2H); $^{13}$C NMR (75 MHz, (CD$_3$)$_2$C(O)): δ 29.2, 32.0, 38.6, 81.3, 116.0, 120.4, 124.5, 130.6, 137.6, 138.8, 146.4, 154.0, 159.0, 173.3; MS (EI): m/z 371 (M$^+$, trace), 191 (M$^+$ - OArOCH$_3$ - C(CH$_3$)$_3$, 44%), 124 (HOArOCH$_3$, 100%); HRMS(EI) calcd for C$_{31}$H$_{33}$NO$_5$ 371.1733, found 371.1728.

3-(4-[(4-Methoxyphenoxo)carbonyl]amino)phenylpropanoic acid (3.68). The t-butyl ester 3.67 (0.090 g, 0.243 mmol) was dissolved in a solution of TFA in dichloromethane (30% TFA, 5 mL), and the solution was stirred at room temperature for 1 h, at which time the reaction had reached completion (TLC). The solvent was removed by rotary evaporation, the product was redissolved/resuspended in dichloromethane, and the flask was again placed on the rotary evaporator. After repeating this procedure several times to remove most of the TFA, the product was placed under high vacuum for 12 h. The resulting white solid was recrystallized in dichloromethane/ethyl acetate, and was obtained in pure form (0.055 g, 73%). mp = 172-173 °C; $^1$H NMR (200 MHz, (CD$_3$)$_2$C(O)): δ 2.61 (t, J = 7.5 Hz, 2H), 2.90 (t, J = 7.5 Hz, 2H), 3.81 (s, 3H), 6.94 (d, J = 9.1 Hz, 2H), 7.12 (d, J = 9.1 Hz, 2H), 7.23 (d, J = 8.6 Hz, 2H), 7.53 (d, J = 8.6 Hz, 2H), 8.97 (bs, 1H); $^{13}$C NMR (75 MHz, (CD$_3$)$_2$C(O)): δ 31.9, 37.0, 56.9, 116.0, 120.5, 124.5, 130.6, 137.7, 138.9, 146.4, 154.0, 159.1, 174.9; MS (EI): m/z 315 (M$^+$, trace), 191 (M$^+$ - HOArOCH$_3$, 42%), 124 (HOArOCH$_3$, 100%); HRMS(EI) calcd for C$_{17}$H$_{17}$N$_2$O$_5$ 315.1107, found 315.1104.

Tert-butyl 3-[(4-nitrophenoxo)carbonyl]oxy)phenyl)propanoate (3.70). To a cooled and stirred solution of tert-butyl 3-(4-hydroxyphenyl)propanoate (3.69)$^{37}$ (0.200 g, 0.901 mmol, 1.0 eq.) and N-methylmorpholine (0.109 mL, 0.991 mmol, 1.1 eq.) in dry dichloromethane (5 mL) was slowly added p-nitrophenyl chloroformate (0.199 g, 0.991 mmol, 1.1 eq.). Within 15 min, a fine precipitate had formed (N-methylmorpholine
hydrochloride salt). After 2 h, none of the phenol derivative remained (observed by TLC). Dichloromethane (20 mL) was added to the flask, and the organic layer was washed with 0.1 M HCl (20 mL), water (20 mL), brine (20 mL), dried (MgSO₄) and concentrated under reduced pressure to yield a colorless oil. The product was purified via silica gel chromatography (hexanes:ethyl acetate = 85:15, Rf = 0.3), and was recrystallized in hexanes/ethyl acetate to yield pure 3.70 as a white solid (0.227 g, 65%). mp = 58-60 °C; ¹H NMR (200 MHz, CDCl₃): δ 1.42 (s, 9H), 2.55 (t, J = 7.7 Hz, 2H), 2.94 (t, J = 7.7 Hz, 2H), 7.15 - 7.30 (m, 4H), 7.48 (d, J = 9.2 Hz, 2H), 8.30 (d, J = 9.2 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 28.1, 30.4, 36.8, 80.5, 120.5, 121.7, 125.3, 129.6, 139.4, 145.6, 149.0, 151.1, 155.3, 171.9; MS (EI): m/z 387 (M⁺, trace), 331 (M⁺ - C(CH₃)₃ + H⁺, 45%), 57 (C(CH₃)₃, 100%); HRMS(EI) calcd for C₂₀H₂₃NO₇ 387.1318, found 387.1307.

3-(4-[(4-Nitrophenoxycarbonyl)oxy]phenyl)propanoic acid (3.71). The t-butyl ester 3.70 (0.100 g, 0.258 mmol) was dissolved in a solution of TFA in dry dichloromethane (30 % TFA, 5 mL), and the solution was stirred at room temperature for 30 min, at which time the reaction had reached completion (TLC). The solvent was removed by rotary evaporation, the product was redissolved/resuspended in dichloromethane, and the flask was again placed on the rotary evaporator. After repeating this procedure several times to remove most of the TFA, the product was placed under high vacuum for 12 h. Pure product was obtained as a white solid (0.061 g, 74%) following recrystallization in hexanes/dichloromethane. mp = 154-155 °C; ¹H NMR (200 MHz, CDCl₃): δ 2.71 (t, J = 7.3 Hz, 2H), 3.00 (t, J = 7.3 Hz, 2H), 7.15 - 7.35 (m, 4H), 7.49 (d, J = 9.3 Hz, 2H), 8.33 (d, J = 9.3 Hz, 2H); ¹³C NMR (75 MHz, S(O)(CD₃)₂): δ 29.6, 35.0, 120.9, 122.7, 125.4, 129.4,
My1 3-(4-Hydroxyphenyl)propionate (3.72). To a suspension of 3-(4-hydroxyphenyl propionic acid) (0.200 g, 1.20 mmol, 1.0 eq.) in distilled water (0.75 mL) was added a 40% aqueous solution of tetrabutyl ammonium hydroxide (0.79 mL, 1.20 mmol, 1.0 eq.), and the mixture was stirred at room temperature until all of the acid dissolved. Water was removed on a high-vacuum rotary evaporator, and the residue was placed under high vacuum for an additional 12 h, yielding the crude 3-(4-hydroxy-phenyl) propionic tetra-n-butyl ammonium salt as an off-white solid. The salt was dissolved in dry DMF (2 mL), and to this was added a solution of allyl chloride (0.0985 mL, 1.20 mmol, 1.0 eq.) in dry DMF (2 mL), over a period of 2 h, and the reaction was stirred for 12 h. The solution was then poured into water (20 mL) and extracted with ethyl acetate (3×20 mL). The combined extracts were washed with water (20 mL), a solution of NaHCO₃ (5 % aqueous, 20 mL), dried (MgSO₄) and concentrated under reduced pressure. The residue was purified by silica gel chromatography (hexanes:ethyl acetate = 90:10, Rf = 0.3) to yield pure 3.72 as a pale yellow oil (0.185 g, 74%). ¹H NMR (200 MHz, CDCl₃): δ 2.62 (t, J = 7.5 Hz, 2H), 2.90 (t, J = 7.5 Hz, 2H), 4.58 (d, J = 5.5 Hz, 2H), 4.74 (s, 1H), 5.10 - 5.40 (m, 2H), 5.80 - 5.60 (m, 1H), 6.91 (d, J = 8.4 Hz, 2H), 7.07 (d, J = 8.4 Hz, 2H); ¹³C NMR (50 MHz, CDCl₃): δ 30.2, 36.2, 65.2, 115.6, 118.2, 129.3, 132.2, 132.3, 154.5, 173.1; MS (EI): m/z 206 (M⁺, 65%), 123 (100%); HRMS(EI) calcd for C₁₂H₁₄O₃ 206.0943, found 206.0938.

Allyl 3-[4-(((4-nitrophenyl)amino)carbonyl)oxy]phenylpropanoate (3.73). To a stirring solution of 3.72 (0.400 g, 1.94 mmol, 1.0 eq.) and dry triethylamine (0.200 μL, 0.00136 mmol, 0.07 mol%) in dry benzene (4 mL) was added p-nitrophenylisocyanate (0.320
g, 1.94 mmol, 1.0 eq.), gradually over 1 min. Within 5 min, a thick, light yellow precipitate had formed, and did not dissolve even with addition of more dry benzene (5 mL). The solid was filtered and recrystallized in hexanes/ethyl acetate to yield product 3.73 as a yellow solid (0.550 g, 77%). mp = 110-111 °C; $^1$H NMR (200 MHz, CDCl$_3$): δ 2.67 (t, $J$ = 7.7 Hz, 2H), 2.99 (t, $J$ = 7.7 Hz, 2H), 4.59 (d, $J$ = 5.8 Hz, 2H), 5.15-5.35 (m, 2H), 5.80-5.60 (m, 1H), 7.11 (d, $J$ = 8.8 Hz, 2H), 7.26 (d, $J$ = 8.8 Hz, 2H), 7.62 (d, $J$ = 8.8 Hz, 2H), 8.24 (d, $J$ = 8.8 Hz, 2H); $^{13}$C NMR (50 MHz, CDCl$_3$): δ 30.3, 35.7, 65.3, 118.3, 121.4, 125.1, 129.4, 132.2, 138.5, 143.4, 143.5, 143.8, 148.9, 151.5, 172.5; MS (EI): m/z 370 (M$^+$, trace), 165 (OOCCH$_2$CH$_2$ArOH, 63%), 123 (100%); HRMS (EI) calcd for C$_{19}$H$_{18}$N$_2$O$_6$ 370.1165, found 370.1138.

3-[4-(((4-Nitrophenyl)amino)carbonyloxy)phenyl]propanoic acid (3.74). The allyl ester 3.73 (0.200 g, 0.541 mmol, 1.0 eq.) was dissolved in dichloromethane/THF (4 mL, dichloromethane:THF = 1:1), and to this solution was added 5,5-dimethyl-1,3-cyclohexanedione (dimedone) (0.190 g, 1.35 mmol, 2.5 eq.), and the catalyst, tetrakis triphenyolphosphine Pd(0) (0.062 g, 0.054 mmol, 10 mol%). The reaction flask was covered with tin foil, and the clear yellow solution was stirred at room temperature for 12 h. During this time, a yellow precipitate formed, which was filtered, washed with dichloromethane (4 mL), and recrystallized in hexanes/ethyl acetate, to yield pure 3.74 as a yellow solid (0.070 g, 40%). mp = 180-181 °C; $^1$H NMR (300 MHz, (CD$_3$)$_2$C(O)): δ 2.64 (t, $J$ = 7.3 Hz, 2H), 2.95 (t, $J$ = 7.3 Hz, 2H), 7.16 (d, $J$ = 8.4 Hz, 2H), 7.34 (d, $J$ = 8.4 Hz, 2H), 7.87 (d, $J$ = 9.3 Hz, 2H), 8.27 (d, $J$ = 9.3 Hz, 2H); $^{13}$C NMR (75 MHz, (CD$_3$)$_2$C(O)): δ 31.9, 37.0, 120.0, 123.5, 126.8, 131.1, 140.7, 144.8, 147.0, 150.9, 153.5, 175.1; MS (EI): m/z 330 (M$^+$, trace), 164
Benzyl (4-bromophenyl)acetate (3.77). To a suspension of p-bromophenyl acetic acid (1.50 g, 6.98 mmol, 1.0 eq.) in water (5 mL) was added a 40 % aqueous solution of tetrabutyl ammonium hydroxide (4.57 mL, 6.98 mmol, 1.0 eq.). The acid went into solution gradually over a period of 30 min, at which point water was removed on a high-vacuum rotary evaporator. The residual water in the resulting yellow oil was removed by formation of an azeotrope with toluene (4×100 mL) and high-vacuum rotary evaporation, and the residue was placed under high vacuum for 12 h. The thick yellow oil was then redissolved in dry DMF (20 mL), and benzyl bromide was added to this solution (1.20 mL, 10.1 mmol, 1.4 eq.). The reaction was stirred at room temperature for 24 h, at which time the solution was poured into ethyl acetate (100 mL). This solution was extracted with water (100 mL), and the water layer was then extracted with ethyl acetate (2×20 mL). The pooled organic layers were washed with water (50 mL), a solution of NaHCO₃ (5% aqueous, 50 mL), and brine (2×50 mL). After drying the organic layer (MgSO₄), the solvent was removed by rotary evaporation, and the product was purified via silica gel chromatography (hexanes:pentane = 50:50, Rf = 0.4). Following recrystallization in hexanes, compound 3.77 was isolated as a white solid in pure form (1.80 g, 85%). mp = 43-44 °C; ¹H NMR (200 MHz, CDCl₃): δ 3.63 (s, 2H), 5.14 (s, 2H), 7.10- 7.50 (m, 9H); ¹³C NMR (75 MHz, CDCl₃): δ 40.6, 66.7, 121.1, 128.1, 128.3, 128.5, 131.0, 131.6, 132.8, 135.6, 170.8; MS (EI): m/z 304 (M⁺ - H⁺, 7%), 91 (CH₂Ar, 100%); HRMS(EI) calcd for C₁₅H₁₃O₂Br 304.0099, found 304.0105.

Tert-butyl (2E)-3-[4-(2-(benzyloxy)-2-oxoethyl)phenyl]prop-2-enoate (3.78). Compound 3.78 was synthesized according to literature procedures.
[4-(3-Tert-butoxy-3-oxopropyl)phenyl]acetic acid\(^{68}\) (3.79). Compound 3.79 was prepared according to literature procedures.\(^{68}\)

**Tert-butyl 3-[(2-(4-nitrophenoxy)-2-oxoethyl)phenyl] propanoate (3.80).** To a suspension of 3.79\(^{68}\) (0.500 g, 1.89 mmol, 1.0 eq.) in dry dichloromethane (10 mL), was added dicyclohexylcarbodiimide (DCC) (0.507 g, 2.46 mmol, 1.3 eq.), and 1-hydroxybenzotriazole hydrate (HOBt) (0.332 g, 2.46 mmol, 1.3 eq.), followed by a solution of PNP (0.342 g, 2.46 mmol, 1.3 eq.) in dry dichloromethane (10 mL). The reaction mixture was stirred at room temperature for 12 h. The solvent was evaporated under reduced pressure, and the residue was redissolved in ethyl acetate (20 mL). The solution was washed with water (20 mL) and brine (20 mL), dried (MgSO\(_4\)), and solvent was removed by rotary evaporation. The product was purified via silica gel chromatography (hexanes:ethyl acetate = 70:30, \(R_f = 0.5\)) and was obtained as a yellow oil (0.470 g, 64%). \(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta 1.42\) (s, 9H), 2.55 (t, \(J = 7.7\) Hz, 2H), 2.92 (t, \(J = 7.7\) Hz, 2H), 3.86 (s, 2H), 7.15 – 7.35 (m, 6H), 8.24 (d, \(J = 9.1\) Hz, 2H); \(^13\)C NMR (50 MHz, CDCl\(_3\)): \(\delta 28.1, 30.8, 36.9, 41.0, 80.3, 122.2, 125.1, 128.9, 129.2, 130.5, 140.4, 145.6, 155.6, 168.9, 171.9; MS (EI): \(m/z\) 385 (M\(^+\), trace), 329 (M\(^+\) - C(CH\(_3\))\(_3\) + H\(^+\), 5%), 163 (100 %); HRMS(EI) calcd for C\(_{21}\)H\(_{23}\)NO\(_3\) 385.1525, found 385.1510.

3-[(4-[(2-(4-Nitrophenoxy)-2-oxoethyl)phenyl] propanoic acid (3.81). The tert-butyl ester 3.80 (0.380 g, 0.986 mmol) was dissolved in a solution of TFA in dichloromethane (30 \% TFA, 25 mL), and the solution was stirred at room temperature for 2 h, at which time the reaction had reached completion (TLC). The solvent was removed by rotary evaporation, the product was redissolved/resuspended in dichloromethane, and the flask was again placed on the rotary evaporator. After repeating this procedure several times to remove most of the
TFA, the product was placed under high vacuum for 12 h, yielding the product as an off-white solid (0.322 g, 99%). mp = 125-126 °C; \(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta\) 2.70 (t, \(J = 7.5\) Hz, 2H), 2.94 (t, \(J = 7.5\) Hz, 2H), 3.87 (s, 2H), 7.20 - 7.40 (m, 6H), 8.25 (d, \(J = 9.2\) Hz, 2H); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)C(O)): \(\delta\) 31.1, 35.7, 40.9, 123.7, 125.9, 129.4, 130.3, 132.1, 141.0, 146.2, 156.7, 170.1, 174.1; MS (El): \(m/z\) 329 (M\(^+\), trace), 190 (M\(^+\) - HOArNO\(_2\), 18%), 163 (CH\(_2\)ArCH\(_2\)CH\(_2\)COOH, 100%); HRMS(El) calcd for C\(_{17}\)H\(_{15}\)N\(_2\)O\(_6\) 329.0900, found 329.0910.

**Tert-butyl 3-(4-{2-[(4-nitrophenyl)amino]-2-oxoethyl}phenyl)propanoate (3.82).**

To a solution of 3.79\(^{68}\) (0.200 g, 0.756 mmol, 1.0 eq.) and \(p\)-nitroaniline (0.136 g, 0.984 mmol, 1.3 eq.) in dry THF (1 mL) was added diisopropylcarbodiimide (DiPC) (0.096 g, 0.756 mmol, 1.0 eq.), and the reaction was stirred at room temperature for 12 h. The solvent was evaporated, the residue was redissolved in ether, and was then washed with 0.1 M HCl (10 mL), water (10 mL), and brine (10 mL), and dried (MgSO\(_4\)). The product was purified via silica gel chromatography (gradient 100 % chloroform to chloroform:ethyl acetate = 70:30, \(R_f = 0.8\)) and was obtained as an off-white solid (0.192 g, 66%). mp = 107 - 108 °C; \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 1.43 (s, 9H), 2.56 (t, \(J = 7.7\) Hz, 2H), 2.94 (t, \(J = 7.7\) Hz, 2H), 3.75 (s, 2H), 7.25 (m, 4H), 7.43 (bs, 1H), 7.60 (d, \(J = 9.4\) Hz, 2H), 8.16 (d, \(J = 9.4\) Hz, 2H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 28.0, 31.6, 36.8, 44.4, 80.5, 119.0, 124.9, 129.3, 129.4, 131.3, 140.6, 143.5, 169.6, 172.1; MS (El): \(m/z\) 384 (M\(^+\), trace), 328 (M\(^+\) - C(CH\(_3\))\(_3\) + H\(^+\), 100%); HRMS(El) calcd for C\(_{21}\)H\(_{24}\)N\(_7\)O\(_8\) 384.1685, found 384.1676.

**3-(4-{2-[4-(Nitrophenyl)amino]-2-oxoethyl}phenyl)propanoic acid (3.83).** The tert-butyl ester 3.82 (0.163 g, 0.420 mmol) was dissolved in a solution of TFA in dichloromethane (30 % TFA, 15 mL), and the solution was stirred at room temperature for 2
h, at which time the reaction had reached completion (TLC). The solvent was removed by rotary evaporation, the product was redissolved/resuspended in dichloromethane, and the flask was again placed on the rotary evaporator. After repeating this procedure several times to remove most of the TFA, the product was placed under high vacuum for 12 h, yielding the product as yellow crystals (0.133 g, 96%). mp = 212-213 °C; \(^1\)H NMR (200 MHz, (CD\(_3\))\(_2\)C(O)): \(\delta\) 2.59 (t, \(J = 7.4\) Hz, 2H), 2.88 (t, \(J = 7.4\) Hz, 2H), 3.72 (s, 2H), 7.06 - 7.31 (m, 4H), 7.89 (d, \(J = 9.2\) Hz, 2H), 8.19 (d, \(J = 9.2\) Hz, 2H), 9.86 (s, 1H); \(^{13}\)C NMR (75 MHz, (CD\(_3\))\(_2\)S(O)): \(\delta\) 39.4, 44.7, 52.4, 128.2, 134.5, 137.7, 138.6, 142.4, 148.8, 151.6, 154.9, 179.6, 183.2; MS (EI): \(m/z\) 328 (M\(^+\), 31 %), 163 (M\(^+\) - C(O)NHArNO\(_2\), 100%); HRMS(EI) calcd for C\(_{17}\)H\(_{16}\)N\(_2\)O\(_3\) 328.1059, found 328.1058.

**Allyl 3-(4-[(4-nitrophenyl)acetyl]oxy)phenyl)propanoate (3.84).** To a solution of 3.72 (0.400 g, 1.94 mmol, 1.0 eq.) in dry dichloromethane (10 mL) was added dry triethylamine (0.324 mL, 2.32 mmol, 1.2 eq.), and p-nitrophenylacetic acid chloride (0.464 g, 2.32 mmol, 1.2 eq.). The solution became dark brown in color, and was stirred at room temperature for 12 h. The reaction mixture was washed with water (2×20 mL), the aqueous solution was extracted with dichloromethane (2×20 mL), and the organic layer was dried (MgSO\(_4\)) and concentrated using rotary evaporation. The product was obtained as an off-white semi-solid after purification via silica gel chromatography (100% dichloromethane, \(R_f = 0.6\), then repurified in hexanes:ethyl acetate = 70:30, \(R_f = 0.5\); 0.504 g, 70%). \(^1\)H NMR (200 MHz, CDCl\(_3\)): \(\delta\) 2.65 (t, \(J = 7.7\) Hz, 2H), 2.96 (t, \(J = 7.7\) Hz, 2H), 3.98 (s, 2H), 4.58 (d, \(J = 5.8\) Hz, 2H), 5.15 - 5.35 (m, 2H), 5.80 - 6.00 (m, 1H), 6.98 (d, \(J = 8.6\) Hz, 2H), 7.21 (d, \(J = 8.6\) Hz, 2H), 7.57 (d, \(J = 8.6\) Hz, 2H), 8.27 (d, \(J = 8.6\) Hz, 2H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 30.0, 35.4, 40.7, 64.9, 118.0, 121.0, 123.6, 129.2, 130.2, 131.9, 138.2, 140.6,
147.1, 148.7, 168.6, 172.0; MS (EI): m/z 369 (M⁺, trace), 206 (M⁺ - C(O)CH₂ArNO₂ + H⁺, 99%), 165 (C(O)CH₂ArNO₂ + H⁺, 100%); HRMS(EI) calcd for C₂₀H₁₉NO₆ 369.1212, found 369.1215.

3-(4-[[4-Nitrophenyl]acyl]oxy)phenyl)propanoic acid (3.85). To a solution of 3.84 (0.200 g, 0.542 mmol, 1.0 eq.) in dry dichloromethane/THF (5 mL, dichloromethane:THF = 1:1) was added 5,5-dimethyl-1,3-cyclohexanedione (dimedone) (0.110 g, 0.785 mmol, 1.4 eq.), and the solution was stirred at room temperature until all the dimedone had dissolved. The catalyst, tetrakis triphenylphosphine Pd(0) (0.063 g, 0.055 mmol, 10 mol%), was added, and the reaction was covered with foil and stirred for 12 h at room temperature. Additional amounts of dimedone (0.076 g, 0.542 mmol, 1.0 eq.) and Pd catalyst (0.027 g, 0.030 mmol, 5.5 mol%) were added to the reaction, which was stirred for another 8 h at room temperature. The reaction mixture was concentrated by rotary evaporation, and the product was purified via silica gel chromatography (hexanes:ethyl acetate = 40:60, Rf = 0.3), followed by recrystallization from ethyl acetate, to yield a light brown crystalline solid (0.119 g, 67%). mp = 173-174 °C; ¹H NMR (200 MHz, CDCl₃): δ 2.67 (t, J = 7.5 Hz, 2H), 2.96 (t, J = 7.5 Hz, 2H), 3.97 (s, 2H), 7.00 (d, J = 8.6 Hz, 2H), 7.22 (d, J = 8.6 Hz, 2H), 7.57 (d, J = 8.2 Hz, 2H), 8.24 (d, J = 8.2 Hz, 2H); ¹³C NMR (50 MHz, (CD₃)₂C(O)): δ 31.0, 35.9, 41.3, 122.3, 124.3, 130.1, 131.7, 139.8, 142.8, 148.5, 150.4, 169.7, 173.8; MS (EI): m/z 329 (M⁺, trace), 166 (M⁺ - O₂NArCH₂C(O) + H⁺, 68%), 107 (100%); HRMS(EI) calcd for C₁₇H₁₅NO₆ 329.0899, found 329.0909.

Tert-butyl 3-(4-[[4-nitrophenyl]acyl]amino)phenyl)propanoate (3.86). To a suspension of p-nitrophenyl acetic acid (0.213 g, 1.17 mmol, 1.3 eq.), DCC (0.242 g, 1.17 mmol, 1.3 eq.), and HOBt (0.159 g, 1.17 mmol, 1.3 eq.) in dry dichloromethane (5 mL) was
added a solution of tert-butyl 3-(4-aminophenyl)propanoate (3.60)\textsuperscript{66} (0.200 g, 0.900 mmol) in dry dichloromethane (3 mL) over a period of 1 min. Dry DMF (4 mL) was added to increase the solubility of the acid in the solvent, and the mixture was stirred for 48 h. Dichloromethane (15 mL) was added to the reaction mixture, and the organic layer was washed with water (2×15 mL), brine (2×15 mL), and dried (MgSO\textsubscript{4}). After evaporation of solvent under reduced pressure, the resulting off-white solid was purified via silica gel chromatography (hexanes:ethyl acetate = 70:30, R\textsubscript{f} = 0.25, then repurified in dichloromethane:ethyl acetate = 95:5, R\textsubscript{f} = 0.4). Product 3.86 was recrystallized in hexanes/ethyl acetate, and was isolated as a white solid (0.165 g, 47%). mp = 170-172°C; \textsuperscript{1}H NMR (200 MHz, CDCl\textsubscript{3}): \delta 1.41 (s, 9H), 2.50 (t, J = 7.7 Hz, 2H), 2.87 (t, J = 7.7 Hz, 2H), 3.82 (s, 2H), 7.07 (bs, 1H), 7.15 (d, J = 8.6 Hz, 2H), 7.37 (d, J = 8.6 Hz, 2H), 7.54 (d, J = 8.6 Hz, 2H), 8.25 (d, J = 8.6 Hz, 2H); \textsuperscript{13}C NMR (50 MHz, CDCl\textsubscript{3}): \delta 28.1, 30.6, 37.0, 43.9, 80.5, 120.5, 123.9, 128.9, 130.2, 135.7, 137.7, 142.1, 147.5, 167.5, 172.1; MS (EI): m/z 384 (M\textsuperscript{+}, 7%), 328 (M\textsuperscript{+} + H\textsuperscript{+} - C(CH\textsubscript{3})\textsubscript{3}, 100%); HRMS (EI) calcd for C\textsubscript{21}H\textsubscript{24}N\textsubscript{2}O\textsubscript{5} 384.1685, found 384.1691.

3-(4-[[4-Nitrophenyl]acetyl][amino]phenyl)propanoic acid (3.87). The \textit{t}-butyl ester 3.86 (0.143 g, 0.371 mmol) was dissolved in a solution of TFA in dry dichloromethane (30% TFA, 5 mL), and the solution was stirred at room temperature for 2 h, at which time the reaction had reached completion (TLC). The solvent was removed by rotary evaporation, the product was redissolved/resuspended in dichloromethane, and the flask was again placed on the rotary evaporator. After repeating this procedure several times to remove most of the TFA, the product was placed under high vacuum for 12 h. The resulting white solid was recrystallized in ethyl acetate, and was obtained in pure form (0.101 g, 83%). mp = 204-
205°C; ¹H NMR (200 MHz, (CD₃)₂C(O)): δ 2.58 (t, J = 7.5 Hz, 2H), 2.87 (t, J = 7.5 Hz, 2H), 3.89 (s, 2H), 7.20 (d, J = 8.4 Hz, 2H), 7.56 (d, J = 8.4 Hz, 2H), 7.67 (d, J = 8.4 Hz, 2H), 8.21 (d, J = 8.4 Hz, 2H), 9.41 (bs, 1H); ¹³C NMR (75 MHz, (CD₃)₂C(O)): δ 31.9, 37.0, 45.1, 121.2, 121.3, 125.1, 128.4, 130.4, 132.4, 138.3, 139.2, 145.8, 169.4, 174.9; MS (EI): m/z 328 (M⁺, 75%), 106 (100%); HRMS (EI) calcd for C₁₇H₁₆N₂O₃ 328.1059, found 328.1053.

Tert-butyl 3-{4-[(4-nitrobenzoyl)amino]phenyl}propanoate (3.88). To a cooled and stirred solution of tert-butyl 3-(4-aminophenyl)propanoate (3.60)⁶⁶ (0.200 g, 0.904 mmol, 1.0 eq.) and N-methylmorpholine (0.109 mL, 0.991 mmol, 1.1 eq.) in dry dichloromethane (10 mL) was slowly added p-nitrobenzoyl chloride (0.184 g, 0.992 mmol, 1.1 eq.). Within 15 min, a fine precipitate had formed (N-methylmorpholine hydrochloride salt). After 2 h, none of the amine derivative remained (observed by TLC). Dichloromethane (15 mL) was added to the flask, and the organic layer was washed with water (2×20 mL), 1.0 M HCl (2×20 mL), brine (2×20 mL), dried (MgSO₄) and concentrated under reduced pressure to yield an off-white solid. The product was purified first by silica gel chromatography (hexanes:ethyl acetate = 75:25, Rf = 0.3), followed by recrystallization in hexanes/dichloromethane, to yield a white solid (0.238 g, 71%). mp = 164-165 °C; ¹H NMR (200 MHz, (CD₃)₂C(O)): δ 1.41 (s, 9H), 2.54 (t, J = 7.3 Hz, 2H), 2.89 (t, J = 7.3 Hz, 2H), 7.27 (d, J = 8.4 Hz, 2H), 7.77 (d, J = 8.4 Hz, 2H), 8.24 (d, J = 8.8 Hz, 2H), 8.38 (d, J = 8.8 Hz, 2H), 9.78 (bs, = 1H); ¹³C NMR (50 MHz, CDCl₃): δ 28.1, 30.6, 37.0, 80.5, 120.9, 123.8, 128.4, 129.0, 135.6, 138.0, 140.6, 149.8, 163.9, 172.2; MS (EI): m/z 370 (M⁺, 5%), 314 (M⁺ + H⁺ - C(CH₃)_₃, 100%); HRMS (EI) calcd for C₂₀H₂₂N₂O₅ 370.1529, found 370.1517.
3-{4-[(4-Nitrobenzoyl)amino]phenyl}propanoic acid (3.89). The t-butyl ester 3.88 (0.200 g, 0.541 mmol) was dissolved in a solution of TFA in dichloromethane (30 % TFA, 5 mL), and the solution was stirred at room temperature for 2 h, at which time the reaction had reached completion (TLC). The solvent was removed by rotary evaporation, the product was redissolved/resuspended in dichloromethane, and the flask was again placed on the rotary evaporator. After repeating this procedure several times to remove most of the TFA, the product was placed under high vacuum for 12 h. The resulting white solid was recrystallized in methanol, and was obtained in pure form (0.104 g, 61%). mp = 240-241 °C; 1H NMR (200 MHz, CD3OD): δ 2.62 (t, J = 7.4 Hz, 2H), 2.93 (t, J = 7.4 Hz, 2H), 7.26 (d, J = 8.7 Hz, 2H), 7.63 (d, J = 8.7 Hz, 2H), 8.13 (d, J = 8.7 Hz, 2H), 8.37 (d, J = 8.7 Hz, 2H); 13C NMR (75 MHz, (CD3)2S(O)): δ 29.8, 35.2, 120.5, 123.5, 128.4, 129.1, 136.6, 136.7, 140.6, 149.0, 163.6, 173.7; MS (EI): m/z 314 (M+, 37%), 150 (COArNO2, 100%); HRMS(EI) calcd for C18H14N2O5 314.0903, found 314.0900.

3.2.16 Rapid Screen for Catalytic Activity of ST51 with Various Carbamate, Ester, Amide and Carbonate Derivatives

General Procedure. Unless otherwise indicated, ST51 was screened against several carbamate, ester, amide, and carbonate substrates in the following manner: ST51 (5 μM) was incubated with a potential substrate (1 mM) in 5/95 DMSO/appropriate buffer (reaction volume ranged from 20 – 55 μL). Reactions were monitored at 25°C by HPLC, unless otherwise indicated. Control reactions consisted of 1 mM potential substrate in 5/95 DMSO/appropriate buffer, with no antibody present. All reactions were quenched by addition of perchloric acid, and were spun down on a microcentrifuge before injection into the HPLC.
Where possible, the rates of hydrolysis of the potential substrates in the buffer of interest in the absence of antibody were first determined by UV/VIS: 50 μL of 1 mM stock solution of the derivative in DMSO was added to 950 μL of an appropriate buffer solution, and the hydrolysis reaction was monitored at 25°C by continuous scanning of the UV/VIS spectrum. The hydrolysis reaction was followed to several half lives, and from kinetic data collected at a given wavelength(s) (change in absorbance with time), the pseudo-first-order rate constant could be calculated. This data was then used to set up appropriate assay conditions with ST51. This protocol was used for compounds 3.62, 3.66, 3.68, 3.71, 3.74, 3.76, 3.81, 3.83.

Alternatively, the progress of some of the hydrolysis reactions in the absence of antibody was followed by analytical reverse-phase HPLC for a period of time up to 16 h (if no catalysis was observed after this time period, the compound would be considered to be a poor substrate for ST51). The reaction mixture consisted of 1 mM potential substrate in 5/95 DMSO/appropriate buffer, incubated at 25°C (water bath), and the reactions were analyzed periodically for hydrolysis products, ensuring that as little as 5 μM of hydrolysis product could be detected. All reactions were quenched by addition of perchloric acid, and were spun down on a microcentrifuge before injection into the HPLC. Based on the data from these experiments, appropriate assay conditions with ST51 were then devised. This protocol was followed for compounds 3.64, 3.85, 3.87, and 3.89.

**Compound 3.62.** The hydrolysis reaction of compound 3.62 (50 μM) was studied using UV/VIS, in the absence of ST51, in 5/95 dioxane/BIS-TRIS (100 mM BIS-TRIS, 100 mM NaCl, 0.01 % NaN₃, pH 6.0) (λ₊ₑ₅ = 325 nm, 400 nm), by following the reaction to several half-lives. ST51-containing reactions were prepared as described in the General
Procedure, and were monitored by UV-VIS, at a temperature of 10°C, over a time period of 1.5 minutes. However, due to the lability of the compound, both in the stock solution of dioxane and in the buffer used for the experiment, reproducible results could not be obtained.

**Compound 3.64.** A solution of 3.64 (1 mM) in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0) was monitored over a period of 16 h, by analytical reverse-phase HPLC, for the production of hydrolysis products (namely, p-nitrobenzyl alcohol). Compound 3.64 was screened with ST51 in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0) according to the General Procedure, monitoring the formation of p-nitrobenzyl alcohol by analytical reverse-phase HPLC. The conditions used for the HPLC analysis were as follows: 5 μL injection loop; λ<sub>det</sub> = 254 nm; mobile phase = 20/80 acetonitrile/water (0.1 % TFA) for 15 min, ramp to 100% acetonitrile over 5 min, holding 100% acetonitrile for 15 min, ramp back to 20/80 acetonitrile/water (0.1 % TFA) over 5 min, holding this solvent system for 15 min, flowrate = 1 mL/min throughout. The retention time for p-nitrobenzyl alcohol was approximately 9 min. The control and ST51-containing reactions were monitored over a period of 16 h.

**Compound 3.66.** The hydrolysis of a 50 μM solution of 3.66 was monitored, using UV/VIS, in the absence of ST51, in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0) (λ<sub>det</sub> = 270 nm), and the reaction was followed to several half-lives. This carbamate was then screened with ST51 in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 7.0), according to the General Procedure, monitoring the formation of phenol via analytical reverse-phase HPLC: 20 μL injection loop; λ<sub>det</sub> = 280 nm; mobile phase = 75/25 water (0.1 % TFA)/methanol for 25 min, ramp to 100% methanol over 5 min, holding 100% methanol for 10 min, ramp back to 75/25 water (0.1 % TFA)/methanol
over 5 min, holding this solvent system for 15 min; flowrate = 0.9 mL/min for 13 min, 1.5 mL/min for 47 min. The retention time for phenol was approximately 8 - 9 min. The control and ST51-containing reactions were monitored over a period of 2 h.

**Compound 3.68.** The hydrolysis of a 50 µM solution of 3.68 was monitored, using UV/VIS, in the absence of ST51, in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0) (λDET = 290 nm) and the reaction was followed to several half-lives. This carbamate was then screened with ST51, according to the General Procedure, in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0), monitoring the formation of p-methoxyphenol using the same analytical reverse-phase HPLC conditions as in the screening of 3.66 with ST51. The retention time for p-methoxyphenol was approximately 11 min. The control and ST51-containing reactions were monitored over a period of 5 min.

**Compound 3.71.** The hydrolysis of a 50 µM solution of 3.71 was monitored, using UV/VIS, in the absence of ST51, in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0) (λDET = 400 nm) and in 5/95 acetonitrile/ACES (20 mM ACES, pH 6.1) (λDET = 400 nm) and the reaction was followed to several half-lives in both buffer systems. This carbonate was then screened with ST51 in both buffer systems, as per the General Procedure, except that different detection methods were used for the two buffer systems. For the reaction at pH 9.0, the formation of PNP was monitored in the control and ST51-containing reactions by analytical reverse-phase HPLC: 5 µL injection loop, λDET = 325 nm; mobile phase = 20/80 acetonitrile/water (0.1 % TFA) for 20 min, ramp to 100% acetonitrile over 5 min, holding 100% acetonitrile for 15 min, ramp back to 20/80 acetonitrile/water (0.1 % TFA) over 5 min, holding this solvent system for 15 min, flowrate
= 1 mL/min throughout. The retention time for PNP was 14 min. The ST51-containing reaction and control reaction time was 1.5 min. For the reactions at pH 6.1, the ST51-containing and control reactions (100 µL volume) were followed for a period of 10 minutes using a microplate reader detecting at 405 nm, and the maximum concentration of 3.71 used was 500 µM, due to solubility problems with this compound.

**Compound 3.74.** The hydrolysis reaction of a 50 µM solution of 3.74 was monitored via UV/VIS, in the absence of ST51, in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0) (λ_{DET} = 320, 385 nm), and the reaction was followed to several half-lives. Compound 3.74 was screened with ST51, as per the General Procedure, in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0), and the formation of p-nitroaniline was detected by analytical reverse-phase HPLC using the same conditions as for

**Compound 3.71.** The retention time for p-nitroaniline was 10 min. The ST51-containing reaction and control reaction time was 15 min.

**Compound 3.81.** The hydrolysis reaction of a 50 µM solution of 3.81 was monitored over several half-lives via UV/VIS, in the absence of ST51, in 5/95 DMSO/BIS-TRIS (100 mM BIS-TRIS, 100 mM NaCl, 0.01% NaN₃, pH 7.0) and in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0) (λ_{DET} = 400 nm). Compound 3.81 was screened with ST51, according to the General Procedure, in 5/95 DMSO/BIS-TRIS (100 mM BIS-TRIS, 100 mM NaCl, 0.01% NaN₃, pH 7.0), and the formation of PNP was detected by analytical reverse-phase HPLC under the same conditions as were described for the assay of ST51 with 3.71. The retention time for PNP was 14 min. The ST51-containing reaction and control reaction time was 6.33 min.
Compound 3.83. The hydrolysis of a 50 μM solution of 3.83 in 1/99 DMSO/1 M NaOH was monitored by UV/VIS (λ_{DET} = 400 nm) over several half-lives, with no ST51 present. This amide was screened with ST51, as per the General Procedure, in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0), and the formation of p-nitroaniline was detected by analytical reverse-phase HPLC, using the same experimental conditions as in the assay of ST51 with 3.74. The retention time for p-nitroaniline was 10 min. The reactions (ST51-containing, and control) were followed for 24 h.

Compound 3.85. A solution of 3.85 (50 μM) in 5/95 DMSO/bicine (100 mM bicine, 100 mM NaCl, 0.01% NaN₃, pH 9.0) was monitored over a period of 6 h, by analytical reverse-phase HPLC, for the production of hydrolysis products (namely, p-nitrophenylacetic acid). This compound was screened with ST51 using the same experimental conditions as were used with 3.64, except p-nitrophenylacetic acid was detected, having a retention time of 12 - 13 min. The reaction time for the control and ST51-containing samples was 30 min.

Compound 3.87. Compound 3.87 was analyzed using the same experimental conditions as were used with 3.64, except that p-nitrophenylacetic acid was detected, having a retention time of 12 - 13 min.

Compound 3.89. Compound 3.89 was analyzed using the same experimental conditions as were used with 3.64, except that p-nitrobenzoic acid was detected, having a retention time of 14 - 15 min.

3.2.17 Synthesis of Model Tripartate Prodrug Substrate

**Allyl 4-(methylamino)benzoate (3.90).** To a suspension of p-(methylamino)benzoic acid (1.00 g, 6.62 mmol, 1 eq.) in water (20 mL) was added a 40% aqueous solution of tetrabutyl ammonium hydroxide (4.29 mL, 6.62 mmol, 1 eq.), and the mixture was stirred at
room temperature until all of the acid dissolved. The pale brown, clear solution was stirred at room temperature for 30 min. Water was removed on a high-vacuum rotary evaporator, and the residue was placed under high vacuum for an additional 12 h, yielding the crude salt as an off-white solid. The salt was then dissolved in dry DMF (20 mL), and to this was added allyl chloride (0.65 mL, 7.94 mmol, 1.2 eq.), dropwise, with stirring, and the reaction was stirred at room temperature for 12 h. To the solution was then added additional dichloromethane (100 mL), water (100 mL), and the biphasic solution was transferred to a separatory funnel. The organic layer was washed with water (2×100 mL), then with a solution of NaCl (sat'd. aqueous, 50 mL), dried (MgSO₄) and concentrated under reduced pressure. The residue was purified by silica gel chromatography (dichloromethane:hexanes = 85:15, Rf = 0.3) to yield pure 3.90 as a clear, colorless oil (0.96 g, 76%). ¹H NMR (300 MHz, CDCl₃): δ 2.86 (s, 3H), 4.18 (s, 1H), 4.75 (d, J = 4.8 Hz, 2H), 5.23 (d, J = 10.7 Hz, 1H), 5.36 (d, J = 17.7 Hz, 1H), 6.00 (m, 1H), 6.53 (d, J = 8.2 Hz, 2H), 7.88 (d, J = 8.4 Hz, 2H); ¹³C NMR (75 MHz, CDCl₃): δ 29.8, 64.6, 110.8, 117.3, 117.5, 131.3, 132.6, 153.0, 166.4; MS (EI): m/z 191 (M⁺, 48%), 143 (100%); HRMS(EI) calcd for C₁₁H₁₂NO₂ 191.0946, found 191.0947.

Allyl 4-{methyl[(4-nitrophenoxy)carbonyl]amino}benzoate (3.91). To a cooled (ice bath) and stirred solution of 3.90 (0.5352 g, 2.80 mmol, 1.0 eq.) and N-methylmorpholine (0.31 mL, 2.80 mmol, 1.0 eq.) in dry dichloromethane (10 mL) was added p-nitrophenylchloroformate (0.5642 g, 2.80 mmol, 1.0 eq.), and the reaction was allowed to come to room temperature gradually. A white precipitate formed gradually after addition of the chloroformate (N-methylmorpholine hydrochloride salt), and the reaction was stirred at room temperature for 48 h. Dichloromethane (30 mL) was added to the flask, and this solution was washed with 0.1 M HCl (3×30 mL), water (3×30 mL), brine (2×30 mL), dried
(MgSO₄), filtered, and the solvent was removed via rotary evaporation. The residue was purified by silica gel chromatography (hexanes:ethyl acetate = 80:20, Rf = 0.25) to yield pure 3.91 as a light yellow solid (0.64 g, 64%). mp = 86 – 88 °C. ¹H NMR (300 MHz, CDCl₃): δ 3.47 (s, 3H), 4.82 (d, J = 5.5 Hz, 2H), 5.30 (m, 2H), 6.01 (m, 1H), 7.29 (d, J = 8.8 Hz, 2H), 7.42 (d, J = 8.6 Hz, 2H), 8.10 (d, J = 8.2 Hz, 2H), 8.23 (d, J = 9.4 Hz, 2H); ¹³C NMR (75 MHz, CDCl₃): δ 37.8, 65.5, 118.2, 122.0, 124.9, 128.1, 130.4, 131.9, 144.8, 146.2, 151.9, 155.6, 165.2; MS (EI): m/z 356 (M⁺, 15%), 218 (M⁺ - OArNO₂, 100%); HRMS(EI) calcld for C₁₈H₁₆N₂O₆ 356.1008, found 356.1020.

4-{Methyl[(4-nitrophenoxy)carbonyl]amino}benzoic acid (3.92). To a solution of 3.91 (0.42 g, 1.18 mmol, 1.0 eq.) in dichloromethane/THF (1:2 = dichloromethane:THF; 6 mL) was added 5,5-dimethyl-1,3-cyclohexanedione (dimedone) (0.413 g, 2.95 mmol, 2.5 eq.) and the catalyst, tetrakis triphenylphosphine Pd(0) (0.136 g, 0.118 mmol, 10 mol%). The reaction flask was covered with tin foil, and the clear solution was stirred at room temperature for 20 min. During this time, a white precipitate formed, which was filtered, and washed with dichloromethane (10 mL) to yield pure 3.92 as a white solid (0.370 g, 99%). mp = 250 – 252 °C. ¹H NMR (300 MHz, CDCl₃): δ 341 (s, 3H), 7.40 (d, J = 7.8 Hz, 2H), 7.62 (d, J = 7.8 Hz, 2H), 7.98 (d, J = 7.8 Hz, 2H), 8.27 (d, J = 8.3 Hz, 2H); ¹³C NMR (75 MHz, CDCl₃): δ 37.6, 122.9, 125.0, 125.4, 128.4, 130.0, 144.6, 146.1, 151.8, 155.8, 166.6; MS (EI): m/z 316 (M⁺, 16%), 178 (M⁺ - OArNO₂, 100%); HRMS(EI) calcld for C₁₅H₁₂N₂O₆ 316.0695, found 316.0708.

4-Nitrophenyl 4-(hydroxymethyl)phenyl(methyl)carbamate (3.93). To a cooled (ice bath) and stirred solution of benzyltriethylammonium borohydride⁶⁹ (1.97 g, 9.52 mmol, 2.0 eq.) in dry dichloromethane (20 mL) was added over a period of 5 min
trimethylsilylchloride (1.20 mL, 9.46 mmol, 2.0 eq.). This solution was stirred for 20 min at 0 - 4°C. To this was added the acid 3.92 (1.50 g, 4.75 mmol, 1.0 eq.), which did not dissolve until the ice bath was removed and the reaction had warmed to room temperature (approximately 15 min). The pale yellow solution was stirred at room temperature for a period of 2 h, at which point none of the acid remained (TLC). A solution of NaHCO₃ (5% aqueous, 20 mL) was added slowly, and dichloromethane (50 mL) was added to the flask. The organic layer was washed with 0.1 M HCl (25 mL), brine (25 mL), then dried (MgSO₄) and concentrated under reduced pressure to yield a yellow oil. Purification via silica gel chromatography (hexanes:ethyl acetate = 50:50, Rf = 0.3) resulted in pure product as a pale yellow oil (1.00 g, 70%). ¹H NMR (200 MHz, CDCl₃): δ 1.73 (bt, unresolved, 1H), 3.43 (s, 3H), 4.73 (d, J = 3.7 Hz, 2H), 7.20 - 7.50 (m, 6H), 8.23 (d, J = 8.7 Hz, 2H); ¹³C NMR (50 MHz, CDCl₃): δ 38.4, 64.4, 122.0, 124.9, 125.9, 127.7, 140.2, 141.7, 145.1, 152.6, 156.3; MS (EI): m/z 302 (M⁺, 27%), 164 (M⁺ - OArNO₂, 100%); HRMS(EI) calcd for C₁₅H₁₄N₂O₅ 302.0903, found 302.0910.

4-[(4-Nitrophenyl)carbonyl](methyl)amino|benzyl (4-nitrophenyl) carbonate (3.94). To a cooled and stirred solution of the alcohol 3.93 (0.900 g, 2.99 mmol, 1.0 eq.) and p-nitrophenyl chloroformate (0.720 g, 3.59 mmol, 1.2 eq.) in dry THF (10 mL) was added dry pyridine (0.290 mL, 3.59 mmol, 1.2 eq.). A white precipitate formed immediately, and within 5 min the reaction had completed and the product precipitated out of solution. The product was redissolved in dichloromethane (50 mL), and the solution was washed with 0.1 M HCl (2×25 mL), water (25 mL), brine (25 mL), then dried (MgSO₄). Solvent was evaporated under reduced pressure, and the white solid was recrystallized in hexanes/ethyl acetate to yield pure product (1.01 g, 72%). mp = 153-155°C; ¹H NMR (200 MHz, CDCl₃):
\[ \delta 3.46 \text{ (s, 3H)}, 5.31 \text{ (s, 2H)}, 7.20 - 7.55 \text{ (m, 8H)}, 8.20 - 8.30 \text{ (m, 4H)}; ^{13}\text{C NMR (75 MHz, CDCl}_3\text{)}: \delta 38.26, 70.12, 121.68, 122.13, 125.03, 125.26, 126.19, 129.53, 133.00, 142.91, 144.91, 145.38, 152.35, 155.37, 155.90; \text{MS (EI): } m/z \text{ 468 (M}^+ + \text{H}, 11\%), \text{ 329 (M}^+ - \text{OArNO}_2, 92\%), \text{ 123 (100\%); HRMS(EI) calcd for } \text{C}_{22}\text{H}_{17}\text{N}_3\text{O}_9 \text{ 467.096479, found 467.097693.}

\text{Tert-butyl } N\text{-}((4\text{-}[(4\text{-}nitrophenoxy)carbonyl]amino)benzyloxy)carbonyl\text{-}tryptophate (3.95). \text{To a stirring suspension of } L\text{-}tryptophan (Trp) \text{ tert-butyl ester hydrochloride salt (0.254 g, 0.86 mmol, 1.0 eq.) in dry dichloromethane (10 mL) was added dimethylaminopyridine (DMAP) (0.209 g, 1.71 mmol, 2.0 eq.), and within minutes the reaction mixture became clear and colorless. To this solution was added compound 3.94 (0.4 g, 0.86 mmol, 1.0 eq.), at which point the reaction became bright yellow. After stirring at room temperature for 12 h, the reaction was diluted with dichloromethane (50 mL) and washed with a solution of NaHCO\textsubscript{3} (5% aqueous, 2×50 mL), 0.1 M HCl (2×50 mL), and brine (2×50 mL). The organic layer was dried (MgSO\textsubscript{4}), and concentrated under reduced pressure to yield a yellow oil. Following purification via silica gel chromatography (hexanes:ethyl acetate = 60:40; \text{Rf} = 0.3), the desired compound 3.95 was obtained as a brittle, foamy, light yellow solid (0.451 g, 89\%). \text{mp = 76 - 78 \textdegree C.} \text{H NMR (300 MHz, CDCl}_3\text{)}: \delta 1.38 \text{ (s, 9H)}, 3.27 \text{ (m, 2H)}, 3.43 \text{ (bs, 3H)}, 4.60 \text{ (bs, 1H)}, 5.00-5.20 \text{ (m, 2H)}, 5.30 \text{ (bs, 1H)}, 7.00 \text{ (bs, 1H)}, 7.08-7.40 \text{ (m, 8H)}, 7.57 \text{ (d, J = 7.2 Hz, 2H)}, 8.08 \text{ (bs, 1H)}, 8.23 \text{ (bd, 2H); ^{13}\text{C NMR (75 MHz, CDCl}_3\text{)}: } \delta 28.0, 38.5, 55.2, 66.1, 82.2, 110.0, 111.3, 118.8, 119.5, 122.0, 122.3, 123.0, 125.1, 126.1, 127.8, 129.1, 135.6, 136.2, 142.1, 144.9, 152.7, 155.8, 156.1, 171.2; \text{MS (EI): } m/z \text{ 588 (M}^+, 17\%), \text{ 130 (100\%); HRMS(EI) calcd for } \text{C}_{31}\text{H}_{32}\text{N}_4\text{O}_8 \text{ 588.2220, found 588.22232.} \]
**N-{(4-{methyl[(4-nitrophenoxy)carbonyl]amino}benzyloxy)carbonyl}tryptophan (3.96).** The tert-butyl ester 3.95 (0.150 g, 0.25 mmol, 1.0 eq.) was dissolved in dry dichloromethane (3 mL), and to this solution was added anisole (0.055 mL, 0.51 mmol, 2 eq.) and thioanisole (0.060 mL, 0.51 mmol, 2.0 eq.). An aliquot of TFA (1 mL) was added, dropwise with stirring, and the reaction was left to stir at room temperature for 12 h. The solution was then transferred to a 250 mL round-bottom flask, to which was added chloroform (50 mL), and the solvent was removed by rotary evaporation. This procedure was repeated several times in order to remove the TFA from the solution. After placing the sample under vacuum for 3 h, the reaction mixture was purified via silica gel chromatography (chloroform:methanol = 90:10, Rf = 0.3) to yield a pale yellow crystalline solid (0.080 g, 59%). $^1$H NMR (400 MHz, CDCl$_3$): δ 3.15-3.45 (m, 5H), 4.66 (bs, 1H), 4.85-5.14 (m, 2H), 5.41 (bs, 1H), 6.81 (bs, 1H), 6.88-7.24 (m, 9H), 7.48 (bd, 2H), 8.16 (bd, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$): δ 27.7, 38.6, 55.0, 67.0, 109.6, 111.6, 118.7, 119.8, 122.4, 123.4, 125.3, 126.4, 127.8, 129.2, 136.3, 142.3, 145.2, 152.8, 153.9, 156.2, 176.5; MS (Electrospray) 531 (M$^+$ - H$^+$, 5%), 229 (100%).

**3.2.18 Preparation of Standard Curves for Detection and Quantitation of PNP and Tryptophan (Trp) by HPLC**

The standard curves previously prepared for detection of PNP (Section 3.2.5) could not be used due to the fact that the HPLC conditions (solvent, flowrate, etc.) had been significantly changed. A series of stock solutions of PNP and Trp were prepared (0 µM to 28 µM, in 5/95 DMSO/bicine [100 mM bicine, 100 mM NaCl, 0.01 % NaN$_3$, pH 9.0]; PNP and Trp present in equal concentrations), and to an aliquot of each of these solutions (15 µL) was added a solution of PNA (42 µM, 10 µL, in 5/95 DMSO/bicine [100 mM bicine, 100 mM
NaCl, 0.01 % NaN₃, pH 9.0); internal standard) and concentrated perchloric acid (3 µL). Using a 20 µL injection loop, these samples were analyzed via reverse phase analytical HPLC: \( \lambda_{\text{det}} = 280 \text{ nm} \) for 10 min, followed by \( \lambda_{\text{det}} = 325 \text{ nm} \) for 50 min; mobile phase = 75/25 water (0.1 % TFA)/methanol for 25 min (flowrate = 0.9 mL/min for 13 min, 1.5 mL/min for 47 min), ramp to 100% methanol over 5 min, holding 100% methanol for 10 min, ramp back to 75/25 water (0.1 % TFA)/methanol over 5 min, holding this solvent system for 15 min. Retention times for the analytes were as follows: Trp = 8 - 9 min; PNA = 11 - 12 min; PNP = 16 - 17 min. Plots of concentration of Trp/PNA vs. peak area ratio of Trp/PNA, and concentration PNA vs. peak area ratio of PNP/PNA were found to be linear. These standard curves were used for the detection of PNP and Trp in small-scale antibody-catalyzed and uncatalyzed reactions with the model prodrug substrate 3.96.

3.2.19 Rapid Screen for Catalytic Activity with ST51 and Z-Tryptophan (3.97)

The same protocol was used as described for ST51 and substrate 3.54 (Section 3.2.6), except that Z-tryptophan (3.97) was the substrate, and the final [ST51] was 5 µM. The concentration of tryptophan in the solution was determined by reverse-phase analytical HPLC (as outlined in Section 3.2.18).

3.2.20 Detailed Kinetic Studies with ST51 and Model Prodrug Substrate 3.96

An aliquot (4.5 µL) of a stock solution of the model prodrug substrate 3.96 (1.54 mM - 6.67 mM) in DMSO was added to bicine buffer (100 mM bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0) and the solution was mixed well. A solution of the antibody in bicine buffer (100 mM bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0) was added, so that the final volume was 90 µL and the final concentration of antibody was 5 µM. The final concentration of DMSO was 5%, and the final concentration of 3.96 ranged from 0.077 mM to 0.333 mM in

183
the antibody-containing reactions. The control reactions consisted of a solution of 0.077 mM to 0.333 mM 3.96 in 5/95 DMSO/bicine buffer (as above), with no antibody present. These solutions were mixed gently and were maintained at 25°C in a water bath. At various time intervals, the solutions were gently mixed, and an aliquot (15 μL) of each reaction mixture was withdrawn. To this was added a solution of PNA (10 μL of 42 μM in 5/95 DMSO/bicine buffer; internal standard), and concentrated perchloric acid (3 μL). This solution was mixed well, precipitated antibody was removed via centrifugation (microcentrifuge), and the concentrations of PNP and Trp in the solution were determined by analytical reverse-phase HPLC (as outlined in Section 3.2.18). The reactions were followed to approximately 5% completion, and plots of the concentration of PNP and Trp over time exhibited excellent linearity and similarity to one another. The initial velocities of the reactions at the various substrate concentrations were determined by taking the slopes of these plots. This procedure was repeated in duplicate for each substrate concentration. Lineweaver-Burk plots were generated using Grafit.
3.3 RESULTS AND DISCUSSION

3.3.1 Prodrug Design

Before embarking upon the time-consuming and costly process of raising antibodies for the objectives outlined in Section 3.1.3, we first carried out some simple kinetic studies with various water-soluble carbamate substrates (Table 3.3.1). We wished to determine the stability of these compounds under various conditions, as well as ascertain that the desired cascade reaction of the type shown in Scheme 3.1.17 (Section 3.1.3) would indeed occur in a bis-carbamate substrate. Bis-carbamate 3.42 was first examined as a model system. The rate of hydrolysis of this compound was studied by monitoring the production of N-methyl-4-nitroaniline by spectrophotometry. This carbamate was found to be very stable and did not exhibit any significant breakdown at pH 8.0. In 1 M NaOH, this compound exhibited a half-life of 26 minutes. Although N-methyl-4-nitroaniline was indeed being produced in the reaction, it was not clear if it was being formed as a result of an initial reaction at the N,O-aryl carbamate moiety followed by the cascade reaction, or by direct hydrolysis of the O-benzylic carbamate linkage. To determine this, we examined carbamate 3.43, which mimics the N,O-aryl carbamate portion of compound 3.42, and carbamate 3.44, which mimics the O-benzylic carbamate moiety of 3.42, and determined their rates of hydrolysis. As was expected, neither of these carbamates exhibited any breakdown at pH 8.0, so their hydrolysis reactions were studied in 1 M NaOH. The hydrolysis of carbamate 3.43 was monitored spectrophotometrically by following the production of p-hydroxybenzoic acid, while the formation of N-methyl-4-nitroaniline was monitored for carbamate 3.44. Carbamate 3.44 had a half-life that was almost identical to that of the bis-carbamate 3.42, while that of carbamate 3.43 was about 3.5 times greater than the bis-carbamate 3.42. These results
suggested that 3.42 was undergoing hydrolysis more readily at the O-benzylcarbamate moiety. Thus, we still did not know if the cascade reaction was indeed occurring.

### Table 3.3.1. Hydrolysis of Carbamates 3.42 - 3.46

<table>
<thead>
<tr>
<th>Compound Structurea</th>
<th>Hydrolysis Conditionsb</th>
<th>t½</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Structure 3.42" /></td>
<td>1 M NaOH</td>
<td>28 min</td>
</tr>
<tr>
<td><img src="image" alt="Structure 3.43" /></td>
<td>1 M NaOH</td>
<td>86 min</td>
</tr>
<tr>
<td><img src="image" alt="Structure 3.44" /></td>
<td>1 M NaOH</td>
<td>30 min</td>
</tr>
<tr>
<td><img src="image" alt="Structure 3.45" /></td>
<td>50 mM KPi, pH 8.0</td>
<td>27 min</td>
</tr>
<tr>
<td><img src="image" alt="Structure 3.46" /></td>
<td>50 mM KPi, pH 8.0</td>
<td>25 min</td>
</tr>
</tbody>
</table>

*aConcentration of substrate was 50 µM; *bReactions were carried out in 1% DMSO.
To ascertain if the cascade reaction would indeed occur, it was decided to use more reactive $N$-$H$ carbamates in our model system. The hydrolysis reaction of bis-carbamate 3.45 was followed by monitoring the rate of production of $N$-methyl-4-nitroaniline. At pH 8.0, this compound exhibited a half-life of only 27 minutes. Since it had already been demonstrated that the $O$-benzylic carbamate in 3.44 was very resistant to hydrolysis, these results confirmed that the cascade reaction did indeed occur. To gain information as to the rate of the cascade reaction vs. the rate of the initial hydrolysis reaction, the $N$-$H$ carbamate 3.46 was prepared. The rate of hydrolysis of 3.46 was studied by monitoring the production of $p$-hydroxybenzoic acid. This compound exhibited a half-life that was almost the same as compound 3.45, which was determined by monitoring the cascade reaction. These results demonstrated that the cascade reaction was fast compared to the initial hydrolysis reaction.

The difference in stability of $N$-methyl carbamates and $N$-$H$ carbamates can be attributed to their differing mechanisms for hydrolysis. While $N$-$H$ carbamates generally hydrolyze via an E1cB mechanism, $N$-methyl carbamates must hydrolyze by a $B_{AC2}$ mechanism. These mechanisms are illustrated in Scheme 3.3.1. In the E1cB process, the conjugate base of the carbamate derivative is the reactive species, therefore $N$-methyl carbamates cannot follow this mechanism of hydrolysis. The rate-determining step for this mechanism is elimination of $^7OR_3$ from the anion to give the isocyanate intermediate. This then reacts rapidly with water or hydroxide ion to yield the unsubstituted carbamate, which decarboxylates to produce an amine. In the case where $R_1$ is a methyl group, the $B_{AC2}$ mechanism of hydrolysis occurs. This involves rate-determining attack of hydroxide ion on the ester to give the tetrahedral intermediate, which then decomposes rapidly to the unsubstituted carbamate. Thus, cleavage of the C-$OR_3$ bond is not involved in the TS of the
rate-determining step.\textsuperscript{74} From the descriptions of the two mechanisms, it is apparent that while the hydrolysis rate constants of $N$-$H$ carbamates should be highly dependent on the nature of the leaving group (\textsuperscript{3}OR$_3$), this is not the case for carbamates hydrolyzing \textit{via} the $B_{Ac2}$ mechanism.\textsuperscript{56,71-73}

\begin{center}
$E1cB$ mechanism - $R_1 =$ H only
\end{center}

\begin{center}
$B_{Ac2}$ mechanism - $R_1 =$ methyl, or H (if leaving group is poor)
\end{center}

\textbf{Scheme 3.3.1.} Mechanism of hydrolysis of $N$-$H$ and $N$-methyl carbamates.

Thus, as the pKa of the leaving group conjugate acid (HOR$_3$) of an $N$-$H$ carbamate increases (as the leaving group becomes poorer), the rate-determining decomposition of the conjugate base of the carbamate ester ($R_2$N(C(O)OR$_3$) becomes progressively more difficult, until the transition-state free energy equals that for the rate-limiting step of the $B_{Ac2}$ mechanism, the formation of the tetrahedral intermediate. As the pKa of the conjugate acid of the leaving
group (HOR$_3$) further increases (above 12.5 – 17 for N-aryl carbamates), there is a changeover in mechanism from ElcB to B$_{AC2}$. The ratio of rates of ElcB hydrolysis (R$_1$ = H) and B$_{AC2}$ hydrolysis (R$_1$ = methyl) of otherwise identical carbamates is approximately $10^8$. Therefore, the free energy difference between these mechanisms is approximately 13 kcal per mole, with B$_{AC2}$ hydrolysis being the more energetically demanding process.

This explains why the N-H carbamates studied (compounds 3.45 and 3.46) are much more reactive than the N-methyl carbamates examined (compounds 3.42 – 3.44).

Having examined the hydrolysis behavior of various types of carbamate compounds, and having ascertained that the desired cascade reaction for our tripartate prodrug system did indeed occur, we went on to pursue our objective of abzyme-catalyzed N-methyl carbamate hydrolysis of a tripartate prodrug.

### 3.3.2 Transition State Analogue (TSA) Design and Synthesis

The next step was to construct the TSA, compound 3.41, for the reaction outlined in Scheme 3.1.17 (Section 3.1.3). The TSA used to elicit our carbbamase antibodies was synthesized according to Scheme 3.3.2. The synthesis of the TSA 3.41 was based on a procedure developed in our lab by Dr. Mei-Jin Chen for the production of N, O-arylphosphoramidates. Tricoordinate phosphite chemistry was used to synthesize the O-alkyl N,O'-arylphosphoramidate derivative 3.49 in a one-pot procedure by reacting p-nitrophenol (PNP) and an aniline derivative 3.47 with methyl dichlorophosphite to form phosphoramidate 3.48, which upon oxidation with m-chloroperbenzoic acid (m-CPBA) gave the fully protected phosphoramidate 3.49. The methyl ester was deprotected by reaction with lithium bromide in butanone (reflux), to yield 3.50. The final product 3.41 was obtained in
pure form through hydrolysis of the allyl protecting group with lithium hydroxide. Later, an attempt was made

\[
\begin{align*}
\text{H}_3\text{COCl} & \quad \text{1. DIPEA (2.2 eq.), Et}_2\text{O, -78°C, 10 min} \\
& \quad \text{2. p-nitrophenol (1.02 eq.), Et}_2\text{O, -78°C, 2 h}
\end{align*}
\]

![Chemical structure](image)

\[
\begin{align*}
\text{H}_3\text{COCl} & \quad \text{1. DIPEA (2.2 eq.), Et}_2\text{O, -78°C, 10 min} \\
& \quad \text{2. p-nitrophenol (1.02 eq.), Et}_2\text{O, -78°C, 2 h}
\end{align*}
\]

Scheme 3.3.2. Synthesis of TSA 3.41.
Scheme 3.3.3. Attempted synthesis of TSA 3.41 using an allyl protecting group on phosphorus.
to shorten the TSA synthesis by using an allyl protecting group on the phosphate, as shown in Scheme 3.3.3. The idea here was to improve the synthesis by removing the allyl protecting group on both the phosphate and carboxylate of 3.52 in a single step by a Pd-catalyzed reaction between dimedone (a weak nucleophile) and the allyl groups. It was hoped that the final product would precipitate out of solution during the reaction. However, this was unsuccessful due to decomposition of the product in the final deprotection step, probably due to the sensitivity of phosphoramidates to trace amounts of acid (numerous spots appeared on the TLC with time).

3.3.3 Monoclonal Antibody (mAb) Production and Screening

TSA 3.41 was conjugated to bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) via its N-hydroxysuccinimide ester, which was prepared in situ by reaction of 3.41 with N-hydroxysulfosuccinimide in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Scheme 3.3.4). The degree of conjugation to the carrier protein was determined using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay of Habeeb. This procedure was carried out with the BSA-3.41 solution, but not the KLH conjugate, since KLH solutions are slightly precipitous which makes it difficult to obtain quantitative results with this protein.

![Scheme 3.3.4. Conjugation of TSA 3.41 to carrier proteins BSA and KLH.](image-url)
The concentration of BSA in the BSA-3.41 conjugate solution was first determined via the bicinchoninic acid (BCA) protein assay, using a kit designed for this purpose (Pierce). To determine the number of free amino groups, the 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay of Habeeb was utilized. TNBS reacts readily with the primary amino groups of amino acids in basic aqueous solutions, forming brightly colored yellow adducts, and there are roughly 33 solvent-accessible lysine residues per molecule of BSA. The BSA-3.41 conjugate reacts to a lesser extent with TNBS than unmodified BSA at the same concentration, thus the number of TSA-occupied lysine residues can be estimated by measuring the absorbance of these two solutions.

It was calculated that roughly 13 molecules of TSA 3.41 were attached per molecule of BSA. Since the conjugation was successful with BSA, it was assumed that this procedure was also successful with KLH. These two conjugates were sent to Melanie Lea, a technician in the laboratory of Professor Alex Marks at the Banting and Best Research Institute, University of Toronto, where monoclonal antibodies were obtained against the KLH conjugate using standard hybridoma technology. In brief, Balb/c mice were hyperimmunized with the KLH conjugate. When the antibody titer in the sera was sufficiently high as determined by Enzyme-Linked ImmunoSorbant Assay (ELISA), their spleens were removed and fused with myeloma cells using polyethyleneglycol to form hybridoma cells. The BSA conjugate was used for all antibody screenings to avoid obtaining antibodies that were against KLH rather than the TSA. Hybridoma cells were propagated, and those producing mAbs exhibiting a high affinity for the TSA were subcloned by limiting dilution. A total of 64 cell lines survived this process. Isotyping of these antibodies revealed that all were of the IgG class.
If an antibody possesses high affinity for the hapten, this is no guarantee that the antibody will be catalytically active. The antibody affinity may be a consequence of binding interactions to residues on the analogue not central to mimicking the TS. Green and coworkers have suggested that substances representing TS structures but which are different from the immunizing hapten can be used in a simple method for selecting catalytic monoclonal antibodies that exhibit turnover and specificity. Given that only the binding interactions that are present in the TS structure will contribute directly to catalysis, only antibodies that bind these elements with high affinity are likely to exhibit catalytic activity. Green suggests executing competitive inhibition binding assays with the mAbs and "short", or truncated, TSAs that contain all the unique elements of the TS but do not contain other non-essential elements that may be common to TS, substrate and products. Through these competitive inhibition binding assays, antibodies with high affinity for the truncated TSAs can be easily identified, and can be expected to bind the TS of the desired reaction more strongly than those antibodies that exhibit lower affinities for the "short" TSAs. These mAbs would have the highest potential for catalysis and displaying turnover.

Competitive inhibition binding studies were therefore carried out, to ensure that the antibodies that bound 3.41 were recognizing the "business end" of the TSA. The truncated TSA, compound 3.53, possessed the characteristics of the TSA 3.41, but lacked the linker chain to BSA. Thus, antibodies that had their binding to 3.41 strongly inhibited by the presence of 1 mM of the truncated TSA 3.53 were known to bind only the portion of the TSA that mimicked the transition state of the N-methyl carbamate hydrolysis reaction. A total of 32 of the original 64 cell lines produced antibodies that fell into this category. Quantities of these antibodies sufficient for catalytic screenings were obtained by injecting these cell lines
into mice, followed by harvesting of the resulting ascitic fluid and purification of the mAbs using a protein A column.

Selection for antibodies that recognized only the "business" portion of the TSA had other advantages in addition to issues of catalysis. First, central to the success of our prodrug system is the requirement that the abzyme must not recognize the "drug" portion of the prodrug substrate, so as to allow for the activation of a variety of different drugs by the same abzyme. Second, there is a significant cost issue when attempting to obtain in pure form large numbers of antibodies from ascitic fluid. Thus, by narrowing down the number of antibodies that may have the desired properties, the costs are also reduced.

\[
\begin{align*}
\text{Li}^+\text{O} & \quad \text{O} \\
\text{H}_3\text{C} & \quad \text{N} \\
\quad & \quad \text{3.53}
\end{align*}
\]

**Compound 3.53**: Truncated TSA used for competitive inhibition binding studies.

Initial catalytic screenings were carried out at pH 9.0 (5/95 DMSO/bicine buffer, pH 9.0, 25 °C) since it has been well-documented in the literature that almost all hydrolytic antibodies exhibit greater activity at basic pH. Thus, the purified antibodies were dialyzed against bicine buffer, pH 9.0, and were screened for catalytic activity. We chose not to perform the initial screen for catalytic activity with a bis-carbamate substrate of type 3.37 (Scheme 3.1.17, Section 3.1.3) for the following reason. If none of the antibodies hydrolyzed a bis-carbamate substrate, the lack of catalysis could be attributed to the "drug" portion of the substrate interfering with substrate binding. However, it is possible that an antibody that did not display catalytic activity with a bis-carbamate substrate might catalyze the hydrolysis of a
simpler monocarbamate substrate. Thus, the antibodies were initially screened using the N-methyl carbamate substrate 3.54.\(^{78}\)

![Scheme 3.3.5. Initial screen for catalytic activity of antibodies raised to TSA 3.41.](image)

To minimize costs (in terms of antibody usage), it was essential that the screenings were carried out on as small a scale as possible. Thus, it was decided to follow the reactions by analytical reverse-phase HPLC, since this would allow us to set up and monitor very small scale reactions, and detect amounts as low as 1 \(\mu\)M PNP. However, before screening the antibodies with 3.54, we first had to determine the reactivity of 3.54. Due to the stability of this substrate, this could not be accomplished by direct observation of the hydrolysis reaction at pH's less than 13. Instead, the hydrolysis of 3.54 was followed by incubating the carbamate in basic solutions ranging from 0.10 M NaOH to 1.0 M NaOH, and pseudo-first-order rate constants were measured by following the hydrolysis reaction to several half-lives using spectrophotometry. These pseudo-first-order rate constants were then plotted vs. hydroxide ion concentration, shown in Figure 3.3.1.
Figure 3.3.1. Pseudo-first-order rate constants for the uncatalyzed hydrolysis of substrate 3.54 vs. hydroxide ion concentration

From the slope of this plot, the second order rate constant was calculated to be $2.31 \times 10^{-2} \text{ M}^{-1} \text{ min}^{-1}$. Assuming linearity to pH 9.0 and 10.0, we estimated the pseudo-first-order constants for the uncatalyzed hydrolysis reactions at pH 9.0 and 10.0 to be approximately $1.4 \times 10^{-5} \text{ h}^{-1}$ and $1.4 \times 10^{-4} \text{ h}^{-1}$, respectively. Thus, compound 3.54 has a half life of roughly 5.7 years at pH 9.0.

Before screening for catalysts, standard curves were prepared for the detection of PNP. A similar compound, p-nitroaniline (PNA), was used as an internal standard, and these compounds were separated and detected easily by analytical reverse-phase HPLC. Two standard curves were prepared, one over a small PNP concentration range (0 μM to 10 μM) (Figure 3.3.2a) and one over a larger range of PNP concentrations (0 μM to 50 μM) (Figure 3.3.2b). The plots of concentration of PNP/PNA vs. peak area ratio of PNP/PNA exhibited the same linear relationship for both ranges of concentrations studied, and these standard curves were used for the detection of PNP in the reaction mixtures.
The abzyme reactions were followed for a time period of approximately 40 h at pH 9.0, and during this time no detectable breakdown of the substrate 3.54 was observed in the absence of antibody (although the substrate was found to be contaminated with approximately 0.1 % PNP). Several of the antibodies were found to exhibit modest catalytic activity with substrate 3.54, some of which are listed in Table 3.3.2.
Table 3.3.2. Results from the initial screen for catalytic activity with selected antibodies and substrate 3.54

<table>
<thead>
<tr>
<th>mAb</th>
<th>[PNP] (µM) after 40 h reaction time</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST51</td>
<td>31</td>
</tr>
<tr>
<td>ST30</td>
<td>15</td>
</tr>
<tr>
<td>ST24</td>
<td>7</td>
</tr>
<tr>
<td>ST46</td>
<td>7</td>
</tr>
<tr>
<td>ST43</td>
<td>6</td>
</tr>
<tr>
<td>ST47</td>
<td>6</td>
</tr>
<tr>
<td>ST35</td>
<td>5</td>
</tr>
<tr>
<td>ST42</td>
<td>5</td>
</tr>
</tbody>
</table>

A plot of competitive inhibition values (from the competitive binding studies of the antibodies with the truncated TSA 3.53) vs. catalytic activity of the antibodies (expressed in terms of [PNP] (µM) after a 40 h reaction period) was prepared, in order to determine if there was any correlation between these parameters. From the plot, it is difficult to ascertain whether there is indeed a correlation between the competitive binding studies and catalytic activity. One one hand, two of our three best antibody catalysts (ST51 and ST24) exhibit two of the four highest competitive inhibition values. As well, the binding of ST30 to the TSA (3.41) was inhibited by the truncated TSA (3.53) about half as much as ST51, and this antibody catalyst exhibits approximately half as much catalytic activity. However, one of the antibodies that was strongly inhibited by the truncated TSA 3.53 did not show any significant activity. Thus, although it appears that some correlation may exist between the affinity of the antibodies for the truncated TSA and the catalytic activity of the most active abzymes, perhaps a more detailed study of the type performed by Green,37 using progressively smaller...
and smaller truncated TSAs, would have to be done before any correlation could be determined with certainty.

![Graph](image)

**Figure 3.3.3.** Competitive inhibition values vs. catalytic activity of mAbs.

Competitive inhibition values were determined by Melanie Lea, at the Banting and Best Research Institute, University of Toronto. An ELISA was performed with the antibodies and the BSA-3.41 conjugate, whereby the BSA-3.41 conjugate was used to coat the wells of an ELISA plate, an antibody solution was incubated in each well, and the amount of bound antibody was detected using an antibody-enzyme conjugate. The absorbance of the final enzyme assay corresponded directly to the amount of antibody bound to the BSA-3.41 conjugate coating the well. The competitive inhibition values in the above plot represent the ratio of absorbances of this final assay, when the ELISA was carried out in the absence and presence of 1 mM truncated TSA 3.53, respectively. The largest competitive inhibition values are therefore exhibited by antibodies that had their binding to the BSA-3.41 conjugate strongly inhibited by the presence of the “short” TSA 3.53.

### 3.3.4 Is Enhanced Hydrolysis a Result of Abzyme Catalysis?

The following query is one of the most important questions that must be addressed for any abzyme project: *Is the activity a result of antibody catalysis or a contaminating enzyme?*

Since abzymes usually exhibit activities that are often several orders of magnitude less than enzymes, it is possible that the activity found during a preliminary screening of a potential abzyme may be a result of a small and almost imperceptible amount of contaminating enzyme and not the antibody.
There are two common approaches to establish if activity is indeed a result of antibody catalysis. One is to determine whether the activity is inhibited by stoichiometric quantities of the TSA. If catalysis by these antibodies is a result of the abzymes themselves, one would expect their activity to be strongly inhibited by the TSA to which they were raised. To test for this, inhibition studies were carried out with ST51, ST30, and ST24 (our three best abzymes, as determined by the initial screen for catalytic activity) and the TSA (3.41). Reaction mixtures containing 4 μM antibody and 1 mM substrate 3.54 in 5/95 DMSO/bicine pH 9.0 were incubated at 25°C in the presence and absence of 10 μM 3.41.

![Analytical reverse-phase HPLC traces for antibody-containing reactions after a 46 h time period with and without TSA 3.41.](image)

**Figure 3.3.4.** Analytical reverse-phase HPLC traces for antibody-containing reactions after a 46 h time period with and without TSA 3.41.
Figure 3.3.4. Analytical reverse-phase HPLC traces for antibody-containing reactions after a 46 h time period with and without TSA 3.41.
The results in Figure 3.3.4 show that the catalytic activities of antibodies ST51, ST30, and ST24 with substrate 3.54 have been completely inhibited by approximately stoichiometric amounts of the TSA 3.41. The concentration of PNP produced in each antibody-containing reaction in the absence of the TSA is consistent with what was observed in the initial screen for catalytic activity. In the control reaction, with no antibody present, no breakdown of the substrate or TSA was observed over 46 h. The trace amounts of PNP observed in the HPLC traces are due to a slight (0.1%) contamination of the substrate 3.54 with this substance.

The other method for determining if activity is a result of antibody catalysis is to convert the mAbs into pure Fabs and compare their activities. For reasons of cost and time, we decided to focus our attention on ST51, our best abzyme. Scheme 3.3.6 outlines the general procedure for comparing the catalytic activity of mAbs and Fabs.

**Scheme 3.3.6.** General scheme for comparing catalytic activity of mAb to Fab.

ST51 was purified from ascites fluid using a Protein A column. Protein A specifically binds to the constant region of mAbs. If the activity of the purified mAbs is a result of a contaminating enzyme, then this enzyme would have to also bind to the Protein A
column and elute with the mAb. Samples of ST51 mAb were analyzed by SDS-PAGE and were found to be pure by Coomassie staining (Figure 3.3.5).

![Figure 3.3.5. Analysis of ST51 mAb by SDS-PAGE.](image)

SDS-PAGE was performed in a 7.5% acrylamide gel according to the method developed by Laemmli. Lane 1 contains molecular weight standards ranging from 25,000-150,000 da (Sigma); Lane 2 contains non-reduced ST51 mAb (expected MW=150,000 da); Lane 3 contains reduced ST51 mAb (expected MWs=50,000 and 25,000 da). The gel was stained with Coomassie Brilliant Blue R, destained in 7/7/86 methanol/acetic acid/water, and vacuum-dried at 80 °C.

ST51 mAb was then converted into a Fab. As discussed in Section 3.1.1, proteolysis by papain cleaves an antibody molecule at the hinge portion of the molecule (shown in Scheme 3.3.7), producing two Fab fragments and one Fc fragment. The papain used for proteolysis of ST51 was immobilized on a support of cross-linked 6% beaded agarose, and was easily separated from the digested antibody sample. Following papain digestion, ST51 Fab was purified on a Protein A column. Samples of ST51 Fab were analyzed via SDS-PAGE and were found to be very pure (Figure 3.3.6).
Figure 3.3.6. Analysis of ST51 Fab by SDS-PAGE.

SDS-PAGE was performed in a 7.5% acrylamide gel according to the method developed by Laemmli. Lane 1 contains molecular weight standards ranging from 25,000-100,000 da (Sigma); Lane 2 contains non-reduced ST51 Fab (expected MW=50,000 da). The gel was stained with Coomassie Brilliant Blue R, destained in 7/7/86 methanol/acetic acid/water, and vacuum-dried at 80 °C.

Scheme 3.3.7. Proteolysis by papain produces two Fab fragments and one Fc fragment.

The Fab portion of an antibody molecule retains all the substrate binding specificity of the entire antibody molecule, since this segment of the immunoglobulin contains the
hypervariable sequences. The Fc fragment contains the constant region of the original mAb. Therefore, the Fc fragment retains the ability to bind to Protein A, but the Fabs will not bind to a Protein A column. Since the ST51 Fab fragments were purified via a Protein A column, if any enzyme had contaminated the ST51 mAb sample and survived the papain digest, then this enzyme would be expected to bind to the Protein A column again, as it did originally, and the resulting ST51 Fab should be free of contaminating enzyme.

ST51 Fab was screened for catalytic activity, and was found to possess the same activity as ST51 mAb. If the catalytic activity of ST51 mAb was a result of a contaminating enzyme, then the Fab should have exhibited significantly less activity than the mAb. However, ST51 Fab and mAb exhibited the same catalytic activity, which strongly indicates that the catalytic activity observed in ST51 samples was due to the antibody and not to a contaminating enzyme. It is possible that the papain digestion used to generate the Fab also acted upon a contaminant, and in a region that does not affect its activity but does affect its ability to bind Protein A. However, it is unlikely that a contaminant would undergo papain digestion without losing at least some activity. We believe that the results of the TSA inhibition studies and the Fab study very strongly support the notion that the catalytic activity observed was a result of the antibody and not a contaminating enzyme. More evidence supporting this is given in Section 3.3.8.

In addition to a contaminating enzyme, another possible problem is catalysis by serum albumins. Indeed, recent studies by Kirby et al.⁷⁹ have shown that some reactions are catalyzed almost as well, or sometimes more efficiently, by BSA than by catalytic antibodies. Although the above experiments with the TSA and Fab would seem to indicate that catalysis is not a result of contaminating serum albumins, we examined whether BSA was capable of
catalyzing the hydrolysis of compound 3.54. No catalytic activity was observed with BSA and substrate 3.54. That the catalytic activity observed with ST51 is not due to contaminants from the ascites is also supported by the fact that similarly purified antibodies from other cell lines were unable to catalyze the hydrolysis of substrate 3.54.

3.3.5 pH, Turnover, and Stability Studies

Before performing more detailed kinetic studies, ST51 was rapidly screened at pH 10.0 and 7.4. Reactions containing ST51 and its N-methyl carbamate substrate 3.54 were incubated at pH 7.4, 9.0 and 10.0, and the catalytic activity of ST51 under these conditions was monitored over time by detecting the production of PNP by analytical reverse-phase HPLC. Negligible background hydrolysis of the substrate 3.54 was observed regardless of the pH. The results of these studies are summarized in Figure 3.3.7.

![Figure 3.3.7. Catalytic activity of ST51 mAb with substrate 3.54 at various pH.](image)

The rate of production of PNP at basic pH was considerably greater than that at pH 7.4. Although the level of activity at pH 7.4 was extremely low, this does not necessarily mean that the rate enhancement at pH 7.4 is less than that at pH 9.0 or 10.0. It is possible that the reactive species is hydroxide ion and thus, as the pH is decreased from pH 9.0 to 7.4, both the
uncatalyzed reaction and catalyzed reaction decrease proportionately. In other words, the rate enhancement at pH 7.4 may be equal to, or perhaps even better than, the enhancement in the reaction rate at pH 9.0 or 10.0. However, this may be insufficient to see significant quantities of PNP because of the high stability of the substrate at pH 7.4. We believe that this may indeed be the case, since the substrate has a half-life of approximately 570 years at pH 7.4, so a rate enhancement of at least $10^3$ is required to see any PNP produced at all over the time course of these experiments. We should also point out that there are other explanations. The difference in ST51 activity between pH 9.0 and 10.0 does not appear to be proportional to hydroxide concentration. Thus, it is also possible that the abzyme requires for activity the ionization of a general base or active site nucleophile that has a pKa in the basic region. However, the lack of correlation between catalytic activity at pH 9.0 and 10.0 and hydroxide concentration could also be due to slight denaturation of ST51 at pH 10.0.

The linearity of the results in Figure 3.3.7 would seem to indicate that ST51 is not strongly product inhibited, and, at pH 9.0 and 10.0, multiple turnovers are observed. The fact that ST51 exhibits multiple turnover is significant since many hydrolytic abzymes are heavily product inhibited and often exhibit only a single turnover. The ability of ST51 to undergo multiple turnover may be due to the fact that carbamate hydrolysis yields CO$_2$, as opposed to a negatively charged carboxylic acid found with ester or amide hydrolysis. Thus, products of carbamate hydrolysis reactions should experience minimal charge interactions with positively-charged residues in the active site that were generated in response to the negatively-charged TSA.

Stability studies on ST51 at pH 9.0 and 10.0 were also performed. Stock solutions of ST51 at pH 9.0 and pH 10.0 were incubated at room temperature over a period of several
days. Aliquots were removed from these solutions at roughly 24 h intervals and added to a solution of substrate 3.54 to examine for activity. Figure 3.3.8 clearly illustrates that ST51 is a remarkably stable abzyme, exhibiting minimal loss of catalytic activity even after several days of incubation at pH 9.0 and 10.0.

![Graph showing catalytic activity over time](image)

**Figure 3.3.8.** Catalytic activity of ST51 with 3.54 after incubation at pH 9.0 and 10.0.

### 3.3.6 Determination of Michaelis-Menten Parameters with ST51 and Substrate 3.54

Due to the slow rate of the antibody-catalyzed reaction below pH 9.0, we were unable to obtain full pH profiles (pH vs. k\text{cat} or k\text{cat}/K_m) for the reaction. Michaelis-Menten parameters (k\text{cat} and K_m) could only be determined for ST51 at pH 9.0 and 10.0. The abzyme was first examined with substrate 3.54 at pH 10.0, since this was the pH at which the antibody seemed to demonstrate maximum catalytic activity. The antibody-catalyzed reaction obeys saturation kinetics, and it was determined that ST51 exhibited a K_m of approximately 1.3 ± 0.2 mM, a V_{max} of 2.5 ± 0.3 μM/h, and a k\text{cat} of 0.25 ± 0.03 h^{-1} at pH 10.0 (Figure 3.3.9). Thus, this abzyme turns over about once every four hours at its maximal velocity under these reaction conditions. The value of k\text{cat}/K_m is (1.92 ± 0.37) × 10^{-1} μM^{-1} h^{-1}.
1, or approximately $0.053 \pm 0.010 \text{ M}^{-1} \text{ s}^{-1}$. The rate enhancement, or $k_{\text{cat}}/k_{\text{uncat}}$, at this pH was 1800-fold.

![Lineweaver-Burk plot for ST51 and substrate 3.54 at pH 10.0.](image)

**Figure 3.3.9.** Lineweaver-Burk plot for ST51 and substrate 3.54 at pH 10.0.

At pH 9.0 (see Figure 3.3.10), the $K_m$ value exhibited by ST51 with substrate 3.54 is considerably lower at $266 \pm 15 \mu\text{M}$. The $V_{\text{max}}$ was calculated to be $0.91 \pm 0.04 \mu\text{M/h}$, thus the $k_{\text{cat}}$ was $0.091 \pm 0.004 \text{ h}^{-1}$, and ST51 exhibits turnover about once every eleven hours at its maximal velocity at pH 9.0. The value of $k_{\text{cat}}/K_m$ was $(3.4 \pm 0.2) \times 10^{-4} \mu\text{M}^{-1}\text{h}^{-1}$, or approximately $0.094 \pm 0.006 \text{ M}^{-1} \text{ s}^{-1}$, and the rate enhancement at this pH was 6500-fold.

While enzymes are capable of rate accelerations of up to $10^{17}$ over the background abzymes are generally able to accelerate reactions to a maximum of about $10^5$- to $10^7$-fold above the rate of the uncatalyzed reaction.\textsuperscript{17,81} The typical range of rate enhancements for antibody-catalyzed reactions, however, is usually on the order of 10 to $10^3$-fold,\textsuperscript{17,18,81} and the values of $k_{\text{cat}}/k_{\text{uncat}}$ obtained for ST51 and substrate 3.54 fall within this range.
Figure 3.3.10. Lineweaver-Burk plot for ST51 and substrate 3.54 at pH 9.0.

The term $k_{cat}/K_m$ can provide some measure of catalytic efficiency of abzymes, as in the case of enzymes. The values for $k_{cat}/K_m$ are typically in the range of $10^5$ to $10^6$ M$^{-1}$ s$^{-1}$ for enzymes, however, for the majority of catalytic antibodies, these values generally range from $1$ to $10^3$ M$^{-1}$ s$^{-1}$. In the case of ST51 and substrate 3.54, $k_{cat}/K_m$ is quite small (0.053 M$^{-1}$s$^{-1}$ at pH 10, and 0.094 M$^{-1}$s$^{-1}$ at pH 9), thus this antibody is not a very efficient carbanase abzyme.

There are some general, inherent reasons why antibodies are limited in their catalytic efficiency. First, transition state analogues (TSAs) cannot be perfectly designed to match every feature of the transition state of a reaction. The transition state may contain partially broken or formed bonds, distorted bond angles, partial charges, etc. and these features cannot be mimicked exactly by a stable molecule. As well, while an enzyme can bury its active site within its structure, lessening or removing the effect of the polar solvent on the reaction, the location at which a substrate (or TSA) binds to an antibody, in comparison, is quite exposed to the surrounding solution. This may create a less favorable environment for catalysis. Antibodies also have a strong affinity for aromatic residues, and thus binding may be focused
on certain features of the TSA that are less essential to transition state stabilization than others. If most of the binding energy arises from interactions with structural features that are present in both the substrate and the TSA, the antibody will not stabilize the transition state relative to the ground state. As well, on average, only 10% of the immune repertoire is sampled when catalytic antibodies are obtained through a hybridoma approach, and although enzymes have become optimized in their catalytic activities over millions of years, antibody binding is optimized over a period of weeks to months. All of these factors contribute to the lower efficiency of antibodies in comparison to enzymes.

Given the current limitations of the catalytic antibody field, the choice of $N$-methyl carbamate hydrolysis as an enzyme-catalyzed reaction is quite ambitious. Blackburn has selected several criteria for choosing a reaction for antibody catalysis, and these criteria are as follows: the reaction should (1) have a slow but measurable spontaneous rate under the conditions to be used with the enzyme, (2) be well-analyzed in terms of its mechanism, (3) have as small a number of reaction steps as possible, (4) be easily monitored, and (5) exhibit a transition state that can be mimicked by a stable TSA, that is straightforward to design and synthesize. In the case of our enzyme-catalyzed reaction, all but the first of these criteria are met. As discussed in Section 3.3.1, $N$-methyl carbamates are incredibly stable to hydrolysis, and the spontaneous rate of the uncatalyzed reaction was too slow to be measured over the time-scale of the ST51-catalyzed reaction at both pH 9.0 and 10.0. It is therefore quite impressive that we were able to obtain an $N$-methyl carbamase enzyme, as this is a highly challenging reaction for antibody catalysis. ST51 represents the first and only catalytic antibody to date capable of catalyzing the hydrolysis of an $N$-methyl carbamate.
Indeed, this is one of the most difficult hydrolytic reactions of a carbonyl derivative (ester, amide, carbamate) ever catalyzed by an antibody obtained using the TSA approach.

Although several researchers have reported abzymes capable of hydrolyzing $N$-H carbamates, the closest approximation to ST51 is catalytic antibody DF8-D5, described in Section 3.1.2, which was raised by Wentworth et al. DF8-D5 catalyzed the hydrolysis of carbamates of the type 3.29 shown in Section 3.1.2, and evidence suggested that the abzyme-catalyzed reaction proceeded by the highly disfavoured $B_{Ac2}$ mechanism, rather than the more favoured $E1cB$ process found for the uncatalyzed reaction. The rate enhancement obtained with substrate 3.54 and ST51 at pH 9.0 (6500) is approximately 20 times greater than that obtained by Wentworth et al. for the hydrolysis of their $N$-H carbamate (3.29) when $X = NO_2$ with DF8-D5 ($k_{cat}/k_{uncat} = 300$). However, when DF8-D5 is screened with other $N$-H carbamate substrates ($X = Br, F, and OMe$), it exhibits higher rate enhancements than the ST51-catalyzed hydrolysis of substrate 3.54.

3.3.7 Determination of the $K_D$ for the TSA and the Relationship Between $k_{cat}/k_{uncat}$ and $K_m/K_D$

The rate enhancement for an antibody-catalyzed reaction can be related to the ratio of equilibrium constants for the complex between the abzyme and substrate and the abzyme and the TSA to which it was raised, through the application of a thermodynamic cycle derived from transition state theory (Scheme 3.3.8). These equilibrium constants can be estimated as $K_m$ for the substrate and $K_f$ for the TSA.
To investigate the relationship between the rate enhancement for the STSI-catalyzed hydrolysis of substrate 3.54, and the abzyme’s affinity for the TSA(3.41) relative to its substrate (3.54), it was first necessary to determine the $K_i$ value for the TSA. Since the abzyme-catalyzed reaction proceeded very slowly, this precluded the determination of the $K_i$ for the TSA via Michaelis-Menten kinetics. Instead, we chose to obtain this value using an indirect competitive ELISA. The theory behind this assay is described well by Friguet et al. but will be recounted here briefly for the sake of clarity. Scheme 3.3.9 illustrates the general approach. If a monoclonal antibody $\text{Ab}$ is incubated at a constant concentration ($i_o$) with various concentrations ($a_o$) of antigen $\text{Ag}$ for an appropriate period of time, each of the solutions should reach equilibrium. The amount of “unbound” antibody in the liquid phase (i.e. antibody which has not bound to the antigen in solution – (i)) can then be monitored by an indirect ELISA, through the use of an anti-Ab antibody-enzyme conjugate (Y-ENZ in Scheme 3.3.9). The amount of “free” antibody in solution (not in the form $\text{Ab}*\text{Ag}$) can only be quantified accurately if there exists a linear relationship between antibody concentration and the absorbance of the final enzymatic assay. To establish this correlation, various known
Detect by indirect ELISA

Add the preincubated solution of Ab (Y) and Ag to wells

Scheme 3.3.9. Indirect detection of “free” antibody in solution by ELISA.
amounts of antibody are added to coated wells under experimental conditions identical to those used in the equilibrium binding experiment. A linear dependence between the antibody concentration and absorbance of the final assay allows the determination of the free antibody concentration at equilibrium, provided that the total antibody concentration is known.

Thus, if the antibody at total concentration $i_0$ is incubated with the antigen at a given concentration $a_0$, the "free" antibody concentration at equilibrium (not bound to antigen in the equilibrated solution), $i$, will be related to the absorbance $A$ measured in the ELISA by the relationship

$$ \frac{ii_0}{i_0} = \frac{A}{A_o} \quad (\text{Eqn. 3.3.1}) $$

where $A_o$ is the absorbance measured for the antibody in the absence of antigen. This assumption can only be made if the ELISA gives a response proportional to the free antibody concentration throughout the concentration range investigated in the affinity measurements.

The "free" antibody concentration at equilibrium, $i$, can be determined using Eqn. 3.3.1 only if there is no readjustment of the equilibrium in the liquid phase during the incubation of the Ab/Ag mixture in the coated wells. If large amounts of the antibody bind to the coated wells, this will certainly disrupt the equilibrium. The amount of antibody bound to the coated antigen in the ELISA must represent only a small fraction (no more than 10%) of the free antibody initially present in the liquid phase. Failure to fulfil this requirement has led to values of the dissociation constant overestimated by orders of magnitude.$^8$

Thus, two requirements exist for the determination of equilibrium dissociation constants using indirect competitive ELISA. The relationship between antibody concentration and absorbance of the final assay step (enzyme assay) must be linear, and less
than 10% of the total amount of antibody in solution should remain trapped by the antigen coating the plate during the incubation of the antibody in the coated wells.

The experimental variables involved in determining the $K_D$ of TSA 3.41 and ST51 included: the amount of antigen used to coat the wells of the microplate, the length of time and temperature for coating, the amount of antibody to be used for incubating with the bound antigen, the temperature and time for incubating the antibody with the antigen, as well as the amount of anti-antibody enzyme conjugate used and the temperature and length of time for incubating this conjugate in the wells of the microplate.

We first had to test whether the relationship between ST51 concentration and absorbance of the final assay step (enzyme assay) was linear for the experimental conditions we were considering using for determining the $K_D$ of TSA 3.41 and ST51. Briefly, a 96-well microplate (Nunc Maxisorp) was coated with 80 $\mu$L of 1.25 $\mu$g/mL BSA-3.41 conjugate, in 50 mM potassium phosphate buffer (pH 7.45) for 15 h, covered, at 4°C, washed with 150 mM NaCl, and blocked with BSA in tris-buffered saline (TBS). After blocking, solutions of ST51 (0 to 0.5 nM) were prepared in 100 mM bicine, 100 mM NaCl, 0.01% NaN$_3$, pH 9.0, supplemented with 10 mg/mL BSA. (Addition of BSA is absolutely necessary, so as to prevent nonspecific binding of the antibody to the sample containers used for diluting the solutions.) These solutions were incubated at room temperature for 1 h (to represent the time that would be given for preincubation of ST51 with TSA 3.41), then 150 $\mu$L of each solution was transferred into the wells of the microtitre plate directly following the blocking step. After covering the microplate and incubating at room temperature for 1 h, the microplate was then washed with Millipore water, dried, and, to detect the bound ST51, an aliquot of goat-anti-mouse (Fc) alkaline phosphatase conjugate in blocking solution (100 $\mu$L of 0.25 $\mu$g/mL)
was added to each well. The microplate was covered and incubated at room temperature for 1.7 h, and the plate was then washed with 150 mM NaCl, 50 mM Tris, 0.05 % Tween-20, pH 7.4 (TBS/Tween), rinsed with 10 mM diethanolamine, 0.5 mM MgCl₂, pH 9.5 (assay buffer), and thumped dry. A solution of p-nitrophenyl phosphate (PNPP) in assay buffer (2 mg/mL) was prepared immediately before use. This solution was added to the wells of the microplate, and the development of yellow color was monitored over time using a microplate reader, detecting at 405 nm. The resulting absorbance values were corrected for background absorbance of blanks/controls and were plotted against the concentration of ST51 that had been incubated in the microplate (see Figure 3.3.11).

![Graph](image)

**Figure 3.3.11.** The relationship between [ST51] and absorbance of the final assay is linear for the indirect ELISA assay for determining the K_D of TSA 3.41 and ST51.

We then tested whether or not <10% of the total amount of ST51 in solution remained trapped by the antigen (BSA-3.41) coating the plate during the incubation of the antibody in the coated and blocked wells, using the experimental conditions previously described for carrying out the indirect ELISA. After incubating the antibody at various known
concentrations in the coated and blocked wells of one microplate, for the appropriate time, the contents of each well were transferred into other newly coated and blocked wells in a different microplate. These solutions were then incubated in these wells for the same amount of time, concurrently with wells containing solutions of the same concentration of ST51 that had not been previously incubated in the first microplate. In all wells, the antibody trapped in the ELISA was detected as previously described. The terms $A_1$ and $A_2$ represent the absorbance of the final enzymatic assay obtained for the well containing the antibody solution that had not been previously incubated in the first microplate, and the antibody solution of the same concentration that had been previously incubated in the first microplate, respectively. The fraction of antibody retained in the well in the first microplate is given by

$$f = \frac{(A_1 - A_2)}{A_1} \quad \text{(Eqn. 3.3.2)}$$

and as long as $f < 0.10$, then the amount of antibody trapped in the ELISA represents only a small fraction of the free antibody, and the ELISA therefore does not significantly displace the Ab-Ag equilibrium. For the experimental conditions described above for carrying out our indirect ELISA, this requirement was satisfied (see Table 3.3.3), therefore it was determined that these conditions could be used for determining the $K_D$ of TSA 3.41 and ST51 by indirect competitive ELISA.
Table 3.3.3. Percentage of ST51 trapped by the wells of a coated and blocked microplate.

<table>
<thead>
<tr>
<th>[ST51] (nM)</th>
<th>OD (405 nm) of final assay for ST51 solution NOT preincubated in microplate</th>
<th>OD (405 nm) of final assay for ST51 solution preincubated in microplate</th>
<th>% ST51 bound by wells of microplate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.835</td>
<td>0.798</td>
<td>4</td>
</tr>
<tr>
<td>0.4</td>
<td>0.652</td>
<td>0.662</td>
<td>0</td>
</tr>
<tr>
<td>0.3</td>
<td>0.528</td>
<td>0.526</td>
<td>0.4</td>
</tr>
<tr>
<td>0.2</td>
<td>0.355</td>
<td>0.366</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.206</td>
<td>0.201</td>
<td>2</td>
</tr>
</tbody>
</table>

Having determined that our experimental conditions satisfied both requirements for establishing the validity of Eqn. 3.3.1, we were then able to use the method developed by Friguet et al.\textsuperscript{65} to determine the $K_D$ for TSA 3.41 and ST51. The experimental procedure used to obtain this value is described well in Section 3.2.14. The conditions used were essentially the same as those described above, with one exception. The TSA 3.41 at various concentrations (0.3125 nM to 100 nM) was mixed with a constant amount of ST51 (0.5 nM), in 100 mM bicine, 100 mM NaCl, 0.01% NaN\textsubscript{3}, pH 9.0, supplemented with 10 mg/mL BSA. These solutions were preincubated at room temperature for 1 h, before transfer of a 150 µL aliquot to the coated and blocked wells of the microplate.

The concentrations of bound antibody $x$ and free antigen $a$ at equilibrium are calculated from the mass conservation equations shown in Scheme 3.3.9.

\[
x = i_o - i \quad \text{(Eqn. 3.3.3)}
\]
\[
a = a_o - x \quad \text{(Eqn. 3.3.4)}
\]

where $a_o$ is the total concentration of antigen, and $i_o$ and $i$ are the total and "free" (not in form Ab*Ag) antibody site concentrations. Friguet et al.\textsuperscript{65} then relate the values $x$, $a$, and $i_o$ to the dissociation constant $K_D$ of the equilibrium by the Scatchard equation (Eqn. 3.3.5).
\[
\frac{x}{a} = \frac{1}{K_D} (i_o - x) \quad \text{(Eqn. 3.3.5)}
\]

Since Eqn. 3.3.1 has been shown to be true, \(x\) and \(a\) are therefore related to the absorbances measured in the ELISA:

\[
x = i_o \left( \frac{A_o - A}{A_o} \right) \quad \text{(Eqn. 3.3.6)}
\]

\[
a = a_o - i_o \left( \frac{A_o - A}{A_o} \right) \quad \text{(Eqn. 3.3.7)}
\]

Then the Scatchard equation (Eqn. 3.3.5) can be rewritten as:

\[
\frac{A_o - A}{a_o - i_o \left( \frac{A_o - A}{A_o} \right)} = \frac{1}{K_D} \left( 1 - \frac{A_o - A}{A_o} \right) \quad \text{(Eqn. 3.3.8)}
\]

and if \(v = \frac{A_o - A}{A_o} \quad \text{(Eqn. 3.3.9)}\)

then \[\frac{v}{a} = \frac{1}{K_D} - \frac{1}{K_D} (v) \quad \text{(Eqn. 3.3.10)}\]

The Scatchard plot of the binding of ST51 to the TSA 3.41, as measured by the indirect competitive ELISA method, is shown in Figure 3.3.12. From the Scatchard analysis, it was determined that the \(K_D\) for ST51 and the TSA 3.41 is approximately 13 nM. This is well within the suggested range of dissociation constants that can be calculated by this method.\(^{65}\)
We were able at this point to investigate the relationship between the rate enhancement for the ST51-catalyzed hydrolysis of substrate 3.54, and the abzyme's affinity for the TSA(3.41) relative to its substrate (3.54). The relationship $k_{cat}/k_{uncat} = K_m/K_i$ has been shown to be valid for enzymes with TSA inhibitors\textsuperscript{88} and catalytic antibodies with their TSA immunogens.\textsuperscript{85,89,90} When our absolute values of $k_{cat}/k_{uncat}$, and $K_m/K_D$ are compared for the ST51-catalyzed hydrolysis of substrate 3.54 and the TSA 3.41 to which ST51 was raised, $k_{cat}/k_{uncat}$ is about a factor of three lower than one would expect, based on the preferential binding ST51 exhibits for its TSA relative to its substrate. This discrepancy could be arising due to the fact that the $K_m$ for ST51 and the substrate 3.54 and the $K_D$ value for ST51 and the TSA 3.41 were obtained using very different methods.

Attempts were made to determine the $K_m$ value for the substrate using the indirect competitive ELISA method. However, when the BSA-3.41 conjugate was used to coat the microplate wells, the affinity of ST51 for this antigen was so much higher than its affinity for the substrate 3.54 that, even after preincubation of ST51 with very high concentrations of 3.54, no competitive inhibition was observed. When a BSA-substrate(3.54) conjugate was
prepared and used to coat the microplate wells, the affinity of ST51 for this antigen was so low that much higher concentrations of antibody had to be used (approximately 80-fold increase in antibody binding sites) to see any color develop in the final assay, even at very high concentrations of the antibody-enzyme conjugate. Large quantities of antibody would therefore have to be consumed to work out another set of experimental conditions for determining the $K_D$ for ST51 and substrate 3.54 by this method.

Thomas$^{18}$ has recently compared reactivity and transition state binding data for enzyme reactions and for catalytic antibody reactions. It is interesting to note that the values obtained for $(K_m/K_i)$ for the best competitive inhibitors of selected enzymes,$^{18}$ where the inhibitors are thought to be reasonable TSAs, fall far short of the rate enhancements imparted by the enzymes. This serves to illustrate that there is considerable room for improvement in even our best attempts to design accurate TSAs. In the case of the catalytic antibodies compared by Thomas,$^{18}$ in several of the examples he cites values for $(K_m/K_i)$ for the substrates and TSAs to which the antibodies were raised, that are greater than the rate enhancements exhibited by the abzymes, as seems to be the case for ST51. It could therefore be quite possible that the discrepancy we observe between $k_{cat}/k_{uncat}$ and $K_m/K_D$ is not due to the two very different methods we used to obtain these values. It could be quite simply that, as has been noted for other catalytic antibodies, the difference in binding energy between ST51 and its substrate 3.54 and TSA 3.41 is not efficiently converted to catalysis.

3.3.8 Studies with Other Potential Substrates

It is not unusual for a hydrolase catalytic antibody to catalyze the hydrolysis of different classes of substrates. For example, the first reported carbamase abzyme, antibody 33B4F11 (Schultz and coworkers)$^{84}$ was capable of catalyzing the hydrolysis of carbamate...
3.55 (Scheme 3.3.10) following Michaelis-Menten kinetics, as well as the hydrolysis of the

$p$-nitrophenyl ester 3.56. This catalytic antibody was raised to a phosphonate TSA (3.57).

Scheme 3.3.10. Catalytic antibody 33B4F11 catalyzes the hydrolysis of both carbamate 3.55
and ester 3.56.

As well, antibody 43C9, reported by Benkovic et al. and raised to TSA 3.59 (Scheme
3.3.11),91,92 has been shown to accelerate the hydrolysis of a $p$-nitroanilide (3.58a), in
addition to catalyzing the hydrolysis of a series of aromatic esters (3.58b-f). Therefore, ST51
was screened for its ability to catalyze the hydrolysis of other carbonyl derivatives such as
esters, amides, and $N$-$H$ carbamates. Such studies might provide information as to the
mechanism of the abzyme.

Carbamate 3.62, which is the $N$-$H$ analogue of substrate 3.54, was first prepared and
examined as a substrate (Scheme 3.3.12). It was hoped that studies with 3.62 would yield
information concerning the necessity of the methyl group on the nitrogen of the carbamate
for catalysis. Carbamate 3.62 was prepared in an overall 29% yield by reacting amine 3.60 with p-nitrophenylchloroformate in the presence of N-methylmorpholine to give the protected carbamate 3.61, followed by deprotection of the t-butyl ester using trifluoroacetic acid (TFA) (Scheme 3.3.12).

**Scheme 3.3.11.** Abzyme 43C9\textsuperscript{91,92} catalyzes the hydrolysis of both p-nitroanilide 3.58a and esters 3.58b-f.

*Stability studies with carbamate 3.62 indicated that it was extremely reactive and exhibited a half-life of only 7 minutes at pH 6.0 (Table 3.3.4). Although we were unable to detect any significant increase in hydrolysis in the presence of ST51 at this pH, the lability of this compound made it very difficult to accurately measure the difference in the amount of PNP being produced in the presence and absence of antibody.*
Table 3.3.4. Kinetic Studies Performed with Carbamates 3.62-3.68.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reaction Conditions*</th>
<th>Half-life</th>
<th>Catalysis with ST51?b</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Image] 3.62</td>
<td>100 mM BIS-TRIS, 100 mM NaCl, 0.01 % NaN3, pH 6.0 (5% dioxane)</td>
<td>7 min&lt;sup&gt;c&lt;/sup&gt;</td>
<td>inc.&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>![Image] 3.64</td>
<td>100 mM Bicine, 100 mM NaCl, 0.01 % NaN3, pH 9.0</td>
<td>no breakdown observed over 16 h&lt;sup&gt;d&lt;/sup&gt;</td>
<td>No</td>
</tr>
<tr>
<td>![Image] 3.66</td>
<td>100 mM Bicine, 100 mM NaCl, 0.01 % NaN3, pH 7.0</td>
<td>30 min&lt;sup&gt;c&lt;/sup&gt;</td>
<td>d.n.d.&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>![Image] 3.68</td>
<td>100 mM Bicine, 100 mM NaCl, 0.01 % NaN3, pH 9.0</td>
<td>≈ 2 days&lt;sup&gt;h&lt;/sup&gt;</td>
<td>No</td>
</tr>
</tbody>
</table>

*All reactions contain 5% DMSO, unless otherwise indicated; All ST51 reactions were performed using 5 μM ST51 and 1 mM carbamate compound, followed by HPLC unless otherwise indicated; <sup>c</sup>monitored by UV; <sup>d</sup>monitored by HPLC; <sup>inc</sup> = inconclusive; <sup>d.n.d.</sup> = did not determine; <sup>ST51</sup> reaction monitored by UV-VIS; <sup>estimate kinetic data at pH 9.0.

Since carbamate 3.62 was too labile for accurate kinetic studies, the O-benzylic carbamate 3.64, a much less labile N-H carbamate, was prepared and examined as a ST51 substrate (Scheme 3.3.12). Compound 3.64 was prepared in an overall yield of 59% in a manner similar to that described for carbamate 3.62 except p-nitrobenzyl chloroformate was used instead of p-nitrophenyl chloroformate. Unlike compound 3.62, this compound is very stable towards hydrolysis and did not exhibit any detectable decomposition in bicine buffer at pH 9.0 over a 16 h period. This carbamate most likely hydrolyzes by a B<sub>AC</sub>2 mechanism. ST51 did not catalyze the hydrolysis of this compound.
It is not unusual for abzymes that catalyze the hydrolysis of p-nitrophenyl esters or N-H carbamates to also catalyze the hydrolysis of the unnitrated analogues and even methoxy analogues. Consequently, we prepared the unsubstituted and p-methoxy-substituted analogues of 3.62 (Scheme 3.3.12). Carbamates 3.66 and 3.68 were prepared in overall yields of 65% and 63% respectively by first reacting amine 3.60 with either phenylchloroformate or p-methoxyphenylchloroformate in the presence of N-methylmorpholine to give unsubstituted carbamate 3.65 and p-methoxycarbamate 3.67. Removal of the t-butyl protecting group using TFA/CH₂Cl₂ yielded the desired carbamates 3.66 and 3.68. Carbamate 3.66 exhibited a half-life of only 30 minutes at pH 9.0 while at pH 7.0 it had a half-life of approximately 2 days. Since the background reaction was quite high at pH 9.0, studies with ST51 were performed at pH 7.0. However, no catalysis was observed at pH 7.0. The methoxy-substituted carbamate 3.68 exhibited a half-life of 2 hours at pH 9.0. However, once again, no catalysis with ST51 was observed.

We also screened carbonate 3.71 and N-H carbamate 3.74 with ST51 (Table 3.3.5). Carbonate 3.71 was prepared in an overall 48% yield by reacting phenol 3.69 with p-nitrophenylchloroformate in the presence of N-methylmorpholine to give the protected carbonate 3.70, followed by deprotection of the t-butyl protecting group with TFA (Scheme 3.3.13). Carbamate 3.71 was quite hydrolytically labile, exhibiting a half-life of only 12 minutes in bicine buffer at pH 9.0. Surprisingly, some modest catalysis was observed with ST51 at this pH, however, accurate data could not be obtained due to the high background reaction. However, at pH 6.1 in 20 mM ACES buffer, carbonate 3.71 exhibited a half-life of 4.8 hours.
Scheme 3.3.13. Synthesis of compounds 3.71 and 3.74.

In the presence of 5 µM ST51 and 500 µM 3.71, in 20 mM ACES, pH 6.1, the initial rate of the reaction was increased by a very modest 2-fold under these conditions. It is highly likely that this carbonate has a higher Kₐ for ST51 than substrate 3.54 and that, at 500 µM, ST51 is
not saturated with the carbonate. Consequently, the rate enhancement may be greater than what we see here using 500 μM carbonate. We attempted to perform more detailed kinetic studies using higher concentrations of the carbonate substrate, however, it precipitated out of solution at concentrations greater than 500 μM. If the rate enhancement is indeed as high with the carbonate substrate at pH 6.0 as it is with substrate 3.54 at pH 9.0 (6500-fold), then the $K_m$ for the carbonate substrate would have to be very high indeed, since at 500 μM carbonate, it is clear that the rate enhancement is not even close to 6500-fold. However, it is possible that ST51 is more effective, both in terms of substrate binding and catalysis, at pH 9.0 than at pH 6.0.

**Table 3.3.5. Kinetic Studies Performed with Carbonate 3.71 and Carbamate 3.74.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reaction Conditions</th>
<th>Half-life</th>
<th>Catalysis Observed With ST51?</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="3.71" /></td>
<td>100 mM Bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0</td>
<td>12 min$^c$</td>
<td>Yes</td>
</tr>
<tr>
<td>3.71</td>
<td>20 mM ACES, pH 6.1</td>
<td>4.8 h$^c$</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>5% CH₃CN$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><img src="image" alt="3.74" /></td>
<td>100 mM Bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0</td>
<td>1 h$^c$</td>
<td>No reaction</td>
</tr>
</tbody>
</table>

$^a$All reactions contain 5% DMSO as co-solvent unless otherwise indicated; $^b$ST51 reactions were performed using 5 μM ST51 and 1 mM "substrate" unless otherwise indicated, followed by HPLC unless otherwise indicated; $^c$monitored by UV; $^d$This reaction contained 500 μM substrate, monitored by UV-VIS (microplate reader).

Carbamate 3.74 was prepared in an overall yield of 23% by first reacting the tetrabutylammonium salt of the carboxylate residue of $p$-hydroxyphenylacetic acid with allylchloride to form the allyl ester 3.72 (Scheme 3.3.13). Reaction of this phenol with $p$-
nitrophenylisocyanate in the presence of triethylamine gave carbamate 3.73. Removal of the allyl protecting group with dimedone in the presence of a catalytic amount of a Pd catalyst gave the desired carbamate 3.74. Carbamate 3.74 exhibited a half-life of approximately 1 hour in bicine buffer at pH 9.0, suggesting that it reacts by an $E_{1cB}$ mechanism. No catalysis was observed with ST51. In conjunction with the results from the studies with carbonate 3.71 and $N$-methyl carbamate substrate 3.54, these results suggest that an oxygen at position Z (see structure 3.75 below) is important for binding and/or catalysis with ST51. It could be that the lack of activity with 3.74 is due to the poorer leaving group ability of the nitroanilide anion compared to the $p$-nitrophenoxy anion, assuming ST51 is not capable of general acid catalysis.

![Structure 3.75](image)

Even with all of the above substrate studies, it is difficult to ascertain whether the methyl group on the nitrogen atom of the carbamate ($N$-CH$_3$ at position Y in compound 3.75 shown above, Z = oxygen atom) is essential for catalysis. The fact that catalysis is observed with the carbonate substrate suggests that this methyl group is not essential; however, to what degree remains unknown. The results with carbamate 3.62 were inconclusive. The lack of catalysis with carbamates 3.64, 3.66 and 3.68 may have nothing to do with the lack of this methyl group. In the case of carbamate 3.64, the presence of the extra methylene unit could affect binding. Alternatively, the poorer leaving group ability of $p$-nitrobenzyl alcohol (or its alkoxide) compared to the $p$-nitrophenoxy anion may be the reason. The results with carbamates 3.66 and 3.68 may be due to leaving group ability reasons or the need for a nitro
group for binding. This latter explanation is further illustrated by the observation that N-methyl carbamate 3.76,\(^7\) which we estimated has a half-life of approximately 1.9 years at pH 10.0, was not a substrate for ST51 in CHES buffer, pH 10.0.

![Chemical structure of 3.76](image)

3.76

We also screened esters 3.81 and 3.85 and amides 3.83, 3.87 and 3.89 as ST51 substrates (Table 3.3.6). Synthesis of ester 3.81 (Scheme 3.3.14) involved protection of p-bromophenyl acetic acid as a benzyl ester by reacting its tetrabutylammonium salt with benzyl bromide. A Heck reaction between aryl bromide 3.77 and \(\tau\)-butyl acrylate, using the procedure of Tilley et al.\(^6\) gave alkene 3.78 which was then subjected to hydrogenation to give acid 3.79. A DCC coupling between acid 3.79 and p-nitrophenol yielded ester 3.80, which upon deprotection of the \(\tau\)-butyl ester using TFA gave the desired ester 3.81 in an overall yield of 27%. Ester 3.81, which hydrolyzes by an \(E_{1cB}\) mechanism,\(^9\) exhibited a half-life of only 32 minutes at pH 9.0 and 80 minutes in BIS-TRIS buffer at pH 7.0. No rate enhancement was found in the presence of ST51.

Ester 3.85 was prepared in an overall yield of 47% by reacting allyl ester 3.72 with p-nitrophenylacetic acid chloride in the presence of triethylamine to give ester 3.84, followed by deprotection of the allyl ester using dinedone and a Pd catalyst (Scheme 3.3.15). Ester 3.85 is much more stable than ester 3.81, exhibiting only 10% hydrolysis after 30 minutes at pH 9.0. Again, no rate enhancement was found in the presence of ST51.
Table 3.3.6. Kinetic Studies Performed with Esters 3.81 and 3.85 and Amides 3.83, 3.87, and 3.89.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reaction Conditions</th>
<th>Half-life</th>
<th>Catalysis Observed with ST51?</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.81</td>
<td>100 mM Bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0</td>
<td>32 min³</td>
<td>d.n.d.⁶</td>
</tr>
<tr>
<td>3.85</td>
<td>100 mM Bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0</td>
<td>&lt; 10% hydrolysis after 30 minutes⁴</td>
<td>No</td>
</tr>
<tr>
<td>3.83</td>
<td>1 M NaOH (1% DMSO)</td>
<td>16 min³</td>
<td>d.n.d.⁶</td>
</tr>
<tr>
<td>3.87</td>
<td>100 mM Bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0</td>
<td>= 3 years⁵</td>
<td>No</td>
</tr>
<tr>
<td>3.89</td>
<td>100 mM Bicine, 100 mM NaCl, 0.01 % NaN₃, pH 9.0</td>
<td>no breakdown observed over 16 h⁴</td>
<td>No</td>
</tr>
</tbody>
</table>

*All reactions contain 5% DMSO, unless otherwise indicated; ⁴All ST51 reactions were performed using 5 μM ST51 and 1 mM "substrate", followed by HPLC; ⁵monitored by UV; ⁶monitored by HPLC; ⁷d.n.d. = did not determine; ⁸half-life estimated from results obtained in 1 M NaOH
Scheme 3.3.15. Synthesis of compound 3.85.

These results suggest that a heteroatom, or at least two oxygens, at positions Z and Y (see structure 3.75 below) is essential for binding and/or catalysis.

The p-nitroanilide derivative 3.83 was prepared in an overall 27% yield by coupling acid 3.79 to p-nitroaniline using diisopropylcarbodiimide (DIPC) followed by deprotection of the t-butyl ester using TFA (Scheme 3.3.14). The half-life of this amide, in bicine buffer at pH 9.0, was estimated to be approximately 3 years. No catalysis with STS1 was observed. Amide 3.87 was prepared in an overall 39% yield by coupling p-nitrophenylacetic acid with amine 3.60\(^6\) in the presence of dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazol hydrate (HOBut) followed by deprotection of the t-butyl ester with TFA. Finally, amide 3.89
was prepared in an overall 43% yield by reacting amine 3.60 with $p$-nitrobenzoyl chloride in the presence of $N$-methylmorpholine to give the protected amide 3.88, followed by deprotection of the $t$-butyl ester using TFA (Scheme 3.3.16). These two highly stable amides were not acted upon by ST51. The results with these amides and ST51 may be due to poor binding or poor leaving group ability.

Scheme 3.3.16. Synthesis of compounds 3.87 and 3.89.
One final word should be said concerning these substrate studies. It is likely that some of the substrates screened against ST51 would be readily hydrolyzed by proteases or esterases. The fact that our abzyme solution did not catalyze their hydrolysis lends further credence to the supposition that the activity we have seen with ST51 preparation is indeed a result of antibody catalysis and not a contaminating enzyme. It should be noted that failure to observe catalytic activity with ST51 with the more stable potential substrates does not necessarily mean that they are not accepted by our catalytic antibody. It is possible that if these reactions were followed for an even longer period of time, some catalysis would be observed. However, this would involve reaction times of weeks, perhaps months, and this is simply not practical. Nevertheless, it can be concluded that ST51 is a very specific catalytic antibody, and appears to be most effective when catalyzing the hydrolysis of the substrate (3.54) that most closely resembles the TSA (3.41) to which this antibody was raised. It is interesting to speculate as to some of the possible ways that the antibody is able to catalyze hydrolysis of N-methyl carbamate 3.54. Perhaps the antibody has a pocket that accommodates the methyl group on the nitrogen of the carbamate.

3.3.9 Activation of a Model “Prodrug” Substrate by ST51

From our results with ST51, it appears that the possibility of obtaining an abzyme that could catalyze the hydrolysis of an N-methyl carbamate prodrug with sufficient rate enhancements for effective prodrug activation is unlikely. Prodrugs based on N-methyl carbamate moieties are too hydrolytically stable for abzyme catalysis. Nevertheless, it was still important to address the issue concerning whether or not it would be possible to obtain an abzyme that could activate a tripartate prodrug by the cascade reaction outlined in Scheme 3.1.17 (Section 3.1.3). Triggering of such a cascade reaction had never before been
demonstrated with an abzyme. By demonstrating that this could be achieved with ST51, this would open the door for the development of other abzymes that activate tripartate prodrugs by cascade reactions. If the activation of such a system through N-methyl carbamate hydrolysis is impractical, then perhaps hydrolysis of other moieties that are more amenable to antibody catalysis could be utilized, such as ester or N-H carbamate hydrolysis.

The first step was designing and executing a synthesis of the tripartate substrate. Ideally, we wished to design a synthetic route such that, if necessary, would allow us to readily construct a variety of model "prodrugs" with relative ease from a common intermediate. Such a synthesis was achieved and is outlined in Scheme 3.3.17 - 3.3.18, where an intermediate of type 3.94 (Scheme 3.3.17) can be used as a common intermediate in prodrug synthesis. The tetrabutyl ammonium salt of p-(methylamino)benzoic acid was treated with allyl chloride to produce allyl ester 3.90 in 76% yield. Reaction of the amino group of 3.90 with p-nitrophenylchloroformate in the presence of N-methylmorpholine gave the N-methyl carbamate 3.91 in 64% yield. Deprotection of the allyl ester using dimedone and a Pd catalyst in THF/CH$_2$Cl$_2$ gave carboxylic acid 3.92 in a 99% yield. The selection of THF/CH$_2$Cl$_2$ as solvent for this reaction was important as it resulted in the precipitation of the product from the reaction mixture during the course of the reaction. No chromatographic purification of the highly polar product was necessary as pure product could be obtained by simple filtration of the reaction mixture. Selective reduction of the acid moiety in 3.92 to alcohol 3.93 was achieved in an 70% yield using triethylbenzylammonium borohydride and trimethylsilylchloride. Compound 3.93 was reacted with p-nitrophenylchloroformate to form carbonate 3.94 in 72% yield, which is suitably activated for attaching amine-bearing compounds by the reactive p-nitrophenylcarbonate portion of the molecule. Compound 3.94
is a solid compound, it can be chromatographed on silica, and is stable over several months without any detectable decomposition.

Scheme 3.3.17. Synthesis of activated carbonate intermediate 3.94 for prodrug synthesis.

The next step was to attach a suitable amine-bearing moiety or drug to 3.94 to form the model prodrug. Since we simply wanted to determine whether or not the cascade reaction could be triggered by ST51, it was not necessary to use an actual drug to make the
model "prodrug". Indeed, any amine-bearing molecule would suffice so long as it met certain criteria. First, it had to bear no resemblance to the N-methyl carbamate portion of the prodrug, otherwise inhibition of the carbamase antibody might occur. Second, it must be relatively easy to detect and quantitate by HPLC. We wished to use HPLC for these studies since this would allow us to monitor simultaneously the formation of the "drug" and p-nitrophenol (PNP) production. Third, the presence of a charged moiety would be necessary to enhance the overall solubility of the prodrug in aqueous solutions. Finally, it had to be relatively cheap, readily available, and should allow for facile construction of the model system from intermediate 3.94. Tryptophan was chosen to represent the "drug" portion of the prodrug, as it satisfied all of these requirements. Thus, a model "prodrug" was prepared by reacting t-butyl-protected tryptophan with compound 3.94 in the presence of dimethylaminopyridine (DMAP) to give "prodrug" precursor 3.95 in 89% yield. Removal of the t-butyl protecting group using TFA/CH₂Cl₂ in the presence of cation scavengers yields the desired model system 3.96 in 59% yield (Scheme 3.3.18).
Scheme 3.3.18. Synthesis of model prodrug 3.96.

Before beginning the HPLC kinetic studies with ST51 and the model prodrug substrate 3.96, standard curves were prepared for simultaneous detection of PNP and L-tryptophan (Trp), the hydrolysis products of the reaction (Scheme 3.3.20). As before, PNA was used as an internal standard, and these molecules were separated and detected via analytical reverse-phase HPLC. The plots of concentration of PNP/PNA vs. peak area ratio of PNP/PNA, and concentration of Trp/PNA vs. peak area ratio of Trp/PNA were linear. The standard curves previously prepared for detection of PNP (Figures 3.3.2a and 3.3.2b) could not be used due to the fact that the HPLC conditions (solvent, flowrate, etc.) had been significantly changed. The following standard curves were used for the detection of PNP and Trp in the reaction mixtures (Figures 3.3.13a and 3.3.13b).
Figure 3.3.13a. Standard curve for the detection of Trp for prodrug substrate 3.96 hydrolysis reaction.

Figure 3.3.13b. Standard curve for the detection of PNP for prodrug substrate 3.96 hydrolysis reaction.

ST51 was first screened with Z-tryptophan (3.97) at pH 9.0 (Scheme 3.3.19) for catalytic activity. No catalysis was observed. Thus, any release of free tryptophan from the model prodrug 3.96 (above and beyond the background) could therefore be attributed to hydrolysis at the N-methyl carbamate functionality followed by a spontaneous cascade.
reaction, as described previously. This would confirm the remote prodrug activation abilities of ST51.

Scheme 3.3.19. ST51 does not catalyze the hydrolysis of Z-tryptophan (3.97).

ST51 was screened with 3.96 at pH 9.0 (bicine buffer) in the same manner as substrate 3.54. The antibody-catalyzed reaction obeys saturation kinetics at pH 9.0, and equimolar quantities of PNP and Trp were detected for the duration of the time the reactions were monitored. No breakdown of prodrug 3.96 was observed in the control reactions (no ST51 present) in the range of substrate concentrations studied. Therefore, the cascad reaction following N-methyl carbamate hydrolysis does occur, and remote activation of a model prodrug substrate by ST51 has been successfully demonstrated.

The $K_m$ determined for 3.96 by monitoring Trp, $139 \pm 5 \mu M$, was within experimental error of the $K_m$ determined by monitoring the production of PNP, $137 \pm 7 \mu M$. (Figures 3.3.14a and Figure 3.3.14b). Interestingly, compound 3.96 exhibits a $K_m$ that is not quite half that of monocarbamate 3.54, which exhibited a $K_m$ of $266 \mu M$ at pH 9.0. The closeness of the $K_m$ values for these two substrates illustrates that ST51 is not recognizing the drug portion of the prodrug to any significant extent, which validates our hapten design.
Figure 3.3.14a. Lineweaver-Burk plot for ST51 and substrate 3.96 at pH 9.0, using data obtained from monitoring production of Trp.

Figure 3.3.14b. Lineweaver-Burk plot for ST51 and substrate 3.96 at pH 9.0, using data obtained from monitoring production of PNP.

The $V_{\text{max}}$'s determined for 3.96 by monitoring both Trp and PNP were almost identical. According to the Lineweaver-Burk plots for Trp and PNP $V_{\text{max}}$ was $0.72 \pm 0.02$ μM/h, and $0.78 \pm 0.03$ μM/h, respectively. These results indicate that the production of PNP is slow compared to the resulting cascade reaction that produced the tryptophan. The value
for $k_{\text{cat}}$ is approximately 0.075 h$^{-1}$, or $2.1 \times 10^{-5}$ s$^{-1}$, thus, this abzyme turns over about once every thirteen hours at its maximal velocity under these reaction conditions. ST51-catalyzed hydrolysis of 3.96 is slightly slower than that of 3.54 (0.91 µM/h at pH 9.0). Assuming that the spontaneous rate of hydrolysis of the $N$-methyl carbamate moiety in 3.96 is similar to that in 3.54, then the rate enhancement ($k_{\text{cat}}/k_{\text{uncat}}$) with substrate 3.96 is about 5000-fold.

The value for $k_{\text{cat}}/K_m$ is approximately $5.4 \times 10^{-4}$ µM$^{-1}$h$^{-1}$, or 0.15 M$^{-1}$ s$^{-1}$. The efficiency of ST51 with substrate 3.96 is therefore slightly greater than with substrate 3.54, but still somewhat below the average range for abzyme-catalyzed reactions. The $K_m$ values for ST51 and 3.96 fall within the range exhibited by current antibody-catalyzed prodrug activation systems, and our $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values fall just slightly below the reported ranges to be discussed in further detail in Section 3.3.10.

Of particular note is the fact that ST51 can catalyze the hydrolysis of compound 3.96 with more than one turnover. This was of some concern to us since it was possible that the highly reactive intermediate 3.99 (Scheme 3.3.20) could react with a residue in the active site and potentially shut down the abzyme after a single turnover. However, the fact that more than one turnover is observed suggests that intermediate 3.99 is either reacting with a residue in the antibody-combining site that is not essential for catalysis or diffuses out of the active site and reacts with the solvent, buffer or other components in the reaction mixture.
Scheme 3.3.20. Hydrolysis of model prodrug substrate 3.96 by ST51.

X = NH, O, S
inactivation, 1 turnover
(not observed)

no inactivation, multiple turnover
(observed)
3.3.10 Conclusions, Perspectives, and Future Work

Our research with ST51 has been an important contribution to the field of catalytic antibodies for two major reasons. First, ST51 is the first abzyme capable of catalyzing the hydrolysis of an N-methyl carbamate, a highly challenging reaction for antibody catalysis. Indeed, this is one of the most difficult hydrolytic reactions on a carbonyl derivative (ester, amide carbamate) ever catalyzed by an antibody using the TSA approach. Second, this abzyme is also capable of remote activation of a model prodrug substrate. It is unfortunate that sufficient catalytic activity is not observed with ST51 at physiological pH. While this precludes the use of this abzyme for antibody-targeted prodrug activation, many avenues are open for future work stemming from this project.

In order for ST51 to be used for ADAPT, its catalytic activity would have to be improved. The Fab fragments of our ST51 abzyme have been sent to Professor Emil Pai, a crystallographer at the University of Toronto, who is in the process of obtaining the x-ray crystallographic structure of ST51 Fab with the TSA to which it was raised (compound 3.41). It is hoped that this crystal structure will highlight the features that afford ST51 its N-methyl carbamase activity.

It is possible that the catalytic activity of ST51 could be improved through site-directed and random mutagenesis. Site-directed mutagenesis has been used by various groups to improve the catalytic power of their abzymes, and to glean information as to the means by which their antibodies exhibit catalytic activity. For example, Schultz and coworkers\(^\text{94}\) have generated seven site-specific mutations in the genes that encode the variable region of the heavy chain domain (\(V_H\)) of their phosphocholine-binding antibody S107. Abzyme S107, like abzyme MOT167 discussed in Section 3.1.1, Scheme 3.1.4, is
capable of catalyzing the hydrolysis of \( p \)-nitrophenyl \( N \)-trimethylammonioethyl carbonate.\(^{95,96}\) Two key residues, Tyr33 and Arg52 of the heavy chain, were thought to be essential in stabilizing the TS of the reaction through electrostatic and hydrogen bond interactions.\(^{97,98}\) To investigate the roles of these residues in catalysis, three Arg52 mutants (R52K, R52Q, R52C) and four Tyr33 mutants (Y33H, Y33F, Y33E, Y33D) of abzyme S107 were generated. The histidine mutant had an 8-fold higher \( k_{\text{cat}} \) for the carbonate substrate relative to the wild-type antibody.

In a more recent example, Schultz\(^{99}\) has used site-directed mutagenesis again with catalytic antibody 39-A11, raised to the TSA 3.103, which catalyzes the Diels-Alder reaction of substrates 3.100 and 3.101, shown in Scheme 3.3.21, to give the cycloadduct 3.102.

\[
\begin{align*}
\text{HOOC} & \stackrel{\text{CO} \text{NH}}{\text{C}} \text{C} & \text{HOOC} & \stackrel{\text{CO} \text{NH}}{\text{C}} \\
\text{\text{3.100}} & & \text{\text{3.101}} \\
\text{39-A11} & & \\
\text{3.102} \\
\end{align*}
\]

Based on crystal structure data, several site-directed mutants of abzyme 39-A11 were generated, in an attempt to improve van der Waals packing interactions with the TSA 3.103. The most active mutant, Val\textsuperscript{91}Tyr, resulted in an order of magnitude increase in $k_{\text{cat}}$.

It is also possible that the activity of ST51 could be improved by random mutagenesis. Wells and coworkers\textsuperscript{100} have attempted to use random mutagenesis to improve the activity, of their abzyme, 17E8, that catalyzed the hydrolysis of various amino acid phenyl esters. Their approach was to use mutagenesis and phage display to optimize the affinity of 17E8 for the TSA to which it was raised (Compound 3.105, Scheme 3.3.22), reasoning that increasing the antibody's affinity for the TSA might improve its catalytic efficiency with its substrate 3.104.

\[
\text{3.104} \xrightarrow{17E8} \text{3.105} + \text{phenylamine}
\]

Scheme 3.3.22. Reaction catalyzed by abzyme 17E8.

Wells and his group randomly mutated antibody residues that were either in direct contact with or in close proximity to the hapten, according to their crystal structure of 17E8 complexed with the hapten 3.105 to which it was raised. One of the mutants that had a lower
affinity for the hapten than 17E8 actually exhibited a 2-fold increase in $k_{cat}$. This clone, clone 3.1, contained four substitutions relative to the parent sequence – three of which formed the base of the antibody combining site, and one of which made no direct contact with the hapten. Further substitutions on an amino acid distant from the active site in this mutant led to a 5-fold increase in catalytic activity.

Rather conflicting views have been expressed with respect to the requirements for the kinetic parameters $K_m$ and $k_{cat}$ in ADEPT and ADAPT.\textsuperscript{44} It has been suggested that high $k_{cat}$ values and low $K_m$ values are required for an efficient ADEPT system.\textsuperscript{101} However, it has conversely been suggested that a low turnover number for catalysis in ADEPT can proffer an advantage, in that there is a prolongation of the period of time for drug delivery.\textsuperscript{49} Pharmacokinetic analysis of antibody-enzyme systems in ADEPT for cancer treatment indicates that a high $K_m$ and a low $k_{cat}$ could increase the selectivity for tumour cells.\textsuperscript{102} It is thought that for ADEPT systems a $k_{cat}$ of 1.0 s\textsuperscript{-1} provides optimum selectivity and reduces the toxicity in non-tumour cells.\textsuperscript{49} ADAPT, unlike ADEPT, often exhibits low catalytic power of the abzyme catalysts and high $K_m$ values.\textsuperscript{49} Therefore, catalytic antibodies, with their tendencies for low $k_{cat}$ values and high $K_m$ values, have excellent potential for therapeutic application.

Based on our kinetic data for substrate 3.54 (Section 3.3.3), this N-methyl carbamate substrate for ST51 has a half-life of approximately 570 years at pH 7.4. A rate enhancement of at least $10^3$ is required to see any PNP produced at all over a period of many hours, and a far greater rate enhancement would be required for a site-directed or randomly mutated form of ST51 to function as a proper catalyst for ADAPT. The question we must then ask ourselves is: \textit{Can we enhance the catalytic activity of ST51 to this degree through the use of}
site-directed or random mutagenesis? In the examples cited herein, and in other attempts to optimize the activity of specific antibody catalysts using these methods, improvements beyond one or two orders of magnitude have not been observed. It is therefore extremely unlikely that mutagenesis studies on STS1 would enhance the catalytic activity of this abzyme to an extent (to a $k_{cat}$ of 1 s$^{-1}$) where it could be used for ADAPT.

Three examples of antibody-catalyzed prodrug activation have been previously discussed in Section 3.1.2, however, the kinetic parameters obtained for these catalysts will be reviewed here for comparison. Fujii and coworkers isolated abzyme 6D9, which was capable of catalyzing the hydrolysis of a chloramphenicol ester prodrug (Scheme 3.1.12) with a rate enhancement of 1800. The value obtained for $k_{cat}$ was 0.002 s$^{-1}$, and $K_m$ and $k_{cat}/K_m$ values were 64 μM and 312.5 M$^{-1}$s$^{-1}$, respectively. The catalytic antibody (49.AG.659.12) obtained by Schultz et al., which catalyzed the hydrolysis of an ester prodrug of 5-fluorodeoxyuridine (Scheme 3.1.13), exhibited a rate enhancement of roughly 1000. Values obtained for $k_{cat}$ and $K_m$ were 0.0005 s$^{-1}$ and 218 μM, respectively, and $k_{cat}/K_m$ was 2.3 M$^{-1}$s$^{-1}$. Blackburn and coworkers characterized $N$-H carbamase abzyme EA11-D7, which activated a nitrogen mustard prodrug (Scheme 3.1.14), and the value for $k_{cat}$ for this abzyme was 0.03 s$^{-1}$, while $K_m$ and $k_{cat}/K_m$ values were 201 μM and 156 M$^{-1}$s$^{-1}$, respectively. Thus, of these three reports of antibody-catalyzed prodrug activation, the most promising would seem to be the system devised by Blackburn and coworkers, since their abzyme exhibits the highest turnover ($k_{cat}$). However, each of these three systems requires that the antibody recognize the drug portion of the prodrug, so our tripartate prodrug activation system would offer a significant advantage over these earlier reports of antibody-catalyzed prodrug activation, for the reasons discussed in Section 3.1.2.
Quite simply, \textit{N}-methyl carbamate substrates are too stable to be used for ADAPT. As mentioned previously, Blackburn and coworkers have shown that it is possible to obtain abzymes that are capable of catalyzing the hydrolysis of \textit{N}-\textit{H} carbamates with \( k_{\text{cat}} \)'s approaching that of \( 1.0 \text{ s}^{-1} \). Perhaps it would prove more fruitful to pursue abzyme-catalyzed hydrolysis of \textit{N}-\textit{H} carbamates for incorporation into our tripartate remote prodrug activation system. An example of such a tripartate antibody-catalyzed prodrug activation system is shown in Scheme 3.3.23. In this system, 3.106, the specifying portion of the prodrug contains \textit{R}-methylbenzylamine, however the linker has changed slightly, with an oxygen where the \textit{N}-methyl group was located in the previous model prodrug 3.96. Prodrug substrate 3.106 would exhibit a half-life on the order of days, as opposed to years for \textit{N}-methyl carbamate substrates 3.54 and 3.96. Detailed kinetic studies would have to be carried out with this potential prodrug substrate (3.106) before proceeding to the point of TSA synthesis and antibody production. These studies would verify that it has sufficient stability for ADAPT application, and that the desired cascade reaction, proceeding through intermediate 3.107, will occur with the modified linker moiety. Recent studies reported by Springer \textit{et al}.\textsuperscript{103,104} with similarly constructed prodrugs liberate the free drug via the 1,6-elimination mechanism, and a number of different prodrugs could be constructed from a common intermediate molecule, compound 3.109.
Scheme 3.3.23. Relatively labile prodrug substrate 3.106 and TSA 3.108 for our ADAPT system.

During the course of our research with N-methyl carbamase abzymes and the development of our tripartate prodrug activation system, a report was published by Shabat et
Shabat and coworkers\textsuperscript{105} based their prodrug strategy on a sequential retro-aldol-retro-Michael reaction that was catalyzed by abzyme 38C2.\textsuperscript{108} Their antibody-catalyzed prodrug activation system is illustrated in Scheme 3.3.24. Any number of drugs possessing free amines, hydroxyls, or thiols, where their toxicity is significantly decreased when these functionalities are masked, could be candidates for antibody-catalyzed prodrug activation by 38C2. Shabat \textit{et al.}\textsuperscript{105} have demonstrated the ability of aldolase antibody 38C2 to catalyze the activation of a prodrug such as \textbf{3.110}, which undergoes first an abzyme-catalyzed retro-aldol reaction to produce \textbf{3.111}, followed by a retro-Michael transformation to yield the free drug \textbf{3.112}. Both prodoxorubicin and procamptothecin have been activated by this abzyme, releasing the free drugs to kill colon and prostate cancer cell lines. The abzyme-catalyzed reaction of antibody 38C2 with prodoxorubicin followed Michaelis-Menten kinetics with $k_{\text{cat}} = 0.000029 \text{ s}^{-1}$, $K_m = 43 \text{ M}$, and a rate enhancement of $>10^5$. The \textit{in vitro} testing of their antibody-catalyzed prodrug activation system indicated that cell growth was significantly slower when abzyme 38C2 was added with the prodrug. This abzyme also demonstrated a long \textit{in vivo} half-life when administered to mice.
Scheme 3.3.24. Prodrug activation by antibody 38C2 via a tandem retro-aldol-retro-Michael reaction.

The $k_{cat}$ value exhibited by abzyme 38C2 with its substrate prodoxorubicin (3.110) is the lowest $k_{cat}$ reported to date for an antibody-catalyzed prodrug activation system at or near physiological pH, and is only slightly larger than the $k_{cat}$ for ST51 and its model prodrug substrate 3.96. Catalytic antibody 38C2 turns over only once every 10 hours at physiological pH. Shabat et al. mention improving the masking linker and attempting to enhance the catalytic activity of 38C2 in order to increase the value of $k_{cat}$ for this abzyme and its prodrugs – which will be necessary if this antibody is to enter into clinical trials.

It will also be interesting to see whether antibody 38C2 is able to exert toxic effects in vivo. For Shabat's assay of the in vivo activity of abzyme 38C2, mice were injected with a certain amount of the antibody in PBS, and blood samples were obtained every 24 hours and tested for catalytic activity with 100 μM of a fluorescent substrate. Catalytic activity was observed by Shabat and coworkers after several days of incubation of the antibody in vivo. While this would seem to indicate that the antibody does not experience any significant inhibition by endogenous ketones in vivo, all that can be said definitively is that the abzyme was not irreversibly inhibited by diketones or other potential inhibitors. The use of a
relatively large concentration of a fluorogenic substrate in the assay for catalytic activity would make it difficult to observe any competition by ketone bodies present in the blood.\textsuperscript{5} This abzyme is known to have an extremely wide substrate specificity,\textsuperscript{107,108} and among its substrates are acetone and various other ketones which may be present in living systems. This feature which allows for the application of abzyme 38C2 to generic prodrug activation may therefore be problematic in a therapeutic setting.

In the tripartate, “remote” prodrug activation system we have devised, our antibodies will specifically recognize a carbamate moiety and there should hopefully be little or no interaction between our abzymes and endogenous molecules. It should be possible to build an array of prodrug molecules from a single stable precursor, such as precursor 3.109, all of which can be activated by a single abzyme. It is hoped that with a new TSA and model prodrug design, we will be able to obtain an abzyme that can be applied to the activation of tripartate prodrugs in ADAPT, under physiological conditions.
REFERENCES


(2) Pauling, L. Am. Sci. 1948, 36, 51.


(43) Lerner, R. A. Hospital Practice 1993, 53.


(59) Compound 3.47 was kindly provided by Dr. Mei-Jin Chen, a former post-doctoral fellow in the Taylor Group.

(66) Compound 3.60 was kindly provided by Professor Scott Taylor.

(67) Compound 3.69 was kindly provided by Professor Scott Taylor.


(70) Compounds 3.42-3.46 were kindly provided by the Batey Group, University of Toronto.


(77) The truncated TSA 3.53 was kindly provided by Dr. Mei-Jin Chen, a former post doctoral fellow in the Taylor Group.

(78) Carbamate substrate 3.54 was kindly provided by Dr. Mei-Jin Chen, a former post doctoral fellow of the Taylor Group.


263