Targeting Clinically Viable Protein Tyrosine Phosphatases with Rationally Designed, Small Molecule Inhibitors and Artificially Induced Protein-Membrane Anchors

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Chemistry
University of Toronto

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Abstract
Protein tyrosine phosphatases (PTPs) regulate the phosphorylation state of many important signaling molecules and are increasingly viewed as integral components of signal transduction cascades. PTPs have been implicated in the development of type 2 diabetes, breast cancer and neurodegenerative disorders, and thus have been extensively explored as a potential therapeutic target. This thesis explores the functional components required for engineering novel modalities to successfully target protein tyrosine phosphatase 1B (PTP1B), the most well studied member of the large family of PTPs. In the first part of this thesis, a screen of a library of compounds identified potent salicylic acid inhibitors with promising single digit micromolar activity. In silico computational analysis drove the design and synthesis of a second generation of disalicylic acid inhibitors, and new, more selective and potent inhibitors have been identified. The second part of this thesis explores the first ever application of artificially induced protein-membrane anchors designed to target PTPs.
Acknowledgments

First and foremost, I would like to thank Professor Patrick T. Gunning. I remember walking into your office for the first time asking you for an opportunity to work in your laboratory. I elaborated and said that “I wanted to get my hands wet” in research and I wanted to see what it was all about. And so I thank you deeply for allowing me to completely submerge myself in this field. I feel honored and blessed to have been given such an opportunity. I will move forward in my life so proud to have worked with such a renowned professor and researcher such as yourself. Thank you for providing me with a platform to learn, to collaborate and exchange knowledge with others, and more importantly to grow as a person in every way.

I wholeheartedly dedicate this thesis to my family. Mama and Tato, you have sacrificed so much in your life to provide and care for me. I thank you for being outstanding role models, as everything I know today about having a strong work ethic, I have learned from you. You are truly so inspirational. Although I know you had not always understood why I would come home exhausted, be working on the weekends and on the holidays, you have never questioned my passion for science. To my little Zoriana, thank you for always being so understanding, for always being there to make me laugh, and for being the bright, shining star that you. Even though you are growing up to be a fine young lady, you will always be my little baby sister.

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Contributions of Authors

The work described within this dissertation has been reported in two peer-reviewed publications. The introduction is an original composition. Chapters 2, and 3 are based heavily on my published or in preparation manuscripts. The final chapter consists of my own concluding thoughts and reflections.

Chapter 2:

For the first portion of the chapter (section 2.1.1), library synthesis, characterization and computational analysis was reported previously in published work by Dr. Haftchenary, Dr. Fletcher, Dr. Shahani and Dr. Page. Dr. Haftchenary and Daniel Ball are necessarily accredited for developing the rationale of this project. Members of the Tremblay lab, Dr. Isabelle Aubry and Melissa Landry conducted the biological work. For the second portion of the chapter (2.1.2), discussions between myself, Dr. Haftchenary, Daniel Ball and also the work I performed during my undergraduate thesis set the precedence for this library. I was responsible for synthesizing the majority of the building blocks and for the full characterization of the library. Dr. Haftchenary is necessarily accredited for the design, synthesis and computational analysis of the library. Dr. Andrew Lewis provided advice and performed the preliminary biological work. Members of the Tremblay lab, Dr. Isabelle Aubry and Melissa Landry conducted the enzymatic screenings. The Tremblay lab also generously provided the required proteins for in-house experiments.

Chapter 3:

Dr. Haftchenary is necessarily accredited for inventing this project. I was responsible for the design, synthesis and characterization of the entire library presented in this chapter. I invented the three novel scaffolds subsequent to the one discovered by Dr. Haftchenary. Discussions between myself and Dr. Haftchenary has greatly influenced the development of this project and his continuous advice, assistance in synthesis and suggestions is also necessarily accredited. Dr. Saleem from the Macdonald lab aided in performing vanadium NMR experiments. Discussions between myself, Dr. Gradinau, Yuchong Li (PhD Candidate from the Gradinaru lab), Dr. Haftchenary, and Dr. Andrew Lewis set the precedence for a variety of our in vitro experiments. Yuchong Li is necessarily accredited for performing all of the in vitro experiments. Isabelle Aubry provided us with GFP-tagged plasmid for our in vitro experiments and Anna Shahmuradyan (PhD Candidate) from the Krull lab aided in the spectroscopic studies.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AcOH</td>
<td>Acetic acid</td>
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<tr>
<td>AML</td>
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</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>Philadelphia chromosome</td>
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<td>BPO</td>
<td>Benzyol peroxide</td>
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<tr>
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<td>Bixperoxo (1,10-phenthrolene) oxovanadate (V)</td>
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<tr>
<td>CML</td>
<td>Chronic myelogenous leukemia</td>
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<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HBTU</td>
<td>Tetramethyl-N,N,N',N'-urofonyl phosphate</td>
</tr>
<tr>
<td>HRMS</td>
<td>High-resolution mass spectrometry</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput screening</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitor concentration at half maximal</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared spectroscopy (or)</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus Kinase2</td>
</tr>
<tr>
<td>JH2</td>
<td>JAK homology domain2</td>
</tr>
<tr>
<td>K_i</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>LAR</td>
<td>Leukocyte antigen related</td>
</tr>
<tr>
<td>LRMS</td>
<td>Low-resolution mass spectrometry</td>
</tr>
<tr>
<td>LUV</td>
<td>Unilamellar vesicle</td>
</tr>
<tr>
<td>LWTP</td>
<td>Low molecular weight protein tyrosine phosphatase</td>
</tr>
<tr>
<td>M</td>
<td>Molar, mol/L</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MKP</td>
<td>Mitogen activating protein kinase phosphatase</td>
</tr>
<tr>
<td>MLV</td>
<td>Mulilamellar vesicles</td>
</tr>
<tr>
<td>mPTP</td>
<td>Mycobacterium tuberculosis PTP</td>
</tr>
<tr>
<td>Na3VO4</td>
<td>Sodium orthovanadate</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NRPTP</td>
<td>Non-receptor protein tyrosine phosphatase</td>
</tr>
<tr>
<td>Ob-R1</td>
<td>Long form leptin receptor</td>
</tr>
<tr>
<td>OMP</td>
<td>Orotidine monophosphate</td>
</tr>
<tr>
<td>PCC</td>
<td>Pyridinium chlorochromate</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>pIR</td>
<td>Phosphorylated insulin receptor</td>
</tr>
<tr>
<td>P-loop</td>
<td>Phosphate-binding loop</td>
</tr>
</tbody>
</table>
| PMA          | Protein Membrane Anchorag
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>PP</td>
<td>Protein phosphatase</td>
</tr>
<tr>
<td>PRL (A/S)</td>
<td>Phosphatases of regenerating liver (active mutant)</td>
</tr>
<tr>
<td>pSer</td>
<td>Phosphoserine</td>
</tr>
<tr>
<td>pSTAT</td>
<td>Phosphorylated STAT</td>
</tr>
<tr>
<td>PTB</td>
<td>Protein tyrosine binding (domain)</td>
</tr>
<tr>
<td>PTEN</td>
<td>Tensin homologue deleted on chromosome 10</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PTPβ</td>
<td>Protein tyrosine phosphatase alpha</td>
</tr>
<tr>
<td>PTP-PEST</td>
<td>Protein tyrosine phosphatase-proline-glutamate-serine-threonine-rich sequence</td>
</tr>
<tr>
<td>pThr</td>
<td>Phosphothreonine</td>
</tr>
<tr>
<td>pTyr</td>
<td>Phosphotyrosine</td>
</tr>
<tr>
<td>Q-loop</td>
<td>Aspartate-binding loop</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPTP</td>
<td>Receptor protein tyrosine phosphatase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2</td>
</tr>
<tr>
<td>SHP-2</td>
<td>SH2 domain containing protein tyrosine phosphatase</td>
</tr>
<tr>
<td>TBAF</td>
<td>Tetramethylammonium fluoride</td>
</tr>
<tr>
<td>TBTU</td>
<td>O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate</td>
</tr>
<tr>
<td>TC-PTP</td>
<td>T-cell protein tyrosine phosphatase</td>
</tr>
<tr>
<td>TEMPO</td>
<td>Tetramethylpiperidin-1-yl)oxidanyl</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TMR</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>VHR</td>
<td>Vaccinia H-1 related</td>
</tr>
<tr>
<td>VOCl₃</td>
<td>Vanadium oxytrichloride</td>
</tr>
<tr>
<td>VO-OHpic</td>
<td>3-hydroxypicolinate vanadium (V)</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Targeting Enzymes for Therapeutic Intervention

1.1.1 Enzymes as Drug Targets

The synthesis of biomolecules, proteins, nucleic acids, polysaccharides, lipids, as well as all aspects of intermediate metabolism and intercellular communication involve a sequential series of chemical reactions that maintain a cell’s critical function and homeostasis. Ironically, a vast majority of these essential biochemical reactions proceed at uncatalyzed rates that are often too slow to sustain life. For example, the essential building blocks of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are synthesized from the formation of uridine monophosphate (used as a monomer in RNA) via the decarboxylation of orotidine monophosphate (OMP). However, measurements of the rate of OMP decarboxylation have estimated the half-life of this chemical reaction to be 78 million years\(^1\). Fortunately, the enzyme OMP decarboxylase fulfills this cell-critical function and enhances the rate of OMP decarboxylation by \(10^7\)-fold\(^2\). Evidently, enzyme catalysis is vital for the majority of biological processes and the sustainability of life. Unfortunately, genetic mutations can often lead to the over-expression of enzymes within the cell. This over-expression disrupts the homeostasis or disturbs the internal conditions and often leads to various malignancies. Alternatively, point mutations can ultimately enhance or abolish catalytic efficiency, to which either of these mechanisms can lead to aberrant disease states\(^3\). Thus, enzymes are unmistakably attractive targets for drug discovery because of their essential roles in life processes and are therefore targeted for pharmacological intervention of numerous diseases.

![Figure 1.1](image)

**Figure 1.1.** Depiction of the high proportion of enzymes (47 %) in the total distribution of marketed drug. GPCRs: G-Protein Coupled Receptors. Adapted from Ref [4].
The attractiveness of enzymes as therapeutic targets is evident in the high proportion of currently marketed drugs targeting this particular class of proteins. A survey taken by Hopkins and Groom reported that nearly half of all marketed small molecule drugs inhibit enzymes as their molecular target, as shown in Figure 1.1. The clinical viability of enzymes as drug targets results not only from the essentiality of their catalytic activity but also from the fact that enzymes, by nature, are highly amendable to inhibition by low molecular weight, drug-like molecules. More specifically, enzymes contain “druggable” binding pockets, which have evolved to bind their respective native substrates. These physiological ligands are typically low in molecular weight and bind to the pocket in a reversible, non-covalent manner, with moderate binding affinity in the µM to mM range. The elements of molecular recognition between proteins and their physiological ligands are largely mediated through the cumulative effects of multiple, weak, reversible chemical forces of hydrogen bonds, salt bridges, van der Waals forces, and hydrophobic interactions. A definitive example of these elements lies in the first ever selective and potent tyrosine kinase inhibitor, imatinib (Gleevec), which is a BCR-ABL inhibitor used in the treatment of multiple cancers, most notably chronic myelogenous leukemia (CML). Imatinib utilizes some of the fundamental chemical forces to mimic the interaction of the BCR-ABL protein and its native substrate, adenosine triphosphate (ATP) (Figure 1.2). The same types of interactions also mediate interactions between protein binding sites and drug molecules for many other therapeutically relevant enzymes. This example indicates a clear advantage of enzyme active sites as targets for drug binding, as it is evident that the bound molecule or drug only needs to disrupt a small number of critical interactions within the active site to be an effective inhibitor.

Lastly, it is important to note that the active site(s) located in many clefts and cervices are not the only binding pockets featured on the enzymes surface, as allosteric sites are also present. They are defined as sites on the protein other than the active site that also bind natural substrates and communicate and affect the enzyme active site in a way which modulates the catalytic activity. Thus, the presence of allosteric pockets in addition to the active site(s) provides another route for interfering with enzyme activity and overall, substantially adds to the attractiveness of enzymes as molecular targets.
1.1.2 Targeting Enzymes: Kinases and Phosphatases

In any given moment, a cell is exposed and bombarded by several extracellular signals that simultaneously transduce a multitude of intracellular signals that lead to the amplification or termination of specific biological responses\(^9\). The tight balance between the activation and deactivation of these intracellular signaling pathways is strictly regulated by the primary communication tool for the cell, phosphorylation. This processes is governed by protein kinases (PKs) and a diverse range of protein phosphatases (PPs), enzymes that catalyze protein phosphorylation and dephosphorylation, respectively\(^{10}\). Approximately one-third of proteins encoded by the human genome are presumed to be phosphorylated during their life cycle\(^{11}\). Structural changes imparted by protein phosphorylation can regulate protein function. Phosphorylation acts as a molecular switch for many regulatory events in signaling pathways that drive cell division, proliferation, differentiation, and apoptosis\(^{12}\). Consequently, the well-studied PKs are currently the pharmaceutical industry’s second largest drug target, with approximately 30 distinct kinase-targeted drugs being investigated in Phase I clinical trials. Figure 1.3 showcases a selected array of FDA approved kinase inhibitors that are currently

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**Figure 1.2.** Left: ATP binding in the active site of BCR-ABL making critical intermolecular hydrogen bonding interactions. Right: Imatinib binding in the active site of BCR-ABL and mimicking the critical hydrogen bonds and hydrophobic or van der Waals intermolecular interactions\(^8\).
treating renal cancer, acute CML, and chronic phase or accelerated CML, respectively for compounds 1.1-1.3\textsuperscript{13}.

As briefly mentioned earlier, Imatinib, discovered in the late 1990s by scientists at Ciba-Geigy became the first ever rationally designed selective and potent small molecule inhibitor targeting an oncogenic kinase. Imatinib is a BRC-ABL tyrosine-kinase inhibitor used to treat multiple cancers, including chronic myelogenous leukemia, a cancer of white blood cells caused by the reciprocal translocation between chromosomes 9 and 22 which produce the oncogenic BRC-ABL gene fusion\textsuperscript{14}. The mutated BCR-ABL gene encodes unregulated, cytoplasm-targeted tyrosine kinases, which allow the cells to proliferate without being regulated by cytokines. Due to the crucial role of BCR-ABL’s dysregulated kinase activity in carcinogenesis, researchers focused their efforts on the development of BCR-ABL inhibitors for the treatment of CML. Imatinib’s potent \emph{in vivo} activity, clinical viability, and successful clinical trials in CML patients resulted in one of the fastest FDA approved drugs to date\textsuperscript{15}. Significant progress has been made over the last two decades to elucidate the significance of PKs in human disease. However, unlike PKs, PPs, until recently were believed to play an insignificant role in cell signaling. There existed a historical dogma that phosphatases were not an attractive drug target for therapeutics and were falsely under-appreciated as housekeeping enzymes with broad specificity as compared to their PK counterparts. A growing body of evidence firmly dispels the perception of PTPs as being complacent housekeeping enzymes\textsuperscript{16}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{kinase_inhibitors.png}
\caption{Figure 1.3. FDA approved kinase inhibitors currently treating a variety of diseases\textsuperscript{13}.}
\end{figure}
1.2 Protein Tyrosine Phosphatases

1.2.1 Notoriously Large Family of Protein Tyrosine Phosphatases

The superfamily of 107 PTPs can be classified into three sub-groups or types based on the sequential organization of their PTP domains (Figure 1.4). Type I cysteine-based PTPs represent the largest group with 100 members. This group can be further subdivided into the 38 classical phosphotyrosine (pTyr)-specific PTP’s and the 61 VH1-like or dual specificity protein phosphatases (DSPs) subfamilies. The pTyr-specific PTPs can be further subdivided into the transmembrane receptor like PTPs (RPTPs) and the intracellular non-receptor PTPs (NRPTPs). The DSP subfamily is the most diverse group of phosphatases because of their promiscuous substrate specificities. This group includes phosphothreonine(pThr)/pTyr specific mitogen-activated protein (MAP) kinase phosphatases (MPKs), the pThr/pTyr or mRNA-specific atypical DPS, the pThr-specific slingshots, and many more (Figure 1.4). The type II PTP family is represented by only a single gene that encodes a pTyr-specific low molecular weight PTP (LMPTP), and last but not least the type III PTP family comprises the pThe/pTyr-specific cell division cycle 25 (Cdc25) PTPs.

![Protein tyrosine phosphatase CX_5R superfamily](image)

**Figure 1.4.** Overview of the large protein tyrosine phosphatase CX_5R superfamily.

Despite the large family of proteins, the presence of variable protein domains reflects the location and role particular of the PTP in the cell. Trans-membrane or RPTPs such as leukocyte antigen related (LAR), PTPalpha (PTPα), PTPsigma (PTPσ), and cytoplasmic domain 45 (CD45) contain two cytoplasmic domains, a catalytically active D1 domain adjacent to the plasma membrane and a distal D2 domain (which is either inactive or has negligible catalytic activity). In addition, a signal trans-membrane spanning domain, extracellular domains such as
immunoglobulin-like and fibronectin type III domains that are similar to the extracellular domains of cellular adhesion molecules, are also present on this subfamily of phosphatases\textsuperscript{20}. On the other hand, the intracellular or NRPTPs such as T-cell PTP (TC-PTP), Src-homology 2 domain-containing PTPase 2 (SHP-2), SHP1, PTP-proline-, glutamate-, serine-, and threonine-rich sequence (PTP-PEST) and the well studied PTP1B, all contain one catalytic domain and highly variable localization domains. These domains largely participate in protein-protein interactions (PPIs) that serve as regulatory regions to directly and spatially control protein enzymatic activity\textsuperscript{17,21}. DSPs, such as MPKs also contain a single PTP domain additionally are decorated with an assortment of non-catalytic motifs that participate in substrate recognition\textsuperscript{22}. DSP’s catalytic pocket adopts a shallower profile (topology) that allows promiscuous and non-selective catalytic dephosphorylation of multiple substrates such as pTyr, pThr and pSer\textsuperscript{23}. Lastly, LMPTPs play significant roles in regulation of cell growth initiated through growth factor receptors and have been shown to dephosphorylate the platelet-derived growth factor receptor, insulin receptor, and the fibroblast-growth factor receptor\textsuperscript{24,25,26}.

Although PTPs are a structurally large and diverse family of tightly regulated and highly specific enzymes with various regulatory functions, all PTPs share a highly conserved catalytic motif. The following section will explore this key motif, as it mediates an important catalytic mechanism in PTP enzyme activity. The ~280 residue PTP catalytic domain consists of a sophisticated spatial arrangement of several α/β structured loop regions, shown in Figure 1.5\textsuperscript{27}. The phosphate-binding loop (P-loop) contains the conserved PTP signature motif CX$_5$R (where X represents any amino acid). By projecting the positively charged P-loop residues into the pocket, this provides a positively charged microenvironment that ensures this catalytic pocket has exceptionally high affinity for tetrahedral oxyanions (a phosphate group)\textsuperscript{28}. This environment also substantially lowers the pKa of the catalytic cysteine through stabilizing the thiolate anion (Cys215-S$^-$). Typically cysteine residues within proteins have a pKa value of ~8.5. However, this unique environment confers an unusually low pKa of ~4.5 that allows the enzymatic reaction to efficiently proceed at physiological pH (~7.2)\textsuperscript{5}. Approximately 30-40 residues upstream of the P-loop lies the conserved tryptophan-proline-aspartate or “WPD” loop. This acts as a flexible gate to the active site and has been observed in a variety of “open” or inactive conformations and in “closed” or active conformations.
Figure 1.5. Spatial arrangement of all components in the catalytic domain of PTP1B. Conserved residues important for catalysis are highlighted in ball-and-stick representation: P-loop: catalytic Cys215 and invariant Arg221, WPD-loop: catalytic acid/base Asp181, Q-loop: conserved Gln262, p-Tyr-loop: Tyr46 and E-loop: conserved Glu115. The various WPD loop and P-loop structure and conformations are also highlighted. Catalytic cysteine and aspartate residues are shown in stick representation, conserved tryptophan and proline are shown in line representation; tryptophan–proline–aspartate. Adapted from Ref [27].

Conveniently, upon substrate binding the WPD-loop closes around the active site in order for the catalytic aspartate to be in a position to participate in catalysis. The glutamine-binding loop (Q-loop) is present in all classical PTPs as it and contains a 97% conserved glutamine residue that has been shown to coordinate to a water molecule during substrate hydrolysis. The pTyr-recognition loop (pTyr-loop) contains the pTyr-recognition motif lysine, asparagine, arginine, and tyrosine (KNRY), where the tyrosine is 84% conserved. This residue is positioned in such a way that provides a considerable depth of ~9Å to the catalytic pocket that allows for only a pTyr substrate to bind. In addition, this conserved tyrosine forms edge to face π-π stacking interactions with the pTyr substrate. Thus, both the depth of the pocket and the strong stacking interactions ensures a protected and gated environment that prevents any promiscuous substrates from
binding to this active site pocket. Lastly, the glutamic acid-binding loop (E-loop) contains multiple conserved residues that appear to coordinate the dynamics of the WPD-loop which further provides favourable-substrate-bound conformations\textsuperscript{30,28}.

1.2.2 Mechanism of Catalysis

As mentioned previously, all PTPs active sites are characterized by the signature amino acid motif HC(X)\textsubscript{2}G(X)\textsubscript{2}R[S/T], where X represents any amino acid. This highly conserved motif confers a common catalytic mechanism in PTP activity, represented in Figure 1.6. The general mechanism begins with the stabilization of a negatively charged phosphate substrate via hydrogen bonding between the highly conserved Arg221 located in close proximity to the P-loop. Substrate binding confers further conformational changes in the enzyme that when shifted from the open to closed state, brings the WPD loop over the substrate-binding pocket. In this “closed” or active conformation, Asp181 becomes positioned near the pTyr substrate and forms a hydrogen bond with the phenolic oxygen atom. Overall, pTyr residue is an amphipathic molecule; the phosphorylated end of the tyrosine is polar, highly charged and binds to the polar catalytic site and the phenol ring is non-polar and repelled by the polar catalytic site. However, residues such as Tyr46 and Phe182 form favourable hydrophobic interactions with the phenyl ring that ensures the phosphorylated end is securely placed in the catalytic cleft\textsuperscript{31}. Once these favourable interactions are made, a nucleophilic attack by Cys215 results in the formation of a covalent thiophosphoryl intermediate stabilized by ionic interactions via the positively charged Arg221 and the negatively charged Asp181. Subsequently, Asp181 deprotonates a coordinated water molecule which then attacks the thiophosphoryl intermediate, thereby releasing the cysteine thiolate anion and the phosphate group. The enzyme regenerates after dissociation of the product\textsuperscript{28,32}. 
1.2.3 Exploring the Therapeutic Relevance of PTPs

Validation of PTPs as therapeutically relevant enzymes and as clinically viable targets began in 1993. In a breakthrough study Zhai, Y. and coworkers successfully cloned the intracellular PTP1B protein and demonstrated that it was up-regulated in human breast epithelial cells and breast cancer\textsuperscript{33}. In latter work, Wiener et al. evaluated PTP1B expression at both the messenger RNA (mRNA) and protein level in 29 human mammary tumors. Overexpression was found in 72% of the tissues in tumors as compared to normal epithelial cells, with a maximal expression of PTP1B occurring in 38% of the tumors\textsuperscript{34}. Most recently, Abbott Laboratories demonstrated that antisense oligonucleotides designed to down regulate expression of PTP1B normalized blood glucose and improved insulin sensitivity without changing the regular diet of mice\textsuperscript{35}. Shortly after, nearly every other major pharmaceutical company launched a program in order to identify potent PTP1B inhibitors\textsuperscript{36}. These data completely revolutionized the biological significance of all PTPs, but more specifically conveyed the significance of PTP1B. Overall, the validation of PTP1B as a clinically important target strongly influenced numerous academic research groups and pharmaceutical companies to focus on the development of molecular inhibitors of PTP1B. The biological and therapeutic role of PTP1B will be discussed in the following section.
1.2.3.1 Role of PTP1B in Diabetes, Mellitus, and Obesity

Diabetes is recognized as the world’s most common metabolic disorder, affecting people of all ages globally. The World Health Organization projects that diabetes will be the 7th leading cause of death in 2030. Unfortunately, approximately 90% of the population have Type II diabetes which is acquired throughout life, the remaining 10% of the population have Type I diabetes which is primarily caused by genetics. Type II diabetes is a human disease characterized by insulin resistance and impairment in insulin secretion. It is the result of excess body weight spanning from physical inactivity and a poor diet that leads to high blood sugar levels and consequently obesity. Obesity is defined medically as a state of increased body weight, more specifically adipose tissue that consequentially produces adverse health complications. Obesity largely contributes to insulin resistance and more importantly, is a health factor that contributes to the pathogenesis of various human diseases. Although both Type II diabetes and obesity are treatable, currently there is no cure. Therefore, understanding of the molecular events that lead to the pathogenesis of diabetes and obesity is critical for the rational treatment of these diseases.

![Figure 1.7](image)

**Figure 1.7.** Schematic view of PTP1Bs role in the insulin signaling pathways. IR: Insulin Receptor, IRS: Insulin Receptor Substrate, PI3K: Phosphoinositide 3-kinase, PDK1: PI3K-dependent kinase 1, PSK3: Glycogen synthase kinase 3, GLUT4: vesicle used to transport glucose into cell, Akt: protein kinase. Adapted from Ref [39].
The discovery of PTP1B from biochemical, genetic and pharmacological studies as a negative regulator of the insulin and leptin receptor pathways has highlighted this enzyme as an attractive drug target in metabolic disorders\textsuperscript{40}. Insulin, a hormone that plays a critical role in the maintenance of glucose homeostasis and in the regulation of carbohydrate, lipid and protein metabolism, exerts its pleiotropic biological effects by binding to the insulin receptor (IR) on peripheral target tissue cells, including but not limited to adipose, liver, and muscle. This results in activation of kinase mediated signal cascades\textsuperscript{41}. The insulin receptor is a prototypical receptor PTK composed of two $\alpha$ and two $\beta$ subunits. The binding of insulin to the IR induces a conformational change that results in the $\beta$ subunits undergoing autophosphorylation on at least six tyrosine residues\textsuperscript{42}. The activated IR then carries out the phosphorylation of several downstream protein substrates referred to as the insulin receptor substrates (IRS)\textsuperscript{43}. Further activation of a plethora of key downstream enzymes leads to the activation of protein kinase Akt, which is essential for the insulin-stimulated GLUT4 translation to the plasma membrane (Figure 1.7)\textsuperscript{44}. Akt activation prompts circulating glucose to be up taken by cells leading to the synthesis of the short-term energy storage glycogen and synthesis of fatty acids and proteins, critical to cell survival. PTP1B can associate and dephosphorylate activated IR or IRS’s, which then leads to the shut down of glucose homeostasis. It is well established that circulating glucose in blood is converted to triglycerides and stored in adipose tissue, as a non-metabolic storage instrument of energy. This process ultimately leads to excess weight gain and subsequently diabetes and/or obesity.

More conclusive evidence for the role of PTP1B as a major negative regulator of the IR signaling pathway was provided by groundbreaking PTP1B knockout studies\textsuperscript{45}. These studies concluded that PTP1B-deficient mice (PTP1B$^{+/}$) displayed phenotypes with a normal (if not heightened) lifespan as compared to the wild type controls. Mice lacking functional PTP1B displayed increased or prolonged phosphorylation of IR and IRS in the skeletal muscle and liver. Interestingly, PTP1B$^{-/-}$ mice proved to retain a healthy body weight in the presence of a high fat and calorie diet as compared to the control wild type subjects. Knockout specimens were further shown to be protected against weight gain by significantly lower triglyceride levels when placed on a high-fat diet\textsuperscript{46}. It is important to note that although PTP1B plays a very critical role in insulin signaling and is the main focus of this thesis, rPTP-$\alpha$, LAR, and SHP-2 are the other major phosphatases that can also participate in the insulin signaling cascade\textsuperscript{47}.
Figure 1.8. A detailed schematic view of PTP1Bs role in the leptin signaling pathways. ACC: Acetyl coenzyme A carboxylase, FAS: Fatty acid synthase, ACD: Acyl-coenzyme A dehydrogenase, CPT-1: Carnitine palmitoyl transferase 1, Ob-R1: Long form leptin receptor, PTP1B: Protein Tyrosine Phosphatase 1B, STAT3: Signal Transducers and Activators of Transcription 3. Adapted from Ref [39].

Moreover, in addition to insulin resistance, leptin resistance may be overcome by inhibiting the PTPs that negatively regulate leptin signaling. Leptin is a hormone whose primary role is to enhance tolerance to dietary fat and to maintain fatty acid homeostasis\textsuperscript{48,49}. Leptin initiates its action by binding to the Ob-R1 receptors on the liver, muscle and non-adipose tissues. This receptor then proceeds to dimerise, or come in close proximity with its dimeric counterpart, upon leptin engagement and activates the leptin receptor associated Janus Kinase (JAK2) through phosphorylation. Activated JAK2 then proceeds to phosphorylate a critical tyrosine residue on Ob-R1, which provides a docking site for the SH2 (Src-homology 2) domain of Signal Transducer and Activator of Transcription 3 protein (STAT3)\textsuperscript{50}. Once STAT3 docks proximal to JAK2, it becomes phosphorylated, dimerizes, and translocates into the nucleus wherein it binds to DNA and influences the transcription of proteins important for lipid homeostasis\textsuperscript{51}. However, both SHP2 and PTP1B have been associated with the leptin pathway and are highly plausible
candidates for leptin resistance factors. When leptin action is lacking in cells, the lipid content of liver, muscle and other non-adipose tissues increases, causing lipotoxicity from an over accumulation of fatty acid derivatives. Similarly with PTP1B−/− in insulin studies, it was suspected that mice lacking PTP1B might show increased leptin sensitivity, resistance to obesity when fed a high-fat diet and display increased energy expenditure. These hypotheses have been verified by results that have shown PTP1B−/− mice to be hypersensitive to leptin and thus exhibit more resistance to obesity. With these findings, it appears that both insulin and leptin resistance may be overcome by inhibiting the PTPs that negatively regulate insulin and leptin signaling, respectively. Moreover, this confers the importance of PTPs, specifically PTP1B, in metabolic disorders.

### 1.2.3.2 Elucidating the Significance of PTPs in Cancer

Although PTPs carry significant roles in cellular signaling cascades in Type II diabetes and obesity, there are a number of PTP genes that have been identified as critical oncogenes in human cancers. These candidates are classified as either oncogenic, negative regulator, or tumor suppressor/promoter PTPs. Many oncogenic PTPs have been found to play a casual important role in metastasis, oncogenesis, and progression. Cdc 25 A, B, and C belonging to the DSPs, have been shown to regulate cyclin-dependent kinases (CDKs), which are key participants in cell division and checkpoint pathways in the cell cycle. Overexpression of Cdc25A or B inactivates these kinases in malignant cells and eventually induces cell cycle arrest, ultimately leading to apoptosis. Both of these phosphatases have been reported in various human cancers, including but not limited to breast, ovarian, prostate, lung, glioma, neuroblastoma and non-Hodgkin’s lymphoma. MKP-1, SHP-2 and phosphatases of regenerating liver (PRL) have also been shown to be oncogenic PTPs. Secondly, PTPs that are negative regulators, such as SHP-1, have been shown to negatively regulate cytokine signaling and immune cell activation. More specifically of anti-tumor immune cells, including T cells, natural killer cells, dendritic cells, and macrophages. Therefore, SHP-1 is an attractive target for increasing the antitumour activity of cytokines and immune cells. Lastly, since PTPs are the biochemical counterparts of PTKs which often function as oncogenes, it was anticipated that PTPs would presumably act as tumor suppressors. A recent study identified six PTPs that were frequently inactivated by mutations in colorectal cancer cells, indicating that PTPs could potentially act as tumor suppressors. In
addition, RPTPs have been shown to dephosphorylate and inactivate STAT3, which has been shown to play a master regulatory role in oncogenesis\textsuperscript{61}. Furthermore, a rather controversial outlook on the role of PTP1B in cancer has recently been described by our collaborator Michel L. Tremblay, which suggests that PTP1B can exert both tumor suppressing and tumor promoting efforts, depending on the substrate involved and the cellular context (Figure 1.9). However, further work must be done in order to dissect the role of PTP1B in different \textit{in vivo} tumorigenesis models to help identify which types of human cancers could be accelerated or suppressed by PTP1B inhibition\textsuperscript{62}. Overall the increased significance of PTPs in signal transduction and the flurry of discoveries that link their aberrant activity to pathologies, have firmly validated PTPs as a therapeutic target. The next section will explore current approaches and techniques utilized to develop molecular inhibitors of PTP1B, the most well studied member of the PTP family.

**Figure 1.9.** Controversial roles of PTP1B; substrates and the effects posed for both roles as a tumor suppressor and tumor promoter. ER: C-terminal tail anchored in the endoplasmic reticulum, PR: Proline-rich motif, CD: Catalytic Domain\textsuperscript{30}. 
1.2.4 Current Approaches to Targeting PTP1B

The emerging pathogenic implications of uncontrolled activity for members of the PTP family has identified them as novel drug targets, particularly PTP1B. However, there are significant technical challenges with PTP inhibitor design since all PTPs share a high degree of sequence similarity and structural topology. For example, a typical panel of PTPs sequence identity and similarity to PTP1B can be viewed in Table 1.1. TC-PTP has the highest degree of similarity to PTP1B and contains identical residues in the YRD region (common grouping region of amino acid residues in PTPs containing tyrosine, arginine, and aspartic acid within the active site). The crystal structures are almost identical where TC-PTP shows 72% sequence identity to PTP1B in its catalytic domain, and 100% sequence identity in their active sites (root mean square deviation between TC-PTP and PTP1B is 1.82 Å). Thus, selectivity is one of the major issues in the development of PTP1B inhibitors as drugs. In addition, due to the structural and chemical properties of PTP active sites, most PTPs lack “druggable” binding pockets that are amendable to bind low molecular weight, drug-like inhibitors.

Table 1.1. A Typical PTP1B Selectivity Panel\textsuperscript{65}.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Identity</th>
<th>Similarity</th>
<th>Type</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTP1B</td>
<td>100%</td>
<td>100%</td>
<td>Intracellular</td>
<td>PTP</td>
</tr>
<tr>
<td>TCPTP</td>
<td>72%</td>
<td>85%</td>
<td>Intracellular</td>
<td>PTP</td>
</tr>
<tr>
<td>CD45</td>
<td>38%</td>
<td>48%</td>
<td>TM type 1</td>
<td>PTP</td>
</tr>
<tr>
<td>PTP-LAR</td>
<td>42%</td>
<td>51%</td>
<td>TM type 2</td>
<td>PTP</td>
</tr>
<tr>
<td>PTPα</td>
<td>40%</td>
<td>51%</td>
<td>TM type 3</td>
<td>PTP</td>
</tr>
<tr>
<td>SHP-2</td>
<td>39%</td>
<td>52%</td>
<td>Intracellular</td>
<td>PTP</td>
</tr>
<tr>
<td>Cdc25c</td>
<td>13%</td>
<td>24%</td>
<td>Intracellular</td>
<td>Dual-specificity</td>
</tr>
</tbody>
</table>

For example, PTP1B contains three potential binding pockets located in and around its catalytically active domain. The positively charged primary phosphate-binding pocket is the most polar region of the protein, as it contains the conserved signature motif that accommodates negatively charged aryl phosphate anions. Located nearby is a larger and more shallower second phosphate-binding pocket, exhibiting a lower but still significant affinity for aryl phosphates\textsuperscript{66}. Due to the lack of a catalytically active cysteine residue, this site is completely non-catalytic.
Also, it is important to note that this secondary phosphate-binding site is not conserved in PTP1B, nor amongst the entire family of PTPs. Lastly, there is a large flat region that can occasionally accommodate negatively charged substituents, but overall is decorated with hydrophobic residues that produce an overall non-polar environment. This site is also known as the allosteric site and is recognized to control the WPD-loop by determining when it adopts the catalytically active or “closed” conformation\textsuperscript{67}. Inherently, the highly charged primary phosphate binding site and flat, solvent exposed secondary phosphate binding site significantly increase the difficulty of designing inhibitors that first, display high affinity for PTP1B and second, that are drug-like and are cell permeable. Despite these difficulties, a plethora of research programs have targeted both the active sites independently and simultaneously, as well as the allosteric site, with a variety of scaffolds and small molecule inhibitors that will briefly be explored in the following chapter.

\textbf{Figure 1.10.} Structural topology of PTP1B; active site or primary phosphate binding pocket, second site or secondary phosphate binding pocket and allosteric site, highlighted in yellow, blue and green, respectively, (PDB: 1NWL).
1.2.4.1 Inhibitors of PTP1B: Approaches, Successes and Complications

Natural products have been a rich source of PTP1B inhibitors and a good starting point for the development of synthetic analogues. The first ever reported inhibitor was derived from the natural product, 4-Isoavenaciolide, which was isolated from a fungal strain and identified as an irreversible inhibitor of vaccinia H-1 related (VHR) phosphatase with an half maximal inhibitory concentration (IC$_{50}$) of 1.2 µM$^{68}$. In addition, the tetronic acid derivative was isolated from a Streptomyces strain and identified as a competitive inhibitor of the DSPs VHR and CD45. Impressively, it arrests cell-cycle progression of mammalian cells in the G$_1$ phase$^{69}$. Apart from natural products, more rational approaches have been successfully incorporated in the development of PTP1B inhibitors, such as molecular modeling based on X-ray crystallographic structures of PTPs. Utilizing these techniques, efforts were directed towards the development of competitive PTP inhibitors that have been realized through the replacement of the phosphate portion of peptidic substrates with nonhydrolyzable phosphate mimetics$^{32}$. A pTyr mimetic is intended to represent the chemical and structural properties of a pTyr group. Ideally, incorporating a non-hydrolyzable phosphate such as a phosphonate, most efficiently mimics the natural substrate. Although similar in structures, phosphonates do not mimic the diatomic character of the phosphate group at physiological pH simply as a consequence of their pKa’s. Shown in Figure 1.11, at physiological pH, the phosphonate would be monoprotonated, yielding only one negative charge as compared to the phosphate ester, which would be fully deprotonated. This subtle replacement of the oxygen with a methyl group substantially changes the electrochemical properties. Since oxygen is more electronegative than a carbon, it inductively withdraws electrons away from the neighbouring phosphate and oxygen, thus stabilizing the negative charge and reducing the pKa of the functionality$^{70}$.

**Figure 1.11.** The protonation states of phosphate and phoshonate at physiological pH. The respective pKa’s are noted beside each hydroxyl group on the phosphate and phosphonate moieties$^{70}$. 
The first ever pTyr mimetic was reported to incorporate electron withdrawing substituents such as fluorines on the methyl group. The difluoromethylphosphate (DFMP) effectively stabilizes the anionic charge and withdraws inductively to reduce the pKa of the phosphonate to 6.7. Like the phosphate moiety, this mimetic presents a double anionic character at physiological pH. Unfortunately, whilst less anionic in character than a phosphate ester, the main drawback of dianionic pTyr mimetics is their inherent lack of cell-membrane permeability. Despite their strong inhibition of phosphatases in vitro, they are not biologically active in cell-based assays. Thus, academic groups and pharmaceutical companies have attempted to develop more drug-like and less negatively charged pTyr mimetic groups. Since it is beyond the scope of this thesis to document every effort, we have selected to report on several notable mimetics and their application in inhibitors.

Researchers at Merck Frosst developed a series of DFMPs substituted with a deoxybenzoin side chain which showed strong nanomolar (nM) PTP1B inhibition. Moreover compound 1.4 (Figure 1.12), with an ortho-bromo substituent on the phenyl ring proved to be orally bioavailable (IC$_50$= 0.12 µM). The lack of information about the binding mode of these inhibitors impaired a further improvement in selectivity. In addition, researchers at Pharmacia developed the 2-carboxymethoxybenzoic acid group (1.6) as a replacement of the O-malonyltyrosine moiety (1.5). To overcome problems caused by poor cell permeability, one carboxylic acid group was replaced by a tetrazole unit as a carboxyl group bioisostere (1.7). The resulting compound 1.7 showed PTP1B inhibition ($K_i$ = 2 µM) and modest cellular activity. Furthermore, researchers at Novo Nordisk identified a similar structure, 2-(oxalylamino)benzoic acid (1.8). Based on this structure, analogues were developed with sub- µM IC$_{50}$ values for PTP1B and possessed modest oral bioavailability in rats. Overall, a plethora of pTyr mimetics have replaced phosphate moieties in scaffolds in order to develop more orally bioavailable, cell permeable and reversible inhibitors of PTP1B. Unfortunately, most of the mimetics mentioned, and the many others that exist, exhibited limited to negligible activity against PTPs. It is hypothesized that most pTyr mimetics fail to incorporate certain structural and chemical properties of a phosphate group, such as physical structure, shape and chemical charge. Salicylic acid, one notable and more drug-like pTyr mimetic, will be discussed in detail in the following section of this thesis.
1.2.4.1.1 Salicylic Acid as a pTyr Mimetic in PTP1B Inhibitors

Derived from willow bark, salicylic acid is a monohydroxybenzoic acid that is most known for its anti-inflammatory and analgesic effects in Aspirin. It is administered as the prodrug acetylsalicylic acid, where it is transformed to the active salicylic acid metabolite. There are a variety of properties that contribute to its mimicry of pTyr. Firstly, the salicylic acid is similar in structure to pTyr, as it contains an ortho-disubstituted benzene ring with a carboxylic acid and phenol. In addition, there is a bond dipole present in both the phosphate group and the carboxylic acid functionality of the salicylic acid. Although the phosphate functionality on pTyr is polar, with an electronegativity difference between P and O of 1.25, the carboxylic acid functionality is not much different with the electronegativity difference between the C and O of 0.89. A larger electronegativity between a bond dipole attributes to slightly higher polarity as compared to the smaller electronegativity, which typically attributes to a lower polarity. As a consequence, the salicylic acid is slightly less polar than a phosphate group. This in turn improves cell permeability and bioavailability as compared to the phosphate functionality. Secondly, as mentioned earlier, negative charges are imperative to pTyr mimetics and since the salicylic acid has a pKa of 2.97, it does not encounter problems with being singly ionized at physiological pH. The extent of ionization present at physiological pH can be estimated using the Henderson Hasselbalch equation, \[ \text{pH} = \text{pKa} + \log \left( \frac{[\text{A}-]}{[\text{HA}]} \right) \]. If the physiological pH is approximately 7.2 and the pKa is 2.97, then the ratio of 16982 for [A-]/[HA]. This conveys that there is undoubtedly a substantial amount of the deprotonated species than the protonated present at
physiological pH. Lastly, because these two groups have such similar characteristics, it has been hypothesized that the binding in the active site of PTP1B would be fairly similar, and would have somewhat similar bonding configurations to the residues in the primary phosphate-binding site, despite their geometrical differences (Figure 1.13). Concluding the overall discussion of the salicylic acid, it is apparent that this molecular arrangement has electrostatic and steric properties necessary to achieve a highly viable pTyr mimetic. Overall, the salicylic acid is evidently less charged, more cell-permeable and thus more orally bioavailable in comparison to most pTyr mimetics previously mentioned.

**Figure 1.13.** Left: Proposed binding mode of salicylic acid (red) in the primary phosphate-binding pocket of PTP1B. Water molecules (blue) and other residues bound to salicylic acid are removed for simplicity. Right: Structural comparison of the phosphate ion and salicylic acid moiety.

### 1.2.4.2 Two-Site or ‘Bidentate’ Binders as Selective PTP1B Inhibitors

As already mentioned, all PTPs share a high degree of structural conservation in the active site and designing inhibitors with both high affinity and selectivity for PTP1B poses a challenge. In this respect, the discovery by Zhang and co-workers of a second proximal non-catalytic binding which showed less homology among the phosphatases is of particular importance for the development of more selective inhibitors of PTP1B. In this site, a second pTyr forms ionic interactions with Arg24 and Arg254, polar interactions with Met258 and Gln262, and van der Waals contacts with Ile219, Asp48 and Arg254. Coupled with this discovery, PTP1B substrate specific studies have shown that pTyr alone is not sufficient for high-affinity binding and residues flanking the pTyr are important for PTP substrate recognition. These studies provide a
molecular basis for addressing and manipulating PTP inhibitor potency and specificity, and suggested a novel paradigm for the design of potent and specific inhibitors; namely bidentate ligands that bind to both the active site and the second aryl phosphate binding site. Since the second site is less conserved among the large family of PTPs, a bidentate ligand that binds the two sites has a better chance to be more selective than a classical active-site binding compound. The rationale for the enhanced affinity and potency of bidentate inhibitors is based on the principle of additivity of free energy of binding. Thus, the strategy of linking molecules that independently are known to bind to PTP1B was, at the time of the discovery, a novel and innovative way to create potent and selective PTP1B inhibitors. Naturally, researchers began by linking a variety of active-site-directed PTP inhibitors, but unsurprisingly were reported to possess high charge density and generally were not drug-like. Since then, several strategies have been applied to improve the cell permeability by replacing active site binders with pTyr mimetics. Currently, there are a variety of sophisticated bidentate scaffolds targeting PTPs and a selected array are showcased in Figure 1.14 (1.9-1.11). Thus, rationally designed bidentate inhibitors hold immense potential to gain selectivity handle among this large family of structurally similar proteins and will be further explored in a following section.

**Figure 1.14.** A selected array of bidentate PTP1B inhibitors. \( K_i \) values for compounds 1.9, 1.10 and 1.11 are 2.4, 3 and 22 nM, respectively.
1.2.4.3 Allosteric Site Binders as PTP1B Inhibitors

To circumvent the issue of improved selectivity and bioavailability, targeting the allosteric site has recently been described for PTP1B. Small molecules that are able to occupy this site and stabilize PTP1B in an inactive conformation can thereby inhibit their catalytic capabilities. Unlike the pTyr binding active site, the allosteric site is not well conserved and is substantially less polar. Compounds 1.12-1.14 are examples of allosteric inhibitors, with IC$_{50}$’s of 1.6, 350, 8 µM, respectively (Figure 1.15)\(^{40}\). Inhibitors targeting the allosteric site are more likely to be cell permeable since they do not contain negative charges and as a result have acceptable pharmacological properties. However, the main drawback of allosteric binders is their poor solubility and drug-like characteristics. Since the allosteric pockets are largely hydrophobic, most inhibitors targeting this site are functionalized with large hydrophobic moieties that suffer from aggregation problems\(^{78}\). Nonetheless, targeting the allosteric site may present an alternative strategy for developing selective inhibitors that have potential for improving their pharmacological properties.

\[ \text{Figure 1.15. A selected array of PTP1B allosteric binders}^{39}. \]

1.2.4.4 Vanadate and Vanadium-based PTP Inhibitors

At the turn of the 20\(^{th}\) century, vanadium salts were prescribed for a variety of clinical conditions such as anaemia, tuberculosis and diabetes\(^{79}\). Peroxovanadium complexes were originally synthesized in the late 1970s with the purpose of understanding the chemistry and bonding of the peroxo group, a functional group consisting of two oxygen atoms bonded together with a single bond. Interest in the use of vanadate derivatives for the treatment of diabetes were rekindled in the late 1970s and early 1980s as a result of the demonstration that vanadate (V$^{5+}$) solutions produced insulin-like effects in rat diaphragms and isolated adipocytes \textit{in vitro}\(^{80}\). The combination of vanadate (V$^{5+}$) and hydrogen peroxide (H$_2$O$_2$) in water, which independently are both weak insulin mimetics, led to a synergy of insulin-like effects \textit{in vitro}. These results were due to a chemical reaction that resulted from the peroxide ion forming a complex with the vanadium,
creating aqueous peroxovanadates. The poor stability of aqueous peroxovanadates and the multitude of species in complex equilibrium enthused research chemists to prepare many new, stable and structurally defined peroxovanadium complexes\textsuperscript{81}. The introduction of ancillary ligands, a ligand that is not typically contributing to the chemistry of the complex, helps to confer greater kinetic stability compared with vanadate or aqueous peroxovanadates. These complexes have proved to be 100-1000 times more effective as inhibitors of PTPs \textit{in vitro} than sodium orthovanadate (Na\textsubscript{3}VO\textsubscript{4})\textsuperscript{82}. Although the full mechanism underlying the insulin-like actions of these complexes has not been elucidated, inhibition of PTPs and the subsequent enhancement of cellular phosphorylation appear to be the most relevant effects in explaining of insulin mimesis\textsuperscript{83}. Insulin mimesis is essentially any external stimulant that produces cellular effects similar to that of insulin.

![Figure 1.16. Mechanism of inhibition vanadate and peroxovanadium complexes 1.15 and 1.16\textsuperscript{81}.

Vanadate appears to act as a phosphate analogue, mimicking the transition state and behaving as a competitive inhibitor of PTPs, specifically PTP1B\textsuperscript{82,84}. On the other hand peroxovanadium complexes and aqueous peroxovanadates are irreversible PTP inhibitors, acting on the well-conserved critical cysteine residue and inhibiting its critical action in the mechanism of catalysis\textsuperscript{85}. Currently, some of the most potent vanadium based inhibitors exhibit extremely high affinity for PTPs. For example, inhibitor 1.15, also known as potassium bisperooxo (1,10-phenanthroline) oxovanadate (V) or bpV(phen), potently inhibits PTP1B with an IC\textsubscript{50} of 900 nM. Another example would be 3-hydroxypicolinate vanadium (V) or VO-OHpic (1.16, Figure 1.16) which selectively and potently inhibits phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (IC\textsubscript{50} = 35 nM)\textsuperscript{86,87}. Evidently, vanadium complexes hold immense therapeutic potential due to their convincing inhibitory effects against PTPs. Such potent and selective inhibitory effects could prove useful in the treatment of a variety of metabolic disorders. Chapter 3 of this thesis will explore the incorporation of vanadate-based inhibitors in a novel approach to targeting PTPs.
1.3 Concluding Remarks

Enzymes are the most popular drug targets currently on the market. Although kinases have had a great deal of success with many drug candidates entering the clinic and persevering as FDA approved drugs, PTPs have had much less success. However, their important roles in a variety of metabolic disorders such as type II diabetes and obesity have been studied and it is significantly understood that targeting this class of enzymes holds immense therapeutic potential to modulate the progression of many disorders. Unfortunately, constructing potent and selective inhibitors has thus far been a daunting task due to their considerable similarities in PTP morphology and active sites that are termed “undruggable”. Nonetheless, many research programs and pharmaceutical companies have elucidated the discovery of a variety of successful PTP inhibitors. However, their lack of drug-like characteristics such as high anionic charge, large molecular weight, poor bioavailability and cell permeability characteristics has severely hindered the advancement of any candidates into the clinic. Thus, it is this author’s opinion that exploring PTPs, specifically PTP1B, with rationally designed small-molecules holds immense potential as most drug discovery programs have thus far relied on techniques such as High-throughput screening (HTS) assays, which identify non-rationally designed binders. Computationally inspired and rationally designed small-molecules that simultaneously target both the active site and neighbouring pocket of PTP1B could potentially overcome the hurdles of PTP1B inhibitors and derive potent and selective molecular binders. In addition, the convincing inhibitory concentrations of vanadium compounds against PTPs could hold immense application and potential in the design and development of novel scaffolds. The work summarized herein has provided insight into the structural requirements of PTP1B binders and has showcased the design of clinically relevant PTP1B inhibitory agents.
1.4 Central Aims of This Research

In the field of medicinal chemistry, enzymes are not by any means a novel drug target, as they date back to the 1970s when the first ever oncogene was shown to be a kinase. Their biochemical counterparts, phosphatases were discovered a decade later. It was not until 1999 when the Tremblay research group identified PTP1B as a negative regulatory of the IR and more importantly highlighted its therapeutic importance in type II diabetes and obesity. This sparked an interest from various pharmaceutical companies that took on multiple research programs to target this therapeutically relevant enzyme, once thought to be merely a housekeeping protein. However, it was soon evident that issues of creating and designing potent and selective PTP inhibitors was a formidable task, let alone clinically viable drug candidates. This research work has effectively challenged the many technical issues that originate with creating selective and potent PTP inhibitors. By employing computational aided rational design, this work has furnished a selection of rationally designed small-molecule inhibitors that successfully, selectively and potently target clinically viable PTPs. In addition, research efforts have developed strategically designed vanadium-based protein membrane anchors as PTP inhibitors that hold promising therapeutic and diagnostic potential. The methodologies described in this thesis have further highlighted that the challenges with targeting PTPs can be overcome and selectivity can be achieved. The results from this investigation emphasize the therapeutic relevance of this class of enzymes and certainly disapprove them as “undruggable” drug targets.
2 Rationally Designed, Potent Salicylic Acid based PTP1B Inhibitors

A tremendous amount of progress has been made towards the development of potent and selective PTP1B inhibitors ever since their therapeutic importance in diabetes and cancer has been discovered. Drug discovery programs have traditionally focused on targeting the active site or the primary phosphate-binding active site with pTyr mimetics. Although there are a variety of pTyr mimetics published in literature, the salicylic acid contains particular chemical and physical properties that surpass the mimetic capabilities of many other derivatives. With this in mind, our group first applied the salicylic acid moiety as a pTyr mimetic in a STAT3 inhibitor (compound 2.1, Figure 2.1). Further optimizations of this inhibitor led to the identification of 2.3 (IC$_{50}$ = 19 µM). According to our computational studies the salicylic acid moiety mimics the desired pTyr705 of STAT3 and makes favourable interactions in the SH2 domain pocket of another STAT3 homodimer protein, while simulating the highly charged aryl phosphate group. Due to the success of incorporating the salicylic acid as a pTyr mimic in our STAT3 inhibitors and given the importance of pTyr mimicry in PTP inhibition, we decided to evaluate our existing library of compounds furnished with this appendage against a variety of phosphatase platforms (this library of compounds was originally synthesized to target STAT3). We hypothesized that
this would identify if our compounds were appropriate scaffolds for targeting the PTP family of proteins.

**Figure 2.1.** Compounds 2.1-2.3 bound to STAT3 SH2 domain and respective IC₅₀ values are shown below. Salicylic acid (red) is present on all three of these STAT3 inhibitors and is evidently making favourable interactions in the SH2 domain of STAT3. Values were identified via EMSA and Western Blots of nuclear extracts.

We assessed for PTP activity by conducting a high throughput enzyme activity screen of a library of tri-substituted purine-based inhibitors, a variety of inhibitors appended with the salicylic acid moiety and finally a series of carboxylic acid mimetics (sulphonamides, oxalic acids, tetrazoles, masked acids and salicylic acid moieties). To measure the PTP activity, the screen employed a fluorometric kinetic activity-based assay to measure the rate of 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP; Invitrogen, Carlsbad, CA) hydrolysis. DiFMUP is a substrate hydrolyzed by PTPs via the removal of a phosphate group. The dephosphorylated product, 6,8-difluoro-4-methylumbelliferyl (DiFMU) produces a fluorescent signal that can be measured. If the inhibitors are tight binders and prevent the PTP from removing the phosphate group from the substrate, the fluorescent signal decreases. The rate of the reaction is thus determined from the amount of fluorescence emitted, which is then converted to IC₅₀ values. Our top lead inhibitors exhibited IC₅₀’s in the single digit µM range against several phosphatase isoforms, including PTP1B, TC-PTP and PTPσ. Encouragingly, there was no observed inhibition of PTP-LAR, PRL2 A/S or MKPX, which are all closely related PTPs that share more than 75%
sequence homology. Interestingly, all potential lead compounds had shared a common core structure that contained a salicylic acid moiety. Thus, we perceived that our result belayed the critical pTyr mimetic potential of this moiety as PTP1B active site binders.

Integrating the overall discussion of PTP1B active site binders possessing selectivity and potency, or lack thereof, literature has presented studies to which many examples of bidendate inhibitors have been employed as a strategy to increase binding affinity by probing or targeting the second phosphate-binding site. However, these inhibitors were quickly dismissed when tested in whole cells due to suffering from poor cell permeability, bioavailability, and generally displaying poor drug-like characteristics. Thus to date, there still stands a lack of drug candidates targeting PTPs, and more specifically PTP1B\textsuperscript{39}. Herein, this chapter will describe the first ever reported computationally aided, novel rationally designed di-salicylic acid inhibitors. This chapter will first describe the methodologies employed to identify our first generation, potent salicylic acid-based PTP1B inhibitors. The second part of this chapter will explore the rationale and computational optimization of our first generation inhibitors aimed to improve potency and selectivity. This second generation library applied the utility of tethered di-salicylic acid-based inhibitors that further optimize the first generation potent \textit{in vitro} bimodal compounds that extend the intramolecular distance between the salicylate moieties. The methodologies described herein further denote more information about the structural characteristics of PTPs pertaining to their activities.

\textbf{Figure 2.2.} A closer view to the active site of PTP1B. Highlighted is the primary and secondary phosphate binding sites which are approximately 20.7 Å apart, (PDB: 1NWL).
2.1.1 Identification of Our First Generation, Potent Salicylic Acid-Based Inhibitor of PTP1B

2.1.1.1 Preliminary screening of the library

A diverse chemical library of compounds designed to target SH2 domain through pTyr mimicry were evaluated for inhibitory activity against the NRPTPs PTP1B and TC-PTP, the RPTPs PTPσ and PTP-LAR, the DSPTPs PRL2 A/S (active mutant) and MKPX, as well as human cysteine-based protease papain (used as a control for oxidation). From this screen, six lead compounds were identified (2.4a-f). The inhibition of PTP1B, TC-PTP and PTPσ enzymatic activity at 10 μM inhibitor concentration are shown in Figure 2.3. The trials were conducted with vanadate (VO₄³⁻) as a positive control. At 10 μM compounds 2.4a-f inhibited PTP1B, TC-PTP and PTPσ by more than 80% compared to the DMSO control. Interestingly, 2.4a-f negligibly inhibited RPTP PTP-LAR and the DSP PRL2 A/S, MKPX phosphatases as well as the protease papain.

![Figure 2.3](image_url)

**Figure 2.3.** High-throughput kinetic enzyme activity screen for compounds 2.4a-f against the phosphatases; PTP1B, TC-PTP, PTPσ (D1D2), PTP-LAR (D1D2), the DSPTPs; PRL2 A/S (active mutant) and MKPX, and the protease papain is present as a negative control and vanadate as the positive. Graph bars represent inhibition (1 = complete inhibition, 0 = no inhibition).
This evidence supports the hypothesis that compounds 2.4a-f target the active site of the RPTPs and NRPTPs and that the salicylic acid might in fact be acting as a pTyr mimetic. MKPX and PRL2 A/S are DSPs that recognize pTyr, pThr and pSer. However, it would appear that our molecular scaffold precludes interaction with the active site of these enzymes. Observed selectivity may be a result of unfavorable scaffold interactions with the surface residues of the catalytic pocket of PTP-LAR, MKPX, and PRL2 A/S which are generally much larger and generally deeper (~3Å deeper) to accommodate for pThr and pSer residues.

2.1.1.2 Dose-dependent inhibition of PTPs and IC\textsubscript{50} Determination

Our next step was to evaluate our lead inhibitors at various concentrations to quantify relative levels of PTP1B, TC-PTP and PTP\textalpha inhibition and to identify phosphatase selective compounds. The results are presented as the mean value ± SE in Table 2.1. IC\textsubscript{50} values for 2.4a-f ranged between 3.9 and 6.3 µM for all PTPs tested except for PTP-LAR, MKPX and PRL2 A/S, against which the compounds showed no inhibition at the concentrations tested. Interestingly, these inhibitors also displayed equipotent inhibition of PTP\textalpha, another clinically important PTP target which has been firmly associated with Crohn’s disease and neurodegenerative disorders\textsuperscript{56}.

Unfortunately, candidates 2.4a-f did not show appreciable selectivity between PTP1B, TC-PTP or PTP\textalpha. Thus, only compounds 2.4a-c were selected for further biological evaluation against PTPs as previous work demonstrated limited effects against other SH2 domain containing proteins (STAT1/3/5) and kinase enzymes. Dose dependent enzyme activity rate curves for compounds 2.4a, 2.4b, 2.4c are shown in Figure 2.4. The limited specificity between PTP1B and TC-PTP is unsurprising given their 72% sequence similarities. It was however quite encouraging to observe equipotent inhibition of PTP\textalpha, since this enzyme is a RPTP and structurally very dissimilar to PTP1B and TC-PTP. Another interesting result was that inhibitors potently targeting PTP\textalpha did not inhibit PTP-LAR, as shown in Figure 2.3, a member of the same subclass of RPTPs.
Table 2.1. Activity summary of 2.4a-f determined for various phosphatase enzymes. IC$_{50}$ values were calculated for PTP1B, PTP$_{D1D2}$, TC-PTP and PRL2 A/S through dose dependent enzymatic assays using DiFMUP as substrate. Data present for PTP-LARD1D2, MKPX and papain are from initial kinetic experiments.

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* Based on initial enzyme screen
2.1.1.3  *In vitro* biological data

To validate the activity of our top inhibitors within a cellular context, 2.4a-c were tested against the most well characterized and validated therapeutic target, PTP1B. As already discussed, signal transduction pathways within the insulin signaling circuit are heavily reliant on the phosphorylation state of the IR. Insulin, when present binds on the IR on the extracellular side of the plasma membrane and instigates phosphorylation of the IR (pIR). pIR is a direct substrate for PTP1B and as such the IR phosphorylation state is largely dependent on the activity levels of PTP1B within the cytoplasm. Monitoring pIR levels is therefore useful as a reporting system for PTP1B activity. Chinese hamster ovary (CHO)-IR cells over-express the IR and upon stimulation by insulin become phosphorylated. Cells were incubated with 1, 5, and 10 µM inhibitor concentrations in serum free media then stimulated with insulin for 15 minutes. Western analysis determined the relative levels of pIR, as shown in Figure 2.5. Encouragingly, 2.4b and 2.4c elicited a pronounced effect on the pIR signal relative to the DMSO control, indicated by the blue arrow in Figure 2.5A. The densitometry graph shows the dose-response effect of pIR/IR compared to the DMSO control (Figure 2.5B). Moreover, a dose-dependent increase in pIR levels that correlated well with PTP1B IC$_{50}$ values obtained previously indicated that 2.4a-c directly inhibited the enzymatic activity of PTP1B.

![Figure 2.4.](image)

*Figure 2.4.* Dose-dependent curves of inhibitor compounds 2.4a-c. Inhibitors tested against the phosphatases PTP1B, PTPσ, and TC-PTP. Individual plots overlay inhibitor specific data for the three phosphatases.

Furthermore, cytotoxocities of 2.4a-c were determined by xCELLigence systems (Roche). CHO-IR cells were seeded in 96-well E-plates and the electrical impedance across the surface of electrodes in the bottom of the well was measured over 4 days. Electrical impedance in the xCELLigence system correlates with differences in cell growth, cell size, number and degree of
attachment to the surface of the electrode (defined at the Cell Index- CI value). The readouts of these studies over time are visualizations of cellular activity and by extension a reflection of cytotoxicity using a label free analytical technology. At concentrations ranging between 0.4 and 10 µM, compounds 2.4a-c showed no impedance of cellular proliferation rates. At elevated (50 µM) concentrations compounds 2.4a showed marked cytotoxicity. However, even at this high concentration, 2.4b and 2.4c continued to induce CHO-IR cell responses that trended with the DMSO and salicylic acid control data, where only slight reductions in cell viabilities were observed. These results, coupled with the dose-dependent relationship of PTP1B inhibition described in the Western analysis, reveal compounds 2.4a-c to be a new class of PTP inhibitors.

Recently, Zhou et al. published a series of benzofuran-based salicylic acid compounds targeting Mycobacterium tuberculosis PTP (mPTPB) in which they determined a non-competitive mechanism of action. Their inhibitor was also shown to target PTP1B and TC-PTP (IC50 = 19 ± 1.5 and 22 ± 2.5 µM, respectively) with ~15-fold selectivity towards mPTPB. Furthermore, He et al. reported bicyclic benzofuran and indole-based salicylic acid compounds as inhibitors of a variety of PTPs.

**Figure 2.5.** (A) Western blot analysis of the phosphorylated insulin receptor in the presence of 1, 5, and 10 µM concentrations of compounds 2.4a-c. (B) Densitometry graph showing the dose-response effect of 2.4a-c on the relative levels of pIR and IR compared to the DMSO control. 2.4a, 2.4b, 2.4c presented as the mean of 4 replicate trials and salicylic acid and vanadate the mean of 3 replicate trials.
Figure 2.6. Michaelis-Menten rate data as a function of substrate concentration for 2.4a, 2.4b, 2.4c and vanadate. Curves represent the hydrolysis of DiFMUP and subsequent fluorescent detection at 450 nm (excitation of 358 nm). Below each curve show Lineweaver-Burke transformations. For all three inhibitors, $V_{\text{max}}$ decreases as $K_{\text{m}}$ increases as a function of substrate concentration, indicating mixed inhibition. Michaelis-Menten control for vanadate and experimental data.
Interestingly, X-ray crystallographic studies of these inhibitors showed that the salicylic acid functionality embedded in the active site of Lyp1 and SHP2 PTPs. Given the relative structural similarities between 2.4a-f and the inhibitor published by Zhou et al. and He et al., it is probable that our observed activities are as a result of analogous binding modes between the inhibitor and protein. In order to further explore our hypotheses, we performed computational docking studies that demonstrated compound 2.4c embedded its salicylic acid moiety within the PTP1B active site in close proximity (3.8 Å) to the key catalytic residue, Cys215 (Figure 2.7). Also, the quinoline portion of compound 2.4c makes favourable π-π interactions with the adjacent Phe182 residue, with the nitrogen engaged in a hydrogen bond interaction with the peptide backbone of the protein. Finally, we explored the kinetic activities of 2.4a-c via classical Michaelis-Menten studies, shown in Figure 2.4. Briefly, kinetic experiments were monitored spectroscopically by measuring DiFMUP hydrolysis and the change in light attenuation at 405 nm. Lineweaver-Burk graph plots were generated using the GraphPad Prism software. The analysis of this graph revealed that 2.4a and 2.4b were mixed inhibitors of PTP1B ($K_i = 2.1 \pm 0.6 \mu M$ and $4.0 \pm 1.4 \mu M$ for 2.4a and 2.4b, respectively). Compound 2.4c also exhibited mixed inhibition. Although not the most ideal results from the Michaelis-Menten kinetic studies, observed activity levels for 2.4a-c are approximately equipotent with published inhibitors. Therefore, optimization of lead structures can be undertaken to furnish enhanced inhibitors with improved selectivity as compared to 2.4a-c.

Figure 2.7. Docking studies of candidate 2.4c with PTP1B shows the embedment of the salicylic acid group within the PTP1B active site and the favourable binding interactions with residues Cys215 and Phe182 (PDB: 1NWL).
2.1.2 Second Generation Bidendate Di-salicylic Acid Inhibitors

In our first study we previously screened a large library of diverse compounds against a panel of PTPs and successfully identified a number of single digit µM inhibitors of PTP1B, TC-PTP and PTPσ. Common to our top hits is the salicylic acid moiety, which as previously described is a well-established pTyr mimetic. Although our studies determined that the IC₅₀ values that were equipotent with published inhibitors, we achieved selectivity against very structurally similar PTPs. In order to optimize our most potent inhibitor containing a single salicylic acid, we decided to explore the second aryl-phosphate binding site of PTP1B. Despite being catalytically inactive it is in close proximity to the catalytic site. This physical feature of PTP1B has profound implications for the design of a molecular inhibitor. As already mentioned, potent and selective PTP1B inhibitors can be created with further exploitation of the second or peripheral phosphate-binding site. Thus the following section of this chapter focuses on improving the potency and selectivity of our monosalicylate PTP1B inhibitors. This section will explore the design, synthesis, and evaluation of a new library of PTP1B-targeting inhibitors equipped with a second salicylic acid group with the aim to access the second pTyr binding site. The results herein demonstrate the utility of tethered di-salicylic acid-based inhibitors for targeting the PTP family of proteins.

2.1.2.1 Strategically Constructing Di-Salicylic Acid Inhibitors

In order to determine the best position to incorporate the second salicylic acid to our most potent monosalicylate inhibitor, we synthesized and screened a small library of compounds (Figure 2.8). We opted to replace the cyclohexylbenzyl moiety with salicylic acid and the structurally similar benzoic acid functionalities at the nitrogen-alkyl position to afford compounds 2.28 and 2.24, respectively. In addition, we placed the salicylic acid on the nitrogen-sulfonamide position and furnished compound 2.20. These compounds were tested in a similar fashion as previously described utilizing a DiFMUP assay against PTP1B, TC-PTP and PTPσ. We discovered that compound 2.20, containing the salicylic acid placed at the nitrogen-sulfonamide position, exhibited the highest degree of inhibition of PTP relative to any other compounds tested with low µM inhibitory concentrations. Interestingly, removal of the cyclohexyl benzene from our scaffold completely abolished PTP activity. Although we anticipated that attaching an additional salicylic acid would decrease inhibitory concentrations, we observed equipotent values with this line of inhibitors. Thus, in order to further discern what was occurring on a molecular level
between the compound and the active site of PTP1B, we performed *in silico* computational docking studies that unveiled an intriguing piece of data. As shown in Figure 2.8, once docked in the active site of PTP1B, the salicylic acid in compound 2.20 makes favourable interactions in the primary active site or phosphate-binding site.

**Figure 2.8.** Small library of our first-generation di-salicylic acid inhibitors, compounds 2.20, 2.24 and 2.28. Data for compound 2.20 is shown in the table and the corresponding docking image is shown on the right (PDB: 1NWL).

Unsurprisingly, the cyclohexylbenzyl moiety on the nitrogen-alkyl position is making favourable hydrophobic interactions with the side pocket containing residues Ile219, Gly259 and Val49 which explains the high IC\textsubscript{50} values of compounds without this functional group. Encouragingly, the positioning of the second salicylic acid in the active site is comparable to other salicylic acid inhibitors published in literature. However, it appears that the second salicylic acid was not probing or interacting with the second phosphate-binding pocket, but merely a few angstroms away from interacting with the desired pocket (highlighted in red in Figure 2.8). This result set the precedence for further optimization of this first generation di-salicylic acid inhibitor to feature a tethered salicylic acid group that extends further away from the core scaffold. We hypothesized that extension of the second salicylic acid will allow for this moiety to probe and make favourable interactions at the secondary site, thus potentially increasing the potency and improving selectivity. To rapidly prepare compounds with a series of linker lengths, we employed copper catalyzed click-chemistry (Table 2.2)
**Table 2.2.** Chemical structures of the Click chemistry library of tethered di-salicylic acid derivatives **2.38a-j.**

2.1.2.2 Synthesis of the Library

The synthesis of this library commenced by constructing the core scaffold outlined in Scheme 2.1. Starting from commercially available methyl ester glycine hydrochloride, tosilation and Boc protection of the sulfonamide nitrogen afforded compound 2.6. Boc protection was performed in order to protect the sulfonamide nitrogen from acting as a nucleophile in the subsequent peptide coupling reaction. Lastly, conversion to the corresponding acid occurred by treatment with lithium hydroxide monohydrate, in a 3:1 solvent ratio of THF to H$_2$O. Cyclohexylbenzaldehyde was synthesized starting from commercially available 4-methy cyclohexylbenzoate. This starting material was converted to the corresponding cyclohexylbenzalcohol utilizing harsh lithium aluminum hydride reducing conditions. Converting the methyl ester directly to the aldehyde is non-trivial, as most reducing agents are not capable of selectively reducing to the aldehyde.
Thus, complete conversion of the methyl ester to the alcohol and re-oxidation to the aldehyde using Swern oxidation conditions was a more facile and viable synthetic route.

Scheme 2.1. a) $p$-TsCl, DIPEA, CH$_3$CN, 0°C to r.t, 1 hr, 91%; b) Boc$_2$O, DMAP$_{cat}$. 0°C to r.t, DCM, 8-10 hrs., 80-90%; c) LiOH(H$_2$O), THF: H$_2$O (3:1), 16 hrs, r.t, 55%; d) $p$-TsCl, DIPEA, CH$_3$CN, 0°C to r.t, 1 hr, 91%; e) LiOH(H$_2$O), THF: H$_2$O (3:1), 16 hrs, r.t, 79%; f) i) LiAlH$_4$, THF, 0°C to r.t 5-8 hours; ii) EtOAc quench iii) 0.1 M HCl, 0°C, 79%; g) i) COCl$_2$, DCM, -78°C ii) DMSO, DCM, -78°C iii) cyclohexylbenzalcohol, C$_{13}$H$_{16}$O (2.10) iv) DIPEA, DCM -78°C, 16 hrs, 90%; h) BnBr, KOtBu, DMF, 0°C to r.t, 16 hrs. 41-48% i) NaBH(OAc)$_3$, AcOH, DCE, r.t, 16 hrs, 56-90%; j) 2.7, Cl$_2$PPh$_3$, CHCl$_3$, 125°C, 40 min, MW, 71%.
**Scheme 2.2.** a) SOCl₂, DMAP\textsubscript{(cat)}, Acetone, 71% b) NBS, Bz₂O₂, CCl₄, reflux, 16 hrs, 53%; c) 2.17, Cs₂CO₃, DMF, 82% d) TFA: H₂O (9:1), 1 hr, 75% e) 20% Pd/C H₂, 2:1 THF:MeOH, 16 hrs, 71% f) Cs₂CO₃, DMF, 90%; g) Aldehyde, AcOH, 4Å MS, CH₃CH₂Cl₂, 30 min; then Na(OAc)₃BH, R.T., 12 hrs, 77 – 98%; h) PPh₃Cl₂, 100 °C Microwave assisted heating, CHCl₃, 30 min, 60-87%; i) 20% Pd/C H₂, 2:1 THF:MeOH, 16 hrs, 90%; j) Cs₂CO₃, DMF, 90%; k) Aldehyde, AcOH, 4Å MS, CH₃CH₂Cl₂, 30 min; then Na(OAc)₃BH, R.T., 12 hrs, 77 – 98%; l) PPh₃Cl₂, 100 °C MW, CHCl₃, 30 min, 60-87%; m) 20% Pd/C H₂, 2:1 THF:MeOH, 16 hrs, 81%.
Scheme 2.3. a) COCl(CH2)nBr, DIPEA, CHCl3, 1 hrs, 85-95%; b) NaN3, DMF, 50°C, 16 hrs, 80-84%; c) SO2Cl-R, DIPEA, CH2Cl2, 16 hrs, 91-93%; d) BOC2O, DMAP, 0 °C to r.t. CH2Cl2, 4 hrs, 85-88%; e) LiOH(H2O) 3:1, 16 hrs, 71-81%; f) PPh3Cl2, 100 °C MW, CHCl3, 30 min, 60-87%; g) C3H7Br, Cs2CO3, DMF, 16 hrs, 71-77%; h) Sodium ascorbate (10% molar), CuSO4 (2% molar), t-BuOH:H2O (1:1), 35°C, 72 hrs, 59-68%; i) 20% Pd/C, H2, 16 hrs, 56-89%.
Furthermore, 4-amino salicylic acid was selectively benzyl protected and used in a reductive amination reaction with compound 2.11 to afford the secondary amine, compound 2.13. Due to the generation of hydrochloride acid (HCl) during peptide coupling, the removal of the Boc group is accomplished simultaneously in this step. Lastly, although numerous other peptide-coupling reagents were employed, utilizing PPh₃Cl₂ as a peptide-coupling reagent yielded the highest yields, incomparable to other peptide-coupling reagents such as TBTU, HBTU and DCC. If handled and stored properly, this reagent works effortlessly to afford the desired product in excellent yields. In order to install the salicylic moiety on the sulfonamide nitrogen position of the core scaffold (compound 2.20), 4-formyl salicylic acid was first protected utilizing the acetonide protecting group. Next, the methyl position was brominated using NBS in small portions and catalytic amounts of benzoyl peroxide (Bz₂O₂). This reagent was added in catalytic amounts in order to limit the di-brominated species forming. As shown in Scheme 2.3, compound 2.17 was installed on the core scaffold following the removal of the acetonide and the benzyl protecting group(s) to afford final compound 2.20. Attachment of the benzoic and salicylic acids on the nitrogen-alkyl positions was accomplished by first benzyl protecting 4-formyl benzoic acid and 4-formyl salicylic acid to afford compounds 2.22 and 2.23, respectively. These building blocks were subsequently reacted with compound 2.12 to afford two different secondary amines, compounds 2.26 and 2.27. The secondary amines were both separately peptide coupled with compound 2.9 utilizing conditions previously mentioned and lastly, benzyl groups were globally cleaved using 20% Pd/C to afford the final products (2.24 and 2.28).

Previous efforts to synthesize tethered di-salicylic acid compounds were made but with limited success. Firstly, we attempted to functionalize the salicylic acid moiety using a linker directly off the core structure. However, due to the localized pair of electrons on the sulfonamide nitrogen that imparts a low pKa and relatedly lower nucleophilicity and reactivity, we were unable to functionalize directly from this position. We established that this sulfonamide nitrogen was only nucleophilic enough to attack activated positions where there are two activating, or electron withdrawing substituents drawing electrons away from the carbon atom, increasing its electrophilicity. Secondly, we attempted to functionalize the salicylic acid to a linker to which then would be installed to the core scaffold. Similarly, the poor nucleophilicity of the 4-amino salicylic acid limited our success. Moving forward, in order to quickly and effortlessly synthesize a library of compounds with an extended linker that moved the salicylic acid moiety
away from the core scaffold with increasing carbon chain lengths, we opted to use copper catalyzed click chemistry driven syntheses shown in Scheme 2.3. We initially experienced poor yields (~42%) when using room temperature conditions but increasing reaction temperatures to 35°C considerably increased our yields (59-68%) and afforded us with the desired products. With these optimized conditions, a total of 10 compounds were synthesized which featured an extended salicylic acid moiety with linker lengths ranging from one to five. We chose not to pursue extensions greater than five. Since a carbon-carbon length is approximately 1.54 Å and the distance from the sulfonamide nitrogen to the secondary phosphate-binding pocket is approximately 12 Å, we assumed that a linker length varying from one to five would be adequate to probe our pocket of interest.

2.1.2.3 Preliminary Screening of Bidendate Disalicylic Acid Inhibitors

In order to analyze our second-generation disalicylic acid inhibitors, we employed the previously described fluorometric kinetic activity-based assay to measure the rate of DiFMUP hydrolysis normalized to a DMSO control. The results showed that at 10 µM concentrations compounds 2.38a, 2.38f and 2.38h-j potently suppressed TC-PTP, PTP1B and PTPσ quite efficiently, with percent inhibition ranging from 70-90% (Figure 2.9).

Figure 2.9. High-throughput kinetic enzyme activity screen for compounds 2.4c, 2.38a-j against the variety of phosphatases. Graph bars represent percent inhibition (100% = complete inhibition, 0% = no inhibition).
Consistent with our results from our previous study with compound 2.4c, these compounds also had little to no effect on PRL2 A/S, PTP-LAR (LAR(D1D2)), MKPX, or CD45(D1D2). We hypothesized the selectivity was a result of unfavourable scaffold interactions with the binding surface residues of the catalytic pocket of LARD1D2, MKPX, CD45D1D2 and PRL2 A/S. Since the results from the tolyl derivatives were not as consistent in comparison to the quinoline counterparts we did not investigate the tolyl derivatives in any further experiments. This could be attributed to their poor solubility and less favourable binding interaction with the PTP1B active site as determined by modeling studies.

Figure 2.10. Right: Tabulated IC₅₀ values for top compounds 2.38f-j are shown.

Having observed greater relative inhibition with the quinoline analogies against the receptor-like PTPσ and non-receptor PTP1B, compounds 2.38f-2.38j were evaluated at various inhibitor concentrations to quantify relative levels of PTP1B and PTPσ inhibition. Selected candidates were further evaluated against a panel of other PTPs to scope the degree of selectivity among the PTP family members. Dose dependent enzyme activity rate curves for compounds 2.38f-2.38j are shown in Figure 2.10. IC₅₀ values were determined to range between 570 nM and 17.2 µM; the IC₅₀ values for each compound are presented. From this study we identified 2.38f and 2.38h...
which in addition to being greater than 2-fold more potent than 2.4c, they displayed a 4-fold selectivity for PTP1B over PTPσ. Additionally, we identified compound 2.38i, a nanomolar PTPσ inhibitor with 4-fold selectivity over PTP1B.

In attempt to rationalize the achieved selectivity, we observed that generally inhibitors that contained shorter linker lengths exhibited substantial selectivity towards PTP1B over PTPσ. On the other hand, inhibitors that contained longer linker lengths exhibited selectivity towards PTPσ over PTP1B. As mentioned previously, obtaining even subtle levels of selectivity between PTPs is of paramount importance since most PTP binders lack specificity and suffer from off-target effects. Thus to discern the binding orientations and determine the rational behind the selectivity of these inhibitors, we performed in silico computational docking studies with PTP1B. Encouragingly, once we examined inhibitor 2.38f (n=1, quinoline derivative) docked in the active site of PTP1B, we determined this inhibitor makes favourable interactions in both the primary and secondary phosphate-binding sites.

**Figure 2.11.** Docking studies of candidate 2.38f (blue) with PTP1B shows the salicylic acid embedded in the primary phosphate binding site and the second salicylic acid making favourable binding interactions with residues at the vicinity of the secondary phosphate-binding site (amino acid residues Lys41, Asn44 and Cys47 highlighted in red). The binding mode is similar to SP7343-SP7964 (green), a known pTyr mimetic and potent inhibitor of PTP1B (PDB: 1NWL).
As shown in Figure 2.11, the salicylic acid functionalized from the nitrogen-sulfonamide is still bound to the primary phosphate-binding site but the second extended salicylic acid is now probing the polar secondary phosphate-binding site and making favourable hydrogen bond interactions with residues Lys 41 and Cys 47. In addition, the cyclohexyl benzene moiety has maintained its ability to engage in favourable hydrophobic interactions with the adjacent hydrophobic pocket (residues Ala217, Gly259, Ale219, and Val49). Interestingly, we determined through our computational docking studies that the primary and secondary phosphate-binding sites in PTP1B are approximately 21 Å apart. In addition, analysis of PTPσ revealed a proposed neighbouring pTyr binding pocket (Figure 2.12, residues Lys1417 and Asn1420) that was situated approximately 27 Å away from the active site or primary phosphate binding site. Thus, this explains why inhibitors with short linker lengths are more selective towards PTP1B since the distance between the two binding pockets is much shorter than in PTPσ. Similarly, inhibitors with longer linker lengths are more selective towards PTPσ as the distance between the two binding pockets is much greater. Thus, a longer linker length is required to probe these two binding pockets.

Figure 2.12. PTPσ crystal structure identified three main pockets (A, B and C) and an adjacent site (D) which could potentially act as a second binding site (PDB: 2FH7).
In addition, since PTP1B is a NRPTP and PTPσ is a RPTP, their surface properties are significantly different. The active site and proximal site of PTP1B is largely electropositive whereas in PTPσ, the positive electrostatic potentials are mainly localized to the active sites\textsuperscript{27}. These variable electrostatic properties must be a discerning factor to the selectivity being observed. Unfortunately, poor resolution of the crystal structure for PTPσ has impeded docking studies and alternative docking programs are being considered for further investigation. Thus, further docking experiments are required before any further conclusions can be made.

![Figure 2.13](image.png)

**Figure 2.13.** Michaelis-Menten rate data as a function of substrate concentration for 2.38f and 2.4c. Curves represent the hydrolysis of DiFMUP and subsequent fluorescent detection at 450 nm (excitation of 358 nm). Curves below show Lineweaver-Burke transformations. For 2.38f, $K_m$ increases as a function of substrate concentration as $V_{max}$ stays constant, indicative of a competitive inhibitor. For 2.4c, $K_m$ decreases as a function of substrate concentration as $V_{max}$ decreases, indicating mixed inhibition.
Lastly, we performed kinetic experiments to explore the kinetic activity of compound 2.38f via classical Michaelis-Menten studies that were performed as previously mentioned. The analysis of the graph revealed that this compound is a competitive inhibitor of PTP1B ($K_i = 1.83 \pm 0.39 \ \mu M$ Figure 2.13). For consistency, the $K_i$ for compound 2.4c was also determined ($K_i = 5.66 \pm 2.15 \ \mu M$, mixed inhibition). Encouraged by these results, we can be certain that based on the mode of inhibition, our novel, potent and selective di-salicylic acid inhibitor 2.38f is unmistakably binding to the active site on PTP1B. Currently, we are currently performing Michaelis-Menten studies on all of our top inhibitors.

2.2 Conclusion

Discovering potent and selective ‘drug-like’ PTP inhibitors has been a daunting task for medicinal chemists and pharmaceutical companies ever since this diverse and phenomenal class of enzymes has been recognized as a viable clinical targets. We set out to determine if potency and selectivity can be achieved through rationally designed small molecules that contain more orally bioavailable, cell permeable scaffolds and pTyr mimetics. Starting from a large library of salicylic acid or pTyr mimetic based STAT3 inhibitors, we found compound 2.4c that displayed low µM potency in enzymatic functional assays against a few select PTPs. This provided a promising lead for further investigation that led to optimization strategies that identified compounds 2.38f and 2.38h. Excitingly, these inhibitors were greater than 2-fold more potent than our previous lead 2.4c and displayed 4-fold selectivity for PTP1B over PTPσ. In addition, we have discovered compound 2.38i, which astonishingly displays approximately 4-fold selectivity towards PTPσ with an IC$_{50}$ of 570 nM. Although, further comprehensive computational analysis must be completed to determine further details about the binding events, we are exceptionally encouraged to have improved upon the potency as well as the selectivity of our first generation inhibitors. We plan to further investigate these inhibitors in order to assess in vivo efficacy, oral bioavailability, as well as their clinical viability.

2.3 Experimental Methods

Experimental methods for all compounds (intermediates and final) mentioned are available in Chapter 5: Appendix 1.
3 Artifici ally Induced Protein-Membrane Anchorage with Vanadium-Based PTP Inhibitors

3.1 Introduction

By nature, the majority of proteins in eukaryotic cells have evolved to contain a vast array of post-translational modifications that result in physical changes in their characteristics, functionality and most importantly, cellular localization. These post-translational modifications serve to sequester the targeted protein membrane, where it is then subjected to further modification for purposes of either activation and/or deactivation. Depending on the signaling cascade, protein localization can serve to increase effective molarity of the target protein in close proximity to membrane-associated enzymatic binding partners, modify a protein's tertiary conformation, and protein functionality. Although there are a large variety of lipid modifications, they are broadly organized into two categories; lipid modification that occurs in the cytoplasm or on the cytoplasmic face of membranes (N-myristoylation, S-palmitoylation, and prenylation (farnesylation and geranylgeranylation)), and those that occur in the lumen of the secretory pathway (addition of glycosylphosphatidylinositol, addition of cholesterol, N-palmitoylation and O-acylation). For example, the best-characterized lipid modification that occurs in the lumen of the secretory pathway is the attachment of glycosylphosphatidylinositol (GPI) anchors. This particular lipid modification is composed of a phosphatidylinositol connected through a carbohydrate linker to the protein. After the GPI moiety is added to the protein in the endoplasmic reticulum (ER), the protein travels through the secretory pathway to the cell surface, where the PGI anchor tethers the protein to the extra-cellular face of the plasma membrane for excretion.

These elegant and profound, yet simple chemical modifications and the physiochemical effects they generate has inspired numerous researchers to mimic and employ very similar chemical modifications to investigate and/or inhibit cellular pathways by lipitating therapeutics. Lipidated therapeutics have been prepared by simple covalent conjugation of a lipid group (such as those shown in Figure 3.1) to an alcohol, amino, or acid group on a peptide/peptidomimetic inhibitor via amide, ester, and carbamate linkages. In general, they are designed to target a specific protein with the peptidic inhibitor and anchor it within the cell membrane (Figure 3.2). In addition, peptide inhibitor lipidation is also utilized to significantly increase inhibitor cell
penetration and \textit{in vivo} half-life. Although researchers have synthetically prepared ‘prenylated’ inhibitors bearing a hydrophobic appendage for purposes of targeting membrane associated proteins, the concept of sequestering cytosolic signaling proteins to the plasma membrane via small molecule protein membrane anchors (PMAs) was only recently introduced by our group and coworkers\textsuperscript{91}.

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{chart}
\caption{Natural lipidation of proteins in the cell leads to protein-membrane localization. There are several other methods of lipidation, listed on the right side along with the modifying group associated with the modification. Adapted from Ref [71 & 72].}
\end{figure}

They postulated that the induced membrane anchorage of proteins involved in cancer-promoting cell-signaling cascades could hold significant therapeutic value, and thus they applied this methodology against the oncogenic STAT3 protein. Briefly, STAT3 plays a key role in relaying cytokine or growth factor signaling to the nucleus, where it binds to specific DNA-response elements in the promoter region of target genes and thereby induces cancer-promoting gene expression profiles. Constitutively active STAT3 leads to uncontrolled progression of cancer and is present in over 65\% of all primary breast tumors. Thus STAT3 is considered a clinically viable
target for therapeutic intervention\textsuperscript{95}. Most traditional inhibitors seek to disrupt the activated protein dimer from forming, but Gunning et al. inhibited the cellular motility of STAT3 by artificially inducing STAT3-membrane anchorage via the PMA approach\textsuperscript{89}. As shown in Figure 3.3 (1) they designed two different PMAs; PMA1 contained a cholesterol membrane anchor and the potent STAT3-binding peptide sequence and PMA 2 contained a cholesterol membrane anchor, a fluorescein moiety and the potent STAT3-binding peptide sequence. Their proof of concept was assessed utilizing in vitro liposome experiments to determine whether their PMAs could sequester the fluorescently labeled 93kDa STAT3 protein at a lipid membrane. As shown in Figure 3.3, 2C in the presence of PMA1, approximately all of STAT3 was localized to the membrane in multimellar vesicles (MLVs) and large unilamellar vesicles (LUVs). In addition, the induction of STAT3 membrane anchorage and inhibition of nuclear translocation of STAT3 by PMA 1 was determined in MDA-MB-231 breast tumor cells that harbor constitutently activated STAT3. Shown in Figure 3.3(3), they observed complete inhibition of STAT3 nuclear translocation in PMA-treated cells (yellow co-localization on the membrane), whereas untreated MDA-MB-231 cells, activated STAT3 resided predominantly in the nucleus. Overall, they demonstrated that PMA-induced protein localization is a conceptually viable therapeutic strategy and more importantly, presented a novel approach to targeting aberrant signaling proteins associated with human disease.

![Diagram](image)

**Figure 3.2.** Lipidated therapeutics, (1) once embedded in a membrane, can (2) recruit and inhibit soluble, cytosolic proteins to a membrane or, (3) inhibit membrane-bound proteins and increase the cell permeability of molecules. Lipidated therapeutics also (4) increase the cell permeability of polar molecules\textsuperscript{91}. 
In summary, recent advances in this field have envisaged that polar, poorly cell-permeable molecules previously rejected as therapeutics may be recycled as lipo-molecules. This chapter describes the first ever application and the design of lipo-molecules that are rationally designed towards targeting the most well studied member of the PTP family, PTP1B. Our rational and approach for this application will be described in the following section.

**Figure 3.3.** (1) Strategy of protein-membrane anchorage and chemical structures of PMA 3.1 and 3.2. (2) Wide-field fluorescence images of liposomes; A: an MLV prepared with PMA 3.3 (1 mol%), B: an LUV encapsulating tetramethylrhodamine (TMR)-tagged STAT3 in the absence of the inhibitor, C: an MLV encapsulating TMR-tagged STAT3 in the presence of PMA 3.1. Scale bars: 1µ M. (3) Induction of STAT3 membrane anchorage and inhibition of nuclear translocation of STAT3 by PMA 3.1. MDA-MB-231 breast tumor cells were treated with PMA 3.1 for 6 h (25 µ M), immunostained with membrane stain FM-4-64 (red) or anti-STAT3 antibody (green) or stained with DAPI (nucleus, blue), and analyzed by laser-scanning confocal microscopy. Adapted from Ref [70].
3.2 Rationale

We wished to explore the therapeutic potential of applying the principles of protein anchorage to the development of conceptually novel drug modalities that target the large family of PTPs. Our objective was to develop a scaffold that could effectively sequester PTP1B at the plasma membrane and suppress its motility within a cellular environment. As mentioned, PTPs are known for their roles as negative regulators in both insulin and leptin signaling pathways, and have prominent responsibilities associated with the progression of various metabolic disorders and cancers. Although there are approximately 21 different RPTPs that are already bound to the membrane, there are approximately 17 NRPTPs, such as PTP1B and TC-PTP, which are able to freely dephosphorylate many receptors in the cell. Thus, by binding to their active sites and inhibiting their motility through PMA-induced protein-membrane association, can in turn effectively prevent their participation in the many cellular signaling pathways that lead to diabetes, obesity and a variety of cancers (Figure 3.4).

Figure 3.4. An overview of the protein membrane anchorage strategy. Over activated PTP1B dephosphorylates the IR, inhibiting glucose (yellow G) from entering the cell, leading to the progression of diabetes and/or obesity. Vanadium-based PMA inhibits motility of PTP1B in the cell which in turn restores the phosphorylated state of the IR, allows glucose to enter the cell and more importantly stops the progression of a variety of metabolic disorders.

Cumulating the overall discussion of vanadium-based PTP inhibitors, we chose to incorporate their potent capabilities and high binding affinities towards PTPs in our novel PMA inhibitors. We envisaged that these polar, poorly cell-permeable molecules, may be repurposed as potent,
more cell permeable lipo-molecules. Herein, we describe the first ever design, synthesis and application of novel PMAs which target the therapeutically relevant PTP1B. We postulated that the induced membrane anchorage approach could hold significant diagnostic and therapeutic potential that departs from the various traditional inhibitors already described in this thesis.

3.3 Synthesis of PMAs

Our primary objective was to synthesize compound PMA 1 shown in Scheme 3.1, which contains a direct attachment of cholesterol to our ligand of choice, 1,10-phenanthroline. This particular ligand is commercially available as 5-nitro-1,10-phenanthroline and 1,10-phenanthroline-5-amine. We opted to use the latter as we anticipated this would provide us with a reactive nucleophilic center which we could employ in our synthesis. We began by exploiting a variety of different conditions with the objective to convert the hydroxyl functionality on the cholesterol to a leaving group. Unfortunately, all attempts to convert the hydroxyl to a leaving group, such as tosylation, conversion to the bromide, or iodide were unsuccessful. This can be attributed to the level of hindrance at the secondary hydroxyl center which could prohibit a SN2 reaction at this position. Next, we attempted to oxidize the secondary alcohol to a ketone. Unfortunately, utilizing a variety of oxidizing agents, such as pyridinium chlorochromate (PCC), Dess–Martin periodinane, 2,2,6,6-Tetramethylpiperidinyloxyl (TEMPO), to name a few, led to unsatisfactory yields due to the formation of multiple undesired side products. For example, it was determined that the oxidation of cholesterol in DCM yields 50% cholest-4-en-3-one, 10% cholest-4-en-3-dione and 8% cholest-4-ene-3-ol-6-one 96. We determined that this was primarily due to the presence of the double bond in close proximity to the hydroxyl group that was also oxidizing and causing the formation of multiple side products. Thus, we opted for commercially available 5-α-cholestan-3-one that contains no double bond and a ketone instead of the hydroxyl functionality. It is important to note that while devising the synthetic scheme, we had to avoid all hydrolysable functionalities as during the last step, which is the coordination of vanadium to the phenanthroline ligand, a nucleophilic base is introduced. In brief, the coordination step introduces potassium hydroxide, a strong base with the capabilities of reacting and cleaving a variety of functional groups such as carbamates and acetates, for example.

Thus, employing 5-α-cholestan-3-one as our starting material we first exasperated a variety of reductive amination conditions to afford intermediates 3.3. First, after evaluating a variety of
different solvent ratios of MeOH and DCM, we determined that a 9:1 ratio was the ideal mixture. Since the 1,10-phenanthroline-5-amine is a highly polar ligand, soluble in MeOH and the 5-α-cholestan-3-one was inherently soluble in non-polar solvents (such as DCM) we opted to utilize a mixture of DCM and MeOH to ensure that both of our components were thoroughly dissolved. Being that the 1,10-phenanthroline-5-amine is evidently a poor nucleophile, due to the electrons on the primary nitrogen being highly delocalized into the phenanthroline ring, we allowed the reaction mixture to stir for 24 hours at 50°C before adding the reducing agent. This allowed sufficient time for imine formation to occur.

![Graphical representation of the two diastereomers that form during the reductive amination conditions utilized to synthesize compound 3.3.](image)

**Figure 3.5.** Graphical representation of the two diastereomers that form during the reductive amination conditions utilized to synthesize compound 3.3.

Following the addition of the reducing agent, the reaction was given another 24 hours to completely react, yielding compound 3.3 in low but satisfactory yields (51%). A major factor contributing to the yield is the different sides or ‘faces’ of the electrophilic ketone on cholesterol where the 1,10-phenanthroline-5-amine nucleophile can attack (Figure 3.5). Our results indicated that one diastereomer is present in higher proportions as compared to the other. This can be attributed to the fact that the top face of the 5-α-cholestan-3-one is substantially more hindered than the bottom face. This results in the nucleophile being less likely to attack from the top face than from the bottom, generating a higher proportion of the S-stereoisomer. Nonetheless, we successfully obtained a single isomer after we subjected the crude mixture to manual column chromatography.
Scheme 3.1. a) NaBH₃CN, AcOH, 4Å mol. sieves, MeOH:DCM (9:1), 72 hrs, 51%; b) H₂O, V₂O₅, KOH, ancillary ligand, ethanol, ~1 hrs, 45-51%; c) CH₃(CH₂)₁₄COOH, DCM, 100°C MW, 3 hrs, 79%; d) H₂O, V₂O₅, KOH, ancillary ligand, ethanol, ~ 1 hrs, 45-51%; e) KtOBu, BnBr, DMF, 0°C to r.t, 100%; f) CH₃(CH₂)₁₄COOH, DCM, DIPEA, 100°C MW, 3 hrs, 74%; g) 20% Pd/C, MeOH:THF (2:1), 61%; h) H₂O, V₂O₅, KOH, ancillary ligand, ethanol, ~1 hrs, 45-51%.

The final step involved a metallation reaction which yielded the desired bpV complexes in moderate yields (45-51%)⁹⁷. We confirmed the coordination of our metal to our ligand by utilizing ⁵¹ Vanadium (⁵¹V) NMR. The nucleus ⁵¹V has a high natural abundance of 99.75% and a high receptivity, outdating that of ¹³C by a factor of more than 2000. With a spin of -7/2, it belongs to the quadropolar nuclei with a comparatively (and conveniently) low nuclear quadropole moment, giving rise to reasonably narrow resonance lines⁹⁸. As a result, scientists have reported the use of ⁵¹V NMR as a facile and effective method to detect vanadium and the formation of vanadium complexes in organometallic compounds⁹⁹. Thus, we opted to employ
vanadium oxytrichloride (VOCl$_3$) as a standard and calibrated the corresponding peak to 0 ppm, while the presence of any additional peaks were an indication of a vanadium nuclei other than that of the standard. Since a variety of vanadium complexes and their associated chemical shifts for selected coordination types have been previously reported, we were able to discern the presence of our bipyrimidal pentagonal vanadium complex. For compound 3.4, we observed a chemical shift of -760 ppm. In addition, we employed Infrared Spectroscopy (IR) to also confirm the presence of frequency signals corresponding to the vanadium-oxygen and oxygen-oxygen bonds, as compared to literature (shown in Appendix 5, section 6.2). Thus utilizing both techniques, we confirmed that we constructed compound 3.4, the first ever vanadium-based PMA inhibitor synthesized to date.

Furthermore, with the understanding that palmitic acid was also a viable PMA in addition to cholesterol, we decided to synthesize a vanadium-based PMA utilizing palmitic acid as the membrane anchor component. Synthesis of PMA 2 commenced by reacting 1,10-phenanthrolin-5-amine with palmitoyl chloride, to yield compound 3.5 in high yields. Lastly, intermediate was subjected to the previously described coordination reaction which yielded the desired compound 3.6 in 45-51% yield. In order to study the importance of the cholesterol lipid anchor and the phenanthroline ligand in our experiments, we decided to also synthesize PMA 3 containing a palmitoyl anchor and a picolinic ligand. This compound was synthesized in a similar manner (Scheme 3.5). First, 5-aminopyridine-2-carboxylic acid was benzyl protected and then subsequently utilized in a simple SN2 reaction with palmitoyl chloride. After removal of the benzyl group via 20% Pd/C, compound 3.9 was coordinated as previously mentioned to yield the final compound 3.10.
Scheme 3.2. a) SOCl$_2$, EtOH, 70°C, 30 hrs, 100%; b) K$_2$CO$_3$, DMF, r.t, 16 hrs, 73%; c) TFA, DCM, 90%; d) 5-α-cholestan-3-one, THF, C$_5$H$_{10}$NO$_3$P, LiOH, r.t, 16 hrs; e) 20% Pd/C, MeOH:THF (3:1), quantitative yield; f) THF, LiAlH$_4$, 3 hrs, r.t, 45%; g) K$_2$CO$_3$, DMF, r.t, 16 hrs, 89%; h) NaBH$_3$CN, AcOH, 4Å mol. sieves, MeOH:DCM (9:1), 72 hrs total, 71%; i) THF:H$_2$O (3:1), LiOH(H$_2$O), 45°C, 16 hrs, 71%; j) TBTU, DIPEA, r.t, 16 hrs, 60%; k) PPh$_3$Cl$_2$, C$_2$H$_3$N, 100°C, 1 hr, 10% l) H$_2$O, V$_2$O$_5$, KOH, ancillary ligand, ethanol, ~1 hrs, 45-51%.
The fluorescein based PMA was synthesized by first protecting the carboxylic acid functionality on the starting material, fluoresceinamine isomer I (Scheme 3.2). This ensured that this functionality was unable to conjugate to the ring of the compound and form the non-fluorescent isomer. Next the intermediate (3.11) was treated with potassium carbonate and tert-butyl bromoacetate to afford compound 3.12. Cleavage of the tert-butyl functionality with trifluoroacetic acid (TFA) in DCM yielded the free carboxylic acid appendage. We synthesized the diethylamine-cholesterol building block by first utilizing Horner–Wadsworth–Emmons conditions to afford the vinyl cyano intermediate (3.14). In the two consecutive reactions the double bond was reduced under Pd/C hydrogenation conditions and the cyano functionality was reduced utilizing LiAlH$_4$ to yield compound 3.16. Although it was reported by Knölker and coworkers that the hydrogenation step yields a single isomer, however we determined otherwise$^{100}$. Utilizing a 2D Heteronuclear Single-Quantum Correlation (HSQC) experiment, we confirmed the presence of two isomers or diastereomers as products of this reaction. HSQC is frequently used in NMR spectroscopy of organic molecules and is of particular interested in the field of protein NMR. Typically, the resulting spectrum is two-dimensional with one axis for $^1$H and the other for a heteronucleus most often $^{13}$C or $^{15}$N$^{101}$. For our purposes, the spectrum showed us a peak for each unique proton attached to the $^{13}$C heteronucleus being considered. As shown in Figure 3.6, we were aiming to distinguish what components were present in our sample; either we had obtained only one isomer (A) as reported in literature or we had obtained two diastereomes (B). If we had in fact only one single isomer, then the two protons (blue and pink) highlighted on the $^1$H-NMR Spectra would be present on one carbon (or correspond to one carbon peak) on the $^{13}$C-NMR spectra. Unfortunately, as shown and directed with red arrows, the two different protons (and their associated peaks) most definitely correspond to two different carbons (or are present on two different carbons), confirming that both of these proton peaks should integrate to two protons each, and the cholesterol portion to ~90 protons (each cholesterol moiety corresponds to a total of 45 protons in the $^1$H NMR). Fortunately, we were able to successfully separate these two diastereomers utilizing manual column chromatography by employing an isocratic solvent system (98% Hexanes and 2% Ethyl acetate). For simplicity, we decided to proceed with the single isomer to the next step in our synthesis.
Figure 3.6. 2D HSQC Experiment of compound 3.15. Vertically shows the $^1$H-NMR Spectra and horizontally shows the $^{13}$C-NMR Spectra.

Once we had obtained this building block in its pure isomeric form, we employed a peptide coupling reaction utilizing TBTU which provided us with the desired product (3.20) in moderate yields (60%). Furthermore, the 1,10-phenthrolin-5-amine ligand was utilized in a reductive amination reaction with compound 3.17 to afford compound 3.18. Subsequently, the benzyl protecting group was cleaved utilizing a lithium hydroxide hydrolysis reduction, to afford the final product (3.19). Due to a variety of synthetic complications, we were unable to successfully synthesize compound 3.31 in acceptable yields. We are currently evaluating other synthesis routes to obtain candidate 3.31 in more acceptable yields.
3.4 A Spectroscopic Study On The Coordination and Solution Structures Of The Interaction Systems Between The Biperoxidovanadate Complex and The Phenanthroline Ligand

Prior to with the biological evaluation of our novel PMAs, we decided to quantitatively measure the formation constant or equilibrium constant, $K$ (or $K_{eq}$) for the interaction system between bpV (bisperoxovanadate) and the phenanthroline ligand. Although we understood that this metal complex displays a high affinity towards PTPs, we wanted to further discern the affinity of the ligand towards the vanadate metal\textsuperscript{86}. Although there have been similar studies presented in literature for the determination of the formation constant for other ligands, there was unfortunately a lack of precedence for the experimental determination of the affinity of the phenanthroline ligand to the bpV metal\textsuperscript{102}. Thus, in order to quantitatively determine the coordination affinity of the ligand, we analyzed the fluorescence quenching of the ligand with increasing concentrations of bpV. Shown in Figure 3.7 is the fluorescence emission spectra of the 1,10-phenanthroline ligand with increasing concentrations of the bpV metal complex. Upon successive additions of the metal complex, we observed a consistent florescence quenching of the ligand.

![Diagram of bpV and 1,10-phenanthroline interaction](image)

**Figure 3.7.** The fluorescence emission spectra of phenanthroline ($1 \times 10^{-4}$ mol L$^{-1}$) with different concentrations of bpV. [bpV] = 0, 1, 2-12 $\times 10^{-4}$ mol L$^{-1}$ ($\lambda_{310}$ nm).
Since it is understood that the molar ratio between bpV and the phenanthroline ligand in the newly-formed peroxidovanadate complexes is 1:1, the formation constant can be analyzed by using the Benesi-Hildebrand equation. This equation is shown in Figure 3.6, where $K$ is the formation constant, $I_0$ is the initial fluorescence intensity of the free phenanthroline ligand, $I'$ is the minimum fluorescence intensity, and $I$ is the observed fluorescence. According to the equation, the formation constant can be extrapolated from the intercept and the slope of the equation of the straight line from a plot of $1/(I-I_0)$ vs. $1/[bpV]$ (Figure 3.8).

![Benesi-Hildebrand equation](image)

**Figure 3.8.** Fluorescence spectra of the Benesi-Hildebrand plot for the interaction between 1,10-phenanthroline and bpV.

We determined the $K_{eq}$ to be 500 M. This also equates to $K_d$ or dissociation constant of 2.0 mM ($K_d = 1/K_{eq}$). A recent publication by Li. X., *et al* reported the use of density functional calculations to theoretically determine the free energy changes for the formation constant of the 1,10-phenanthroline-5-amine bpV complex. To the best of our knowledge, this is the only report that attempts to determine this value, however experimental evidence to implement these results has yet to be established. In their study Li and coworkers reported a delta G of binding ($\Delta G$) of -6.8 kcal/mol that will give a $K_a$ on the order of $8.6 \times 10^4$ which on conversion to $K_d$ (the reciprocal) equates to $1.17 \times 10^{-5}$ or 12 µM. Thus, we were unable to replicate their theoretical values with utilizing the fluorescence quenching experiment. However, it is in this author’s
opinion that currently there lacks a substantial amount of evidence to further discern any information about the affinities for the metal to the ligand. It has been noted that temperature plays an important factor in the formation of these complexes. Thus, we are currently optimizing our fluorescence experiments in order to determine if we observe a lower dissociation constant at higher temperatures. In addition, there a variety of other factors that affect the stability of these complexes, such as pH and salt concentration, which must be taken into consideration in any further experiments. Overall, these metal complexes have been utilizing in a number of in vitro and in vivo experiments published in a variety of high impact journals, with no reported complications of rapid degradation or de-metallation from the ligand.

We believe that this particular complex holds immense potential and applicability in our novel PTP based PMAs.

3.5 In vitro Supported Lipid Bilayer Studies

In order to assess our proof-of-concept, we developed a series of in vitro immunofluorescence-based experiments that would allow visualization of PMA-induced PTP1B protein localization in lipid models. For the purposes of our experiments, we decided to initially utilize a supported lipid bilayer rather than the traditional multilamellar vesicles (MLVs) to test our preliminary experiments, since supported lipid bilayers are substantially more stable than MLVs. In order to visualize the localization of PTP1B, we decided to utilize a three-component system containing a primary and secondary (fluorescent) antibody, and also our protein of interest, PTP1B. Since we were unable to express, purify and isolate Green Fluorescent Protein (GFP)-tagged PTP1B for visualization purposes we opted to utilize a fluorescent antibody. Thus, we hypothesized that with a supported lipid bilayer, we would be able to simultaneously measure PTP1B-PMA membrane interactions and optimize and fine-tune the concentrations of each component for further experiments on MLVs. Experiments were conducted by assembling a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid bilayer supported on a glass coverslip. We incubated, PTP1B protein, primary PTP1B Antibody (H-135) rabbit polyclonal Immunoglobulin G (IgG) and the secondary Alexa Fluore®488-anti-rabbit antibody. Briefly, the primary antibody is designed to bind directly to PTP1B and the fluorescently labeled secondary antibody is designed to recognize and bind to the primary antibody. This ensures that we are able to visualize the locality of our protein under custom-built total internal reflection (TIRF) microscopes. Several water washes were performed following incubation to remove excess
unbound antibody and protein. As shown in Figure 3.9 (1), the nonspecific absorption of single fluorescently tagged PTP1B proteins to the lipid bilayer was observed by (TIRF) imaging. Since the fluorescence signal was so dim, we increased our signal gain to x500 for improved visualization. This fluorescent signal was similar to the protein adhesion observed for the hydrophilic surface of cleaned glass coverslips, indicating limited to no adhesion of PTP1B on the lipid bilayer. We also determined background fluorescence by incubating ~2 mol % compound 3.4 with the lipid bilayer. We observed no fluorescent background signal with x500 gain (Figure 3.9 (2)). In contrast, when the lipid bilayer was exposed to compound 3.4 (~2 mol %) prior to incubation with both antibodies and PTP1B, we observed a very large amount of fluorescence. The fluorescence intensity was so high that not a single molecule was discernable. Since we were able to visualize the fluorescence without a x500 signal gain, this indicated that there was at least 100 times more fluorescence on the bilayer. Thus, we are exceptionally encouraged by these preliminary supported lipid bilayer results and we are currently optimizing our TIRF imaging in order to better discern and quantify the amount of protein sequestered to the lipid-surface area. Furthermore, since we have an established protocol, we plan on performing MLVs experiments to determine the distribution of our fluoresceinated PMA in micrometer-sized lipid vesicles in the absence and presence of PTP1B.

Figure 3.9. (1) Supported Lipid Bilayer containing 2 µg/mL Alexa Fluore®488-anti-rabbit antibody, 5 µg/mL PTP1B Antibody rabbit polyclonal IgG and 2 µg/mL PTP1B incubated, then washed with water (x500 signal gain). (2) Supported Lipid Bilayer containing ~2mol % compound 3.4 (x500 signal gain). (3) Supported Lipid Bilayer incubated with 2 µg/mL Alexa Fluore®488-anti-rabbit antibody, 5 µg/mL PTP1B Antibody rabbit polyclonal IgG, 2 µg/mL PTP1B and ~ 2mol % compound 3.4, and then washed with water. This image was not under x500 signal gain, indicating that there was at least 100 times more fluorescence on the bilayer.
3.6 *In Vitro*, Whole Cell Localization Studies Utilizing GFP-Tagged PTP1B

In addition to our supported lipid bilayer studies, we performed a variety of *in vitro*, whole cell studies utilizing GFP-tagged PTP1B. We decided to pursue immunofluorescence studies that would determine the ability of PMA 1 to localize PTP1B to the membrane in live cells. First, we expressed GFP-tagged PTP1B in a CHO cell line and studied the distribution of PTP1B in the cell. Due to the nature of this study, we would ideally require the uniform distribution of PTP1B throughout the cell. Only under such circumstances it would be possible to visualize the membrane localization of the protein post treatment with the inhibitor. However, as shown in Figure 3.10, we observed the exact opposite.

![Figure 3.10](image)

**Figure 3.10.** CHO-cells expressing GFP-tagged PTP1B grown in OptiMEM media. Top Row: Cells imaged to visualize basic morphology and localization of GFP-tagged PTP1B prior to introduction of compound 3.4 (PMA 1). Bottom row: Cells imaged after the addition of increasing concentrations of compound 3.4 (PMA 1) and measured time points at 30, 60 and 90 mins, respectively from left to right as indicated.
Coincidently in all three dishes the GFP-tagged PTP1B localized on the membrane, prior to the introduction of the inhibitor (3.4). As mentioned previously, the majority of PTP1B’s substrates are present on the membrane. Additionally, we also determined that our media contained ~10% fetal bovine serum (FBS), which in our case acts as a stimulant for the cells. Therefore, naturally GFP-tagged PTP1B would migrate to the cell membrane and dephosphorylate its many receptors, a process which we are aiming to prevent utilizing our vanadium-based PMA inhibitors. Thus, in order to prevent localization of PTP1B to the membrane prior to treatment of inhibitor, we opted to starve the cells of FBS with plain media in order to see if this would alter the localization of the GFP-tagged PTP1B.

![Figure 3.11. CHO-cells expressing GFP-tagged PTP1B grown in stimulant free media. Left: Cells imaged to visualize morphology and localization of GFP-tagged PTP1B prior to introduction of compound 3.4 (PMA 1). Bottom row: Cells imaged after the addition of 0.05 µg/mL compound 3.4 (PMA 1) and measured at 30 mins. Arrows indicate localization or distribution of GFP-tagged PTP1B.

After we expressed GFP-tagged PTP1B in our regular OptiMEM media and subsequently deprived them of any stimulant, GFP-tagged PTP1B was considerably less localized near the membrane than in our previous experiment. As shown in Figure 3.11, PTP1B appears to be scattered uniformly throughout the cytoplasm. This image indicated that our stimulant-free media had successfully prevented GFP-tagged PTP1B from sequestering to the membrane prior
to the introduction of our PMA 1 inhibitor (3.4). This provided us with an ideal control which we could utilize to accurately determine the affect of our inhibitors in these experiments. Encouragingly, after treatment of the cells with 0.05 µg/mL PMA 1 (3.4), we visualized an increase in membrane localization of GFP-tagged PTP1B on the membrane after 30 minutes. In addition, we also performed a very similar experiment where we allowed a longer incubation period with our inhibitor (Figure 3.12). We determined that a longer incubation period showed no drastic differences to the localization of PTP1B than when incubation time was only 30 minutes.

In summary, the preliminary results are very encouraging and inhibitor 3.4 shows promise as a first in class vandate based inhibitor to be employed in a membrane anchorage tactic. However, a variety of experiments must be performed to further establish the extent of PTP1B anchorage which requires further cellular studies at variable concentrations of 3.4. Additionally, in order to clearly visualize the cellular components we plan on employing two different immunostains, FM-4-64 (red) and DAPI (blue) in order to simultaneously stain the nucleus and the membrane, respectively. This will determine if the localization of the PTP1B is a result of the cells simply changing morphology and reducing in size or a direct result of the PMA inhibitor.

**Figure 3.12.** CHO-cells expressing GFP-tagged PTP1B grown in stimulant free media. Left: Cells imaged to visualize morphology and localization of GFP-tagged PTP1B prior to introduction of compound 3.4 (PMA 1). Bottom row: Cells imaged after the addition of 0.01 µg/mL compound 3.4 (PMA 1) and measured time point at 20 hrs.
3.7 Future Directions

Although our preliminary data is very encouraging, we still have a number of experiments to conduct before we are able to fully discern the binding mode and potential of our novel vanadium-based PMAs. Once we have synthesized our fluorescein-based vanadium PMA, we are planning on utilizing this inhibitor in a variety of our experiments that will visually assess the anchorage capabilities in lipid vesicles. In addition, we plan on utilizing similar strategies in our \textit{in vitro} experiments to further discern the efficacy of our novel PMAs in whole cells. As mentioned, we also plan to study the structural morphology of the CHO cells with nucleus and membrane stains. Furthermore, we are also currently designing and planning Western blot experiments can help analyze the levels of phosphorylated IR and a variety of other direct phosphorylated substrates of PTP1B, such as JAK2 and IRS1. Analyzing the levels of substrates will determine the ability of our PMAs to directly inhibit and more importantly affect the localization of PTP1B in the cell. If we are directly affecting the localization, most phosphorylated receptors should remain in their phosphorylated states, thereby preventing the initiation and/or progression of aberrant disordered or diseased states of cells. It in this author’s opinion that novel approach in targeting PTPs utilizing artificially induced protein membrane anchorage holds immense therapeutic potential and grasps immediate relevance to the field of lipo-therapeutics.

3.8 Experimental Methods

Experimental methods for all compounds (intermediates and final) mentioned are available in Chapter 6: Appendix 2.
4 Concluding Remarks

This thesis has been concerned with the development of scaffolds designed to target the most popular and attractive biochemical class in drug discovery, enzymes. More specifically, this work has incorporated a multifaceted approach that has led to the design, synthesis and evaluation of targeted-molecular inhibitors of therapeutically relevant PTPs.

Chapter 2 explored the development of salicylic acid based pTyr mimicking inhibitors. Screening a large library of >70 salicylic acid based compounds, we identified six lead candidates which inhibited select PTP members with low single µM activities ranging from 3.6-6.3 µM. All lead candidates contained the pertinent pTyr mimetic salicylic acid at the N-alkyl position. Although we observed promising activity, our lead candidates failed to display selectivity amongst PTPs. In order to discern the binding mode of the lead candidate 2.4c, we performed computational docking analysis using Genetically Optimized Ligand Docking (GOLD) software. This indicated that the salicylic acid of candidate 2.4c was bound to the primary phosphate-binding pocket. In addition, we observed the secondary phosphate-binding site to be approximately 20Å away from the primary phosphate-binding site. We hypothesized that bivalent inhibitors possessing two salicylic acids, one that could bind to the primary site and another that could bind to the secondary site would potentially furnish more selective and potent inhibitors of PTP1B. This approach was explored in the remaining sections of this chapter by the development of a library of tethered disalicylic acid inhibitors. Preliminary screening identified compounds 2.38f and 2.38h, which displayed potency and selectivity for PTP1B with IC₅₀’s of 3.0 and 1.7 µM, respectively. These inhibitors were greater than 2-fold more potent than our previous lead 2.4c and displayed 4-fold selectivity for PTP1B over PTPσ. In addition, we have discovered compound 2.38i, which astonishingly displays approximately 4-fold selectivity towards PTPσ with an IC₅₀ of 570 nM. To emphasize, obtaining selectivity amongst the PTP family has been the goal of numerous pharmaceutical companies and laboratories for many decades. Thus, discovery of these novel inhibitors lends credence to rational drug design and to the field of medicinal chemistry.
Chapter 3 explores the first ever application of applying the principles of protein anchorage to the development of conceptually novel modalities targeting PTPs. Three novel and innovatively designed scaffolds containing vanadium complexes furnished onto lipid anchors were synthesized and evaluated in both supported lipid bilayers and in in vitro whole cells. Supported lipid bilayers indicated that in the presence of compound 3.4, we observed 100 times the amount of fluorescence on the membrane than in the absence of compound 3.4. This indicated that our novel vanadium-based PMA was able to effectively anchor fluorescently tagged PTP1B to the membrane. In the in vitro whole cell studies, we observed a questionable but encouraging result when we treated CHO cells expressing GFP-tagged PTP1B. Post treatment of inhibitor, we observed a substantial amount of GFP-tagged PTP1B sequestered to the membrane. Due to the complex nature of the study, we are inquiring about the morphology of the cells in order to determine if localization of the PTP1B is a result of the cells simply changing morphology and reducing in size or a direct result of the PMA inhibitor. Overall, we demonstrated that PMA-induced protein localization is a conceptually viable strategy that lends credence to the design of lipo-molecules and to the field of lipo-therapeutics.

This work has set to furnish potent and selective agents that target therapeutically relevant proteins, in particular PTP1B. Historically, obtaining selective and potent PTP1B inhibitors, or phosphatase inhibitors in general, seemed like an unattainable goal. With the use of rational drug design driven by in silico computational docking studies, we have successfully challenged this goal, achieved selectivity and maintained potency with our second generation, disalicylic acid inhibitors. These candidates hold immense clinical viability in the treatment of various metabolic disorders and cancers. Our vanadium-based scaffolds have immense diagnostic potential, as they can be an imperative tool for chemical biology and can further elucidate the significance of PTP1B in cellular signaling pathways related to a variety of diseases. Medicinal chemists have made significant progress in the field of phosphatases, and it is in this author’s opinion we are closer than ever in producing candidates with the potential to reach the clinic.
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5 Appendix 1: Experimental Methods for Chapter 2

5.1 Screening of the Library

5.1.1 Reagents

Papain and BSA were purchased from Sigma. HEPES was purchased from Fisher. DiFMUP was purchased from Invitrogen and Z-Phe-Arg-pNA from Cederlane.

5.1.2 Preparation of GST-fusion proteins

GST-PTP were prepared as previously reported\textsuperscript{106}. Purified GST-PTPs used are catalytic domain of PTP1B and TC-PTP, Full length MKPX and PRL2A/S (active mutant) and D1D2 of LAR and Sigma. All PTP were the human form.

5.1.3 Buffer Preparations

Enzyme reactions were performed in 50mM HEPES pH7.0 in which 3mM DTT and 0.1mg/mL BSA were added fresh. DiFMUP was used as substrate for all assays with GST-PTP. For Papain assay the substrate Z-Phe-Arg-pNA was used.

5.1.4 Kinetic Measurements

The hydrolysis of DiFMUP was conducted in black 96-well plates (Corning) in a final volume of 100µL at 25°C. The reaction was monitored by measuring excitation/emission 358/450 (for DiFMUP) or absobrance 405nM (for Z-Phe-ARG-pNA) using Varioskan plate reader (Thermo electron). Kinetic measurements were monitored over 10 minutes in 30 seconds intervals and rates were calculated using the slope (relative unit/min). Enzyme dilution was determined by choosing a reaction rate comprise in a Fluorometric range of 5-15 Fluorescence units/min (DiFMUP) or 0.3 OD405nm units (Z-Phe-ARG-pNA).

5.1.5 Compound Screening

Inhibitors were diluted in DMSO and kinetic reactions were performed in 1% DMSO final. 10uM compound were used for initial screen with DiFMUP as substrate. Compound inhibiting
any of the PTP were selected for a Papain screen to rule out oxidative molecules. A substrate concentration equivalent to the $K_m$ value for each enzyme was used.

5.1.6 IC$_{50}$ Determination Assays

For IC$_{50}$ a serial dilution starting at 30uM was made in assay buffer. IC$_{50}$ reactions were performed in less than 2% DMSO final. IC$_{50}$ values were derived by a sigmoidal dose-response (variable slope) curve using GraphPad Prism software. A substrate concentration equivalent to the $K_m$ value for each enzyme was used for IC$_{50}$ determinations. The reported data are average of at least three independent experiments.

5.1.7 Cell Culture, Inhibitor Treatment and Lysate Preparation

Cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1x gentamicin (Wisent). For FLT3-ITD/PTP1B expression, 293T cells were plated at 1x10$^6$ cells/well in 6-well plates 20 h prior to transfection. Cells were transfected with 0.5 µg hFLT3-ITD-HA DNA, 0.25 µg PTP1B DNA, and 1.5 µl Lipofectamine 2000 (Invitrogen) for 6 h according to the manufacturer’s directions. Following transfection, cells were serum-starved in 0.1% FBS DMEM overnight. For inhibitor treatment, media was then replaced with 0.1% FBS DMEM containing 1% DMSO and 10 µM of compound and cells were incubated a further 6 h. Cells were lysed in mRIPA with complete protease inhibitors (Roche) Vanadate and Sodium fluoride and analyzed by Western blot.

For insulin time-course assays, CHO-IR cells were used. Prior to treatment, cells were serum-starved for 2 h in plain DMEM, then media was replaced with DMEM containing 1% DMSO and 1, 5 or 10 µM of inhibitor. After an extra 3 h incubation, human insulin (Humulin R, Eli Lilly), diluted in starvation media, was added at 10 nM for 15minutes. Cell lysates were prepared and analyzed as described above. Quantification of band intensity was performed by densitometry using ImageJ software (NIH).
5.2 General Synthetic Methods and Characterization of Molecules

5.2.1 Chemical Methods

Anhydrous solvents methanol, DMSO, CH$_2$Cl$_2$, THF and DMF were used directly from their Sure-Seal bottles and were purchased from Sigma Aldrich. 4Å molecular sieves also purchased from Sigma Aldrich, were activated by heating to 300 °C under vacuum overnight. All reactions were performed in oven-dried glassware and were monitored for completeness by TLC using silica gel (visualized by UV light, or developed by treatment with KMnO4 stain or Hanessian's stain). A 400 MHz Bruker NMR was utilized to obtain $^1$H and $^{13}$C NMR spectra in CDCl$_3$, MeOD, or d$_6$-DMSO. All NMR Chemical shifts ($\delta$) are reported in parts per million after calibration to residual isotopic solvent and coupling constants (J) are reported in Hz. Inhibitor purity was evaluated by a Water's rpHPLC prior to biological testing. Analysis by rpHPLC was performed using a Microsorb-MV 300 Å C18 250 mm x 4.6 mm column with eluent flow set at 1 mL/min, and using gradient mixtures of (A) water with 0.1% TFA and (B) an acetonitrile solution containing 10% H$_2$O and 0.1% TFA. Ligand purity was confirmed using linear gradients from 50% A and 50% B to 100% B after an initial 2 minute period of 100% A, and a second linear gradient of 100% A to 100% B. The linear gradient consisted of a changing solvent composition of either (I) 5.2% per minute and UV detection at 254nm or (II) 1.8% per minute and detection at 254nm, each ending with 5 minutes of 100% B. When reporting the HPLC results, retention times for each condition are written followed by their purities in their respective order. Biologically evaluated compounds are > 95% chemical purity as measured by HPLC. Traces of the HPLC results are provided in supporting information.

5.2.2 General Procedures

**General Procedure a (Benzylation Reaction).** 4-amino salicylic acid (1.0 eq.) was dissolved in DMF in a suitable round bottom flask and was cooled to 0°C. Subsequently, potassium tert-butoxide, KOtBu (1.1 eq.) was added in slow additions until dissolved completely, following the drop wise addition of benzyl bromide, BnBr (1.1 eq.). The reaction mixture was brought back to room temperature and was allowed to stir overnight. The reaction mixture was concentrated,
dissolved in distilled water, and extracted with ethyl acetate to remove the DMF. Organic fractions were combined, washed with saturated sodium chloride solution and dried over anhydrous sodium sulfate, Na$_2$SO$_4$. This crude mixture was concentrated *in vacuo* and then dissolved in minimal amounts of DCM to which solid silica powder was added and the resulting suspension was dried under reduced pressure to afford a dried silica adduct. This adduct was then loaded onto the manual column and purified with a mixture of hexanes and ethyl acetate. Desired fractioned were pooled, concentrated *in vacuo*, co-evaporated with chloroform and desolvated under a high-throughput vacuum system to yield the desired product (yields 40-48%).

**General Procedure b (Swern Oxidation).** In a suitable round bottom flask, oxalyl chloride, COCl$_2$ (2.8 eq.) was dissolved in DCM and cooled to -78°C (temperature was attained by using dry ice in acetone). DMSO (4.7 eq.) was added drop wise in 1-2 mL portions to solution, strictly maintaining a nitrogen atmosphere Cyclobenzylalcohol (1.0 eq.) was added, followed by $N,N$-diisopropylethylamine, DIPEA (6.0 eq.) and reaction was stirred overnight under nitrogen atmosphere. Reaction mixture was concentrated *in vacuo*, dissolved in distilled water, and extracted with ethyl acetate. Organic fractions were combined, washed with sodium chloride solution and dried over anhydrous Na$_2$SO$_4$. This crude mixture was concentrated *in vacuo* and then dissolved in minimal amounts of DCM to which solid silica powder was added and the resulting suspension was dried under reduced pressure to afford a dried silica adduct. This adduct was then loaded onto a manual column and purified with a mixture of hexanes and ethyl acetate. Desired fractioned were pooled, concentrated *in vacuo*, co-evaporated with chloroform and desolvated under a high-throughput vacuum system to yield the desired product (yield 90%).

**General Procedure c (Reductive Amination).** In a suitable round bottom flask, cyclohexyl benzaldehyde (1.1 eq.) was dissolved in DCM or DCE and stirred with 3.0 Å molecular sieves at room temperature. Benzyl protected 4-amino salicylic acid (1.0 eq.) was added to this solution which was allowed to stir for 5 minutes following the addition of glacial acetic acid, AcOH (1.5 eq.). Lastly, sodium triacetoxyborohydride (NaBH(OAc)$_3$) (1.5 eq.) was added to the reaction mixture which was allowed to proceed overnight. Reaction mixture was concentrated *in vacuo*, a standard dH$_2$O/ethyl acetate work up was performed, and then purified by being transferred onto a silica gel column in a Biotage-Isolera™ auto-column for automated flash chromatography or the crude mixture was dissolved in minimal amounts of DCM to which solid silica powder was added and the resulting suspension was dried under reduced pressure to afford a dried silica
adduct. This adduct was then loaded onto the manual column and purified with a mixture of hexanes and ethyl acetate. Desired fractions were collected, concentrated in vacuo, co-evaporated with chloroform and desolvated under a high-throughput vacuum system to yield the desired product (yields 56-90%).

**General Procedure d (Amide couplings via acid chlorides).** To a stirred solution of commercially available acid chlorides (1.0 eq) at 0 °C in DCM (0.1 M) was added the dibenzyl protected 4-amino salicylic acid (1.1 eq) and the reaction stirred for 16 hrs. The reaction was quenched with H₂O and repeatedly extracted with ethyl acetate. The organics were combined and then washed with H₂O and saturated sodium chloride solution, dried over Na₂SO₄ and concentrated in vacuo. The resulting residue was purified using the Biotage-Isolera™ automated column chromatographer in a gradient of EtOAc and Hexane and then dried under reduced pressure.

**General Procedure e (Bromine to Azide Conversions).** To furnish the desired azide species, the coupled material (1.0 eq) from step a were treated with sodium azide (1.2 eq) in DMF (0.1 M) and heated at 50 °C overnight. The reaction was quenched with H₂O and repeatedly extracted with ethyl acetate. The organics were combined and then washed with H₂O and saturated sodium chloride solution, dried over Na₂SO₄ and concentrated in vacuo. The resulting residue was purified using the Biotage-Isolera™ automated column chromatographer in a gradient of EtOAc and Hexane and then dried under reduced pressure.

**General Procedure f (Sulfonylation of the secondary amine).** A solution of the methyl amino (1.0 eq) and DIPEA (3.0 eq) were dissolved in anhydrous acetonitrile and cooled to 0 °C before the addition of sulfonyl chloride (1.1 eq). The resultant solution was allowed to stir overnight at R.T. The solvent was removed and the residue redissolved in CH₂Cl₂. The organics were then washed sequentially with 0.1 M HCl, saturated NaHCO₃ and saturated sodium chloride solution. The organics were then dried over Na₂SO₄ and concentrated in vacuo. The material was carried forward without any purification.

**General Procedure g (Boc protection).** A rapidly stirred solution of sulfonylated amine (1 eq) and di-tert-butyl dicarbonate (BOC₂O; 1.0 eq) in anhydrous DMSO was briefly cooled over ice under an N₂ atmosphere. After 5 min (or sooner if the DMSO begins to freeze), the reaction flask
was removed from the ice bath and catalytic DMAP (0.05 eq) was added. The septum was then immediately equipped with a venting needle. After stirring for 30 min at room temperature, TLC indicated the reaction was complete. The reaction mixture was diluted with water and repetitively extracted into EtOAc. The EtOAc layers were combined and washed with water, dried on anhydrous Na₂SO₄, filtered and concentrated to afford the Boc protected intermediate. The resulting residue was purified using the Biotage-Isolera™ automated column chromatographer in a gradient of EtOAc and Hexane and then dried under reduced pressure.

**General Procedure h (Ester hydrolysis using Lithium Hydroxide).** Methyl or ethyl esters (1.0 eq) were dissolved in a 3:1 mixture of THF:H₂O. Then LiOH (1.1 eq) was added and after 30 minutes the reaction was confirmed to be complete by TLC. The reaction mixture was diluted with water, acidified (pH~5.5) by KH₂PO₄, and continuously extracted into EtOAc. Organic layers were washed with saturated sodium chloride solution, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. Reaction was purified by flash column chromatography using an isocratic solvent system (35:7:1 DCM:MeOH:H₂O).

**General Procedure i (PPh₃Cl₂ promoted peptide Amide Coupling).** To a stirred solution of the acid (1.1 eq) in CHCl₃ (0.1 M) was added PPh₃Cl₂ (2.5 eq). After five minutes of stirring at R.T. the amine (1.0 eq) was added in one portion and the reaction was heated in the microwave at 110 °C for 30 minutes. The reaction was concentrated in vacuo and absorbed directly onto silica for column chromatography purification using a gradient of EtOAc and Hexane.

**General Procedure j (Alkylation of the sulphonamide nitrogen).** To a solution of the prepared peptide coupled intermediate in DMF (0.1 M) was added propargyl bromide (1.1 eq) and Cs₂CO₃ (1.1 eq). The reaction was stirred overnight at R.T and the material was concentrated in vacuo and absorbed onto silica for column chromatography purification using a gradient of EtOAc and Hexane.

**General Procedure k (Click Chemistry Reactions).** The azide building block (1.1 eq) prepared from step c were reacted with the alkyne species (1.0 eq) from step g by treatment with sodium ascorbate (10% molar) and copper sulfate (2% molar) at 35 0 °C for 72 hrs in a 1:1 mixture of t-butanol and water. The reaction was quenched with H₂O and repeatedly extracted with ethyl acetate. The organics were combined and then washed with H₂O and saturated sodium
chloride solution, dried over Na$_2$SO$_4$ and concentrated in vacuo. The resulting residue was purified using the Biotage-Isolera™ automated column chromatographer in a gradient of EtOAc and Hexane and then dried under reduced pressure.

**General Procedure 1 (Global Deprotections via Pd/C).** The benzyl protected (1.0 eq) click products were dissolved in a stirred solution of MeOH/THF (1:2) (0.1 M). The solution was thoroughly degassed and 20% Pd/C (10 mg/mmol) was carefully added to the reaction. H$_2$ gas was bubbled through the solvent for 5 mins before the reaction was put under an atmosphere of H$_2$ gas and stirred continuously for 16 hrs. The H$_2$ gas was evacuated and the reaction filtered through celite to remove the Pd catalyst and then concentrated under reduced pressure. The resulting residue was adsorbed onto silica gel from CH$_2$Cl$_2$ and columned using a Biotage-Isolera™ in a gradient of MeOH and CH$_2$Cl$_2$.

5.2.3 Detailed Synthetic Procedures and Characterizations

**benzyl 4-formylbenzoate (2.21).** Compound 2.21 was synthesized according to general procedure a, yielding the final product as colourless solid (90%). $\delta_H$ (400 MHz, d-(CDCl$_3$)) 5.32 (s, 2H, CH$_2$), 7.29-7.50 (m, 5H, CH), 7.91-8.00 (m, 4H, CH); $\delta_C$ (100 MHz, d-CDCl$_3$) 66.7, 127.1, 127.8, 128.9, 129.8, 130.5, 135.6, 136.2, 141.2, 165.7, 192.3; LRMS (ES+) Calcd for [C$_{15}$H$_{12}$O$_3$ + Na] 263.09 found 263.56.
**Benzyl 2-(benzyloxy)-5-formylbenzoate (2.25).** Compound 2.25 was synthesized according to general procedure a, yielding the final product as pale white solid (90%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 5.12 (s, 2H, CH$_2$), 5.36 (s, 2H, CH$_2$), 7.45-7.87 (m, 10H, CH), 7.94 (s, 1H, CH), 7.99-8.02 (m, 2H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 66.5, 71.2, 115.6, 119.0, 127.3, 127.4, 128.9, 129.1, 130.8, 131.5, 136.1, 136.7, 166.9, 169.8, 191.2; LRMS (ES+) Calcd for [C$_{22}$H$_{18}$O$_4$ + H] 347.12 found 347.05.

**Benzyl 4-amino-2-(benzyloxy)benzoate (2.12).** Compound 2.12 was synthesized according to general procedure a, yielding the final product as a pale yellow solid (41-48%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 4.10-4.15 (brs, 2H, NH$_2$), 5.05 (s, 2H, CH$_2$), 5.35 (s, 2H, CH), 6.19-6.23 (m, 2H, CH), 7.31-7.49 (m, 10H, CH), 7.81-7.86 (m, 1H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 65.8, 70.3, 99.1, 106.7, 108.8, 126.9, 127.6, 127.8, 128.0, 128.4, 134.4, 136.6, 136.7, 152.5, 160.8, 165.8; LRMS (ES+) Calcd for [C$_{21}$H$_{19}$NO$_3$ + Na] 356.13 found 356.33.
2,2,7-trimethyl-4H-benzo[\textit{d}][1,3]dioxin-4-one (2.16). Compound 2.16 was synthesized by adding thionyl chloride (1.5 eq) and catalytic DMAP to a solution of 4-methyl salicylic acid (1.0 eq) in Acetone. The reaction was stirred for 48 hrs and quenched with water. The reaction was quenched with H$_2$O and repeatedly extracted with ethyl acetate. The organics were combined and then washed with H$_2$O and saturated sodium chloride solution, dried over Na$_2$SO$_4$ and concentrated in vacuo. The resulting residue was purified using the Biotage-Isolera™ automated column chromatographer in a gradient of EtOAc and Hexane to yield the final product as pale yellow solid (71%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 1.73 (s, 6H, CH$_3$), 2.39 (s, 3H, CH$_3$), 6.75 (s, 1H, CH), 6.84-6.97 (m, 1H, CH), 7.73-7.97 (m, 1H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 22.1, 26.1, 105.8, 110.8, 117.5, 123.4, 129.4, 147.8, 156.0, 161.2; LRMS (ES+) Calcd for [C$_{11}$H$_{12}$O$_3$ + Na] 215.07 found 215.35.

![Chemical structure of 2,2,7-trimethyl-4H-benzo[\textit{d}][1,3]dioxin-4-one](image)

7-(bromomethyl)-2,2-dimethyl-4H-benzo[\textit{d}][1,3]dioxin-4-one (2.17). Compound 2.17 was prepared by addition of NBS (1.15 eq) and catalytic benzoyl peroxide to a solution of 6.3a in carbon tetrachloride (0.5M). The reaction was stirred at 75°C for 2 hr, allowed to cool to R.T. and further cooled in an ice-water bath for 30 min. The resulting suspension was filtered to remove succinimide and the precipitate was washed with ice-cold CCl$_4$. The filtrate was purified using a gradient of Hexanes and EtOAc to afford the final product as a white solid (53%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 1.70 (s, 6H, tCH$_3$), 4.40 (s, 2H, CH$_2$), 6.71 (s, 1H, CH), 7.10 (d, $J = 8.0$ Hz, 1H, CH), 7.89 (d, $J = 8.0$ Hz, 1H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 25.8, 31.5, 106.8, 113.5, 117.7, 123.2, 129.9, 146.3, 155.7, 160.7; LRMS (ES+) Calcd for [C$_{11}$H$_{11}$BrO$_3$ + Na] 292.99 found 292.07.

![Chemical structure of 7-(bromomethyl)-2,2-dimethyl-4H-benzo[\textit{d}][1,3]dioxin-4-one](image)
4-cyclohexylbenzaldehyde (2.11). Compound 2.11 was synthesized according to general procedure b, yielding the final product as a pale yellow solid (90%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 1.31-1.57 (m, 5H, CH$_2$), 1.70-1.96 (m, 5H, CH$_2$), 2.59 (m, 1H, CH), 7.34 (d, $J$=11 Hz, 2H, CH), 7.80 (d, $J$= 8.36, 2H, CH), 9.95 (brs, 1H, OH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 25.8, 36.6, 34.1, 45.0, 127.6, 129.9, 134.4, 155.2, 192.1; LRMS (ES+) Calcd for [C$_{13}$H$_{16}$O] 188.26 found 211.94.

Benzyl 2-(benzyloxy)-4-((4-cyclohexylbenzyl)amino)benzoate (2.14). Compound 2.14 was synthesized according to general procedure c, yielding the final product as pale yellow solid (56-90%). $\delta_H$ (400 MHz, $d$-(CDCl$_3$) 1.25-1.48 (m, 5H, CH$_2$), 1.73-1.94 (m, 5H, CH$_2$), 2.48-2.52 (m, 1H, CH), 4.30 (s, 2H, CH$_2$), 5.05 (s, 2H, CH$_2$), 5.35 (s, 2H, CH$_2$), 6.68-6.75 (m, 1H, CH), 6.19-6.23 (m, 2H, CH), 6.86-6.92 (m, 1H, CH), 7.16-7.22 (m, 2H, CH), 7.30-7.48 (m, 10H, CH), 7.37-7.4 (m, 2H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 26.1, 26.6, 34.3, 44.1, 47.1, 65.5, 70.2, 97.3, 104.9, 108.1, 125.9, 126.8, 127.2, 127.4, 127.5, 127.8, 128.1, 128.3, 133.9, 134.8, 135.2, 136.6, 136.8, 147.3, 152.8, 160.6, 165.7; LRMS (ES+) Calcd for [C$_{34}$H$_{35}$NO$_3$ + Na] 528.65 found 528.35.
benzyl 2-((benzyl)oxy)-4-((4-((benzyl)oxy)carbonyl)benzyl)amino)benzoate (2.26). Compound 2.26 was synthesized according to general procedure c, yielding the final product as pale yellow solid (77-98%). $\delta_H$ (400 MHz, $d$-(CDCl$_3$) 4.12 (s, 2H, CH$_2$), 5.12 (s, 2H, CH$_2$), 5.56 (s, 2H, CH$_2$), 5.56 (s, 2H, CH$_2$), 5.56 (s, 2H, CH$_2$), 6.12-6.47 (m, 2H, CH), 7.12 (m, 2H, CH), 7.28-7.56 (m, 12H, CH), 7.65 (s, 2H, CH), 7.72-7.82 (m, 3H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 50.2, 66.2, 72.3, 102.3, 106.5, 110.4, 114.5, 117.8, 128.1, 128.5, 129.8, 130.1, 132.1, 134.5, 135.4, 136.1, 137.5, 153.4, 160.1, 162.3, 170.1; LRMS (ES+) Calcd for [C$_{43}$H$_{37}$NO$_6$ + Na] 686.26 found 686.33.

benzyl 2-(benzyl)oxy)-4-((4-((benzyl)oxy)carbonyl)benzyl)amino)benzoate (2.22). Compound 2.22 was synthesized according to general procedure c, yielding the final product as yellow oil (77-98%). $\delta_H$ (400 MHz, $d$-(CDCl$_3$) 4.32 (s, 2H, CH$_2$), 5.21 (s, 2H, CH$_2$), 5.43 (s, 2H, CH$_2$), 4.30
(s, 2H, CH₂), 6.25-6.30 (m, 2H, CH), 7.25-7.50 (m, 17H, CH), 7.70-7.93 (m, 3H, CH); δₐ (100 MHz, d-CDCl₃) 49.0, 65.7, 70.8, 96.7, 104.3, 109.8, 126.8, 127.1, 127.6, 128.9, 129.7, 131.2, 136.7, 136.7, 144.3, 152.3, 161.8, 165.7, 168.4, 169.9; LRMS (ES+) Calcd for [C₃₆H₃₁NO₅ + H] 558.22 found 558.02.

**benzyl 2-(benzyloxy)-4-((N-(4-(benzyloxy)-3-((benzyloxy)carbonyl)benzyl)-2-(N,N-dimethylphenylsulfonamido)acetamido)benzoate (2.27).** Compound 2.27 was synthesized according to general procedure a, yielding the final product as white solid (60-87%). δₐ (400 MHz, d-CDCl₃) 3.15 (s, 3H, CH₃), 2.78 (s, 3H, CH₃), 4.75 (s, 2H, CH₂), 5.03 (s, 2H, CH₂), 5.05 (s, 2H, CH₂), 5.16 (s, 2H, CH₂), 5.31 (s, 2H, CH₂), 3.37 (s, 2H, CH₂), 6.56-6.71 (m, 2H, CH), 6.89-6.97 (m, 1H, CH), 7.21-7.46 (m, 25H, CH), 7.55-7.65 (m, 1H, CH), 7.83 (d, J = 8.0 Hz, 1H, CH); δₐ (100 MHz, d-CDCl₃) 21.6, 29.6, 36.0, 52.4, 66.6, 70.8, 113.8, 114.1, 119.9, 120.6, 126.9, 127.4, 128.1, 128.2, 128.4, 129.4, 131.9, 133.8, 135.2, 135.7, 136.2, 144.7, 157.5, 165.8, 166.9; LRMS (ES+) Calcd for [C₅₃H₄₈N₂O₉S + H] 889. 31 found 889.45.
benzyl 2-(benzylxylo)-4-(N-(4-((benzyloxy)carbonyl)benzyl)-2-(N,4-
dimethylphenylsulfonamido)acetamido)benzoate (2.23). Compound 2.23 was synthesized according to general procedure a, yielding the final product as pale yellow oil (60-87%). $\delta_H$ (400 MHz, $d$-(CDCl$_3$) 2.40 (s, 3H, CH$_3$), 2.83 (s, 3H, CH$_3$), 2.81 (s, 2H, CH$_2$), 3.69 (s, 2H, CH$_2$), 4.83 (s, 2H, CH$_2$), 5.06 (s, 2H, CH$_2$), 5.40 (s, 4H, CH$_2$), 6.64 (s, 2H, CH), 7.13-7.26 (m, 4H, CH), 7.28-7.47 (m, 15H, CH), 7.61 (d, $J=6.7$ Hz, 2H, CH), 7.83 (d, $J=8.1$ Hz, 1H, CH), 7.96 (d, $J=7.2$ Hz, 2H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 21.6, 36.3, 51.5, 52.7, 67.1, 71.3, 113.8, 119.7, 121.0, 126.9, 127.3, 127.9, 128.1, 128.2, 129.4, 129.9, 133.2, 135.2, 135.6, 135.9, 141.7, 143.4, 144.7, 158.8, 165.2, 165.9, 167.1; LRMS (ES+) Calcd for [C$_{46}$H$_{42}$N$_2$O$_8$S + Na] 805.26 found 805.56.

4-(N-(4-carboxybenzyl)-2-(N,4-dimethylphenylsulfonamido)acetamido)-2-hydroxybenzoic acid (2.24). Compound 2.24 was synthesized according to previously reported procedures and the final general procedure I which yielded the final product as pale yellow solid (79%). $\delta_H$ (400
MHz, $d_6$-(CD$_3$)$_2$SO) 2.34 (s, 3H, CH$_3$), 2.73 (s, 3H, CH$_3$), 3.85 (s, 2H, CH$_2$), 4.86 (s, 2H, CH$_2$), 6.58-6.83 (m, 2H, CH), 7.21-7.41 (m, 4H, CH), 7.47-7.64 (m, 2H, CH), 7.67-8.08 (m, 3H, CH);

$\delta_C$ (100 MHz, $d_6$-(CD$_3$)$_2$SO) 21.4, 36.0, 51.2, 52.4, 116.3, 117.9, 127.2, 128.1, 129.7, 129.9, 130.0, 133.6, 135.5, 142.4, 143.4, 145.9, 162.6, 167.2, 167.4, 171.5, 172.5; HRMS (ES−) Calcd for [C$_{50}$H$_{48}$N$_4$O$_{17}$S$_2$ – H] 1039.2456 found 1039.2352; HPLC (I) $t_R = 13.61$ min (99.9%), (II) $t_R = 5.30$ min (98.1%).

4-(N-(3-carboxy-4-hydroxybenzyl)-2-(N,4-dimethylphenylsulfonamido)acetamido)-2-hydroxybenzoic acid (2.28). Compound 2.28 was synthesized according to previously reported procedures and the final general procedure I which yielded the final product as a white solid (81%). $\delta_H$ (400 MHz, $d_6$-(CD$_3$)$_2$SO) 2.33 (s, 3H, CH$_3$), 2.73 (s, 3H, CH$_3$), 3.80 (s, 2H, CH$_2$), 4.74 (s, 2H, CH$_2$), 6.56-6.64 (m, 1H, CH), 6.71 (s, 1H, CH), 7.17 (m, 1H, CH), 7.27-7.37 (m, 2H, CH), 7.43 (m, 3H, CH), 7.67-7.78 (m, 2H, CH); $\delta_C$ (100 MHz, $d_6$-(CD$_3$)$_2$SO) 21.3, 30.8, 31.1, 36.1, 113.7, 116.4, 117.4, 118.2, 127.2, 127.6, 130.1, 131.6, 135.5, 143.5, 146.1, 160.9, 162.6, 167.0, 172.1, 175.8, 176.1, 190.5; HRMS (ES−) Calcd for [C$_{25}$H$_{24}$N$_2$O$_8$S – H] 527.1203 found 527.1355; HPLC (I) $t_R = 14.35$ min (100%), (II) $t_R = 5.99$ min (100%).
benzyl 2-(benzyloxy)-4-(N-(4-cyclohexylbenzyl)-2-(N-((2,2-dimethyl-4-oxo-4H-benzo[d][1,3]dioxin-7-yl)methyl)-4-methylphenylsulfonamido)acetamido)benzoate (2.18). Compound 2.18 was prepared by treatment of 2.15 in DMF (0.1 M) with 2.17 (1.1 eq) and Cs₂CO₃ (1.1 eq). The reaction was stirred overnight at R.T and the material was concentrated *in vacuo* and absorbed onto silica for column chromatography purification using a gradient of EtOAc and Hexane yielding the final product as pale yellow oil (82%). δH (400 MHz, d-CDCl₃) 1.28-1.44 (m, 5H, CH₂), 1.67 (s, 5H, CH₂), 1.81-1.85 (m, 3H, CH₃), 2.05 (s, 1H, CH), 2.50 (s, 6H, CH₃), 3.71 (s, 2H, CH₂), 4.60 (s, 2H, CH₂), 4.68 (s, 2H, CH₂), 4.87 (s, 2H, CH₂), 5.32 (s, 2H, CH₂), 6.42 (s, 1H, CH), 6.50-6.51 (m, 1H, CH), 6.87-6.97 (m, 3H, CH), 7.05-7.13 (m, 3H, CH), 7.28-7.38 (m, 12H, CH), 7.68-7.77 (m, 3H, CH), 7.85 (d, J = 7.9 Hz, 1H, CH); δC (100 MHz, d-CDCl₃) 14.1, 21.6, 25.6, 26.0, 26.7, 34.3, 44.2, 47.2, 51.1, 52.6, 60.2, 106.4, 112.9, 116.6, 119.8, 122.1, 126.8, 126.9, 127.5, 127.9, 128.1, 128.2, 128.4, 129.4, 129.8, 132.9, 133.7, 135.5, 135.5, 136.6, 140.2, 143.5, 145.5, 147.7, 156.2, 158.6, 160.6, 165.2, 166.4; LRMS (ES+) Calcd for [C₅₄H₅₄N₂O₉S + Na] 929.36 found 929.33.
4-(2-(N-(4-carboxy-3-hydroxybenzyl)-4-methylphenylsulfonamido)-N-(4-cyclohexylbenzyl)acetamido)-2-hydroxybenzoic acid (2.20). Compound 2.20 was synthesized according to general procedure 1, yielding the final product as pale yellow solid (71%). \( \delta_H \) (400 MHz, \( d-\text{CDCl}_3 \)) 1.30-1.44 (m, 5H, CH\(_2\)), 1.70-1.88 (m, 5H, CH\(_2\)), 2.49 (s, 3H, CH\(_3\)), 2.65-2.75 (m, 1H, CH), 3.80 (s, 2H, CH\(_2\)), 4.49 (s, 2H, CH\(_2\)), 4.75 (s, 2H, CH\(_2\)), 6.36-6.47 (m, 2H, CH), 6.76-6.85 (m, 2H, CH), 6.94-7.04 (m, 2H, CH), 7.06-7.14 (m, 2H, CH), 7.34 (d, \( J = 7.4 \) Hz, 2H, CH), 7.70-7.83 (m, 4H, CH); \( \delta_C \) (100 MHz, \( d-\text{CDCl}_3 \)) 14.0, 18.8, 20.9, 26.7, 34.3, 44.03, 59.5, 60.4, 105.4, 113.5, 113.8, 114.3, 116.5, 120.6, 126.4, 127.9, 128.7, 129.3, 131.9, 132.8, 133.3, 136.7, 137.8, 142.8, 145.0, 148.6, 162.3, 164.5, 166.7, 171.8; HRMS (ES–) Calcd for \([C_{37}H_{38}N_2O_9S–H]\) 685.2298 found 685.2321; HPLC (I) \( t_R = 23.44 \) min (92.9%), (II) \( t_R = 15.37 \) min (97.9%).

methyl 2-(quinoline-8-sulfonamido)acetate (2.31). Compound 2.31 was synthesized according to general procedure 6, yielding the final product a brown oil (91-93%). \( \delta_H \) (400 MHz, \( d-\text{CDCl}_3 \)) 3.31 (s, 3H, CH\(_3\)), 3.74 (d, \( J = 5.8 \) Hz, 2H, CH\(_2\)), 6.87 (t, \( J = 5.6 \) Hz, 1H, CH), 7.42 (dd, \( J = 4.3 \), 8.4 Hz, 1H, CH), 7.49 (t, \( J = 7.6 \) Hz, 1H, CH), 7.95 (d, \( J = 8.2 \) Hz, 1H, CH), 8.16 (d, \( J = 8.4 \) Hz, 1H, CH), 8.22 (d, \( J = 7.2 \) Hz, 1H, CH); \( \delta_C \) (100 MHz, \( d-\text{CDCl}_3 \)) 44.5, 52.2, 122.4, 125.2, 128.7, 130.3, 133.6, 135.4, 137.0, 142.9, 151.1, 168.9; LRMS (ES+) Calcd for \([C_{12}H_{12}N_2O_4S + Na]\) 303.05 found 303.78.
methyl 2-({\textit{N}}-\text{tert-butoxycarbonyl})quinoline-8-sulfonamido)acetate (2.32). Compound 2.32 was synthesized according to general procedure g, yielding the final product as pale yellow solid (85-88%). $\delta_\text{H}$ (400 MHz, $d$-(CDCl$_3$) 1.20 (s, 9H, CH$_3$), 3.81 (s, 3H, CH$_3$), 4.30 (s, 2H, CH$_2$), 7.53 (dd, $J$= 4.2, 8.37 Hz, 1H, CH), 7.66 (t, $J$= 7.80 Hz, 1H, CH), 8.09 (d, $J$= 8.17 Hz, 1H, CH), 8.25 (m, $J$= 8.57 Hz, 1H, CH), 8.59 (d, $J$= 7.46 Hz, 1H, CH), 8.95-9.03 (m, 1H, CH); $\delta_\text{C}$ (100 MHz, $d$-CDCl$_3$) 27.8, 48.4, 52.7, 84.4, 124.9, 128.9, 133.5, 134.1, 136.5, 137.8, 139.7, 143.5, 150.7, 151.4, 160.8; LRMS (ES+) Calcd for [C$_{17}$H$_{20}$N$_2$O$_6$S + Na] 403.10 found 403.45.

2-({\textit{N}}-\text{tert-butoxycarbonyl})quinoline-8-sulfonamido)acetic acid (2.33). Compound 2.33 was synthesized according to general procedure h, yielding the final product as a pale yellow solid (71-81%). $\delta_\text{H}$ (400 MHz, $d$-(CDCl$_3$) 1.16 (s, 9H, CH$_3$), 4.86 (s, 2H, CH$_2$), 7.63 (dd, $J$= 4.2, 8.5 Hz, 1H, CH), 7.76 (t, $J$= 7.7 Hz, 2H, CH), 8.27 (d, $J$= 8.3 Hz 1H, CH), 8.43 (d, $J$= 8.3 Hz, 1H, CH), 8.54 (d, $J$= 7.4 Hz, 1H, CH); $\delta_\text{C}$ (100 MHz, $d$-CDCl$_3$) 45.6, 77.9, 84.0, 122.2, 124.9, 128.9, 132.9, 134.3, 136.5, 143.1, 147.6, 151.3, 160.6, 174.3; LRMS (ES+) Calcd for [C$_{16}$H$_{18}$N$_2$O$_6$S + H] 367.08 found 367.06.
methyl 2-(4-methylphenylsulfonamido)acetate (2.5) Compound 2.5 was synthesized according to general procedure f, yielding the final product a white powder (91%). \( \delta_H \) (400 MHz, \( d-\text{CDCl}_3 \)) 2.43 (s, 3H, CH\(_3\)), 3.41 (s, 3H, CH\(_3\)), 3.64 (d, \( J = 5.9 \text{ Hz} \), 2H, CH\(_2\)), 7.74-7.80 (m, 2H, CH), 7.86-8.01 (m, 2H, CH); \( \delta_C \) (100 MHz, \( d-\text{CDCl}_3 \)) 22.1, 45.5, 52.2, 135.4, 137.0, 142.9, 151.1, 168.9; LRMS (ES+) Calcd for [C\(_{10}\)H\(_{13}\)NO\(_4\)S + Na] 266.06 found 266.78.

methyl 2-(N-(tert-butoxycarbonyl)-4-methylphenylsulfonamido)acetate (2.6). Compound 2.6 was synthesized according to general procedure g, yielding the final product as white solid (80-90%). \( \delta_H \) (400 MHz, \( d-\text{CDCl}_3 \)) 1.22 (s, 9H, CH\(_3\)), 3.71 (s, 3H, CH\(_3\)), 4.99 (s, 2H, CH\(_2\)), 4.30 (s, 2H, CH\(_2\)), 7.74-7.80 (m, 2H, CH), 7.86-8.01 (m, 2H, CH); \( \delta_C \) (100 MHz, \( d-\text{CDCl}_3 \)) 22.3, 27.8, 48.4, 52.7, 84.4, 122.1, 124.9, 128.9, 129.8, 133.5, 156.7; LRMS (ES+) Calcd for [C\(_{15}\)H\(_{21}\)NO\(_6\)S + Na] 366.11 found 366.45.

2-(N-(tert-butoxycarbonyl)-4-methylphenylsulfonamido)acetic acid (2.7). Compound 2.7 was synthesized according to general procedure h, yielding the final product as a pale yellow solid (55%). \( \delta_H \) (400 MHz, \( d-\text{CDCl}_3 \)) 1.22 (s, 9H, CH\(_3\)), 3.71 (s, 3H, CH\(_3\)), 4.99 (s, 2H, CH\(_2\)), 7.75-
7.85 (m, 2H, CH), 7.89-8.21 (m, 2H, CH); \( \delta_C \) (100 MHz, \( d-\text{CDCl}_3 \)) 22.3, 27.8, 48.4, 84.4, 124.9, 128.9, 129.8, 133.5, 156.7, 175.7; LRMS (ES-) Calcd for [C\(_{14}\)H\(_{19}\)NO\(_6\)S - H] 328.09 found 328.06.

![Structure](attachment:structure.png)

**2-(N,4-dimethylphenylsulfonamido)acetic acid (2.9).** Compound 2.9 was synthesized according to general procedure h, yielding the final product as pale yellow solid (79%). \( \delta_H \) (400 MHz, \( d-\text{CDCl}_3 \)) 2.37 (s, 3H, CH\(_3\)), 2.72 (s, 3H, CH\(_3\)), 3.18 (s, 2H, CH\(_2\)), 4.30 (s, 2H, CH\(_2\)), 7.39 (d, \( J = 8.1 \) Hz, 2H, CH), 7.65 (d, \( J = 8.3 \) Hz, 2H, CH); \( \delta_C \) (100 MHz, \( d-\text{CDCl}_3 \)) 21.1, 34.3, 65.5, 128.1, 130.1, 136.6, 136.8, 172.7; LRMS (ES-) Calcd for [C\(_{10}\)H\(_{13}\)NO\(_4\)S - H] 242.05 found 242.65.

![Structure](attachment:structure.png)

**Methyl 2-(N,4-dimethylphenylsulfonamido)acetate (2.8).** Compound 2.8 was synthesized according to general procedure f, yielding the final product as pale yellow solid (91%). \( \delta_H \) (400 MHz, \( d-\text{CDCl}_3 \)) 2.28 (s, 3H, CH\(_3\)), 2.62 (s, 3H, CH\(_3\)), 3.83 (s, 2H, CH\(_2\)), 4.25 (s, 3H, CH\(_3\)), 7.38 (d, \( J = 8.1 \) Hz, 2H, CH), 7.55 (d, \( J = 8.3 \) Hz, 2H, CH); \( \delta_C \) (100 MHz, \( d-\text{CDCl}_3 \)) 21.1, 34.3, 51.6, 65.5, 128.1, 130.1, 136.6, 136.8, 172.7; LRMS (ES+) Calcd for [C\(_{11}\)H\(_{15}\)NO\(_4\)S + Na] 280.07 found 280.54.
2-(benzyloxy)-4-(N-(4-cyclohexylbenzyl)-2-(4-methylphenylsulfonamido)acetamido)benzoate (2.15). Compound 2.15 was synthesized according to general procedure i, yielding the final product as a white solid (60-85%). \(\delta_H\) (400 MHz, \(d\)-(CDCl\(_3\)) 1.34-1.45 (m, 5H, CH\(_2\)), 1.68-1.86 (m, 5H, CH\(_2\)), 2.37-2.50 (m, 1H, CH), 3.53 (s, 2H, CH\(_2\)), 4.72 (s, 2H, CH\(_2\)), 4.87 (s, 2H, CH\(_2\)), 5.74 (s, 2H, CH\(_2\)), 6.47 (s, 2H, CH), 6.49 (d, \(J=8.2\) Hz, 1H, CH), 6.74-6.90 (m, 2H, CH), 7.11-7.20 (m, 1H, CH), 7.27-7.43 (m, 10H, CH), 7.48-7.63 (m, 2H, CH), 7.67-7.80 (m, 1H, CH); \(\delta_C\) (100 MHz, \(d\)-(CDCl\(_3\)) 27.1, 27.8, 35.3, 44.2, 47.0, 53.1, 64.6, 72.1, 89.0, 113.2, 114.8, 123.4, 126.4, 127.8, 127.9, 128.1, 128.4, 128.7, 129.0, 129.6, 130.6, 133.1, 133.2, 133.7, 137.5, 143.5, 147.6, 151.3, 158.6, 166.5; LRMS (ES+) Calcd for [C\(_{43}\)H\(_{44}\)N\(_2\)O\(_6\)S+ Na] 739.29 found 739.67.
benzyl-2-(benzyloxy)-4-(N-(4-cyclohexylbenzyl)-2-(quinoline-8-sulfonamido)acetamido)benzoate (2.34). Compound 2.34 was synthesized according to general procedure i, yielding the final product as a brown oil (60-87%). $\delta_H$ (400 MHz, $d$-(CDCl$_3$) 1.24-1.41 (m, 5H, CH$_2$), 1.68-1.86 (m, 5H, CH$_2$), 2.37-2.50 (m, 1H, CH), 3.53 (s, 2H, CH$_2$), 4.62 (s, 2H, CH$_2$), 4.83 (s, 2H, CH$_2$), 5.33 (s, 2H, CH$_2$), 6.37 (s, 1H, CH), 6.48 (d, $J= 8.2$ Hz, 1H, CH), 6.74-6.90 (m, 2H, CH), 6.93-7.07 (m, 2H, CH), 7.11-7.20 (m, 1H, CH), 7.27-7.43 (m, 10H, CH), 7.48-7.63 (m, 2H, CH), 7.67-7.80 (m, 1H, CH), 7.95-8.12 (m, 1H, CH), 8.29 (t, $J = 8.4$ Hz, 2H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 26.1, 26.8, 34.3, 44.2, 45.0, 52.7, 67.3, 71.0, 111.3, 113.7, 119.7, 122.3, 125.3, 126.8, 126.9, 127.9, 128.1, 128.2, 128.4, 128.6, 130.6, 133.1, 133.2, 133.4, 136.5, 143.3, 147.6, 151.3, 158.6, 165.3, 166.5; LRMS (ES+) Calcd for [C$_{45}$H$_{43}$N$_3$O$_6$S + H] 754.28 found 754.73.
benzyl-2-(benzyloxy)-4-(N-(4-cyclohexylbenzyl)-2-(4-methyl-N-(prop-2-yn-1-yl)phenylsulfonamido)acetamido)benzoate (2.35). Compound 2.35 was synthesized according to general procedure j, yielding the final product as a brown oil (71-77%). δH (400 MHz, d-CDCl3) 1.34-1.45 (m, 5H, CH₂), 1.68-1.86 (m, 5H, CH₂), 2.37-2.50 (m, 1H, CH), 2.88 (s, 1H, CH), 3.53 (s, 2H, CH₂), 4.12 (s, 2H, CH₂), 4.72 (s, 2H, CH₂), 4.87 (s, 2H, CH₂), 5.64 (s, 2H, CH₂), 6.67 (s, 2H, CH), 6.49 (d, J = 8.1 Hz, 1H, CH), 6.84-6.90 (m, 2H, CH), 6.97-7.17 (m, 2H, CH), 7.19-7.22 (m, 1H, CH), 7.27-7.47 (m, 10H, CH), 7.48-7.65 (m, 2H, CH), 7.67-7.80 (m, 1H, CH); δC (100 MHz, d-CDCl₃) 26.1, 27.9, 33.3, 33.7, 42.4, 44.2, 45.6, 48.0, 53.5, 67.6, 73.1, 90.0, 114.2, 114.8, 124.4, 126.4, 127.9, 128.0, 128.1, 128.5, 128.9, 129.1, 129.6, 130.7, 133.1, 133.2, 133.8, 138.5, 142.5, 147.6, 152.3, 158.6, 167.5; LRMS (ES+) Calcd for [C₄₆H₄₆N₂O₆S + Na] 777.31 found 777.87.
benzyl-2-(benzyloxy)-4-(N-(4-cyclohexylbenzyl)-2-(N-(prop-2-yn-1-yl)quinoline-8-sulfonamido)acetamido)benzoate (2.36). Compound 2.36 was synthesized according to general procedure j yielding the final product as a brown oil (71-77%). δH (400 MHz, d-CDCl3) 1.27-1.46 (m, 5H, CH2), 1.67 (s, 1H, CH), 1.70-1.88 (m, 5H, CH2), 2.40-2.53 (m, 1H, CH), 4.37 (s, 2H, CH2), 4.53 (s, 2H, CH2), 4.78 (s, 2H, CH2), 4.93 (m, 2H, CH), 5.37 (s, 2H, CH2), 6.73 (s, 1H, CH), 6.80 (d, J = 8.2 Hz, 1H, CH), 7.02-7.14 (m, 4H, CH), 7.21-7.43 (m, 10H, CH), 7.54 (t, J = 7.9 Hz, 1H, CH), 7.86 (d, J = 8.2 Hz, 1H, CH), 7.96 (d, J = 8.2 Hz, 1H, CH), 8.11 (d, J = 8.2 Hz, 1H, CH), 8.42 (d, J = 7.5 Hz, 1H, CH); δC (100 MHz, d-CDCl3) 26.0, 26.7, 34.4, 38.1, 44.1, 48.4, 52.5, 60.2, 66.9, 70.6, 72.6, 121.8, 125.1, 126.9, 127.0, 127.8, 128.1, 128.2, 128.4, 128.5, 128.7, 128.8, 132.2, 132.7, 133.3, 133.9, 135.7, 135.7, 143.8, 145.5, 147.5, 150.5, 158.7, 165.5, 167.8; LRMS (ES+) Calcd for [C48H45N5O6S+ Na] 814.30 found 814.56.
Benzyl 2-(benzyloxy)-4-(2-bromoacetamido)benzoate (2.29a). Compound 2.29a was synthesized according to general procedure d, yielding the final product as a colourless oil (95%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 2.85 (s, 2H, CH$_2$), 2.95 (s, 2H, CH$_2$), 3.63 (s, 2H, CH$_2$), 6.33-6.49 (m, 1H, CH), 6.79-7.03 (m, 4H, CH), 7.27-7.34 (m, 5H, CH), 7.34-7.40 (m, 3H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 26.4, 40.1, 66.4, 106.5, 111.9, 114.1, 117.1, 125.7, 127.6, 128.8, 129.0, 133.9, 135.9, 136.1, 160.6, 165.8; LRMS (ES+) Calcd for [C$_{23}$H$_{20}$BrNO$_4$ + Na] 476.05 found 476.56.

Benzyl 2-(benzyloxy)-4-(3-bromopropanamido)benzoate (2.29b). Compound 2.29b was synthesized according to general procedure d, yielding the final product as a colourless oil (90%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 2.85 (t, $J = 6.6$ Hz, 2H, CH$_2$), 3.56 (t, $J = 6.8$ Hz, 2H, CH$_2$), 5.16 (s, 2H, CH$_2$), 5.32 (s, 2H, CH$_2$), 6.13-6.49 (m, 1H, CH), 6.79-7.03 (m, 1H, CH), 7.27-7.34 (m, 4H, CH), 7.34-7.40 (m, 4H, CH), 7.67 (s, 1H, CH), 7.82-7.89 (m, 1H, CH), 8.16-8.31 (m, 1H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 26.4, 40.1, 66.4, 70.4, 104.5, 110.9, 115.1, 127.1, 127.7, 127.9, 128.3, 128.4, 132.9, 135.9, 136.1, 159.6, 165.8; LRMS (ES+) Calcd for [C$_{24}$H$_{22}$BrNO$_4$ + Na] 490.07 found 490.53.
Benzyl 2-(benzyloxy)-4-(4-bromobutamido)benzoate (2.29c). Compound 2.29c was synthesized according to general procedure d, yielding the final product as a colourless oil (85%). $\delta$H (400 MHz, d-CDCl3) 1.94-2.01 (p, $J = 6.4$ Hz, 2H, CH2), 2.40 (t, $J = 6.4$ Hz, 2H, CH2), 3.38 (t, $J = 6.4$ Hz, 2H, CH2), 5.13 (s, 2H, CH2), 5.35 (s, 2H, CH2), 6.21-6.30 (m, 1H, CH), 6.84 (d, $J = 8.7$ Hz, 1H, CH), 7.26-7.34 (m, 10H, CH), 7.36-7.40 (s, 1H, CH); $\delta$C (100 MHz, d-CDCl3) 24.1, 29.6, 50.7, 70.5, 104.5, 106.7, 110.5, 126.8, 127.1, 127.6, 127.7, 127.9, 128.0, 128.4, 132.9, 136.2, 136.1; LRMS (ES+) Calcd for [C25H24BrNO4 + Na] 504.08 found 504.35.

Benzyl 2-(benzyloxy)-4-(5-bromopentanamido)benzoate (2.29d). Compound 2.29d was synthesized according to general procedure d, yielding the final product as a colourless oil (91%). $\delta$H (400 MHz, d-CDCl3) 1.58 (p, $J = 7.1$ Hz, 2H, CH2), 1.78 (p, $J = 7.1$ Hz, 2H, CH2), 2.37 (t, $J = 7.3$ Hz, 2H, CH2), 3.31 (t, $J = 6.5$ Hz, 2H, CH2), 5.13 (s, 2H, CH2), 5.33 (s, 2H, CH2), 6.83 (d, $J = 8.5$, 1H, CH), 7.27-7.51 (m, 9H, CH), 7.61 (s, 1H, CH), 7.75 (s, 1H, CH), 7.86 (d, $J = 8.5$ Hz, 1H, CH); $\delta$C (100 MHz, d-CDCl3) 26.1, 26.6, 34.3, 44.1, 47.1, 65.5, 104.9, 108.1, 122.9, 123.8, 124.2, 124.4, 125.5, 125.8, 127.1, 136.6, 136.8, 147.3, 152.8; LRMS (ES+) Calcd for [C26H26BrNO4 + Na] 518.10 found 518.32.
Benzyl 2-(benzyloxy)-4-(6-bromohexanamido)benzoate (2.29e). Compound 2.29e was synthesized according to general procedure d, yielding the final product a colourless oil (87%).

δ_H (400 MHz, d-CDCl_3) 1.45 (p, J = 7.2 Hz, 2H, CH₂), 1.58 (p, J = 7.4 Hz, 2H, CH₂), 1.71 (p, J = 7.2 Hz, 2H, CH₂), 2.71 (t, J = 7.2 Hz, 2H, CH₂), 3.23 (t, J = 6.7 Hz, 2H, CH₂), 5.17 (s, 2H, CH₂), 5.11 (s, 2H, CH₂), 6.97 (d, J = 8.5 Hz, 1H, CH), 7.17-7.53 (m, 9H, CH), 7.85 (s, 1H, CH), 7.94 (d, J = 8.7 Hz, 1H, CH), 8.17 (s, 1H, CH); δ_C (100 MHz, d-CDCl₃) 27.9, 26.8, 29.0, 38.3, 53.2, 67.8, 70.5, 104.3, 111.0, 114.7, 128.1, 127.9, 129.3, 132.1, 135.9, 126.2, 144.5, 159.6, 166.7, 170.7; LRMS (ES+) Calcd for [C_{27}H_{28}BrNO₄ + Na] 532.12 found 532.19.

Benzyl 4-(2-azidoacetamido)-2-(benzyloxy)benzoate (2.30a). Compound 2.30a was synthesized according to general procedure d, yielding the final product a colourless oil (81%).

δ_H (400 MHz, d-CDCl₃) 2.45 (s, 2H, CH₂), 2.56 (s, 2H, CH₂), 3.23 (s, 2H, CH₂), 6.21-6.47 (m, 1H, CH), 6.76-7.13 (m, 4H, CH), 7.17-7.24 (m, 5H, CH), 7.31-7.40 (m, 3H, CH); δ_C (100 MHz, d-CDCl₃) 26.1, 41.1, 67.4, 107.5, 112.9, 115.1, 118.1, 126.7, 127.6, 129.8, 130.0, 133.9, 135.9, 136.0, 161.6, 164.8; LRMS (ES+) Calcd for [C_{23}H_{20}N₄O₄ + H] 417.14 found 417.98.
Benzyl 4-(3-azidopropanamido)-2-(benzyloxy)benzoate (2.30b). Compound 2.30b was synthesized according to general procedure d, yielding the final product as a colourless oil (84%). δ\textsubscript{H} (400 MHz, d-CDCl\textsubscript{3}) 2.53 (t, J = 6.5 Hz, 2H, CH\textsubscript{2}), 3.65 (t, J = 6.3 Hz, 2H, CH\textsubscript{2}), 5.08 (s, 2H, CH\textsubscript{2}), 5.33 (s, 2H, CH\textsubscript{2}), 6.10-6.30 (m, 1H, CH), 7.27-7.43 (m, 10H, CH), 7.68 (s, 1H, CH), 7.75-7.88 (m, 1H, CH); δ\textsubscript{C} (100 MHz, d-CDCl\textsubscript{3}) 36.5, 46.9, 66.3, 70.3, 104.4, 110.7, 127.1, 127.7, 127.9, 128.4, 132.9, 136.0, 136.1, 142.8, 159.5; LRMS (ES+) Calcd for [C\textsubscript{24}H\textsubscript{22}N\textsubscript{4}O\textsubscript{4} + Na] 453.16 found 453.21.

Benzyl 4-(4-azidobutanimido)-2-(benzyloxy)benzoate (2.30c). Compound 2.30c was synthesized according to general procedure d, yielding the final product as a colourless oil (82%). δ\textsubscript{H} (400 MHz, d-CDCl\textsubscript{3}) 1.98 (p, J = 7.3 Hz, 2H, CH\textsubscript{2}), 2.37-2.47 (m, 2H, CH\textsubscript{2}), 3.38 (t, J = 6.4 Hz, 2H, CH\textsubscript{2}), 5.13 (s, 2H, CH\textsubscript{2}), 5.35 (s, 2H, CH\textsubscript{2}), 6.21-6.30 (m, 1H, CH), 6.84 (d, J = 8.7 Hz, 1H, CH), 7.26-7.34 (m, 10H, CH), 7.36-7.40 (s, 1H, CH); δ\textsubscript{C} (100 MHz, d-CDCl\textsubscript{3}) 24.1, 29.6, 50.7, 66.6, 70.5, 104.5, 106.7, 110.5, 126.8, 127.1, 127.6, 127.7, 127.9, 128.0, 128.4, 132.9, 136.2, 136.1; LRMS (ES+) Calcd for [C\textsubscript{25}H\textsubscript{24}N\textsubscript{4}O\textsubscript{4} + Na] 445.17 found 445.45.
Benzyl 4-(5-azidopentanamido)-2-(benzyloxy)benzoate (2.30d). Compound 2.30d was synthesized according to general procedure d, yielding the final product as a colourless oil (80%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 1.63 (p, $J = 7.1$ Hz, 2H, CH$_2$), 1.71 (p, $J = 7.2$ Hz, 2H, CH$_2$), 2.27 (t, $J = 7.0$ Hz, 2H, CH$_2$), 3.31 (t, $J = 6.4$ Hz, 2H, CH$_2$), 5.13 (s, 2H, CH$_2$), 5.23 (s, 2H, CH$_2$), 6.25 (d, $J = 8.5$, 1H, CH), 7.25-7.50 (m, 9H, CH), 7.57 (s, 1H, CH), 7.85 (s, 1H, CH), 7.96 (d, $J = 8.5$ Hz, 1H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 27.1, 28.6, 34.3, 47.1, 49.1, 67.6, 107.9, 108.1, 122.9, 123.7, 124.4, 124.9, 125.5, 126.8, 127.1, 137.8, 147.3, 152.3; LRMS (ES+) Calcd for [C$_{26}$H$_{26}$N$_4$O$_4$ + H] 489.19 found 489.56.

Benzyl 4-(6-azidohexanamido)-2-(benzyloxy)benzoate (2.30e). Compound 2.30e was synthesized according to general procedure d, yielding the final product as a colourless oil (84%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 1.36 (p, $J = 7.1$ Hz, 2H, CH$_2$), 1.55 (p, $J = 7.2$ Hz, 2H, CH$_2$), 1.66 (p, $J = 7.1$ Hz, 2H, CH), 2.31 (t, $J = 7.3$ Hz, 2H, CH$_2$), 3.23 (t, $J = 6.9$ Hz, 2H, CH$_2$), 5.07 (s, 2H, CH$_2$), 5.31 (s, 2H, CH$_2$), 6.90 (d, $J = 8.5$ Hz, 1H, CH), 7.27-7.43 (m, 9H, CH), 7.75 (s, 1H, CH), 7.84 (d, $J = 8.7$ Hz, 1H, CH), 8.11 (s, 1H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 24.8, 26.6, 28.3, 37.3, 51.2, 66.6, 70.5, 104.3, 111.0, 114.7, 127.1, 127.9, 128.3, 132.1, 135.9, 126.2, 143.5, 159.6, 165.7, 171.7; LRMS (ES+) Calcd for [C$_{27}$H$_{28}$N$_4$O$_4$ + Na] 495.21 found 495.67.
Compound 2.38a was synthesized according to general procedure k, yielding the final product as a colourless oil (59-68%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 1.13-1.37 (m, 5H, CH$_2$), 1.67-1.85 (m, 5H, CH$_2$), 2.03-2.12 (m, 1H, CH), 2.37 (s, 3H, CH$_3$), 4.10 (s, 2H, CH$_2$), 4.60 (s, 2H, CH$_2$), 4.98 (s, 4H, CH$_2$), 5.19 (s, 2H, CH$_2$), 5.34 (s, 4H, CH$_2$), 6.61-6.79 (m, 4H, CH), 6.81-7.07 (m, 4H, CH), 7.20-7.25 (m, 2H, CH), 7.28-7.40 (m, 19H, CH), 7.41-7.46 (m, 2H, CH), 7.57-7.83 (m, 4H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 18.9, 21.6, 26.1, 26.8, 34.3, 44.3, 47.2, 54.1, 63.5, 66.4, 126.8, 127.1, 127.2, 127.4, 127.5, 127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 129.7, 130.9, 131.1, 132.8, 135.5, 135.6, 136.1, 136.3, 141.1, 144.7, 147.6, 150.1, 152.3; LRMS (ES+) Caled for [C$_{69}$H$_{66}$N$_6$O$_{10}$S + H] 1171.45 found 1171.65.
Benzyl 2-(benzyloxy)-4-(3-(4-((N-(2-((3-(benzyloxy)-4-((benzyloxy)carbonyl)phenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)-4-methylphenylsulfonamido)methyl)-1H-1,2,3-triazol-1-yl)propanamido)benzoate (2.37b). Compound 2.37b was synthesized according to general procedure k, yielding the final product as a colourless oil (59-68%). \( \delta_H \) (400 MHz, \( d-\text{CDCl}_3 \)) 1.23-1.36 (m, 5H, CH\(_2\)), 1.77-1.89 (m, 5H, CH\(_2\)), 2.13-2.19 (m, 1H, CH), 2.47 (s, 3H, CH\(_3\)), 2.76 (t, \( J = 6.8 \text{ Hz} \), 2H, CH\(_2\)), 4.10 (s, 2H, CH\(_2\)), 4.55 (s, 2H, CH\(_2\)), 4.60 (t, \( J = 6.4 \text{ Hz} \), 2H, CH\(_2\)), 4.74 (s, 2H, CH\(_2\)), 4.97 (s, 2H, CH\(_2\)), 5.29 (s, 2H, CH\(_2\)), 5.31 (s, 2H, CH\(_2\)), 5.32 (s, 2H, CH\(_2\)), 6.56-6.62 (m, 2H, CH), 6.89 (d, \( J = 8.5 \text{ Hz} \), 2H, CH), 7.0 (d, \( J = 8.0 \text{ Hz} \), 2H, CH), 7.10 (d, \( J = 7.9 \text{ Hz} \), 2H, CH), 7.26-7.45 (m, 21H, CH), 7.55-7.60 (m, 1H, CH), 7.66-7.72 (m, 2H, CH), 7.78 (d, \( J = 7.8 \text{ Hz} \), 2H, CH), 7.83 (d, \( J = 8.4 \text{ Hz} \), 1H, CH); \( \delta_C \) (100 MHz, \( d-\text{CDCl}_3 \)) 18.9, 21.6, 26.1, 26.8, 30.1, 34.3, 44.3, 47.2, 54.1, 63.5, 66.4, 126.8, 127.1, 127.2, 127.4, 127.5, 127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 129.7, 130.9, 131.1, 132.8, 135.5, 135.6, 136.1, 136.3, 141.1, 144.7, 147.6, 150.1, 152.3; LRMS (ES+) Calcd for [C\(_{70}H_{68}N_6O_{10}S + H] 1184.47 found 1185.18.
Benzyl 2-(benzyloxy)-4-(4-((N-(2-((3-(benzyloxy)carbonyl)phenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)-4-methylphenylsulfonamido)methyl)-1H-1,2,3-triazol-1-yl)butanamido)benzoate (2.37c). Compound 2.37c was synthesized according to general procedure k, yielding the final product as a colourless oil (59-68%). δ\textsubscript{H} (400 MHz, d\textsubscript{4}-CDCl\textsubscript{3}) 1.24-1.76 (m, 5H, CH\textsubscript{2}), 1.77-1.99 (m, 5H, CH\textsubscript{2}), 2.13-2.20 (m, 1H, CH), 2.46 (t, J = 7.4 Hz, 2H, CH\textsubscript{2}), 2.97 (s, 3H, CH\textsubscript{3}), 4.20 (s, 4H, CH\textsubscript{2}), 4.35 (t, J = 6.3 Hz, 2H, CH\textsubscript{2}), 4.41 (s, 2H, CH\textsubscript{2}), 4.62 (s, 2H, CH\textsubscript{2}), 4.78 (s, 2H, CH\textsubscript{2}), 5.12 (s, 2H, CH\textsubscript{2}), 5.22 (s, 2H, CH\textsubscript{2}), 5.45 (s, 2H, CH\textsubscript{2}), 6.65-6.99 (m, 4H, CH), 7.01-7.17 (m, 4H, CH), 7.21-7.29 (m, 2H, CH), 7.31-7.45 (m, 19H, CH), 7.47-7.56 (m, 2H, CH), 7.61-7.89 (m, 4H, CH); δ\textsubscript{C} (100 MHz, d\textsubscript{4}-CDCl\textsubscript{3}) 18.9, 21.6, 26.1, 26.8, 30.1, 32.4, 34.3, 41.3, 44.3, 47.2, 54.1, 63.5, 66.4, 70.8, 126.8, 127.1, 127.2, 127.4, 127.5, 127.8, 127.9, 128.1, 128.2, 128.3, 128.4, 129.7, 130.9, 131.1, 132.8, 135.5, 135.6, 136.1, 136.3, 141.1, 144.7, 147.6, 150.1, 152.3; LRMS (ES+) Calcd for [C\textsubscript{71}H\textsubscript{70}N\textsubscript{6}O\textsubscript{10}S + H] 1198.48 found 1198.61.
Benzyl 2-(benzyloxy)-4-(5-(4-((N-(2-((3-(benzyloxy)carbonyl)phenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)-4-methylphenylsulfonamido)methyl)-1H-1,2,3-triazol-1-yl)pentanamido)benzoate (2.37d). Compound 2.37d was synthesized according to general procedure k, yielding the final product as a colourless oil (59-68%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 1.27-1.37 (m, 5H, CH$_2$), 1.60 (p, $J = 6.9$ Hz, 2H, CH$_2$), 1.70-1.83 (m, 5H, CH$_2$), 1.90 (p, $J = 6.9$ Hz, 2H, CH$_2$), 2.10 (t, $J = 7.2$ Hz, 2H, CH$_2$), 2.32-2.45 (m, 1H, CH), 2.49 (s, 3H, CH$_3$), 3.83 (s, 2H, CH$_2$), 4.32 (t, $J = 6.2$ Hz, 2H, CH$_2$), 4.45 (s, 2H, CH$_2$), 4.70 (s, 2H, CH$_2$), 5.00 (s, 2H, CH$_2$), 5.13 (s, 2H, CH$_2$), 5.31-5.32 (m, 4H, CH), 6.63-6.97 (m, 4H, CH), 7.01-7.17 (m, 4H, CH), 7.21-7.29 (m, 2H, CH), 7.31-7.45 (m, 17H, CH), 7.47-7.56 (m, 4H, CH), 7.61-7.89 (m, 4H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 18.9, 21.6, 26.1, 26.8, 30.1, 34.3, 41.3, 44.3, 47.2, 50.1, 54.1, 63.5, 66.4, 67.8, 126.8, 127.1, 127.2, 127.4, 127.5, 127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 129.7, 130.9, 131.1, 132.8, 135.5, 135.6, 136.1, 136.3, 141.1, 144.7, 147.6, 150.1, 152.3; LRMS (ES+) Calcd for [C$_{72}$H$_{72}$N$_6$O$_{10}$S + H] 1213.50 found 1213.43.
Benzyl 2-(benzyloxy)-4-(6-(4-((N-2-((3-benzyloxy)-4-(benzyloxy)carbonyl)phenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)-4-methylphenylsulfonamido)methyl)-1H-1,2,3-triazol-1-yl)hexanamido)benzoate (2.37e). Compound 2.37e was synthesized according to general procedure k, yielding the final product as a colourless oil (59-68%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 1.14 (p, $J = 6.7$ Hz, 2H, CH$_2$), 1.58 (p, $J = 7.5$ Hz, 2H, CH$_2$), 1.67-1.85 (m, 7H, CH$_2$), 2.15 (t, $J = 7.2$ Hz, 2H, CH$_2$), 2.34-2.50 (m, 1H, CH), 4.23 (t, $J = 5.4$ Hz, 2H, CH$_2$), 4.28 (s, 2H, CH$_2$), 4.70 (s, 2H, CH$_2$), 4.96 (s, 2H, CH$_2$), 5.05 (s, 2H, CH$_2$), 5.08 (s, 2H, CH$_2$), 5.10 (s, 2H, CH$_2$), 5.13 (s, 2H, CH$_2$), 6.75 (d, $J = 9.2$ Hz, 1H, CH), 6.78-6.86 (m, 2H, CH), 6.90-7.02 (m, 3H, CH), 7.03-7.16 (m, 3H, CH), 7.20-7.49 (m, 15H, CH), 7.52-7.62 (m, 2H, CH), 7.76-7.90 (m, 3H, CH), 8.00 (d, $J = 8.1$ Hz, 2H, CH), 8.15 (d, $J = 8.5$ Hz, 1H, CH), 8.38 (s, 1H, CH), 8.55 (d, $J = 7.4$ Hz, 1H, CH), 8.58-8.67 (m, 1H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 18.9, 21.6, 26.1, 26.8, 30.1, 34.3, 41.3, 44.3, 47.2, 50.1, 52.1, 54.1, 63.5, 126.8, 127.1, 127.2, 127.4, 127.5, 127.8, 127.9, 128.1, 128.2, 128.3, 128.4, 129.7, 130.9, 131.1, 132.8, 135.5, 135.6, 136.1, 136.3, 141.1, 144.7, 147.6, 150.1, 152.3; LRMS (ES+) Calcd for [C$_{73}$H$_{74}$N$_6$O$_{10}$S + Na] 1249.51 found 1249.11.
Benzyl 2-(benzyloxy)-4-(2-((N-(2-((3-(benzyloxy)carbonyl)phenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)quinoline-8-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)acetamido)benzoate (2.37f). Compound 2.37f was synthesized according to general procedure k, yielding the final product as a colourless oil (59-68%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 1.23-1.41 (m, 5H, CH$_2$), 1.68-1.85 (m, 5H, CH$_2$), 2.29-2.47 (m, 1H, CH), 4.37 (s, 2H, CH$_2$), 4.54 (s, 2H, CH$_2$), 4.62 (s, 2H, CH$_2$), 4.74 (s, 2H, CH$_2$), 4.84 (s, 2H, CH$_2$), 4.93 (s, 2H, CH$_2$), 5.08 (s, 2H, CH$_2$), 5.28-5.32 (m, 2H, CH$_2$), 6.72-6.91 (m, 4H, CH), 6.96-7.01 (m, 2H, CH), 7.13-7.25 (m, 6H, CH), 7.27-7.45 (m, 15H, CH), 7.55-7.65 (m, 2H, CH), 7.74-7.83 (m, 2H, CH), 7.94-8.05 (m, 2H, CH), 8.14 (d, $J = 8.4$ Hz, 1H, CH), 8.48 (d, $J = 7.7$ Hz, 1H, CH), 8.58-8.63 (m, 1H, CH), 8.87 (s, 1H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 14.2, 20.8, 26.0, 26.5, 29.8, 34.4, 44.2, 51.3, 60.4, 66.3, 112.1, 114.2, 116.2, 122.0, 124.9, 126.8, 127.1, 127.3, 127.4, 127.6, 127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 128.9, 132.4, 132.7, 132.8, 133.4, 133.5, 135.6, 136.1, 136.4, 136.5, 137.4, 138.7; LRMS (ES+) Calcd for [C$_{71}$H$_{63}$N$_7$O$_{10}$S + Na] 1230.45 found 1207.15.
Benzyl 2-(benzyloxy)-4-(3-(4-(N-(2-((3-(benzyloxy)carbonyl)phenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)quinoline-8-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)propanamido)benzoate (2.37g). Compound 2.37g was synthesized according to general procedure k, yielding the final product as a colourless oil (59-68%). \(\delta_H\) (400 MHz, \(d\)-CDCl\(_3\)) 1.29-1.43 (m, 5H, \(\text{CH}_2\)), 1.69-1.85 (m, 5H, \(\text{CH}_2\)), 2.37-2.51 (m, 1H, CH), 2.72 (t, \(J = 6.5\) Hz, 2H, \(\text{CH}_2\)), 4.58 (t, \(J = 5.6\) Hz, 2H, \(\text{CH}_2\)), 4.67 (s, 2H, \(\text{CH}_2\)), 4.68 (s, 2H, \(\text{CH}_2\)), 4.96 (s, 2H, \(\text{CH}_2\)), 5.10 (s, 2H, \(\text{CH}_2\)), 5.15 (s, 2H, \(\text{CH}_2\)), 5.32 (s, 2H, \(\text{CH}_2\)), 5.33 (s, 2H, \(\text{CH}_2\)), 6.67-6.77 (m, 1H, CH), 6.89-6.98 (m, 2H, CH), 7.01-7.10 (m, 2H, CH), 7.16-7.41 (m, 2H, CH), 7.42-7.49 (m, 2H, CH), 7.57 (t, \(J = 7.8\) Hz, 2H, CH), 7.70 (s, 1H, CH), 7.72-7.77 (m, 1H, CH), 7.84 (d, \(J = 8.2\) Hz, 1H, CH), 7.97 (d, \(J = 8.1\) Hz, 1H, CH), 8.14 (d, \(J = 8.3\) Hz, 1H, CH), 8.45-8.64 (m, 2H, CH); \(\delta_C\) (100 MHz, \(d\)-CDCl\(_3\)) 26.7, 34.4, 37.7, 44.1, 49.4, 66.4, 67.0, 70.4, 71.4, 72.3, 74.9, 114.0, 121.9, 125.3, 126.9, 127.1, 127.1, 127.6, 127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 132.1, 132.9, 133.3, 135.5, 136.2, 136.3, 136.5, 137.9, 140.7, 142.4, 142.8, 145.2, 147.5, 165.7; LRMS (ES+) Calcd for [C\(_{72}\)H\(_{67}\)N\(_7\)O\(_{10}\)S + H] 1222.46 found 1221.70.
Benzyl 2-(benzoxly)-4-(4-(4-((N-(2-((3-benzoxly)-4-((benzoxly)carbonyl)phenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)quinoline-8-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)butanamido)benzoate (2.37h). Compound 2.37h was synthesized according to general procedure k, yielding the final product as a colourless oil (59-68%). $\delta_H$ (400 MHz, $d$-CD$_3$OD) 1.39-1.47 (m, 5H, CH$_2$), 1.76-1.84 (m, 5H, CH$_2$), 2.39-2.49 (m, 1H, CH), 3.00 (t, $J$ = 6.2 Hz, 2H, CH$_2$), 4.67 (t, $J$ = 6.2 Hz, 2H, CH$_2$), 4.50 (s, 2H, CH$_2$), 4.69 (s, 2H, CH$_2$), 4.84 (s, 2H, CH$_2$), 4.93 (s, 2H, CH$_2$), 5.16 (s, 2H, CH$_2$), 5.25 (s, 2H, CH$_2$), 5.30-5.43 (m, 4H, CH$_2$), 6.51-6.85 (m, 1H, CH), 6.85-6.70 (m, 4H, CH), 6.97-7.04 (m, 2H, CH), 7.09-7.16 (m, 1H, CH), 7.16-7.43 (m, 20H, CH), 7.44-7.52 (m, 2H, CH), 7.53-7.62 (m, 1H, CH), 7.74 (s, 1H, CH), 7.79-7.88 (m, 2H, CH), 7.89-8.19 (m, 3H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 14.1, 20.9, 25.3, 25.9, 26.6, 29.5, 34.2, 44.0, 48.4, 51.9, 60.4, 66.1, 104.5, 111.6, 113.8, 121.9, 125.5, 126.9, 127.0, 127.2, 127.4, 127.7, 127.8, 127.9, 128.1, 128.2, 128.3, 128.4, 128.5, 128.9, 132.7, 132.9, 133.3, 133.5, 135.6, 136.3, 136.9, 143.6, 150.8, 159.8, 165.9, 168.9; LRMS (ES+) Calcd for [C$_{73}$H$_{69}$N$_7$O$_{10}$S + Na] 1258.48 found 1235.78.
Benzyl 2-(benzyloxy)-4-(5-(4-((N-(2-((3-benzyloxy)-4-((benzyloxy)carbonyl)phenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)quinoline-8-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)pentanamido)benzoate (2.37i). Compound 2.37i was synthesized according to general procedure k, yielding the final product as a colourless oil (59-68%). \( \delta_H (400 \text{ MHz, } d-\text{CDCl}_3) \) 1.29-1.39 (m, 5H, CH\(_2\)), 1.60 (p, \( J = 7.1 \) Hz, 2H, CH\(_2\)), 1.68-1.81 (m, 5H, CH\(_2\)), 1.85 (p, \( J = 6.7 \) Hz, 2H, CH\(_2\)), 2.13 (t, \( J = 7.0 \) Hz, 2H, CH\(_2\)), 2.38-2.50 (m, 1H, CH), 3.83 (s, 2H, CH\(_2\)), 4.27 (t, \( J = 6.1 \) Hz, 2H, CH\(_2\)), 4.37 (s, 2H, CH\(_2\)), 4.67 (s, 2H, CH\(_2\)), 4.97 (s, 2H, CH\(_2\)), 5.13 (s, 2H, CH\(_2\)), 5.32 (s, 2H, CH\(_2\)), 5.34 (s, 2H, CH\(_2\)), 6.89-7.00 (m, 3H, CH), 7.02-7.13 (m, 2H, CH), 7.25-7.41 (m, 21H, CH), 7.41-7.48 (m, 2H, CH), 7.58 (s, 1H, CH), 7.72-7.81 (m, 4H, CH), 7.85 (d, \( J = 8.5 \) Hz, 2H, CH), 8.26 (s, 2H, CH); \( \delta_C (100 \text{ MHz, } d-\text{CDCl}_3) \) 21.5, 21.9, 25.9, 26.7, 29.0, 34.4, 36.3, 43.0, 44.1, 47.8, 49.2, 50.2, 52.7, 104.2, 110.7, 114.6, 126.9, 127.1, 127.2, 127.4, 127.5, 127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5, 129.6, 132.8, 133.4, 135.5, 135.7, 136.2, 136.4, 143.4, 159.5, 161.2, 165.6, 167.8, 168.7, 170.8; LRMS (ES+) Calcd for [C\(_{74}H_{71}N_{7}O_{10}S + Na] 1272.49 found 1272.42.
Benzyl 2-(benzyloxy)-4-(6-(4-((N-(2-((3-benzyloxy)-4-((benzyloxy)carbonyl)phenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)quinoline-8-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)hexanamido)benzoate (2.37j). Compound 2.37j was synthesized according to general procedure k, yielding the final product as a colourless oil (59-68%). δ_H (400 MHz, d-CDCl₃) 1.14 (p, J = 6.7 Hz, 2H, CH₂), 1.58 (p, J = 7.5 Hz, 2H, CH₂), 1.67-1.85 (m, 7H, CH₂), 2.15 (t, J = 7.2 Hz, 2H, CH₂), 2.34-2.50 (m, 1H, CH), 4.23 (t, J = 5.4 Hz, 2H, CH₂), 4.28 (s, 2H, CH₂), 4.70 (s, 2H, CH₂), 4.71 (s, 2H, CH₂), 4.95 (s, 2H, CH₂), 5.13 (s, 2H, CH₂), 5.33 (s, 4H, CH₂), 6.75 (d, J = 9.2 Hz, 1H, CH), 6.78-6.86 (m, 2H, CH), 6.90-7.02 (m, 3H, CH), 7.03-7.16 (m, 3H, CH), 7.20-7.49 (m, 17H, CH), 7.52-7.62 (m, 2H, CH), 7.76-7.90 (m, 3H, CH), 8.00 (d, J = 8.1 Hz, 2H, CH), 8.15 (d, J = 8.5 Hz, 1H, CH), 8.38 (s, 1H, CH), 8.55 (d, J = 7.4 Hz, 1H, CH), 8.58-8.67 (m, 1H, CH); δ_C (100 MHz, d-CDCl₃) 24.1, 25.6, 25.9, 26.7, 29.4, 34.4, 36.9, 44.1, 49.9, 66.3, 67.0, 70.3, 104.3, 113.9, 122.0, 125.4, 126.8, 127.0, 127.1, 127.5, 127.8, 128.1, 128.3, 128.5, 128.9, 132.8, 132.9, 133.4, 133.7, 135.6, 135.7, 136.2, 136.3, 136.5, 143.6, 143.8, 150.7, 158.7, 159.5, 165.5, 165.6; LRMS (ES+) Calcd for [C₇₅H₇₃N₇O₁₀S + H] 1264.51 found 1263.61.
4-(2-((N-(2-((4-carboxy-3-hydroxyphenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)-4-methylphenylsulfonamido)methyl)-1H-1,2,3-triazol-1-yl)acetamido)-2-hydroxybenzoic acid (2.38a). Compound 2.38a was synthesized according to general procedure I, yielding the final product as a white solid (56-89%). \(\delta_H\) (400 MHz, \(d_6-(CD_3)_2SO\)) 1.27-1.37 (m, 5H, CH\(_2\)), 1.54-1.61, 1.66-1.77 (m, 5H, CH\(_2\)), 2.35 (s, 3H, CH\(_3\)), 2.37-2.42 (m, 1H, CH), 3.89 (s, 2H, CH\(_2\)), 4.60 (s, 2H, CH\(_2\)), 4.72 (s, 2H, CH\(_2\)), 5.31 (s, 2H, CH\(_2\)), 6.61 (s, 1H, CH), 6.68 (s, 1H, CH), 7.03 (t, \(J = 8.4\) Hz, 3H, CH), 7.10 (d, \(J = 7.9\) Hz, 2H, CH), 7.25-7.34 (m, 3H, CH), 7.61 (d, \(J = 8.1\) Hz, 2H, CH), 7.66-7.76 (m, 2H, CH), 7.96 (s, 1H, CH); \(\delta_C\) (100 MHz, \(d_6-(CD_3)_2SO\)) 26.7, 29.7, 29.9, 31.9, 34.4, 36.3, 42.9, 44.1, 45.7, 104.4, 106.7, 110.8, 124.8, 126.4, 127.0, 127.9, 129.2, 130.9, 131.5, 133.7, 136.6, 143.9, 147.6, 150.7, 171.9, 174.2, 181.6, 188.9; HRMS (ES–) Calcd for [(C\(_{41}\)H\(_{42}\)N\(_6\)O\(_{10}\) – 2H)/2] 404.1341 found 404.1290; HPLC (I) \(t_R = 20.93\) min (95.3%), (II) \(t_R = 12.67\) min (97.5%).
4-(3-(4-((N-(2-((4-carboxy-3-hydroxyphenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)-4-methylphenylsulfonamido)methyl)-1H-1,2,3-triazol-1-yl)propanamido)-2-hydroxybenzoic acid (2.38b). Compound 2.38b was synthesized according to general procedure I, yielding the final product as a white solid (56-89%). δH (400 MHz, $d_6$-(CD$_3$)$_2$SO) 1.28-1.43 (m, 5H, CH$_2$), 1.66-1.84 (m, 5H, CH$_2$), 2.34 (s, 3H, CH$_3$), 2.38-2.46 (m, 1H, CH), 2.89-3.07 (m, 2H, CH$_2$), 3.79-3.91 (m, 2H, CH$_2$), 4.57 (s, 2H, CH$_2$), 4.65 (s, 2H, CH$_2$), 4.73 (s, 2H, CH$_2$), 6.48-6.65 (m, 2H, CH), 6.90-7.10 (m, 5H, CH), 7.19-7.34 (m, 3H, CH), 7.58 (d, $J = 7.9$ Hz, 2H, CH), 7.67-7.82 (m, 3H, CH); δC (100 MHz, $d_6$-(CD$_3$)$_2$SO) 26.4, 29.4, 29.8, 34.1, 36.1, 42.8, 44.0, 45.6, 66.4, 104.2, 106.6, 110.2, 124.7, 126.4, 127.0, 127.9, 129.2, 130.9, 131.5, 133.7, 136.2, 143.6, 147.2, 150.4, 171.6, 174.0, 175.6, 181.3, 188.5; HRMS (ES−) Calcd for [((C$_{42}$H$_{44}$N$_6$O$_{10}$S$-2H)/2] 411.1420 found 411.1098; HPLC (I) $t_R = 20.64$ min (95.3%), (II) $t_R = 12.42$ min (91.9%).
Compound 2.38c was synthesized according to general procedure I, yielding the final product as a white solid (56-89%). $\delta_H$ (400 MHz, $d_6$-(CD$_3$)$_2$SO) 1.19-1.43 (m, 5H, CH$_2$), 1.62-1.82 (m, 5H, CH$_2$), 2.03 (p, $J = 6.9$ Hz, 2H, CH$_2$), 2.33 (s, 3H, CH$_3$), 2.39-2.46 (m, 1H, CH), 3.66 (t, $J = 7.2$ Hz, 2H, CH$_2$), 3.89 (s, 2H, CH$_2$), 4.33 (t, $J = 7.0$ Hz, 2H, CH$_2$), 4.53 (s, 2H, CH$_2$), 4.75 (s, 2H, CH$_2$), 6.67 (d, $J = 8.5$ Hz, 1H, CH), 6.75 (s, 1H, CH), 6.99-7.12 (m, 4H, CH), 7.27-7.34 (m, 3H, CH), 7.53-7.61 (m, 3H, CH), 7.64-7.74 (m, 2H, CH), 7.89 (s, 1H, CH); $\delta_C$ (100 MHz, $d_6$-(CD$_3$)$_2$SO) 21.3, 25.7, 25.9, 30.1, 30.8, 33.4, 34.2, 43.0, 43.7, 49.1, 52.0, 106.1, 107.8, 110.4, 116.3, 118.6, 120.6, 127.4, 127.9, 129.1, 129.8, 131.3, 134.5, 137.1, 142.3, 162.2, 162.5, 171.2, 171.4, 171.9; HRMS (ES–) Calcd for [(C$_{43}$H$_{46}$N$_6$O$_{10}$S – 2H)/2] 418.1498 found 418.1054; HPLC (I) $t_R = 20.76$ min (98.2%), (II) $t_R = 12.52$ min (95.9%).
4-(5-(4-((N-(2-((4-carboxy-3-hydroxyphenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)-4-methylphenylsulfonamido)methyl)-1H-1,2,3-triazol-1-yl)pentanamido)-2-hydroxybenzoic acid (2.38d). Compound 2.38d was synthesized according to general procedure I, yielding the final product as a white solid (56-89%). $\delta_H$ (400 MHz, $d_6$-(CD$_3$)OD) 1.38-1.45 (m, 5H, CH$_2$), 1.57-1.61 (p, $J = 7.3$ Hz, 2H, CH$_2$), 1.74-1.82 (m, 5H, CH$_2$), 1.86-1.94 (m, 2H, CH$_2$), 2.38 (s, 3H, CH$_3$), 2.39-2.41 (m, 1H, CH), 3.07 (t, $J = 7.8$ Hz, 2H, CH$_2$), 3.87 (s, 2H, CH$_2$), 4.35 (t, $J = 6.8$, 2H, CH$_2$), 4.59 (s, 2H, CH$_2$), 4.76 (s, 2H, CH$_2$), 6.51-6.62 (m, 2H, CH), 6.96-7.05 (m, 3H, CH), 7.07-7.12 (m, 2H, CH), 7.27-7.32 (m, 2H, CH), 7.62 (d, $J = 7.9$ Hz, 2H, CH), 7.70-7.81 (m, 4H, CH); $\delta_C$ (100 MHz, $d_6$-(CD$_3$)$_2$SO) 21.6, 25.8, 25.9, 30.3, 30.9, 33.5, 34.5, 43.1, 43.9, 49.3, 52.3, 60.7, 106.7, 107.9, 110.5, 116.4, 118.8, 120.8, 127.5, 127.9, 129.1, 129.8, 131.5, 134.7, 137.3, 142.5, 162.6, 162.9, 171.3, 171.6, 171.9; HRMS (ES−) Calcd for [C$_{44}$H$_{48}$N$_6$O$_{10}$S − H] 425.1576 found 425.1087; HPLC (I) $t_R = 21.00$ min (91.8%), (II) $t_R = 13.37$ min (93.4%).
4-(6-((N-((2-((4-carboxy-3-hydroxyphenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)-4-methylphenylsulfonamido)methyl)-1H-1,2,3-triazol-1-yl)hexanamido)-2-hydroxybenzoic acid (2.38e). Compound 2.38e was synthesized according to general procedure I, yielding the final product as a white solid (56-89%). $\delta^1_H$ (400 MHz, $d_6$(CD$_3$)$_2$SO) 1.36-1.44 (m, 5H, CH$_2$), 1.66-1.75 (m, 2H, CH$_2$), 1.79-1.87 (m, 5H, CH$_2$), 2.15-2.22 (m, 2H, CH$_2$), 2.33 (t, $J = 7.3$ Hz, 2H, CH$_2$), 2.39-2.43 (m, 2H, CH$_2$), 2.44-2.53 (m, 1H, CH), 3.52 (s, 2H, CH$_2$), 3.82 (s, 2H, CH$_2$), 4.32 (t, $J = 7.2$ Hz, 2H, CH$_2$), 4.77 (s, 2H, CH$_2$), 6.39-6.50 (m, 2H, CH), 6.89 (d, $J = 8.6$ Hz, 1H, CH), 7.04-7.09 (m, 2H, CH), 7.09 (m, 2H, CH), 7.16-7.20 (m, 1H, CH), 7.28-7.32 (m, 2H, CH), 7.58-7.62 (m, 2H, CH), 7.66 (s, 1H, CH), 7.77 (t, $J = 8.9$ Hz, 2H, CH); $\delta^1_C$ (100 MHz, $d_6$(CD$_3$)$_2$SO) 21.7, 25.9, 26.0, 30.3, 30.9, 33.5, 34.5, 43.1, 43.8, 49.3, 52.3, 106.9, 107.9, 110.5, 116.6, 118.9, 120.8, 127.5, 128.0, 129.1, 129.8, 131.8, 134.9, 137.4, 142.5, 156.2, 162.9, 171.3, 171.6, 171.9; HRMS (ES–) Calcd for [(C$_{45}$H$_{50}$N$_6$O$_{10}$S – 2H)/2] 432.1654 found 432.2189; HPLC (I) $t_R = 21.43$ min (98.6%), (II) $t_R = 13.39$ min (96.7%).
4-(2-(4-((N-(2-((4-carboxy-3-hydroxyphenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)quinoline-8-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)acetamido)-2-hydroxybenzoic acid (2.38f). Compound 2.38f was synthesized according to general procedure I, yielding the final product as a white solid (56-89%). $\delta_H$ (400 MHz, $d_6$-(CD$_3$)$_2$SO) 1.36-1.42 (m, 5H, CH$_2$), 1.78-1.84 (m, 5H, CH$_2$), 2.40-2.51 (m, 1H, CH), 3.90 (s, 2H, CH$_2$), 4.49 (s, 2H, CH$_2$), 4.57 (s, 2H, CH$_2$), 4.63-4.70 (m, 1H, CH), 6.43-6.52 (m, 1H, CH), 6.55-6.61 (m, 1H, CH), 6.64 (s, 1H, CH), 6.88-6.70 (m, 2H, CH), 7.01-7.21 (m, 7H, CH), 7.33 (s, 1H, CH), 7.34-7.42 (m, 1H, CH), 7.62 (s, 1H, CH), 7.73 (d, $J = 8.7$ Hz, 1H, CH), 7.80 (d, $J = 8.3$ Hz, 1H, CH); $\delta_C$ (100 MHz, $d_6$-(CD$_3$)$_2$SO) 25.4, 26.0, 34.6, 43.4, 45.5, 50.5, 53.9, 60.3, 108.9, 115.6, 115.7, 121.5, 124.5, 126.3, 126.7, 127.8, 127.9, 128.9, 130.7, 131.5, 131.9, 133.9, 136.4, 140.2, 141.9, 145.2, 148.2, 140.9, 164.5, 166.5, 168.5, 171.2; HRMS (ES$^-$) Calcd for [(C$_{43}$H$_{41}$N$_7$O$_{10}$S - 2H)/2] 422.6318 found 424.2543; HPLC (I) $t_R = 21.78$ min (94.9%), (II) $t_R = 13.61$ min (96.0%).
4-(3-(4-((N-(2-((4-carboxy-3-hydroxyphenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)quinoline-8-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)propanamido)-2-hydroxybenzoic acid (2.38g). Compound 2.38g was synthesized according to general procedure I, yielding the final product as a white solid (56-89%). \( \delta_H (400 \text{ MHz}, d_{6}-(\text{CD}_3)_2\text{SO}) 1.51-1.60 (m, 5H, CH_2), 1.66 (m, 5H, CH_2), 2.30-2.36 (m, 1H, CH), 3.00 (t, \ J = 6.1 \text{ Hz}, 2H, CH_2), 3.80 (s, 2H, CH_2), 4.48 (s, 2H, CH_2), 4.67 (t, \ J = 6.2 \text{ Hz}, 2H, CH_2), 5.23 (s, 2H, CH_2), 6.35 (s, 1H, CH), 6.41-6.45 (m, 1H, CH), 6.55-6.60 (m, 1H, CH), 6.74-6.84 (m, 4H, CH), 6.92 (s, 1H, CH), 6.98-7.12 (m, 6H, CH), 7.33 (d, \ J = 6.8 \text{ Hz}, 1H, CH), 7.56 (d, \ J = 8.5 \text{ Hz}, 1H, CH), 7.78 (s, 1H, CH); \delta_C (100 \text{ MHz}, d_{6}-(\text{CD}_3)_2\text{SO}) 24.5, 26.0, 30.8, 34.7, 43.3, 45.8, 49.5, 54.9, 106.2, 113.8, 114.7, 121.8, 122.9, 124.3, 126.4, 127.3, 127.9, 128.8, 130.7, 131.8, 132.0, 133.8, 136.4, 140.7, 141.7, 145.8, 148.3, 150.6, 164.8, 167.0, 171.8, 174.0; HRMS (ES–) Calcd for \([C_{44}H_{43}N_{7}O_{10}S – 2H]/2\) 429.6396 found 431.6793; HPLC (I) \( t_R = 21.59 \text{ min (98.6%)}, \) (II) \( t_R = 13.39 \text{ min (97.6%)}. \)
4-(3-4-((N-(2-((4-carboxy-3-hydroxyphenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)quinoline-8-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)propanamido)-2-hydroxybenzoic acid (2.38h). Compound 2.38h was synthesized according to general procedure 1, yielding the final product as a white solid (56-89%). $\delta_H$ (400 MHz, $d_6$-(CD$_3$)$_2$SO) 1.28-1.36 (m, 5H, CH$_2$), 1.67-1.73 (m, 5H, CH$_2$), 2.05 (p, $J = 7.6$ Hz, 2H, CH$_2$), 2.33 (t, $J = 7.1$ Hz, 2H, CH$_2$), 2.37-2.45 (m, 1H, CH), 3.93 (s, 2H, CH$_2$), 4.36 (t, $J = 7.2$ Hz, 2H, CH$_2$), 4.42 (s, 2H, CH$_2$), 4.79 (s, 2H, CH$_2$), 6.45 (t, $J = 7.8$ Hz, 1H, CH), 6.54 (s, 1H, CH), 6.63-6.70 (m, 1H, CH), 6.74-6.83 (m, 2H, CH), 6.97-7.11 (m, 7H, CH), 7.28-7.37 (m, 2H, CH), 7.64 (m, 3H, CH); $\delta_C$ (100 MHz, $d_6$-(CD$_3$)$_2$SO) 20.4, 25.7, 25.9, 26.8, 33.3, 34.3, 41.4, 43.7, 49.1, 55.4, 106.5, 107.8, 110.4, 113.9, 114.2, 117.3, 118.7, 123.2, 123.9, 126.9, 127.7, 127.8, 127.9, 128.5, 131.2, 131.6, 134.3, 134.5, 142.1, 143.6, 145.7, 146.8, 162.1, 162.5; HRMS (ES−) Calcd for [C$_{44}$H$_{43}$N$_7$O$_{10}$S − H] 436.6474 found 438.7854; HPLC (I) $t_R$ =21.72 min (93.1%), (II) $t_R = 30.53$ min (90.9%).
4-(5-(4-((N-(2-((4-carboxy-3-hydroxyphenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)quinoline-8-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)pentanamido)-2-hydroxybenzoic acid (2.38i). Compound 2.38i was synthesized according to general procedure 1, yielding the final product as a white solid (56-89%). δH (400 MHz, d6-(CD3)2SO) 1.31-1.43 (m, 5H, CH2), 1.52 (p, J = 7.8 Hz, 2H, CH2), 1.63-1.75 (m, 5H, CH2), 1.75 (p, J = 6.8 Hz, 2H, CH2) 2.35 (t, J = 7.4 Hz, 2H, CH2), 2.36-2.49 (m, 1H, CH), 3.22 (s, 2H, CH2), 4.31 (t, J = 7.0, 2H, CH2), 4.42 (s, 2H, CH2), 4.78 (s, 2H, CH2), 6.44 (t, J = 7.9 Hz, 1H, CH), 6.55 (s, 1H, CH), 6.60-6.70 (m, 1H, CH), 6.76 (s, 1H, CH), 6.78-6.88 (m, 1H, CH), 7.01-7.13 (m, 8H, CH), 7.29-7.35 (m, 1H, CH), 7.66-7.74 (m, 3H, CH); δC (100 MHz, d6-(CD3)2SO) 20.5, 25.9, 26.1, 26.8, 33.8, 34.4, 42.4, 43.8, 49.5, 56.4, 57.8, 106.8, 107.8, 110.6, 114.1, 114.2, 117.9, 118.9, 123.4, 123.9, 126.9, 127.7, 127.8, 128.5, 131.2, 131.6, 134.3, 134.5, 142.1, 143.9, 145.9, 146.9, 163.1, 162.9; HRMS (ES–) Calcd for [(C46H47N7O10S – 2H)/2] 443.6552 found 445.7564; HPLC (I) tR = 21.88 min (98.1%), (II) tR = 13.77 min (94.6%).
4-(6-((N-(2-((4-carboxy-3-hydroxyphenyl)(4-cyclohexylbenzyl)amino)-2-oxoethyl)quinoline-8-sulfonamido)methyl)-1H-1,2,3-triazol-1-yl)hexanamido)-2-hydroxybenzoic acid (2.38j). Compound 2.38j was synthesized according to general procedure l, yielding the final product as a white solid (56-89%). $\delta_H$ (400 MHz, $d_6$-(CD$_3$)$_2$SO) 1.35-1.43 (m, 5H, CH$_2$), 1.66-1.78 (m, 5H, CH$_2$), 2.07-2.19 (m, 1H, CH), 2.30 (p, $J = 7.5$ Hz, 2H, CH$_2$), 2.39-2.51 (m, 2H, CH$_2$), 2.69 (t, $J = 6.1$ Hz, 2H, CH$_2$), 3.20-3.24 (m, 2H, CH$_2$), 3.83-3.92 (m, 2H, CH$_2$), 4.28 (t, $J = 7.1$ Hz, 2H, CH$_2$), 4.43 (s, 2H, CH$_2$), 4.73 (s, 2H, CH$_2$), 6.47 (t, $J = 7.9$ Hz, 1H, CH), 6.58 (s, 1H, CH), 6.61-6.73 (m, 1H, CH), 6.78 (s, 1H, CH), 6.79-6.89 (m, 1H, CH), 7.02-7.16 (m, 8H, CH), 7.31-7.39 (m, 1H, CH), 7.69-7.75 (m, 3H, CH); $\delta_C$ (100 MHz, $d_6$-(CD$_3$)$_2$SO) 21.5, 26.1, 26.4, 26.8, 33.9, 34.7, 42.8, 43.9, 49.9, 56.7, 106.9, 107.8, 110.6, 114.7, 114.9, 117.9, 118.9, 123.9, 124.9, 126.9, 127.7, 127.8, 127.9, 128.8, 131.3, 131.9, 134.3, 134.5, 142.6, 143.9, 145.9, 146.9, 163.3, 163.9; HRMS (ES−) Calcd for [(C$_{47}$H$_{49}$N$_7$O$_{19}$S − 2H)/2] 450.6631 found 452.7053; HPLC (I) $t_R = 22.37$ min (94.2%), (II) $t_R = 14.31$ min (93.8%).
6 Appendix 2: Experimental Methods for Chapter 3

6.1 Screening of the Library

6.1.1 Reagents

Small unilamellar vesicles were purchased from Avanti Polar Lipids. Plain and OptiMEM media, as well as PTP1B Antibody (H-135) rabbit polyclonal IgG and Alexa Fluore®488-anti-rabbit was purchased from the Medical Store, University of Toronto. GeneExpresso™ was purchased from Excellgen. pEGFPC2 vector was purchased from Clontech, Mountain View, CA, Luria Broth and kanamycin both purchased from Wisent, and Genaid Plasmid Maxi Kit was purchased from Froggabio.

6.1.2 GFP-tagged PTP1B Plasmid DNA Construct

GFP-tagged PTP1B plasmid was generously donated from Isabelle Aubry from Michel Tremblay Lab at McGill. She obtained GFP-PTP1B WT by cloning PTP1B into the BglII and EcoRI sites of the pEGFPC2 vector. GFP-PTP1B construct was transformed into competent DH5 alpha bacteria and amplified in Luria Broth in the presence of 30uM kanamycin. DNA was purified using the anion-exchange Genaid Plasmid Maxi Kit.

6.1.3 Preparation of Supported Lipid Bilayer

Lipid bilayers were formed by diluting a solution of POPC vesicles to 1 mg/mL in 20 mM phosphate 50 mM NaCl pH 7.2. One portion contained PTP1B Antibody (H-135) rabbit polyclonal IgG and Alexa Fluore®488-anti-rabbit and compound 3,4 and PTP1B and one without PTP1B. 30 µL of vesicle solution was dropped on a cover slide. After incubation for 2 hrs at room temperature, the cover slides were separated and rinsed thoroughly with Millipore water avoiding air bubbles in the vicinity of the supported bilayer on the glass coverslip. Using tweezers, the coverslip was gently shaken underwater to remove excess vesicles. Keeping the bilayer underwater, a second cleaned coverslip was layed over the bilayer-containing coverslip to create a bilayer sandwich.
6.1.4 Buffer Preparations

Most buffers were commercially available and purchased, as mentioned in section 6.1.1. Buffers used in spectrophotometric determination of equilibrium constants were made by dissolving 4.76 g HEPES in 50 mL water and bringing the pH of the solution to 7.0.

6.1.5 Spectroscopic Determination of Equilibrium Constants

A very similar experiment was previously published, and we utilizing the same conditions with the exception of changing the ligand\textsuperscript{102}. To form the interaction systems between bpV and phenenthroline ligands, NH\textsubscript{4}VO\textsubscript{3} and H\textsubscript{2}O\textsubscript{2} were mixed with a 1:5 molar ratio to the bpV solution first. The respective ligand was then added to the solution. Unless otherwise stated, the total concentration of vanadate species is 0.2 M. The equilibrium constants for coordination between bpV and phenanthroline ligands were measured using a fluorescence reader (PTI: Photon Technology International: uses an arc lap as a source for excitation and photomultiplier for detection. The software that analyses and displays the data is FelixGX-4.1.2). A stock solution of the phenanthroline ligand (1 × 10\textsuperscript{-2} M) was prepared by ethanol, and diluted with HEPES buffer solution (0.1 M, pH = 7.0) to 1 × 10\textsuperscript{-4} M for testing, while bpV was diluted by HEPES buffer solution to 2.5 × 10\textsuperscript{-2} M. A 2.5 mL phenanthroline ligand test solution was titrated by successive additions (10 µL) of bpV solution with concentrations varying from 0 to 12 × 10\textsuperscript{-4} M at 25 °C.

6.1.6 Transfecting CHO (mammalian) Cells Utilizing GeneExpresso™ in vitro DNA Transfection Reagent

Firstly, 1 µg of DNA was added into 50 µL of OptiMEM media with High Glucose. This was vortexed and spun briefly to bring the drops to the bottom of the tube. 3 uL of GeneExpresso™ was added into 50 µL of OptiMEM media with High Glucose. This solution was vortexed gently and spun down briefly. The Diluted GeneExpresso™ was added immediately to the diluted DNA solution. This was vortexed immediately and spun down briefly to bring the liquid drops to bottom of the tube. The solution was left undisturbed for 15-20 mins at room temperature to allow the GeneExpresso™-DNA complexes to form. 100 µL GeneExpresso™ -DNA complex was added dropwise into each well (35 mm dish) containing cells and medium. This was mixed
gently by rocking the plate back and forth. The media was changed 14 hours post transfection and the transfection efficiency was checked 24 hours post transfection.

6.1.7 In vitro Whole Cell Studies

Before dosing the cells, they have been grown in FBS-free DMEM medium for 6-12 hours to prevent PTP1B adsorption to the membrane. Cells were dosed by dissolving compound into the FBS-free, phenol red-free DMEM medium to the desired concentration. Then the original medium from the glass-bottom cell culture dish where the cells were growing was removed. Lastly, the new dosed medium was added to the dish. The dosed cells were incubated in the 37°C carbon dioxide (CO₂) incubator for various lengths of time.

6.1.8 Fluorescence set up

Fluorescence imaging and spectroscopy experiments were performed exactly as previously published (and on the exact same equipment)⁹⁷, on custom-built confocal and TIRF microscopes that are capable of hyperspectral detection of single emitters. The technical details for both instruments are described in detail elsewhere⁹⁸. For single-molecule spectroscopy, tunable excitation in the near-infrared and the visible is given by a broadband femtosecond-pulsed laser (Tsunami HP, Spectra Physics) and frequency-doubled its output by nonlinear crystals, respectively. The laser excites the sample at intensities of 50-200 W/cm² upon focusing by a high NA objective (1.4/100X plan-apochromat, Carl Zeiss) to a diffraction-limited spot. The emitted light is collected by the same objective and passed through a 50-μm pinhole and long-pass optical filters to remove out-of-focus fluorescence and scattering contributions. The fluorescence is divided by a beamsplitter into two beams focused onto separate photon-counting detectors (PD5CTC, Optoelectronic Components, Canada), which are read and digitized by a multichannel time-correlated counting module (PicoHarp300, PicoQuant, Germany). Liposomes and lipid bilayers were imaged on a custom-built, inverted wide-field/TIRF microscope². Diode-pumped, solid-state lasers at 473nm (Cobolt Blues MarketTech, USA) and 532nm (NT56-485, Edmund Optics, USA) illuminate the sample at 1-200 W/cm² through a high NA objective (plan- apochromat 1.45 NA/60x Olympus, USA). The emitted fluorescence is captured by the same objective, while the laser scattering is blocked by dichroic mirrors (FF495-Di02 or Di532, Semrock, USA), long- pass (LP-488-RS, Semrock, USA) and band-pass (HQ520/66, Chroma,
USA) filters. The fluorescence images are acquired with a cooled electron-multiplied charge-coupled device (EMCCD, DU-897BV, Andor, USA)\textsuperscript{107}.

6.2 General Synthetic Methods and Characterization of Molecules

6.2.1 Chemical Methods

Anhydrous solvents methanol, DMSO, CH\textsubscript{2}Cl\textsubscript{2}, THF and DMF were used directly from their Sure-Seal bottles and were purchased from Sigma Aldrich. 4Å molecular sieves also purchased from Sigma Aldrich, were activated by heating to 300 °C under vacuum overnight. All reactions were performed in oven-dried glassware and were monitored for completeness by TLC using silica gel (visualized by UV light, or developed by treatment with KMnO\textsubscript{4} stain or Hanessian's stain). A 400 MHz Bruker NMR was utilized to obtain $^1$H and $^{13}$C NMR spectra in CDCl\textsubscript{3}, CD\textsubscript{3}OD, or d\textsubscript{6}-C\textsubscript{2}D\textsubscript{5}OS (DMSO). A 500 MHz Bruker NMR was used to obtain $^{51}$V NMR Spectra in D\textsubscript{2}O. All NMR Chemical shifts ($\delta$) are reported in parts per million after calibration to residual isotopic solvent and coupling constants (J) are reported in Hz. IR measurements were performed using an AVATAR 360-FT-IR reader and data was analyzed using OMNIC software. Inhibitor purity was evaluated by a Water's rpHPLC prior to coordination and biological testing. Analysis by rpHPLC was performed using a Microsorb-MV 300 Å C18 250 mm x 4.6 mm column with eluent flow set at 1 mL/min, and using gradient mixtures of (A) water with 0.1% TFA and (B) an acetonitrile solution containing 10% H\textsubscript{2}O and 0.1% TFA. Ligand purity was confirmed using linear gradients from 50% A and 50% B to 100% B after an initial 2 minute period of 100% A, and a second linear gradient of 100% A to 100% B. The linear gradient consisted of a changing solvent composition of either (I) 5.2% per minute and UV detection at 254nm or (II) 1.8% per minute and detection at 254nm, each ending with 5 minutes of 100% B. When reporting the HPLC results, retention times for each condition are written followed by their purities in their respective order. Biologically evaluated compounds are > 90% chemical purity as measured by HPLC.
6.2.2 General Procedures

General Procedure a: (Metallation of Ligand)

A typical procedure was prepared by adding H\textsubscript{2}O to V\textsubscript{2}O\textsubscript{5} (1.0 eq) and KOH (2.27 eq) in a suitable Erlenmeyer flask followed by H\textsubscript{2}O\textsubscript{2} (30% v/v). The mixture was gently shaken until most of the solids had dissolved (~2 min). The brightly coloured orange solution was allowed to stand for 15-30 min. More H\textsubscript{2}O\textsubscript{2} was added to the orange solution followed immediately by ligand (2.13 eq) dissolved in ethanol. The reaction was stirred for 30 min, while being protected from light, after which ethanol were added dropwise while stirring to effect precipitation of product as a fine yellow solid (45-51% yield). Please note that the amount of reagent added was precisely calculated based on the amount of compound pre-metalation.

6.2.3 Detailed Synthetic Procedures and Characterizations

\textit{N-}(1,10-phenanthrolin-5-yl)palmitamide (3.5). Compound 3.5 was synthesized by dissolving 1,10-phenenthrolin-5-amine in anhydrous DCM in a microwave vessel, following the addition of palmitoyl chloride (1.5 eq.) The reaction mixture was then heated in a microwave reactor at 150 °C for 3 hours. The reaction mixture was then concentrated down \textit{in vacuo}, dissolved in minimal DCM and purified by passing through a silica gel column with a gradient of DMC and Titanic (92% DCM, 7% MeOH, 1% Ammonium hydroxide) (9:1). The fractions were pooled and concentrated \textit{in vacuo} to give a orange solid (79%). \(\delta\text{H} (400 \text{ MHz, } d-\text{CDCl}_3) 0.86 \text{ (t, } J = 6.8 \text{ Hz, } 3\text{H, CH}_3), 1.22 \text{ (s, } 26\text{H, CH}_2), 1.64 \text{ (m, } 2\text{H, CH}_2), 2.35 \text{ (t, } J = 7.7 \text{ Hz, } 2\text{H, CH}_2), 7.28 \text{ (s, } 1\text{H, CH}), 7.37-7.41 \text{ (m, } 2\text{H, CH}), 8.32-8.44 \text{ (m, } 1\text{H, CH}), 8.35-8.83 \text{ (m, } 3\text{H, CH}); \delta\text{C} (100 \text{ MHz, } d-\text{CDCl}_3) 14.1, 22.7, 25.8, 28.6, 28.9, 29.5, 32.6, 38.9, 1202.2, 121.5, 121.6, 124.5, 128.5, 130.5, 132.0, 136.7, 141.2, 149.5, 149.9, 154.7, 179.1; \text{LRMS (ES+)} \text{ Calcd for } [\text{C}_{28}\text{H}_{38}\text{N}_3\text{O} + \text{H}] 434.62 \text{ found 434.12. HPLC (I) } t_R = 14.125 \text{ min (90.0%), (II) } t_R = 14.132 \text{ min (89.1%).}
**N-bisperoxo (1,10-phenanthroin-5-yl oxovandate (V)) palmitamide (3.6)**. Compound 3.6 was synthesized according to general procedure a, yielding the final product as a dark brown solid (45-51%). \( \delta_{\text{V}=O} (500 \text{ MHz, } d-\text{D}_2\text{O}) -710.95 \) (VOCl\(_3\) was utilized as a reference (0 ppm)). IR (KBr) in cm\(^{-1}\): \( \nu(\text{V}=\text{O}) 925; \nu_1(\text{O}-\text{O}) 870; \nu_2(\text{O}-\text{O}) 802; \nu_3(\text{MO}_2) 635; \nu_4(\text{MO}_2) 590. \\

**Benzyl 5-aminopicolinate (3.7)**. Compound 3.7 was synthesized by dissolving 5-aminopyridine-3-carboxylic (1.0 eq) acid in DMF in suitable round bottom flask. K\( \text{t} \text{OBu} \) (1.2 eq) was added in small portions and the reaction was stirred at r.t for 10 mins. The reaction vessel was cooled to 0°C and then BnBr (1.1 eq) was added drop wise. The reaction was brought to r.t and was left to stir overnight. The reaction mixture was concentrated down *in vacuo*, dissolved in distilled water, and extracted with ethyl acetate. Organic fractions were combined, washed with saturated sodium chloride solution and dried over anhydrous sodium Na\(_2\)SO\(_4\). This crude mixture was concentrated *in vacuo* and then dissolved in minimal amounts of DCM to which solid silica powder was added and the resulting suspension was dried under reduced pressure to afford a dried silica adduct. This adduct was then loaded onto the manual column and purified with a mixture of Hexanes:Ethyl acetate (6:1). Desired fractioned were pooled, concentrated *in vacuo*, co-evaporated with chloroform and desolvated under a high-throughput vacuum system to yield the desired product as a pale yellow solid (100%). \( \delta_H (400 \text{ MHz, } d-\text{CDCl}_3) 5.43 \) (s, 2H, CH\(_2\)), \( \delta_H (400 \text{ MHz, } d-\text{CDCl}_3) 5.43 \) (s, 2H, CH\(_2\)),...
7.29-7.49 (m, 8H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 65.7, 122.4, 125.4, 127.1, 127.6, 128.7, 135.6, 136.4, 137.8, 150.6, 169.6; LRMS (ES+) Calcd for [C$_{13}$H$_{12}$N$_2$O$_2$ + H] 229.24 found 229.65.

Benzyl 5-palmitamidopicolinate (3.8). Compound 3.8 was synthesized by dissolving compound 3.7 in anhydrous DCM in a microwave vessel, following the addition of palmitoyl chloride (1.5 eq). The reaction mixture was then heated in a microwave reactor at 150°C for 3 hrs. The reaction mixture was concentrated down in vacuo, dissolved in minimal DCM and purified by passing through a silica gel column with a gradient of Hexanes:Ethyl acetate (6:1). The fractions were pooled and concentrated in vacuo to give a pale yellow solid (79%). $\delta_H$ (400 MHz, $d$-CDCl$_3$) 0.86 (t, $J = 6.8$ Hz, 3H, CH$_3$), 1.22 (s, 26H, CH$_2$), 1.64 (m, 2H, CH$_2$), 2.35 (t, $J = 7.7$ Hz, 2H, CH), 5.37 (s, 2H, CH$_2$), 7.28-7.35 (m, 2H, CH), 7.37-7.41 (m, 2H, CH), 8.05 (d, $J = 9.3$ Hz, 1H, CH), 8.32-8.44 (m, 1H, CH), 8.63 (s, 1H, CH), 9.32 (s, 1H, CH); $\delta_C$ (100 MHz, $d$-CDCl$_3$) 14.4, 22.8, 25.6, 28.6, 28.9, 29.4, 29.6, 31.5, 38.4, 65.7, 124.5, 127.1, 127.6, 128.9, 123.1, 139.2, 149.3, 169.0, 178.9; LRMS (ES+) Calcd for [C$_{29}$H$_{42}$N$_2$O$_3$ + H] 466.65 found 467.21.

5-palmitamidopicolinic acid (3.9). Compound 3.9 was synthesized by dissolving compound 3.8 in a stirred solution of MeOH:THF (2:1). The solution was thoroughly degassed and 20% Pd/C (10 mg/mmol) was carefully added to the reaction. H$_2$ gas was bubbled through the solvent for 5 mins before the reaction was put under an atmosphere of H$_2$ gas and stirred continuously for 16 hrs. The H$_2$ gas was evacuated and the reaction filtered through celite to remove the Pd catalyst and then concentrated under reduced pressure. The resulting residue was adsorbed onto silica gel
from CH₂Cl₂ and columned using a Biotage-Isolera™ in a gradient of DCM:MeOH (3:1). Fractions were pooled and concentrated in vacuo yielding the final product as a pale orange solid (61%). δ_H (400 MHz, d-CDCl₃) 0.86 (t, J = 6.8 Hz, 3H, CH₃), 1.22 (s, 26H, CH₂), 1.64 (m, 2H, CH₂), 2.35 (t, J = 7.7 Hz, 2H, CH), 5.37 (s, 2H, CH₂), 7.28-7.35 (m, 2H, CH), 7.37-7.41 (m, 2H, CH), 8.05 (d, J = 9.3 Hz, 1H, CH), 8.32-8.44 (m, 1H, CH), 8.63 (s, 1H, CH), 9.32 (s, 1H, CH); δ_C (100 MHz, d-CDCl₃) 14.2, 22.8, 25.0, 29.7, 31.8, 37.4, 67.2, 126.0, 128.8, 135.1, 138.6, 140.2, 141.2, 164.8, 173.2; LRMS (ES+) Calcd for [C₂₂H₃₆N₂O₃ – H] 376.53 found 375.45.

5-palmitamido-3-hydroxypicolinate vanadium (V) (3.10). Compound 3.10 was synthesized according to general procedure a, yielding the final product as an orange solid (45-51%). IR (KBr) in cm⁻¹: v(V=O) 965; v₁(O-O) 869; v₂(O-O) 801; v₃(MO₂) 697; v₄(MO₂) 562. δ_VS₁ (500 MHz, d-D₂O) -745 ppm. (VOCl₃ was utilized as a reference (0 ppm)).

N-((8R,9S,10S,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl)-1,10-phenanthroline-5-amine (3.3). Compound 3.3 was synthesized by dissolving 5-α-cholestan-3-one (5.0 eq) and 1,10-phenanthroline-5-amine (1.0 eq) in a 9:1 mixture of MeOH:DCM in a suitable reaction vessel. The reaction mixture was heated to 50°C and shortly after glacial AcOH (2.5 eq) was added dropwise. The reaction was allowed to
react for 16 hrs at 50°C before sodium cyanoborohydride was added slowly. The reaction was allowed to react for an additional 16 hrs. Once the reaction was complete, it was filtered through Celite to remove the cyano salt and concentrated under reduced pressure. The resulting residue was absorbed onto silica gel from CH2Cl2 and columned using Biotage-Isolera™ a gradient of DCM:Titanic (9:1). Fractions were pooled an concentrated in vacuo yielding the final product as a dark orange solid (51%). δH (400 MHz, d-CD3OD) 0.67 (s, 3H, CH), 0.72-2.26 (m, 46H, CH, CH2 CH3), 3.55 (q, J = 3.77 Hz, 1H, CH), 6.67 (s, 1H, CH), 7.34-7.51 (m, 1H, CH), 7.56-7.71 (m, 1H, CH), 7.99 (d, J = 8.2 Hz, 1H, CH), 8.23 (d, J = 8.3 Hz, 1H, CH), 8.82-8.90 (m, 1H, CH), 9.10-9.23 (m, 1H, CH); δc (100 MHz, d- CD3OD) 11.9, 18.5, 20.6, 22.4, 22.7, 23.7, 24.0, 27.9, 28.1, 28.9, 30.7, 31.9, 32.7, 33.0, 33.2, 35.4, 35.6, 35.9, 37.6, 41.5, 46.1, 54.6, 120.4, 121.6, 123.6, 127.8, 130.1, 131.4, 136.5, 146.7, 149.7, 149.9, 154.7 ; LRMS (ES+) Calcd for [C39H55N3 + H] 565.87 found 566.87. HPLC (I) tR = 16.842 min (88.1%), (II) tR = 32.000 min (91.2%).

N-((8R,9S,10S,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl)-bixperoxo (1,10-phenanthroline-5-amine) oxovanadate (V) (3.4). Compound 3.4 was synthesized according to general procedure a yielding the final product as a light brown solid. IR (KBr) in cm⁻¹: ν(V=O) 930; ν₁(O-O) 870; ν₂(O-O) 854; ν₃(MO₂) 635; ν₄(MO₂) 590. δV51 (500 MHz, d-D₂O) -729 ppm (VOCl₃ was utilized as a reference (0 ppm)).
**Benzyl 4-formylbenzoate (3.17).** Compound 3.17 was synthesized by dissolving 4-formyl benzaldehyde (1.0 eq) in DMF in suitable round bottom flask. KtOBu (1.1 eq) was added in small portions and reaction was stirred under r.t conditions for 10 mins. Following this the reaction vessel was cooled to 0°C, BnBr (1.1 eq) was added drop wise. Reaction was allowed to come to r.t and was stirred overnight. The reaction mixture was concentrated down under reduced pressure, dissolved in distilled water, and extracted with ethyl acetate. Organic fractions were combined, washed with saturated sodium chloride solution and dried over anhydrous sodium sulfate. This crude mixture was concentrated in vacuo and then dissolved in minimal amounts of DCM to which solid silica powder was added and the resulting suspension was dried under reduced pressure to afford a dried silica adduct. This adduct was then loaded onto the manual column and purified with a mixture of Hexanes:Ethyl acetate (4:1). Desired fractioned were pooled, concentrated in vacuo, co-evaporated with chloroform and desolvated under a high-throughput vacuum system to yield the desired product as a clear colourless oil (89 %). δ_H (400 MHz, d-CDCl₃) 5.38 (s, 2H, CH₂), 7.31-7.58 (m, 5H, CH), 7.83-8.01 (m, 2H, CH), 8.09-8.30 (m, 2H, CH), 10.08 (s, 1H, CH); δ_C (100 MHz, d-CDCl₃) 67.2, 76.7, 77.0, 77.3, 134.9, 135.4, 139.0, 165.9, 191.5; LRMS (ES+) Calcd for [C₁₅H₁₂O₃ + Na] 240.25 found 360.33.

**Benzyl 4-(((1,10-phenanthrolin-5-yl)amino)methyl)benzoate (3.18).** Compound 3.18 was synthesized by dissolving compound 3.17 and 1,10-phenanthrolin-5-amine (1.0 eq) in a 9:1 mixture of MeOH:DCM in a suitable reaction vessel. The reaction mixture was heated to 50°C and shortly after glacial AcOH (2.5 eq) was added dropwise to the reaction vessel. The reaction
was allowed to stir for 16 hours at 50°C before sodium cyanoborohydride was added slowly. The reaction was allowed to react for an additional 16 hours. Once the reaction was complete, the reaction was filtered through Celite to remove the cyano salt and concentrated under reduced pressure. The resulting residue was absorbed onto silica gel from CH2Cl2 and columned using Biotage-Isolera™ a gradient of DCM:Ultra (Ultra; 25% DCM, 7% MeOH, 1% ammonium hydroxide) (9:1). Fractions were pooled an concentrated in vacuo yielding the final product as a dark orange solid (58%). \[\delta_H (400 \text{ MHz, d}-\text{CD}_3\text{OD}) 4.53 \text{ (s, 2H, CH}_2\text{)}, 5.36 \text{ (s, 2H, CH}_2\text{)}, 6.50 \text{ (s, 2H, CH}_2\text{)}, 7.28-7.48 \text{ (m, 8H, CH)}, 7.49-7.55 \text{ (m, 1H, CH)}, 7.89 \text{ (d, J = 8.6 Hz, 1H, CH)}, 8.02 \text{ (d, J = 8.4 Hz, 1H, CH)}, 8.28-8.35 \text{ (m, 1H, CH)}, 8.81 \text{ (s, 1H, CH)}, 9.09 \text{ (s, 1H, CH)}; \delta_C (100 \text{ MHz, d}-\text{CD}_3\text{OD}) 47.6, 67.0, 100.9, 122.0, 122.1, 123.1, 127.2, 128.0, 128.14, 128.5, 128.0, 129.2, 130.1, 133.7, 135.8, 140.5, 141.5, 143.7, 146.2, 146.5, 149.7, 166.0; LRMS (ES+) Calcd for [C$_{27}$H$_{21}$N$_3$O$_2$ + Na] 442.47 found 443.65.

**Benzyl 4-(((1,10-phenanthroline-5-yl)amino)methyl)benzoate (3.19).** Compound 3.19 was synthesized by dissolving compound 3.18 in a 3:1 mixture of THF:H$_2$O. Once LiOH (1.1 eq) was added, the reaction mixture was heated for 16 hrs at 45°C. The reaction mixture was diluted with water, acidified (pH~5.5) by KH$_2$PO$_4$, and continuously extracted into ethyl acetate. Organic layers were washed with sodium chloride solution, dried over anhydrous sodium Na$_2$SO$_4$ and concentrated under reduced pressure. Reaction was purified by flash column chromatography using solvent system of Marie Celeste ((92% DCM, 7% MeOH, 1% AcOH), 20% Lucy (65% DCM, 25% MeOH, 4% H$_2$O: DMC (7:1). \[\delta_H (400 \text{ MHz, d- CD}_3\text{OD}) 4.70 \text{ (s, 2H, CH}_2\text{)}, 6.57 \text{ (s, 1H, CH)}, 7.48-7.64 \text{ (m, 3H, CH)}, 7.61-7.89 \text{ (m, 1H, CH)}, 7.96 \text{ (d, J = 7.9 Hz, 2H, CH)}, 8.05-8.18 \text{ (m, 1H, CH)}, 8.55-8.72 \text{ (m, 1H, CH)}, 8.80 \text{ (d, J = 8.6 Hz, 1H, CH)}, 8.84-9.15 \text{ (m, 1H, CH)}; \delta_C (100 \text{ MHz, d- CD}_3\text{OD}) 48.7, 120.4, 121.5, 121.6, 123.6, 126.8, 127.6, 128.2, 130.1, 131.1, 136.1, 145.1, 146.1, 149.1, 149.9, ; LRMS (ES+) Calcd for [C$_{27}$H$_{21}$N$_3$O$_2$ - H] 418.47 found 418.45.
**Ethyl 5-amino-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoatehydrochloride (3.11).** Compound 3.11 was synthesized by dissolving 5-aminofluorescein (1.0 eq) in EtOH and carefully adding SOCl₂ (5.0 eq) at 0°C. When the addition was complete, the solution was refluxed (70°C) for 30 hours. The reaction mixture was concentrated down in vacuo where it was then repeatedly co-evaporated with CHCl₃ to dryness to give the compound as a red solid (100%); δH (400 MHz, (CD₃)₂SO) 0.82 (t, J = 7.2 Hz, 3H, CH₃), 3.92 (q, J = 7.2 Hz, 2H, CH₂), 5.86 (brs, 2H, NH₂), 6.32-6.60 (m, 4H, CH), 6.81-6.96 (m, 3H, CH), 7.05-7.12 (m, 1H, CH), 7.33-7.45 (m, 1H, CH); LRMS (ES⁺) Calcd for [C₂₂H₁₇NO₅ + H] 375.1 found 376.1.

**Ethyl 5-amino-2-(6-(2-tert-butoxy-2-oxoethoxy)-3-oxo-3H-xanthen-9-yl)benzoate (3.12).** Compound 3.12 was synthesized by dissolving compound 3.11 (1.0 eq) in DMF and adding K₂CO₃ (3.0 eq), followed by the dropwise addition of tert-butyl bromoacetate (1.1 eq). After stirring for 16 hrs under an N₂ atmosphere, the reaction mixture was poured into a separatory funnel containing ethyl acetate and water. The ethyl acetate layer was collected, and then the aqueous layer was extracted further with ethyl acetate. The organic layers were combined, then washed with saturated NaHCO₃, water, and sodium chloride solution, where it was then dried over anhydrous sodium Na₂SO₄, and concentrated in vacuo. The crude product was dissolved in a minimal amount of DCM and purified by passing through a silica gel column, eluting with a solvent mixture of DCM:MeOH (21:1). Fractions containing product were pooled and
concentrated in vacuo to give an orange solid (73%); $\delta_H$ (400 MHz, (CDCl$_3$)) 0.83 (t, $J = 7.2$ Hz, 3H, CH$_3$), 1.43 (s, 9H, CH$_3$), 3.81-3.93 (m, 2H, CH$_2$), 4.86 (s, 2H, CH$_2$), 5.89 (brs, 2H, NH$_2$), 6.19 (d, $J = 2.0$ Hz, 1H, CH), 6.39 (dd, $J = 10.0$ and 2.0 Hz, 1H, CH), 6.89-7.08 (m, 5H, CH), 7.15 (d, $J = 2.4$ Hz, 1H, CH), 7.36 (d, $J = 2.4$ Hz, 1H, CH); LRMS (ES+) Calcd for [$C_{28}H_{27}NO_7 + H$] 489.2 found 490.2.

2-((9-(4-amino-2-(ethoxycarbonyl)phenyl)-3-oxo-3H-xanthen-6-yl)oxy)acetic acid hydrochloride (3.13). Compound 3.13 was synthesized by dissolving compound 3.12 in DCM followed by the addition of TFA (5 eq) which resulted in the precipitation of the product. After stirring for 6 hrs at room temperature, toluene and benzene was added in order to azeotrope the TFA (15 mL each) and the reaction mixture was subjected to vacuum filtration. The product was then washed with ether and dried under reduced pressure to give a yellow precipitate (100%); $\delta_H$ (400 MHz, (CD$_3$)$_2$SO) 0.79 (t, $J = 14.2$ Hz, 3H, CH$_3$), 3.85 (q, $J = 14.0$ and 13.9 Hz, 2H, CH$_2$), 5.09 (s, 2H, CH$_2$), 7.01 (q, 1H, CH), 7.14-7.19 (m, 2H, CH$_2$), 7.24 (d, $J = 2.3$ Hz, 1H, CH), 7.31 (q, $J = 9.2$ and 9.2 Hz, 1H, CH), 7.45 (d, $J = 2.3$ Hz, 1H, CH), 7.52-7.57 (m, 2H, CH$_2$), 7.64 (d, $J = 2.4$ Hz, 1H, CH); LRMS (ES+) Calcd for [$C_{24}H_{19}NO_7 + H$] 433.1 found 434.1.

(E)-2-((8S,9S,10S,13R,14S)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)dodecahydro-1H-cyclopenta[a]phenanthren-3(2H,4H,10H)-ylidene)acetonitrile (3.14). Compound 3.14 was synthesized by dissolving 5-α-cholestan-3-one in anhydrous THF. After the addition of diethyl
cyanomethylphosphonate (1.1 eq) and LiOH (1.5 eq) the reaction was left to stir overnight at room temperature. The product was then dissolved in ethyl acetate and extracted with water. The organic layer was combined, dried with anhydrous sodium Na$_2$SO$_4$ and concentrated in vacuo. The crude product was dissolved in a minimal amount of DCM and purified by passing through a silica gel column, eluting with solvent mixture (Hexanes: Ethyl acetate (6:1)) yielding the final product as a white solid (95%).

$\delta$H (400 MHz, d-CDCl$_3$) 0.60 (s, 3H, CH$_3$), 0.69-1.84 (m, 44H, CH, CH$_2$, CH$_3$), 5.10 (s, 1H, CH); $\delta$C (100 MHz, d-CDCl$_3$) 11.9, 18.5, 20.6, 22.4, 22.7, 23.7, 24.8, 24.0, 27.9, 28.1, 28.9, 30.7, 31.9, 32.7, 33.0, 33.2, 35.4, 35.6, 35.9, 46.1, 54.6, 56.1, 56.4, 92.4, 119.6; LRMS (ES+) Calcd for [C$_{29}$H$_{47}$N + Na] 410.63 found 410.12.

2-((8S,9S,10S,13R,14S)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl)acetonitrile (3.15). Compound 3.15 was synthesized by dissolving compound 3.14 in minimal amounts of THF in a suitable round bottom flask. The solution was thoroughly degassed and 20% Pd/C was carefully added to the reaction. Following the slow addition of MeOH to the reaction mixture, H$_2$ gas was bubbled through the solvent for 5 mins before the reaction was put under the atmosphere of H$_2$ gas and stirred continuously for 16 hrs. The H$_2$ gas was evacuated and the reaction filtered through Celite to remove the Pd catalyst and then concentrated under reduced pressure. The resulting crude product was dissolved in a minimal amount of DCM and purified by passing through a silica gel column, eluting with an isocratic solvent mixture (Hexanes: Ethyl acetate (49:1)) yielding the final product as a white solid (45%).

$\delta$H (400 MHz, d-CDCl$_3$) 0.60 (s, 3H, CH$_3$), 0.69-1.84 (m, 44H, CH, CH$_2$, CH$_3$), 4.10 (t, $J = 14.5$ Hz, 2H, CH$_2$); $\delta$C (100 MHz, d-CDCl$_3$) 11.9, 18.5, 20.6, 22.4, 22.7, 23.7, 24.0, 27.9, 28.1, 28.9, 30.7, 31.9, 32.7, 33.0, 33.2, 35.4, 35.6, 35.9, 46.1, 54.6, 56.1, 56.4, 92.4, 117.7, 166.6; LRMS (ES+) Calcd for [C$_{29}$H$_{49}$N + H] 411.70 found 412.61.
2-((8S,9S,10S,13R,14S)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)hexadecahydro-1H-cyclopenta[\(\alpha\)]phenanthren-3-yl)ethanamine (3.16). Compound 3.16 was synthesized by dissolving compound 3.15 in anhydrous THF followed by the careful addition of lithium aluminum hydride. The reaction mixture was left to stir for 3 hrs, while being monitored by thin layer chromatophraphy (TLC). Once the reaction was complete, the crude material was filtered through Celite to remove the lithium salt and concentrated under reduced pressure. The resulting residue was precipitated out of solution utilizing cold ether and concentrated HCl to yield the final product as a white solid (45%). \(\delta_H\) (400 MHz, \(d\)-CDCl\(_3\)) 0.67 (s, 3H, CH), 0.87-2.37 (m, 46H, CH, CH\(_2\), CH\(_3\)), 1.77 (m, 2H, CH\(_2\)), 2.15 (m, 2H, CH\(_2\)); \(\delta_C\) (100 MHz, \(d\)-CDCl\(_3\)) 11.9, 18.5, 20.6, 22.4, 22.7, 23.7, 24.0, 27.9, 28.1, 28.9, 30.7, 31.9, 32.7, 33.0, 33.2, 35.4, 35.6, 35.9, 37.6, 41.5, 46.1, 54.6, 56.1, 56.4; LRMS (ES+) Calcd for [C\(_{29}\)H\(_{53}\)N + H] 415.73 found 416.19.
4-(((1,10-phenanthrolin-5-yl)amino)methyl)-N-(2-(((8R,9S,10S,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-3-yl)ethyl)benzamide (3.20). Compound 3.20 was synthesized by dissolving compound 3.13 (1.0 eq) in DMF inside a suitable round bottom flask. After the addition of compound 3.16 (1.3 eq), TBTU (2.5 eq) and DIPEA (5.0 eq) were added to the reaction mixture which was then allowed to stir for 16 hrs at r.t. The product was then dissolved in ethyl acetate and extracted with water. The organic layer was combined, dried with anhydrous sodium Na₂SO₄ and concentrated in vacuo. The crude product was dissolved in a minimal amount of DCM and purified by passing through a silica gel column, eluting with solvent mixture (DCM: MeOH (9:1)) yielding the final product as a dark red solid (60%). \( \delta \)H (400 MHz, \( d\)-CD₃OD) 0.67 (s, 3H, CH), 0.80-1.84 (m, 46H, CH, CH₂, CH₃), 3.22-3.42 (m 2H, CH₂), 3.94-4.07 (s, 2H, CH₂), 6.45-6.61 (m, 2H, CH), 6.69-6.86 (m, 2H, CH), 6.94-7.14 (m, 4H, CH), 7.50 (s, 1H, CH); \( \delta \)C (100 MHz, \( d\)-CD₃OD) 13.5, 13.7, 19.7, 20.7, 23.4, 23.6, 24.2, 24.6, 25.1, 26.5, 28.1, 28.3, 28.9, 29.6, 32.0, 35.5, 35.6, 35.8, 36.1, 36.5, 38.1, 40.2, 40.8, 42.7, 54.9, 56.4, 56.8, 60.1, 67.0, 95.0, 99.0, 108.1, 108.5, 113.4, 115.5, 118.0, 127.0, 128.6, 129.6, 129.7, 134.3, 147.5, 147.9, 154.7, 156.7, 159.9, 161.7, 167.9, 188.8, 185.9; LRMS (ES+) Calcd for [C\(_{49}\)H\(_{66}\)N\(_4\)O + Na] 750.07 found 750.67.