DESIGN AND SYNTHESIS OF P-SELECTIN INHIBITORS TARGETING CANCER GROWTH AND METASTASIS

AND

MICROWAVE ASSISTED RADIO-FLURODENITRATION AND PHARMACOLOGICAL STUDIES OF (4-[18F]-FLUOROPHENYL)-TRIPHENYLPHOSPHONIUM NITRATE (18F-PTPP) AS A CARDIAC POSITRON EMISSION TOMOGRAPHY TRACER

A dissertation presented

by

Roushan Afroze

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In partial fulfillment of the requirements for the degree of

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Northeastern University

Boston, Massachusetts

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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements

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**ABSTRACT**

Selectins are adhesion receptors that bind to the carbohydrate structure of sialyl Lewis\(x\) (sLe\(x\)) and sialyl Lewis\(a\) (sLe\(a\)) and are over-expressed on tumor cells. P-selectin is a member of the selectin family of cell adhesion molecules (CAM). Increased interaction between P-selectin and P-selectin glycoprotein ligand 1 (PSGL-1) promotes cancer cell metastasis, and over expression of cell surface sialic acid, which is associated with tumor progression and metastatic spread. Sialyl molecules on the surface of tumor cells interact with platelets, leukocytes and endothelium through the selectins. Sialic acids are conjugated to the carbohydrate moieties of Sialyl Lewis\(x\) (sLe\(x\)) on PSGL-1 and are important in cellular functions. Sialyl molecules on the surface of on carcinoma cells interact with platelets, leukocytes and endothelium through the selectin of CAM. Therefore, a small molecule inhibitor could inhibit the interaction of P-selectin mediated platelet binding of tumor cells during the early stages of the metastatic process.

Our strategy was to design novel, therapeutically effective sLe\(x\) mimetic selectin inhibitors on the basis of structure-based and calculated physicochemical properties approach. Non-carbohydrate inhibitors have shown better properties over sugar-based inhibitors with respect to absorption, distribution, metabolism and excretion (ADME). A structure-based approach has been used to replace the sugar moiety of sLe\(x\) with fused tricyclic, heterocycles or constrained bicyclic molecules which could block the formation of cover, enabling tumor cell interaction with platelets. Molecular modeling was used to define a diverse collection of non-carbohydrate small and constrained mimics as central scaffolds in the design of new sLe\(x\) analogues. Synthetic protocols for the efficient introduction of C-8 substituents were developed and used to synthesize a set of C-8 substituted pyrazoloquinolinone analogues. In addition, different substituted azepine and diazepine derivatives were generated. Novel, potentially
effective inhibitors of P-selectin were prepared utilizing structure-based design and these may be useful as anti-cancer agents.

Coronary artery disease (CAD) results in reduced blood flow to the heart due to the rupture of an atherosclerotic plaque. Therefore, quantitative myocardial blood flow measurements are needed. A non-invasive imaging technique such as positron emission tomography (PET) is a useful method for quantitation of myocardial blood flow under various physiological conditions. Fluorine-18 has the best imaging characteristics with low positron energy and a half-life of 110 min. Fluorine-18 is useful for complex synthesis and affords a longer time for in vivo analysis. To develop a fluorine-18 labeled PET radiotracer for cardiac imaging, a fluorinated compound (4-[\(^{18}\)F]-fluorophenyl)triphenylphosphonium nitrate) (\(^{18}\)F-PTPP) was synthesized. The compound was designed such that the fluorine atom is conveniently introduced in a short period of time to allow a one-step radiosynthesis with fluorine-18. The microwave-assisted, one step radio-fluorodenitration of \(^{18}\)F-PTPP via nucleophilic aromatic substitution was used.

The time needed for synthesis, purification and formulation of \(^{18}\)F-PTPP was 53 min with an average radiochemical yield 93%. Tissue distribution studies in Sprague-Dawley (SD) rats showed that heart has moderate uptake relative to liver and the uptake ratio between these tissues was found to be 3.3:1. This novel PET imaging modality could be a useful imaging technique that may benefit patients with coronary artery disease.

Molecular modeling methods were used to design pyrazoloquinolinone, azepine and diazepine analogs that may be useful as anti cancer agents. A fast efficient method for the microwave-assisted, one step radiosynthesis of \(^{18}\)F compounds was developed, which could be useful diagnostic technique that may in the future be beneficial for cardiology patients.
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<th>Full Form</th>
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<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetyl</td>
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<tr>
<td>Anhyd</td>
<td>Anhydrous</td>
</tr>
<tr>
<td>Ar</td>
<td>aryl</td>
</tr>
<tr>
<td>Atm</td>
<td>atmosphere(s)</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
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<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
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<tr>
<td>t-Bu</td>
<td>tert-butyl</td>
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<td>°C</td>
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<tr>
<td>Calcd</td>
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<tr>
<td>Cbz</td>
<td>benzyloxy carbonyl</td>
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<td>Concd</td>
<td>concentrated</td>
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<tr>
<td>Cp</td>
<td>cyclopentadienyl</td>
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<tr>
<td>Cy</td>
<td>cyclohexyl</td>
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<tr>
<td>δ</td>
<td>chemical shift in parts per million downfield from tetramethylsilane</td>
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<tr>
<td>DCC</td>
<td>N,N-dicyclohexylcarbodiimide</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-(dimethylamino)pyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>Et</td>
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</tr>
<tr>
<td>FAB</td>
<td>fast-atom bombardment (in mass spectrometry)</td>
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<tr>
<td>FT</td>
<td>Fourier transform</td>
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<tr>
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<td>grams</td>
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<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<tr>
<td>HRMS</td>
<td>high-resolution mass spectrometry</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
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<tr>
<td>LDA</td>
<td>lithium diisopropylamide</td>
</tr>
<tr>
<td>M</td>
<td>multiplet (spectral)</td>
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<tr>
<td>M</td>
<td>moles per liter</td>
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<td>methyl</td>
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<tr>
<td>MS</td>
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<td>NMR</td>
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<td>q</td>
<td>quartet</td>
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<tr>
<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>retention factor</td>
</tr>
<tr>
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<td>room temperature</td>
</tr>
<tr>
<td>s</td>
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<tr>
<td>TFA</td>
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<td>tetrahydrofuran</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>TMS</td>
<td>tetramethylsilane</td>
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<td>UV</td>
<td>ultraviolet</td>
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Chapter 1:
Design and Synthesis of P-Selectin Inhibitors Targeting Cancer Growth and Metastasis
CHAPTER 1

1.1 GENERAL INTRODUCTION

The major cause of death in cancer patients is metastasis, which remains as a major obstacle in cancer therapy.\textsuperscript{1} Common strategies in cancer research are focused on inhibiting tumor growth or killing tumor cells. Only few anti-metastasis drugs are currently available on the market.\textsuperscript{2} Although the mechanism of metastasis is not fully understood, it is thought that the development of new therapies for the treatment of cancer metastasis could be beneficial.\textsuperscript{3} One hypothesis of metastasis involves a positive correlation between overexpression of sialyltransferase (ST) and cancer metastasis.\textsuperscript{4-12} ST’s belong to the glycosyltransferase family and have a number of known activities such as addition of sialic acid to the terminal portions of the sialylated glycolipids.\textsuperscript{13} The sialylated glycolipids are responsible for cell–cell interactions, cell–cell adhesion, and cell metastasis.\textsuperscript{14-17}

Blood-borne metastasis is a multi-step process where cancerous cells initially group together to form a primary tumor. Once the tumor is formed, cells escape from this tumor, travel through the bloodstream or the lymphatic system to new locations remote from the site of the original disease. In 1865 the French physician Armand Trousseau discovered the clinical correlation between platelets and cancer.\textsuperscript{18} Morphological studies have documented the relationship of circulating tumor cells with host platelets. Several animal models support the clinical observation that tumor cell-platelet interactions significantly contribute to cancer metastasis.\textsuperscript{19-23} Multiple sources indicate that P-selectin binds to several human cancers, including
colon, lung, breast, malignant melanoma.\textsuperscript{24-34} However, the mechanism of tumor cell-platelet interactions are not clearly understood.

Some studies suggest that platelets, by adhering to tumor cells, provide a protective shield that masks them from the cytotoxic activity of natural killer cells.\textsuperscript{35,36} Another hypothesis is that platelet activity may be involved in tumor metastasis.\textsuperscript{37} Therefore, inhibition of tumor cell-platelet adhesion may provide a rational basis for the development of novel therapeutic target of metastasis. During metastasis several interactions between platelets and tumor cells occur. In aggregation steps, activated platelets are conjugated with tumor cells, other platelets and leukocytes to form a hetero-aggregate. A number of growth factors such chemokines and proteases may release that support attachment to the endothelium and thereby contribute to metastasis. In coagulation steps, platelets may activate receptors on tumor cells such as, protease-activated receptor 1 (PAR1) and protease-activated receptor 2 (PAR2) and accelerate the liberation of tissue factor, an agent that acts as catalyst in the coagulation step.

In adhesion steps, CAMs such as selectins, mucins, cD44, and other sialyl Lewis X/A-containing glycoconjugates enhance firm attachment that is mediated by integrins and intercellular CAMs. In metastasis steps, intracellular bridges formed by the interaction of multivalent plasma proteins, activate platelets, and then fibrinogen protects tumor cells from the natural killer cell. Additionally, metastasis may be accelerated by thrombin, which is activated by a tissue factor in the blood stream.\textsuperscript{38}

1.1.1 Selectin Adhesion Receptors

To initiate the series of adhesive and signaling events and to regulate inflammation, platelet activation takes place by allowing leukocyte adhesion to the vascular wall. Selectins
mediate the first adhesion step, which is characterized by tethering and rolling of leukocytes on endothelial cells, platelets, or other leukocytes.\textsuperscript{38,39} Selectins are a family of cell adhesion receptors with glycoprotein ligands. Cell adhesion receptors are transmembrane glycoproteins that mediate binding to extracellular matrix (ECM) molecules or to counter-receptors on other cells, with roles in cell-cell recognition and binding. The C-type lectins are P-selectin and L-selectin and they recognize sialylated, fucosylated, and sulfated ligands. The C-type lectin-like domain is at the end of a multidomain extracellular segment extending outward from the cell surface. Selectin dependent cell-cell interaction is responsible for anchoring cells to their surroundings, regulating cell motility, and migration. Selectins also play an active role in the initiation of intracellular signaling pathways and regulation of cell-cell interactions involving monocytes, lymphocytes, platelets, and endothelial cells.\textsuperscript{40}

P-selectin binds to the specific oligosaccharide through the lectin domain. These carbohydrates are found on both cell-surface glycoprotein and glycolipid molecules. Binding to the lectin domain of the selectins with a specific sugar ligand requires extracellular Ca\textsuperscript{2+}; thus they are called \textit{C-type lectins}.\textsuperscript{41}

1.1.2 Classification of Three Selectin Receptors

The selectin family consists of three kinds of proteins, which were identified in the early 1990’s. They are transmembrane glycoproteins and have been designated epithelium (E)-, Platelet (P)-, and Leukocyte (L)-selectin. They are characterized by a NH\textsubscript{2}-terminal C-type lectin domain, a carbohydrate recognition domain (CRD) domain, followed by an epidermal growth factor (EGF)- like domain, a series of complement regulatory (CR) or binding proteins, a transmembrane domain, and a short cytoplasmic tail. Selectins mediate cell–cell adhesion
through interactions of the lectin domains with specific glycoconjugate ligands. This class of proteins are Ca$^{2+}$ dependent and bind carbohydrates.$^{41}$

E-selectin binds to ligands on most leukocytes and mediates rolling for firm attachment after hours of activation by cytokines, which are expressed on the surface of endothelial cells.$^{36,37}$ P-selectin is stored in Weibel-Palade bodies of endothelial cells and $\alpha$-granules of platelets. It is translocated to the cell surface by immediate activation with cytokines.$^{42,43}$ Platelets, upon activation, are expressed on a cytoplasmic membrane. E- and P-selectins are found on platelets and endothelial cells, and they translocate to the cell surface upon activation, and mediate binding or inhibition activities. This plays a significant role for physiological processes such as inflammation and cancer. L-selectin is expressed on the surface of leukocytes, and it binds to ligands on some endothelial cells and on other leukocytes. Additionally, it functions as a lymphocyte homing receptor.$^{35}$ Leukocytes also expressed by the activation of L-selectin and mediate their interactions with endothelial ligands.

### 1.1.3 Ligands to Selectins

In early 1990’s as the selectins were being characterized, a number of carbohydrate ligands were identified.$^{25}$ The binding site of selectin molecules, localized on the lectin domain, were shown to bind to common carbohydrate epitope, sLe$^x$ (1) and sLe$^a$ (2) (Figure 1.1).$^{33–36,44}$
Figure 1.1 Sialyl Lewis \(^x\) (sLe\(^x\)) and Sialyl Lewis \(^a\) (sLe\(^a\))

P-selectin glycoprotein ligand-1 (PSGL-1) is a natural ligand for P-selectin, and is expressed on blood cells. CD24, is another ligand, which is also important for binding of P-selectin to tumor cells. For L-selectin, four possible ligands have been reported. They are glycosylation dependent cell adhesion molecule (GlyCAM-1), CD34, and Mucosal Addressin Cell Adhesion Molecule (MAdCAM-1), and PSGL-1. No known E-selectin ligands have been identified yet. But, another glycoprotein receptor containing the same carbohydrate residue has been reported as E-selectin ligand 1 (ESL-1).\(^{46,47}\) Sulfation of the P-selectin must occur to allow for the interaction with its ligand.\(^{38}\)
The P-selectin molecule binds to each of the two subunits of PSGL-1. The interactions of NH₂-terminal of sLe\(^x\) containing O-glycan and a tyrosine sulfate residue of PSGL-1 to the C-type lectin domain of P-selectin.\(^{38}\) PSGL-1 is a dimeric mucin-like glycoprotein which recognizes the sugar epitope (O-linked glycans).\(^{48,49}\) Cell-cell interactions are an important criteria for normal and pathological processes. Selectins are adhesion receptors that recognize carbohydrate containing CAM’s, which play a significant role in inflammation, the immune response, and cancer. PSGL-1 is present on leukocyte surfaces and binds to P-and L-selectin through the activation of cell adhesion molecules during inflammatory response.\(^{50-52}\) It also accelerates the interaction of activated platelet with cancer cells to form a cloak, and the adhesion of a cloak on cancer cells stimulates endothelial cells. Selectins mediate tumor cell interactions with platelets, leukocytes, and the vascular endothelium.\(^{53}\) P-selectin mediates initial tethering and rolling of leukocytes through the interaction of PSGL-1, resulting in leukocyte arrest both on the endothelium and in tissues.\(^{55,56}\)

1.1.4 Role of Selectins in the Inhibition of Metastasis

Selectins bind to the carbohydrate structure of sLe\(^x\). The clinical diagnosis and metastatic progression of many epithelial carcinomas has been associated with the overexpression of sLe\(^x\). The process of metastasis involves the formation of tumor-platelet-leukocyte emboli and their interactions with the endothelium of distant organs. P-selectin is a reasonable candidate to mediate multiple interactions involving platelets, leukocytes, endothelium, and carcinoma cells, of mucin-producing carcinomas.\(^{3,4}\) P-selectin ligands have been detected on several human carcinomas and cell lines and are thought to play a role in metastasis.\(^{42-44}\)
The importance of the P-selectin mediated cell adhesive interaction in the inflammation, growth, and thrombin development in cancer has been demonstrated by using P-selectin deficient mice.\textsuperscript{46} It has been known from published results that heparins reduce metastasis in several mouse models, but the mechanism of action is still unclear. One of the reasons that heparin reduces metatasis may be inhibition of P-selectin-mediated platelet coating on tumor cells during the initial phase of the metastatic process.

P-selectin and L-selectin also have pathologic roles in many diseases involving inflammation and reperfusion, as well as in carcinoma metastasis.\textsuperscript{7-9} The process of metastasis and homing of leukocytes is a similar, multi-step process. Selectins and their ligands activate leukocyte homing by causing the cell to roll, which then allows for release of chemotactic stimuli and adhesion onto the endothelium followed by migration across the endothelial barrier. Metastasis processes differ in some steps such as shedding of the malignant cells from the primary tumor, entering into the blood stream, tethering onto the endothelium, and migrating of malignant cells to a new site to form colonies.\textsuperscript{55}

1.1.5 Role of Platelets in the Inhibition of Metastasis

Migrating cancerous cells cannot survive for a long time in circulation; therefore any substance that disturbs this process seems to be a critical point for successful metastasis. Selectins are thus a good target to disrupt metastasis due to the fact they prevent silaylation in the early stage of migration. It has been reported that there is a direct relationship with overexpression of sLe\textsuperscript{x} with an enhanced metastatic potential in human cancers.\textsuperscript{59-62} In animal models, it has been shown that the spread of cancerous cells is accelerated by the formation of selectin-mediated binding of tumor cells and platelets.\textsuperscript{60,21,12} It has been demonstrated that
heparins inhibit the interaction of P- and L-selectins to their ligands, and this is the main mechanism by which heparins attenuate metastases. It has been hypothesized that the selectin-mediated inhibition of metastases by heparins is the interruption of platelet aggregation with leukocytes and cancerous cells.

The most likely mechanisms by which heparins attenuate cancer metastase is that heparins interrupt platelet aggregation by inhibiting the selectins mediated interactions of platelets with cancerous cells. The mechanisms by which platelets assist tumor metastasis are not clearly understood. It is known that blood platelets, which are small cellular fragments without a nucleus derived from bone marrow, are involved in the process of cancer metastasis. Some studies suggest that in the metastatic process, malignant cells enter into the bloodstream, attract platelets, bind on the cancer cell surface through the sugar coated mucins and form microemboli. Platelets, leukocytes and tumor cells form microembolis which protect them from the cytotoxic activity of natural killer cells and establish new colonies in other parts of the body.

Activated platelet-derived growth factor has been reported to stimulate the growth of highly metastatic colon carcinoma cells. It has recently been hypothesized that platelets may contribute to tumor-induced angiogenesis. Therefore, elucidating the molecular mechanisms of tumor cell-platelet adhesion may eventually provide a rational basis for the development of novel therapeutic strategies to combat metastasis.

1.1.6 Design of Non-Carbohydrate Small Molecule Inhibitors

In the field of disease therapy, selectins are well-documented therapeutic targets for the prevention and treatment of related diseases. The selectins are well-characterized adhesion
receptors for mucin-type glycoproteins that have the carbohydrate structure sialyl-Lewis\textsuperscript{x}. \textsuperscript{66-71} Several researchers have published on the role of P-selectin in different disease models including cancer growth and metastasis.\textsuperscript{6-9} P-selectin is expressed on the surface of platelets and endothelium of various cancer cells within minutes after stimulation, so our focus was to identify an inhibitor of this target. The inhibitor plays a key role in the early adhesion cascade. Several knockout animal model studies demonstrated that inhibition of P-selectin and PSGL-1 interaction reduces cancer metastasis.\textsuperscript{6-8} Independent studies suggested that heparin attenuates tumor metastasis in mice by reducing the P-selectin interaction with its ligand on the cancer cell surface.\textsuperscript{63} Inhibition of P-selectin interaction has been described as a plausible mechanism for tumor metastasis. From these findings, we sought to develop compounds that would inhibit the expression of both activated endothelial and platelet cells, which play a significant roles in cancer metastasis.

Sugar based sLe\textsuperscript{x} (I) (Figure 1.1) are the physiological ligands of the selectins which served as the lead structure in the search for selectin antagonists.\textsuperscript{72,73} The interactions of selectins with sLe\textsuperscript{x} (I) has been shown by NMR spectroscopy,\textsuperscript{72,74} protein X-ray crystallography,\textsuperscript{75} and molecular modeling. Various groups have searched for potent selectin antagonists for different disease models.\textsuperscript{79} A ligand-based model was used to design inhibitors and to get most favorable interactions on the P-selectin surface.\textsuperscript{80} The best selectin antagonists synthesized to date have still not led to therapeutic applications yet.

In 1998, Varki et al demonstrated that adhesion of cancer cells mediated by P-selectin using P-selectin deficient mice play an important role in the process of metastasis.\textsuperscript{8} They found that leukocyte ligands bind to P- and L-selectin may play important roles to promote tumor
growth. Subsequently in 2007, the same group of scientists revealed that heparin reduces hematogeneous metastasis by inhibiting the availability of P- and L-selectin for interactions.\textsuperscript{76,77}

The recent finding of molecular interactions between P-selectin and PSGL-1 on tumor cells revealed that P-selectin mediates initial interactions of platelets with tumor cells. These results led us to develop a drug-like small P-selectin inhibitor to block carcinoma metastasis. Drug like small molecules need to have oral bioavailability to be effective and this has been described in the literature.\textsuperscript{78} The aim of our research is to synthesize non-sugar based small molecule inhibitors of the PSGL-1 that can modulate selectin mediated events. P-selectin deficiency attenuates tumor growth and metastasis. Non-carbohydrate inhibitors have better properties over sugar based inhibitors such as absorption, distribution, metabolism and excretion (ADME). For this purpose, we have selected a heterocycle that could block the formation of an envelop, enabling tumor cell interaction with white blood cells.

1.2.DESIGN

1.2.1  Rational Design of P-selectin Inhibitor

The structure activity relationships of 3-hydroxy-2-phenylquinoline-4-carboxylic acid \textsuperscript{3} against P-selectin have been documented in the literature.\textsuperscript{69} Molecular modeling studies via docking were performed on the quinoline derivatives in order to identify the interactions of the best selective molecule with the active site of the P-selectin receptor. Molecular docking methods are capable of predicting ligand-protein interactions with known or unknown ligands. Our aim was to predict the 3-dimensional structures of novel mimics to allow for the discovery of new anti-cancer therapeutic agents through structure-based drug design (SBDD). The method used in SBDD is docking study.\textsuperscript{83} Suh docking studies can be used as a tool to define binding
ability and the binding site of a ligand. The core ligand structure was emerged from our ongoing program. The analogs of that structure were used to study the ligand-receptor interactions. The availability of X-ray crystal structure of P-selectin allowed us to explore the SBDD strategy. The docking study gives information to understand the structure-activity relationship of known ligands on the basis of interactions with the active site residues.\textsuperscript{83}

1.2.2 Molecular docking

Molecular modeling is a combination of methodologies used in computational chemistry such as computation of the energy of a molecular system, energy minimization etc. The approach we used to compute the energy of a molecule is a molecular mechanics calculation. Minimization of energy and molecular mechanics were applied to obtain stable structures that were corrected for eventual distortions due to bonds with unfavorable lengths and dihedral angles. Molecular modeling was carried out in Molecular Operating Environment (MOE) software\textsuperscript{85} to identify the interactions and their relative geometry in three-dimensional space. A molecular docking approach was used to determine the structural requirements and binding affinities of the molecule with the protein.

The protein structure used for our molecular docking is the X-ray crystal structure of P-selectin (lectin and EGF domains) is available from the protein data bank (PDB code 1G1Q: http://www.pdb.org/pdb/home/home).\textsuperscript{84} Following reported modeling studies,\textsuperscript{69} we have reconstructed a model of the P-selectin receptor with 3-hydroxy-2-phenylquinoline-4-carboxylic acid 3 in Figure 1.2.
Figure 1.2 The binding conformation of the compound 3 inside the active site of the receptor.

The results were obtained by molecular modeling of ligand interactions with active site amino acids of P-selectin. A search for non-sugar based small molecules was guided by the docking model of the X-ray crystal structure of P-selectin with 3-hydroxy-2-phenylquinoline-4-carboxylic acid. The docking model indicated that the quinoline analogue was anchored to the
D₁ pocket of P-selectin through the hydrogen bonds to the peptides backbone at Lys111 and Arg85 (Figure 1.3). H₁ hydrophobic pocket is formed by amino acids (Glu80, Pro81, Asn82 and Lys84) in P-selectin and interacts with substitution at 8-position of the quinoline ring. The S₁ pocket is located at the protein-solvent interface.

**Figure 1.3** Binding motif of compound 3 with active site amino acids of P-selectin
The docking simulation was performed by using MOE-dock program. The approach we took was to minimize the energy of a ligand in a known binding pocket from different orientations within the binding site. The docking of reported compound 3 was performed to interact with the chosen enzyme residues. They were Arg 85, Lys 111Glu 80, Pro 81, Asn 82 and Lys 84. Following reported modeling studies, we constructed a similar orientation of the ligand and its receptor. The binding conformation of the reported compound was optimized and the bound compound was used as a template. A flexible alignment approach was used for the superimposition of substituted analogs. The alignment methodology has several advantages:

a) A collection of ligands can be aligned at the same time b) conformational and alignment searches can be conducted together, c) superposition and potential energy strain are balanced and d) the output of alignment collected and used for comparison. Our initial approach was to employ MOE/flexible alignment to automatically generate superpositions of the compounds.

With the aim of obtaining the best selective P-selectin receptor antagonist, we proposed the pyrazolo[4,3-c] quinolin-3-one tricycle (Figure 1.5), because it is a derivative of the quinoline skeleton that introduces a different functionality at the 8-position of the pyrazoloquinoline ring. This could establish optimal space filling for this H$_2$ hydrophobic pocket.

Our comparison was driven by the overlay of the X-ray crystal structure of P-selectin with reported quinoline ring in 3 and pyrazoloquinolinone ring in 4 (Figure 1.4). The first strategy focused to fill the hydrophobic pocket formed by amino acids (Glu80, Pro81, Asn82 and Lys84). The second strategy was aimed to introduce different functionalities to get strong hydrogen bond interactions with Lys111 and Arg85. The third strategy targeted on the hydrophobic interactions of substituents at 8 position of 4 with the residues present in H$_1$ pocket of P-selectin.
Figure 1.4 Overlay of structure of 4 (pink) with compound 3 (yellow)
Introducing the iodo handle at the 8-position of the ring would allow us to incorporate various substituents. Superimposing the iodo intermediate on the template we surmised that substitution on the 8-position of molecule 13 had the potential to reach the residues present in the H₁ hydrophobic pocket (Figure 1.6).
Figure 1.6  Overlay of compound 13 (purple) with compound 3 (grey)
1.2.3 Design of Fused Heterocycles

Based on this docking template, we decided to design a series of pyrazoloquinolin-3-ones analogues were emerged from our ongoing research. Pyrazoloquinolin-3-ones 3 are reported to show a variety of biological activities as Chk 1 kinase inhibitors,\textsuperscript{87} and benzodiazepine receptors (BZRs) antagonists.\textsuperscript{85} Our hypothesis was that the different functionality at the 8-position of pyrazoloquinoline ring would afford a favorable interaction with the H\textsubscript{1} hydrophobic pocket (Figure 1.4 and 1.6). We designed some derivatives conforming to the guidelines dictated by the postulated docking model.\textsuperscript{69} The 3D-quantitative structure activity relationship (QSAR) modeling of quinoline derivatives as P-selectin inhibitors has been reported and indicated the structural requirements (lipophilic, electronic and steric) which caused changes in activity.\textsuperscript{81} Based on the SAR of quinoline salicylate inhibitors of P-selectin, the importance of various substitutions on the quinoline skeleton helped to determine ligand affinities for the active site of receptor.\textsuperscript{82}
Literature review, molecular modeling and Lipinski’s rules\textsuperscript{92} were utilized to design analogues of this core structures. Several common molecular descriptors including molecular weight (MW), AlogP, LogD at pH 7.4, hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), rotatable bonds (RB), polar surface area (PSA) and Lipinski Score (HBA and HBD), for compounds were calculated by ChemAxon software (Table 1.1 and 1.2)\textsuperscript{93}
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Table 1.1 Calculated physicochemical properties of several pyrazoloquinolin-3-ones compound classes derived from ChemAxon software.
The pyrazole ring system has been an increasingly popular heterocycle for the synthesis of pharmaceutically active compounds. The outcome of our modeling studies suggested that pyrazoloquinolinone nucleus as an important structural element. The pharmacological profiles of substituted pyrazolo[4,3-c]quinolin-3-ones have been demonstrated in different disease model. Exploration of the steric, electronic effects of the substituents at 8-position of pyrazoloquinolinone ring could afford a useful series of analogues for evaluation as P-selectin antagonists. The synthetic route of key intermediate is depicted in Scheme 1.1.

**Scheme 1.1** Multi-step synthesis of intermediate pyrazoloquinolinone 13

A novel strategy has been developed for the efficient syntheses of diverse arrays of heterocyclic scaffolds. The key elements of this approach involves an alkylation followed by cyclization reaction in which an amine, ester and halogen handles are introduced. Further transformation provides a diverse set of compounds which possess sub-structures found in biologically active natural products or in clinically useful drugs. The design of this synthetic
approach is a significant challenge but should enable rapid and efficient generation of organic
target compounds that are structurally and functionally diverse those possess skeletal
frameworks found in both natural products and drug-like molecules. This scaffold-based
approach is a classical strategy for new lead discovery in medicinal chemistry.

1.2.4 Design of Conformationally Rigorous Heterocycles

Modeling of 8-substituted pyrazoloquinolinone analogs with the P-selectin receptor was
not easily accomplished, due to the structural characteristics of the ligand-binding site, which is
an almost flat binding surface. In the docking analysis a small molecule with large number of
degrees of freedom fit well for receptors or enzymes with deeper cavities. Reasonable docking
algorithms between molecules and the shallow binding surface of the receptor was more
difficult. This was a great challenge for us.

To overcome this challenge we took a new approach to design conformationally restricted
compounds. Rigid compounds have a better chance to improve ligand-receptor interactions by
reducing any unfavorable changes in binding of the ligand. The introduction of conformational
constraints in structures may provide useful information and an understanding of structural
requirements for bioactivity. The rationale of minimizing the entropy through a constraint
ligand structure has been documented in the literature. The conformational entropy is due to
the changes in conformational degrees of freedom in a molecule. Molecules with more rotatable
bonds can move easily from one conformation to another. The entropy increases due to the
number of rotatable bonds which influences binding potency. From thermodynamic point, the
binding affinity $K_a$ is defined in terms of the free energy of binding

$$K_a = e^{-\Delta G / RT}$$
Where $R$ is the gas constant

$T$ is absolute temperature. The free energy of binding $\Delta G$ is defined as

$$\Delta G = \Delta H - T \Delta S$$

where $\Delta H$ is changes in enthalpy and $\Delta S$ is changes in entropy.

Therefore,

$$K_a = e^{-\frac{(\Delta H - T \Delta S)}{RT}} = e^{-\frac{\Delta H}{RT}} \times e^{\frac{\Delta S}{R}}$$

The above equations show that both enthalpy and entropy components have effect on binding affinity. The enthalpy related to strength of interactions such as van der Waals, hydrogen bonds, etc of the ligand with the protein. The entropy related to changes in solvation and conformational entropy. Thermodynamics suggest that there are three ways to optimize binding affinity such as by making $\Delta H$ negative, $\Delta S$ more positive or pre-shaped to the geometry of the binding site. The approach we took to focus on conformationally constrained molecule that is pre-shaped to the shallow binding surface of the receptor. Upon binding, conformationally constrained ligand became immobilized by its rotatable bond and carries free energy penalty due to the loss of conformational entropy. Given the new interest in a small molecule pan-selectin antagonist, we focused our attention to optimize the binding affinity by making highly hydrophobic and rigid molecule. We used structure based drug design approaches in our research. Conventional structure based minimum energy active conformations were obtained using the Molecular Operating Environment (MOE) program.\textsuperscript{85}
Aydt et al. demonstrated that one carboxylic group in Bimosiamose, (Figure 1.8) is important for selectin binding.\textsuperscript{87} Based on this information we focused our research on a series of conformationally restricted compounds containing an ester as a prodrug. Presence of an ester in a molecule allows introducing different functionalities. \textit{In vivo} the ester bond is readily hydrolysed by ubiquitous esterases present in the blood, liver and other organs and tissues.\textsuperscript{77}

Benzodiazepine and pyrido[2,3-\textit{b}]azepine cores also emerged from our ongoing research are important classes of heterocyclic skeletons occurring in a number of bioactive molecules for a variety of biological targets.\textsuperscript{106-108} Because of their unique structural features and biological activities,\textsuperscript{110-112} we decided to explore fused azepine and diazepine cores by modeling with known structure-based model (Figure 1.9 and 1.10). The pharmacological effects of 1H-pyrido[3,2-b]azepine and 1H-pyrido[3,2-b]diazepine, fused six-membered heterocycles are currently unknown. The rational to make fused small molecules could lead to acceptable pharmacokinetic and pharmacodynamic properties in a P-selectin inhibitor. Superimposition of azepine scaffold AZ within the ligand/receptor template is shown in Figure 1.9. The seven-membered ring B in AZ is overlapped with the B ring of 3. The lone pair of electrons of the
carbonyl oxygen in the ring could interact and carbonyl oxygen of ester is not interacted to $D_1$ site of the receptor in the Figure 1.10. Azepine ring does not fit well on the receptor because lack of hydrogen bond interactions which play important role in the binding.

**Figure 1.9** Superimposition of azepine AZ (blue) with compound 3 (brick-red)
When compared with diazepine core, it is clearly shown that substituted diazepine fits

**Figure 1.10** Superimposition of substituted diazepine DZ (green) with compound 3 (grey)
Scheme 1.2 Diversification of nitrogen heterocycles to obtain SAR data
better in the template ligand-receptor complex in Figure 1.10. There is a hydrogen bonding between substituted urea nitrogen and the arginine residue of the D1 pocket of the receptor which form 3-centered bond interactions with carbonyl oxygen of ring amide. There is a potential interaction between carbonyl oxygen of substituted urea with the lysine residue of D1 pocket by hydrogen bond formation. The fused six membered ring may interact with hydrophobic region and 4-chlorophenyl exposed in the solvent site. Representative examples presented in Scheme 1.2 clearly establish the merits of rigid molecules for the facile synthesis of diverse collections of fused-ring nitrogen heterocycles. Significantly, these scaffolds bear orthogonal functionality that may be selectively exploited for further manipulation and diversification to create novel sets of compounds having substructures found in biologically active natural products and clinically useful drugs. Hence, this chemistry would appear to have considerable potential for generating novel drug leads and tools for studying biological pathways and for drug discovery research.
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**Table 1.2** Calculated physicochemical properties of several azepine and diazapine compound classes derived from ChemAxon software
1.3. EXPERIMENTAL

1.3.1 Computational Chemistry

Molecular mechanics compute the energy of a system as a function of the nuclear position without counting electrons. Different parameters used to define potential energy function to consider electronic environment of the system. Forcefield is a term which includes sets of equations and parameters used to define electrostatic and Van der Waals interactions in the potential surface of a molecule. For the modeling and figures the software “Molecular Operating Environment (MOE), 2011.10” Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2011. was used, applying the Merck molecular force field 94x (MMFF94x) according to Halgren. Molecular simulations such as stochastic conformational searches were performed in two steps.

1. Conformations were minimized using the MMFF94x forcefield field in Figure 1.11

![Energy Minimize Parameter](Image)

**Figure 1.11** Energy minimize parameter
The molecule was minimized by force field MMFF94x and solvation in gas phase. The default parameters of MOE were utilized Figure 1.12. The charges and hydrogen atoms were fixed to obtain optimum minimization energy conformation of the ligand.

**Figure 1.12** MMFF94x parameter

2. Stochastic conformational search is the single lowest energy conformer
The Figure 1.13 show the lowest energy conformation of a ligand from a stochastic conformational search performed with the following standard parameters: Root mean square (RMS) gradient: 0.005, energy cutoff: 7, Root mean square deviation (RMSD) limit: 0.25, conformation limit: 10000, iteration limit: 10000, molecular mechanics (MM) iteration limit: 500, with added chiral constraints, calculating forcefield partial charges (Figure 1.12)

**Figure 1.13** Conformational search parameter
The output of conformational search saved in MOE database contain the following data as in Figure 1.14.

Mol is a molecular conformation

E is the potential energy of the conformation in kcal/mol.

dE is the strain energy of the conformation (in kcal/mol). This is defined to be the energy of the conformation minus the smallest conformational energy seen within the chiral compounds annotated in kcal/mol.

Chi is an integer indicating the chiral class of the conformation.

In conformational search the first entry is the conformer with lowest energy. Minimum energy conformer having the lowest dE value was loaded to MOE window. Ligand is ready for docking.

![Figure 1.14](image)

Figure 1.14 The output of conformational search
Docking techniques is designed to find the most suitable conformation of ligand and its receptor. Before the docking, preparation steps were done by removing water molecules, addition of hydrogen atoms and charges. Further minimization was done using MOE 2011.10(MMFF94x). During the docking process, the receptor was made rigid and the ligand was left free to rotate to find the most suitable conformation. Triangle matcher helps to get the best conformation as placement method. Triangle matcher is a parameter which allows random ligand’s movement at active site of an enzyme to the best bonding orientation. The applied

**Figure 1.15** Docking parameter
scoring function was London dG, which is useful for calculating the binding free energy. The docking result analysis was based on Low $\Delta G_{\text{binding}}$ ($S$) values mean the most stable conformation when ligand bound with enzyme. X-ray crystal structures of P-selectin with PDB Code 1G1Q was used with default parameters in Figure 1.15.

Docking Analysis

The output of docking simulation was saved in MOE database contain the following data (Figure 1.16). This database was then analyzed to study the docking results. Analysis was carried out by comparing the binding energy between ligand and protein.

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Figure 1.16 The output of docking simulation
Mol is molecular field pose mseq is one means single ligand used for docking, S is the final score, E_conf is the energy of the conformer, E_place is the score from the placement stage, E_score1 is the score from the rescoring stage. Top molecule is the best scorer in S and E-score field. Molecular alignment in database is sorted by RMSD. The quality of a docked pose can be analysed by comparing the relative geometry of the original structure. The difference between two conformations is measured by computing root-mean square deviation (RMSD)\(^8\)

The following equation used to calculate RMSD:

\[
\text{RMSD} = \sqrt{\frac{1}{N} \sum_{i=1}^{N} \delta_i^2}
\]

Where \(N\) is the total number of atoms in the molecule

\(\delta\) is the distance between each pair of corresponding atoms

**Docking Result Visualization**

From above docking database the top best conformer used for visualization of molecular alignment. Open graphics object manager window, highlighted ligand interactions and apply. The following image was obtained and used as a ligand-receptor template.
Superimposition by Flexible Alignment Tool

Following reported modeling studies, we constructed ligand–receptor complex according to Ananthula et al. The orientation of the reported compound was optimized and the bound compound was used as a template. Flexible alignment tool was used for alignment of analogs with the template. The molecule was minimized by force field MMFF94x and solvation in gas phase. The charges and hydrogen atoms were fixed to obtain optimum minimization energy conformation of the ligand. Flexible alignment tool was selected as it enables all atom flexibility and conformational search during the generation of molecular alignments. Minimum energy
conformer of each ligand was used for overlay. The flexible alignment was done on the template. The default parameters of MOE were utilized for the alignment. Flexible alignment tool was performed by MOE to automatically generate superpositions of the ligands using default parameter (Figure 1.18). So, the bound ligand was kept fixed and designed ligands were flexibly aligned to the template. Therefore, the designed molecules were subjected to flexible alignment using descriptors like aromaticity, H-bond acceptor/donor groups, LogP, molar refractivity and volume in Figure 1.19.

**Figure 1.18** Flexible alignment parameter
Figure 1.19 Flexible alignment settings

The method has several advantages: a) there are no limits on the number of ligands that can be aligned; b) both conformational and alignment searches are conducted simultaneously; c) the competing goals of feature superposition and potential energy strain are balanced in a natural way; and d) a collection of alignments is output for comparison. The candidate ligands were
arranged to interact with the selected enzyme residues which were Lys111, Arg85, Pro81, Glu80, Asn82 and Lys84.

The output of flexible alignment contains the following data (Figure 1.20):

U is the average strain energy of the molecules in the alignment in kcal/mol.

F is the total mutual similarity score of the configuration

S is the alignment score of the configuration.

Lower values are intended to indicate better alignments.

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**Figure 1.20** The output of flexible alignment

58
Highlight the aligned structures having the lowest dS and dF values to MOE window.

The following image obtained for comparison of ligand interactions for binding pocket.

**Figure 1.21** Flexible alignment visualization image
1.3.2 Optical Rotation

Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Na-D optical rotations were extrapolated from the measured Hg values (546 and 578 nm) with the help of Drude’s equation.\(^8^9\)

\[ [\alpha]_D^{25} = \frac{100.a}{l.c} \]

D = D-line of the sodium lamp
a = observed optical rotation
c = concentration in g/100 ml
l = path length in dm

Methanol was used as a solvent for measurement.

1.3.3 Liquid Chromatography/Mass Spectrometry (LC/MS)

Compound purity was determined by analysis of the diode array UV trace of an LC/MS spectrum using the following procedure. Compounds were dissolved in DMSO, methanol, or acetonitrile, and the solutions were analyzed using an Agilent 1100 LC interfaced to a micromass Waters Micromass Z-spray Mass Detector (ZMD). The compounds were analysed on a Phenomenex Luna column [C18, 50 x 4.6mm, 5um] on a Hewlett-Packard HP 1100 using the following gradients:
Formic acid (FA) Method:

The compounds were eluted with 5% acetonitrile/water/0.1% formic acid (mobile phase A) and 100% acetonitrile/0.1% formic acid (mobile phase B) with a flow rate of 1.5ml/min. The 5min cycle consisted of a gradient of 100% A to 100% B in 3.5min; 100% B for 1min; 100% B to 100% A in 0.1min; then re-equilibration with mobile phase.

LC/MS FA Purity method:

The compounds were analysed on a Phenomenex Luna column [C18, 150 x 4.6mm, 5um] on a Hewlett-Packard HP 1100 using the following gradients:

Formic acid (FA) Purity Method:

The compounds were eluted with acetonitrile (generally either 5%, 20% or 40%) /water/0.1% formic acid (mobile phase A) and 100% acetonitrile/0.1% formic acid (mobile phase B) and a flow rate of 1.0ml/min. The 16 min cycle included a 10min gradient of 100% A to 100% B; 100% B for 2 min; then re-equilibration to 100% A.

1.3.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

Proton (\(^1\)H NMR) and carbon-13 (\(^{13}\)C NMR) spectra were recorded in the solvent reported on a Bruker 300 MHz Avance 1 or 400 MHz Avance 2 (5 mm QnProbe) using residual solvent peaks as the reference.

1.3.5 Thin Layer Chromatography (TLC)

Reactions were monitored by TLC using glass plates coated with silica gel 60 F254 (Merck). The spots were detected under short wavelength UV light (254 nm) or stained with a molybdate solution (a 0.02 M solution of ceric ammonium sulfate and ammonium molybdate tetrahydrate in aqueous 10% H\(_2\)SO\(_4\)). The plates were then heated for 2 min at 155 °C.
1.3.6 Flash chromatography

Purifications were done by flash chromatography using an ISCO system using prepacked 200-400 mesh silica gel 60 column. Thin layer chromatography was used to monitor column fractions.

1.3.7 High Resolution Mass Spectroscopy (HRMS)

HRMS: High resolution mass spectroscopy was obtained on a Micromass 70-VSE mass spectrometer(UIUC Mass Spectrometry Facility, IL).

1.3.8 Hydrogenations

Hydrogenations under pressure were performed in a Parr shaker (Parr Instrument Company, Moline, IL, USA) in 500 mL bottles.

1.3.9 Melting Points

Melting points were taken on a MEL-TEMP II micro melting point apparatus and are uncorrected.
A) By conventional heating

To a 50 mL round bottomed flask equipped with a condenser was added 4-ido aniline 6 (7 mmol, 1.4 mL) and diethyl 2-(ethoxymethylene)-malonate 5 (7 mmol, 1.5 g). The resulting solution was heated at 120-130 °C in an oil bath and stirred for 5 h. The resulting mixture was cooled to room temperature and a light yellow solid formed. The solid was filtered and recrystallized from absolute ethanol to give 2-(4-iodophenylamino)methylene malonate intermediate. The malonate intermediate 7 was taken up in diphenyl ether (4 mmol, 6.0 mL) in a 50 mL round bottomed flask. The suspension was heated at 225 °C in an oil bath and allowed to stir for 4 h. The resulting solution was cooled to room temperature and brown solid formed. The solid was filtered and washed with (2 × 5 mL) ether. The compound was isolated to give 7 (1.2 g, 55%) as a white solid. $^1$H NMR (DMSO-d$_6$, 400 MHz) δ 1.29 (q, 3H), 4.21 (t, 2H) 7.45 (d, 1H), 8.01 (d, 1H), 8.42 (dd, 1H), 8.58 (d, 1H), 12.42 (NH).
B) By microwave heating

A mixture of 4-iodo aniline 6 (7 mmol, 1.4 mL) and diethyl 2-(ethoxymethylene)-malonate 5 (7 mmol, 1.5 g) was added in a 10 mL CEM reaction tube. The tube was purged with argon and sealed with a CEM stopper. The sealed tube was irradiated at 120 °C in a microwave reactor (280W) for 20 min. After cooling to room temperature light yellow solid formed on the side of the tube. The solid was filtered and recrystallized from absolute ethanol to give 7 (2.1g, 79%) as a light yellow solid. $^1$H NMR (CDCl$_3$, 300 MHz) $\delta$ 1.35 (m, 6H), 4.27 (m, 4H) 7.09 (m, 2H), 7.45 (m, 2H), 8.43 (d, 1H) 10.95 (NH); MS m/z (M+1) 290
Synthesis of ethyl 6-iodo 4-oxo-1,4-dihydroquinoline-3-carboxylate (8)

A mixture of compound 7 (4 mmol, 1.5 g) and diphenyl ether (4 mmol, 6.0 mL) was placed in a CEM reaction tube. The tube was purged with argon and sealed with CEM stopper. The tube was heated to 210 °C for 30 min at 280W in a CEM microwave oven. The mixture was cooled to room temperature and brown solid formed on the inner wall of the tube. The resulting solid was filtered, washed with ether (2 × 30 mL). The solid was purified by ISCO using 60% ethyl acetate in hexane to give ethyl 6-iodo 4-oxo-1,4-dihydroquinoline-3-carboxylate 8 (1.5 g, 64%) as a white solid.
A) POCl₃ Chlorination

*Synthesis of ethyl 4-chloro 6-iodo quinoline-3-carboxylate (9)*

A mixture of ethyl 6-iodo 4-oxo-1,4-dihydroquinoline-3-carboxylate (8, 1.0 g, 2.9 mmol) and phosphorus oxychloride (5.9 mL, 15 mmol) was heated at 100 °C for 2 h. The reaction mixture was cooled to room temperature, poured into the 400 mL of ice-water stirred vigorously. Heat evolved upon pouring. A yellow precipitate formed. The resulting precipitate was collected by filtration to provide 9 (0.77 g, 72.0%) as a yellow solid. $^1$H-NMR (CDCl₃): 2.36–2.44 (m, 2H), 3.81 (t, $J = 6.2$ Hz, 2H), 4.06 (s, 3H), 4.37 (t, $J = 6.0$ Hz, 2H), 7.42(s, 1H)
A) SOCl₂ Chlorination

A mixture of ethyl 6-iodo 4-oxo-1,4-dihydroquinoline-3-carboxylate (8, 1.0 g, 2.9 mmol) and thionyl chloride (5.3 mL, 73 mmol) was heated at reflux for 1.5 h. The solution was concentrated and co-evaporated with dichloromethane (3×10 mL) to remove excess thionyl chloride. The pH of the residue was adjusted to 7 with saturated aqueous sodium bicarbonate. The resulting precipitate was collected by filtration to provide 9 (0.98 g, 93% yield).

Synthesis of 8-iodo-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (11)

Ethyl 4-chloro 6-iodo quinoline-3-carboxylate (9, 0.2 g, 0.6 mmol) and phenylhydrazine 10 (0.07 mL, 0.7 mmol) were mixed with 5.0 mL dimethylformamide into a 10 mL Biotage reaction tube, purged with argon, then sealed with Biotage steel stopper. The tube was irradiated at 130 ºC for 45 min. Upon cooling a orange-yellow precipitate formed on the bottom of the tube. The solid was collected by filtration to provide compound (11) (190 mg, 90%, MS (M+1) 387. The solid was used in next step without purification.
8-Iodo-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (11, 0.13 g, 0.34 mmol) was dissolved in anhydrous DMF (5 mL) and cesium carbonate (0.328 g, 1.0 mmol) was added, followed by slow addition of benzyl bromide 12 (0.06 mL, 0.50 mmol). The reaction was stirred at room temperature overnight and then quenched with satd. ammonium chloride (7 mL) and extracted with EtOAc (3×10 mL). The combined organic extracts were washed with brine, dried over Na$_2$SO$_4$, and concentrated. The crude product was purified by ISCO using 20% EtOAc in hexane to provide 13, (82 mg, 51%) as a yellow oil. LCMS:(FA) 478 (M+1); $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 9.16 (d, J = 2.1 Hz, 1H), 8.71 (d, J = 1.8 Hz, 1H), 8.10 (dd, J = 8.9, 1.8 Hz, 1H), 7.99 (t, J = 8.2 Hz, 2H), 7.60 – 7.56 (m, 3H), 7.39 (m, 2H), 7.15 (t, J = 7.4 Hz, 1H), 7.04 (t, J = 7.5 Hz, 2H), 6.62 (d, J = 7.5 Hz, 2H), 5.20 (s, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 161.14, 151.00, 148.69, 147.35, 140.00, 134.49, 133.07, 132.21, 131.80, 129.82, 129.15, 128.77, 128.38, 127.66, 124.67, 119.04, 115.31, 113.46, 92.80, 56.24, 53.79, 28.70, 14.40. HRMS calculated for C$_{23}$H$_{17}$N$_3$O I (M + H$^+$) 478.0409, found 478.0416.
Synthesis of 8-(1-benzothiophene-2-yl)-5-benzyl-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (15)

5-Benzyl-8-iodo-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (13, 0.1 g, 0.2 mmol) and thianaphthene-3-boronic acid 14 (52.2 mg, 0.29 mmol) were stirred with 2.5 mL dimethylformamide in a 10 mL Biotage reaction tube. Dichloro-[1,1’-bis(diphenylphosphino)ferrocene]palladium(II) (8.6 mg, 0.01 mmol) and potassium carbonate (87.0 mg, 0.63 mmol) were added followed by the addition of 0.57 mL of water. The reaction mixture was purged with argon, and sealed with Biotage steel stopper. The tube was irradiated at 110 ºC for 30 min. Upon cooling water was added to dark orange-yellow solution. The reaction mixture was extracted with EtOAc (3×10 mL). The combined organic layers were washed with brine, dried over Na₂SO₄, and concentrated. The crude product was purified by ISCO flash chromatography using 20% EtOAc in hexane to provide 15 (51 mg, 50%).

LCMS (M+1) 484; ¹H NMR (400 MHz, DMSO) δ 9.05 (s, 1H), 8.72 (d, J = 1.6 Hz, 1H), 8.31 (d, J = 8.7 Hz, 1H), 8.19 (dd, J = 8.7, 1.8 Hz, 1H), 8.15 – 8.12 (m, 2H), 7.93 – 7.89 (m, 1H), 7.63 – 7.61 (m, 4H), 7.50 – 7.45 (m, 3H), 7.16 – 7.12 (m, 3H), 6.74 (dd, J = 7.8, 1.5 Hz, 2H), 5.39 (s, 2H); HRMS calculated for C₃₁H₂₁N₅O₅ (M + H⁺) 484.1486, found 484.1484.
**Synthesis of 8-(1-benzothiophene-2-yl)-5-benzyl-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (17)**

![](image)

5-Benzyl-8-iodo-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (13, 0.1 g, 0.2 mmol) and benzofuran-2-ylboronic acid 16 (52.2 mg, 0.29 mmol) were stirred with 2.5 mL dimethylformamide in a 10 mL Biotage reaction tube. Then dichloro-[1,1’-bis (diphenylphosphino)ferrocene]palladium(II) (8.6 mg, 0.01 mmol) and potassium carbonate (87.0 mg, 0.63 mmol) were added followed by the addition of 0.57 mL of water. The reaction mixture was purged with argon, and sealed with Biotage steel stopper. The tube was irradiated at 110 ºC for 30 min. Upon cooling water was added to the dark orange-yellow solution. The resulting aqueous solution was extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine, dried over Na$_2$SO$_4$, and concentrated in vacuo. The crude product was purified by ISCO flash chromatography using 20% EtOAc in hexane to provide 17 (59 mg, 55%) LCMS (M+1) 468; $^1$H NMR (400 MHz, DMSO) $\delta$ 9.03 (s, 1H), 8.97 (s, 1H), 8.48 (dd, J = 8.8, 1.7 Hz, 1H), 8.27 (d, J = 8.8 Hz, 1H), 7.86 (s, 1H), 7.79 – 7.73 (m, 1H), 7.72 – 7.62 (m, 5H), 7.49 (dd, J = 10.0, 4.3 Hz, 1H), 7.45 – 7.37 (m, 1H), 7.33 (dd, J = 10.7, 4.2 Hz, 1H), 7.18 – 7.09 (m, 3H), 6.82 – 6.77 (m, 2H), 5.46 (s, 2H); HRMS calculated for C$_{31}$H$_{21}$N$_3$O$_2$ (M + H$^+$) 468.1704, found 468.1712.
Synthesis of 5-benzyl-8-[(3-chlorophenyl)ethynyl]-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (19)

A 50 mL round bottom flask was charged with 13 (0.100 g, 0.20 mmol), and anhydrous acetonitrile (3.0 mL). A solution of bis(triphenylophosphine)palladium(II) chloride (10 mol %), and CuI (20 mol %), in CH$_3$CN (2 mL) was added, followed by the addition of DIEA (0.073 mL, 0.42 mmol). The yellow reaction mixture was stirred for 5 min at room temperature. Then 3-chloro-1-ethynylbenzene 18 (0.031 mL, 0.25 mmol) was added to the flask and the black reaction mixture was stirred at 100 ºC for 1 h. LC/MS showed the reaction was complete. The solution was washed with water and extracted with ethyl acetate (3 × 15 mL). The combined organic extracts were washed with brine, dried over Na$_2$SO$_4$, and concentrated. The crude product was purified by ISCO using 80% EtOAc in hexane to provide 19 (53 mg, 50%) as a brown oil.

LCMS (M+1) 485; $^1$H NMR (400 MHz, CDCl$_3$) δ 9.09 (s, 1H), 8.46 (s, 1H), 8.21 (d, J = 8.7 Hz, 1H), 7.90 (dd, J = 8.7, 1.7 Hz, 1H), 7.54 – 7.52 (m, 3H), 7.51 (d, J = 1.8 Hz, 1H), 7.42 (dt, J = 7.3, 1.5 Hz, 1H), 7.37 – 7.32 (m, 2H), 7.31 – 7.30 (m, 1H), 7.29 – 7.25 (m, 1H), 7.12–7.06 (m, 1H), 6.98 (dd, J=10.4, 4.8 Hz, 2H), 6.58 – 6.54 (m, 2H), 5.21 (s, 2H). $^{13}$C NMR (101 MHz,
CDCl₃ δ 161.44, 152.18, 149.29, 147.63, 134.86, 134.82, 134.05, 132.45, 132.07, 131.94, 130.29, 130.22, 130.02, 129.63, 129.35, 128.95, 128.66, 127.76, 126.33, 124.82, 124.70, 122.32, 117.45, 113.82, 90.75, 90.05, 56.69. HRMS calculated for C₃₁H₂₁N₃O Cl (M + H⁺) 486.1382, found 486.1373.

Synthesis of 4-(1-benzothiophen-3-yl)-2-methylbut-3-yn-2-amine (22)

A 5mL Biotage microwave tube was charged with 3-bromobenzo[b]thiophene (20, 0.100 g, 0.5 mmol), bis(triphenylohosphine)palladium(II) chloride (10 mol %), and CuI (20 mol %), DIEA (0.350 mL, 0.34 mmol) and 1,1-dimethylpropargyl amine 21 (0.56 mmol, 0.060 mL) were dissolved with anhydrous DMF (2.5 mL). The reaction mixture was stirred and degassed with argon for 5 min. Then the reaction mixture was irradiated with microwave at 100 °C for 30 min. LC/MS showed the reaction was complete. The solution was washed with water and extracted with ethyl acetate (3 × 15 mL), and the combined organic extracts were washed with brine, dried over Na₂SO₄, and concentrated. The crude product was purified by ISCO using 20% EtOAc in hexane to provide 22 (83 mg, 80%) as a brown oil.

LCMS: (FA) (M+1) 199; ¹H NMR (300 MHz, CDCl₃): 1.57 (s, 6H); 1.78 (s, 2H); 7.40 (m, 1H); 7.45 (m, 1H); 7.50 (s, 1H); 7.83(d, 1H); 7.88 (d, 1H).
Synthesis of ethyl 5-benzyl-3-oxo-2-phenyl-3,5-dihydro-2H-pyrazolo[4,3-c]quinoline-8-carboxylate (23)

A mixture of 13 (265 mg, 0.55 mmol), palladium(II) acetate (5.6 mg, 0.025 mmol), and bis(triphenylohosphine)palladium(II) chloride (50.1 mg, 0.05 mmol) was dissolved in 10 ml DMF. Then 1 ml of EtOH and 0.5 ml of triethylamine were added under argon. The atmosphere was changed to carbon monoxide under ballon, and the reaction was conducted at 75 °C for 16h. The reaction mixture was checked by LC/MS. The reaction mixture was messy and no product peak was observed by LC/MS. But TLC showed new spot and no starting material remained. The solvent was evaporated to dryness, and the residue was dissolved in 10 ml DCM. The DCM layer was washed with (2 × 15 ml) of water, 1N hydrochloric acid and brine. The organic layer was separated, dried on sodium sulfate and evaporated. The crude reaction mixture was purified by ISCO using 10% CH$_3$OH in DCM. Analysis of the isolated product by LC/MS and $^{1}$HNMR did not support the structure of the desired compound.
Synthesis of ethyl 5-benzyl-8-[(2-chlorophenyl)amino]-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (25) (Trial 1)

\begin{center}
\includegraphics[width=0.8\textwidth]{synthesis.png}
\end{center}

Pd(OAc)$_2$, Xanphos with sodium butoxide-catalyzed amination: An oven-dried 5 mL Biotage microwave tube equipped with a magnetic stirring bar was charged with Pd(OAc)$_2$ (1 mol%), Xantphos (2 mol%) and t-BuONa (1.4 mmol). The tube was capped with a rubber septum, evacuated, and then flushed with argon. This cycle was repeated three times. o-Chloroaniline 24 (1.2 mmol), 8-iodo-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (13, 1.0 mmol) and toluene (4 mL) were then successively added by syringe. The tube was stirred at room temperature for 5 min and then placed into a microwave cavity and heated at 100 °C for 30 min. The reaction mixture was checked by LC/MS. The reaction mixture was messy, no product peak was observed by LC/MS.
Synthesis of ethyl 5-benzyl-8-[(2-chlorophenyl)amino]-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (25)(Trial 2)

\[
\begin{align*}
\text{I} & \quad \text{N} \quad \text{N} \quad \text{Cl} \\
\text{N} & \quad \text{N} \quad \text{N} \quad \text{O} \\
\text{13} & \quad \text{N} \quad \text{N} \quad \text{O} \\
\end{align*}
\]

\[
\begin{align*}
\text{Cl} & \quad \text{N} \quad \text{N} \quad \text{O} \\
\text{24} & \quad \text{N} \quad \text{N} \quad \text{O} \\
\text{25} & \quad \text{N} \quad \text{N} \quad \text{O} \\
\end{align*}
\]

Pd(OAc)\text{\textsubscript{2}}, Xanphos with Cs\text{\textsubscript{2}}CO\text{\textsubscript{3}}-catalyzed amination: An oven-dried 5 mL Biotage microwave tube equipped with a magnetic stirring bar was charged with Pd(OAc)\text{\textsubscript{2}} (1 mol%), Xantphos (2 mol%) and Cs\text{\textsubscript{2}}CO\text{\textsubscript{3}} (1.4 mmol). The tube was capped with a rubber septum, evacuated, and then flushed with argon. This cycle was repeated three times. o-Chloroaniline 24 (1.2 mmol), 8-iodo-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one( 13, 1.0 mmol) and dioxane (4 mL) were then successively added by syringe. The tube was stirred at room temperature for 5 min and then placed into a microwave cavity and heated at 110 °C for 30 min. The reaction mixture was checked by LC/MS. The reaction mixture was messy, no product peak was observed by LC/MS.
**Synthesis of methyl-5-bromothiophene-2-carboxylate (27)**

A stirred solution of 5-bromothiophene-2-carboxylic acid (26, 4.8 mmol, 1.0 g) in conc. sulfuric acid (9.9 mmol, 0.53 mL) and 5 mL of methanol was stirred at 90 °C for 15 h. LC/MS showed the reaction was complete. The reaction mixture was cooled to room temperature and basified with 1N NaOH, white solid formed. The solid was collected by filtration, washed with methanol, dried and to provide 27 (0.8 g, 70%) as a white solid. $^1$H NMR (400 MHz, DMSO): 3.81 (s, 3 H); 7.36 (d, 1H); 7.65 (d, 1H).
**Synthesis of [5-(dihydroxyboryl)-2-thiophenecarboxylic acid (28)**

To a 100 mL round bottomed flask [5-(methoxycarbonyl)-2-thienyl]boronic acid (27, 0.1 g, 0.5 mmol), tetrahydrofuran (1 mL) and water (1 mL) were added. Then lithium hydroxide (0.26 g, 1.08 mmol) was added and the reaction mixture was stirred overnight at room temperature. LC/MS showed complete reaction. The reaction mixture was quenched with 0.1 M HCl and extracted with ethyl acetate (3×15 mL). The combined organic extracts were washed with brine, dried over Na$_2$SO$_4$ and concentrated to a yellow solid. The crude solid was purified by trituration with hexane. The solid was isolated by filtration and dried in a vacuum oven to provide 28 (75 mg, 80%) as a yellow solid.

$^1$H NMR (400 MHz, DMSO): 7.75 (d, 1H); 7.87 (d, 1H); 12.98 (s, 1H). MS (M+1) 172
Synthesis of methyl 5-(5-benzyl-3-oxo-2-phenyl-3,5-dihydro-2H-pyrazolo[4,3-c]quinolin-8-yl)thiophene-2-carboxylate (29)

5-Benzyl-8-iodo-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (13, 0.1 g, 0.2 mmol) and [5-(dihydroxyboryl)-2-thiophencarboxylic acid (28, 0.055 g, 0.29 mmol) were stirred with 2.5 mL dimethylformamide in a 10 mL Biotage reaction tube. Then dichloro-[1,1′-bis(diphenylphosphino)ferrocene]palladium(II) (8.6 mg, 0.01 mmol) and potassium carbonate (87.0 mg, 0.63 mmol) were added followed by the addition of 0.57 mL of water. The reaction mixture was purged with argon and sealed with Biotage steel stopper. The tube was irradiated at 110 °C for 30 min. Upon cooling the the dark orange-yellow solution was diluted with water and extracted with EtOAc (3×10 mL). The combined organic extracts were washed with brine, dried over Na₂SO₄, and concentrated. The crude product was purified by ISCO flash chromatography using 20% EtOAc in hexane to provide 29 (46 mg, 42%) as a brownish solid.

LCMS: (FA) (M+1) 492. ¹H NMR (300 MHz, CDCl₃) δ 9.17 (s, 1H), 8.53 (d, J = 1.9 Hz, 1H), 8.29 (d, J = 8.8 Hz, 1H), 8.11 (dd, J = 8.8, 1.6 Hz, 1H), 8.01 (s, 1H), 7.84 (dd, J = 3.9, 0.5 Hz, 1H), 7.63 (t, J = 7.6 Hz, 3H), 7.57 (dd, J = 6.5, 2.2 Hz, 1H), 7.45 (dd, J = 4.0, 0.4 Hz, 1H), 7.15
(d, J = 7.5 Hz, 1H), 7.06 (dd, J = 13.9, 6.6 Hz, 2H), 6.71 (d, J = 7.3 Hz, 2H), 5.28 (s, 2H), 3.97 – 3.88 (m, 3H).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 162.51, 161.09, 152.09, 149.46, 147.18, 134.75, 134.41, 133.66, 132.48, 132.30, 129.75, 129.04, 129.01, 128.79, 128.07, 127.60, 125.13, 124.71, 119.81, 117.48, 113.32, 56.32, 52.55. HRMS calculated for C$_{29}$H$_{22}$N$_3$O$_3$S (M + H$^+$) 492.1384, found 492.1382.

Check 13C NMR, missing 2 carbon peaks

**Synthesis of 4,4,5,5-tetramethyl-2-(3-phenoxyphenyl)-1,3,2-dioxaborolane (31)**

![Synthesis diagram](image)

A 20 mL Biotage reaction tube was charged with 13 (0.1 g, 0.2 mmol), 4,4,5,5-tetramethyl-2-(3-phenoxyphenyl)-1,3,2-dioxaborolane (30, 0.087 g, 0.3 mmol). To the mixture was added 2.5 mL dimethylformamide, followed by the addition of dichloro-[1,1’-bis(diphenylphosphino)ferrocene]palladium(II) (8.6 mg, 0.01 mmol) and potassium carbonate (87.0 mg, 0.63 mmol) and 0.57 mL of water. The reaction mixture was purged with argon and sealed with Biotage steel stopper. The tube was irradiated at 130 °C for 1 h. Upon cooling the dark orange-yellow solution was diluted with water and extracted with EtOAc (3×10 mL). The combined organic extracts were washed with brine, dried over Na$_2$SO$_4$, and concentrated. The
crude product was purified by ISCO flash chromatography using 20% EtOAc in hexane to provide 31 (40 mg, 40%) as an oil.

LCMS: (FA) [M+1] 520; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 9.13 (s, 1H), 8.45 (d, J = 1.8 Hz, 1H), 8.28 (d, J = 8.8 Hz, 1H), 8.07 (dd, J = 8.7, 1.9 Hz, 1H), 7.99 (s, 1H), 7.63 – 7.57 (m, 2H), 7.53 (t, J = 7.9 Hz, 3H), 7.39 – 7.31 (m, 4H), 7.23 (dd, J = 4.4, 2.4 Hz, 5H), 7.01 (t, J = 7.6 Hz, 2H), 6.62 (d, J = 7.3 Hz, 2H), 5.28 (s, 3H).

**Synthesis of 2-(3-Bromophenyl)pyridine (34)**

A 50 mL round bottomed flask was charged with 2-bromopyridine (32, 0.4 mL, 4 mmol), water (4.4 mL), ethanol (3.2 mL), 1,2-dimethoxyethane (10 mL), 3-bromophenylboronic acid (33, 0.84 g, 4.2 mmol), and tetrakis(triphenylphosphine) palladium(0) (50 mg, 0.04 mmol). The reaction mixture was degassed with argon and heated at 90 °C for 15 h. LC/MS showed the reaction was complete. Evaporated the solvent, water was added to the residue and extracted with EtOAc (3×10 mL). The combined organic extracts were washed with brine, dried over Na$_2$SO$_4$, and concentrated. The crude product was purified by ISCO flash chromatography using 100% EtOAc in hexane to provide 34 (735 mg, 70%) as an oil.
Synthesis of (3-pyridin-2-ylphenyl)boronic acid (35)

A solution of n-BuLi in hexane was added to a stirred solution of 2-(3-bromophenyl)pyridine (34, 0.730 g, 3.1 mmol) in tetrahydrofuran (5 mL) at -78 °C. The reaction mixture was stirred at -78 °C for 1 h. Then trimethyl borate (1.8 mL, 16 mmol) was added dropwise at same temperature and stirred at -78 °C to room temperature for 1.5 h. LC/MS showed the reaction was complete. 3 M HCl (4 mL) was added to the reaction mixture, and the reaction mixture was stirred for 30 min. The solvent was evaporated, water was added to the residue and extracted with EtOAc (3×10 mL). The combined organic extracts were washed with brine, dried over Na₂SO₄ and concentrated to yellow oil. The crude product was purified by ISCO flash chromatography using 100% EtOAc in hexane to provide 35 (421 mg, 68%) as a yellow oil.

LCMS: (FA) [M+1] 200; ¹H NMR: (300 MHz, CDCl₃) δ 8.73 (dd, J = 3.4, 1.3 Hz, 1H), 8.19 (t, J = 1.7 Hz, 1H), 8.02 – 7.98 (m, 2H), 7.76 – 7.75 (m, 1H), 7.63 (s, 1H), 7.51 – 7.47 (m, 2H), 2.08 (d, J = 6.9 Hz, 1H).
Scheme 1.3 Synthetic route of the known scaffold 36

The ethyl 2-((2R,3S)-3-(tert-butoxycarbonylamino)piperidin-2-yl)acetate (35) was synthesized by known procedure (Scheme 1.3).
Synthesis of azepine analogues:

**Synthesis of methyl 4-((2R,3S)-3-(tert-butoxycarbonylamino)-2-(2-methoxy-2-oxoethyl)piperidin-1-yl)-4-oxobutanoate (38)**

To a mixture of 36 and 3-(carbomethoxy-propionyl chloride (37, 0.103 mL, 0.8 mmol) in DCM (3.0 mL), DIEA (0.401 mL, 2.3 mmol) was added followed by the addition of DMAP (93.8 mg, 0.77 mmol). The solution was stirred at room temperature for 18 h. TLC using 50% EtOAc in hexane was developed by p-anisaldehyde stain which showed yellow spot. 1N HCl solution was added to the reaction mixture, and the solution was extracted with EtOAc. The organic phase was washed with saturated NaHCO₃ solution, then with brine. The organic layer was dried on Na₂SO₄ and evaporated. The residue was purified by ISCO flash chromatography using 0-30% EtOAc in hexane to provide 38 (190 mg, 62%) as an oil.

**LCMS:** (FA) [M+1] 387; **¹H NMR:** (400 MHz, CDCl₃) δ 5.23 – 5.09 (m, 1H), 4.65 – 4.51 (m, 2H), 4.11 (m, 2H), 3.69 (d, J = 4.7 Hz, 3H), 2.87 (dd, J = 15.5, 10.0 Hz, 1H), 2.70 (dt, J = 6.1, 4.2 Hz, 2H), 2.60 – 2.57 (m, 1H), 2.58 – 2.53 (m, 1H), 1.92 – 1.80 (m, 1H), 1.73 (t, J = 10.3 Hz,
1H), 1.62 – 1.53 (m, 2H), 1.43 (d, J = 7.8 Hz, 9H), 1.27 – 1.21 (m, 3H); HRMS calculated for \( \text{C}_{19}\text{H}_{32}\text{N}_2\text{O}_7 \) (M + H\(^{+}\)) 401.2291, found 401.2288. \([\alpha]_{D}^{25} = -12.9\)

**Preparation of (1S,10aR)-methyl 1-(tert-butoxycarbonylamino)-6,9 dioxodecahydropyrido[1,2-a] azepine-8-carboxylate (39)**

To a solution of methyl 4-((2R,3S)-3-(tert-butoxycarbonylamino)-2-(2-ethoxy-2-oxoethyl)piperidin-1-yl)-4-oxobutanoate (38, 190 mg, 0.47 mmol) in ethanol (6.0 mL), was added 5 M sodium ethoxide in ethanol (0.330 mL, 1.65 mmol). The reaction mixture was stirred, at 68 \( ^{\circ} \text{C} \), for 8 h. The solution was allowed to cool to room temperature and then concentrated to give an oily residue. The residue was then dissolved in a mixture of water and ethyl acetate. The organic layer was separated, dried over MgSO\(_4\), filtered and evaporated. The crude product was purified by ISCO flash chromatography using 0-50% EtOAc in hexane to provide 39 (0.116 g, 69%) as a light brown oil.

LCMS: (FA) \([\text{M+MeOH}]^{+}\) 387; \(^1\text{H NMR:} \) (400 MHz, CDCl\(_3\)) \( \delta \) 7.20 – 7.16 (m, 1H), 4.37 (d, J = 9.3 Hz, 1H), 4.19 – 4.09 (m, 2H), 3.35 – 3.23 (m, 1H), 2.99 (dd, J = 9.3, 2.7 Hz, 1H), 2.74 – 2.66 (m, 2H), 2.57 (td, J = 12.0, 2.8 Hz, 1H), 2.35 (d, J = 7.5 Hz, 2H), 2.08 – 2.00 (m, 1H), 1.90
(d, J = 21.4 Hz, 1H), 1.69 (dd, J = 8.5, 4.8 Hz, 1H), 1.48 – 1.42 (m, 9H), 1.26 (m, 3H). [α]$_{D}^{25}$ = -13.4

**Preparation of** (1S,10aR)-methyl 1-amino-6,9-dioxodecahydropyrido[1,2-a]azepine-8-carboxylatemethyl (40)

![Chemical Reaction Diagram]

(1S,10aR)-methyl-(tert-butoxycarbonyl)amino)-6,9-dioxodecahydropyrido[1,2-a]azepine-8-carboxylate (39, 100 mg, 0.30 mmol) was dissolved in 1.0 mL dioxane. 4 M HCl in dioxane (300 mL) was added and the reaction mixture was stirred at room temperature for 2h. The reaction mixture was concentrated to dryness and washed with dichloromethane to give 40 (40 mg, 60 %) as a white powder.

LCMS: (FA) [M+MeOH]$^+$ 468; $^1$H NMR: (400 MHz, DMSO) LCMS: (FA) ES$^+$ [M + MeOH] 386; $^1$H NMR: (400 MHz, MeOD) δ 5.17 (d, J = 5.9 Hz, 1H), 3.90 (m, 2H), 3.66 (t, J = 2.0 Hz, 2H), 3.62 (s, 1H), 2.62 (dd, J = 8.9, 4.3 Hz, 3H), 2.14 (dd, J = 8.5, 4.3 Hz, 2H), 1.87 (m, 2H), 1.74 – 1.68 (m, 2H).
Preparation of (1S,10aR)-methyl 1-((benzo[b]thiophen-2-ylmethyl)amino)-6,9-dioxodecahydro pyrido[1,2-a]azepine-8-carboxylate (42)

Benzothiophene-2-carboxaldehyde (41, 86 mg, 0.5 mmol) was added to a solution of (1S,10a R)-methyl 1-(tert-butoxycarbonyl)amino)-6,9-dioxodecahydropyrido[1,2-a]azepine-8-carboxylate (40, 90 mg, 0.40 mmol) in methanol (2.5 mL) and triethylamine (50 mL, 0.30 mmol). The reaction was stirred at room temperature for 1 h, then sodium cyanoborohydride (33 mg, 0.5 mmol) and acetic acid (100 mL, 1.5 mmol) were added keeping pH ~5. The reaction mixture was stirred overnight, quenched with H₂O (0.1 mL). The reaction mixture was extracted with DCM and then washed with brine. The crude product was purified by flash chromatography using 0-10% of MeOH in DCM. The desired fractions were combined and evaporated to provide 42 (51 mg, 36 %) as a white powder.

¹H NMR: (300 MHz, MeOD) δ 7.81 (dd, J = 7.8, 1.2 Hz, 1H), 7.71 – 7.66 (m, 1H), 7.33 – 7.22 (m, 3H), 5.49 (s, 2H), 4.86 (s, 4H), 4.45 (d, J = 14.7 Hz, 1H), 4.15 – 4.02 (m, 2H), 3.70 – 3.65 (m, 1H), 3.58 – 3.41 (m, 1H), 3.21 (dd, J = 11.4, 3.9 Hz, 1H), 2.70 – 2.56 (m, 1H), 2.24 – 2.11 (m, 1H), 2.02 (s, 2H), 1.87 (dd, J = 15.1, 6.6 Hz, 1H), 1.71 – 1.60 (m, 1H), 1.48 – 1.43 (m, 2H).

[α]²⁵_D = -12.7
Synthesis of (1S,10aR)-methyl 1-(3-(naphthalen-1-yl)-1-(naphthalen-1-ylcarbamoyl)ureido)-6,9-dioxodecahydropyrido[1,2-a]azepine-8-carboxylate (44)

A 50 mL round bottomed flask was charged with (1S,10aR)-methyl 1-(tert-butoxycarbonyl)amino)-6,9-dioxodecahydropyrido[1,2-a]azepine-8-carboxylate (40, 100 mg, 0.45 mmol), DMF (3 mL) and triethylamine (60 mL, 0.43 mmol). To this solution 1-naphthylisocyanate (43, 73 mg, 0.43 mmol) was added and the reaction mixture was stirred at room temperature for overnight. LC/MS showed a dialkylation product formed. The reaction mixture was evaporated to remove DMF. Remaining traces of DMF were removed by co-evaporation with toluene. The residue was diluted with EtOAc (5 mL), and water (5 mL) and extracted. The organic phase was washed with brine, dried over Na₂SO₄ and evaporated. A white solid was obtained. The solid was dissolved in DCM and triturated with hexane. The white solid was filtered and dried in vacuum oven overnight to provide 44 (170 mg, 70 %) as a white powder.

LCMS: (FA) [M+10]+ 588; ¹H NMR: (300 MHz, DMSO) δ 9.64 (s, 2H), 8.43 (s, 2H), 8.27 (s, 1H), 8.08 (m, 3H), 7.95 (d, J = 6.7 Hz, 2H), 7.73 (s, 1H), 7.62 – 7.61 (m, 2H), 7.49 (s, 2H), 7.39 (s, 1H), 5.02 (s, 1H), 4.34 (d, J = 13.6 Hz, 1H), 3.73 (d, J = 13.1 Hz, 1H), 3.60 (t, J = 10.3 Hz,
1H), 3.40 (s, 1H), 3.10 (d, J = 11.2 Hz, 1H), 2.90 (s, 1H), 2.82 – 2.75 (m, 1H), 2.74 (d, J = 0.5 Hz, 1H), 2.67 (d, J = 7.0 Hz, 1H), 2.00 (s, 1H), 1.79 (s, 1H), 1.51 (s, 1H).

**Synthesis of ethyl 2-((2R,3S)-3-(tert-butoxycarbonylamino)-1-(naphthalen-1 ylcarba moyl)piperidin-2-yl)acetate (46)**

A 50 mL round bottomed flask was charged with ethyl (2R, 3S)-3-Boc-amino-2-piperidineacetate (36, 300 mg, 0.001 mol) and DCM (15 mL). 1-naphthylisocyanate (45, 200 mg, 0.001 mol) was added and the reaction mixture was stirred at room temperature for overnight. LC/MS showed the reaction was complete. The solvent was removed and the residue was diluted with EtOAc (5 mL) and water (5 mL) and extracted. The organic phase was washed with brine, dried over Na$_2$SO$_4$ and evaporated. The solid was dissolved in DCM and the crude product was purified by ISCO using 30% EtOAc in hexane to provide 46 (294 mg, 63%) as a white solid.

LCMS: (FA) [M + 1] 456; $^1$H NMR: (400 MHz, CDCl$_3$) δ 8.37 (s, 1H), 8.12 (d, J = 8.3 Hz, 1H), 7.87 – 7.83 (m, 1H), 7.76 (d, J = 7.4 Hz, 1H), 7.63 (d, J = 8.2 Hz, 1H), 7.56 – 7.52 (m, 1H), 7.51 (dd, J = 4.6, 1.5 Hz, 1H), 7.46 (dd, J = 9.1, 6.7 Hz, 2H), 5.16 (d, J = 7.4 Hz, 1H), 4.84 (d, J
\[ \text{H NMR: (300 MHz, DMSO)} \delta \ 11.3 \text{ Hz, 1H}, \ 4.38 \text{ (d}, J = 13.4 \text{ Hz, 1H}), \ 4.30 \text{ (q}, J = 7.1 \text{ Hz, 2H}), \ 3.13 \text{ (dd}, J = 17.1, 11.6 \text{ Hz, 1H}), \ 2.84 \text{ (d}, J = 12.3 \text{ Hz, 1H}), \ 2.63 \text{ (dd}, J = 17.0, 2.3 \text{ Hz, 1H}), \ 1.86 \text{ (d}, J = 2.6 \text{ Hz, 2H}), \ 1.60 \text{ (s, 2H), 1.34 (m, 3H), 1.29 (s, 9H).} \]

\[ \text{C NMR: (400 MHz, DMSO)} \delta \ 171.92, 156.46, 135.70, 135.60, 133.72, 127.93, 125.92, 125.66, 125.49, 125.28, 123.83, 122.90, 121.40, 120.15, 119.99, 111.98, 77.82, 60.33, 54.82, 54.26, 34.17, 28.08, 23.24, 19.42, 13.84. \]

HRMS calculated for C\textsubscript{25}H\textsubscript{34}N\textsubscript{3}O\textsubscript{5} (M + H\textsuperscript{+}) 456.2498, found 456.2506. \[ \alpha \] \text{D} = -10.9
**Synthesis of ethyl 2-((2R,3S)-3-amino-1-(naphthalen-1-ylcarbamoyl)piperidin-2-yl)acetate hydrochloride (47)**

Ethyl 2-((2R,3S)-3-(tert-butoxycarbonylamino)-1-(naphthalen-1-ylcarbamoyl) piperidin-2-yl)acetate (46, 275 mg, 0.604 mmol) was dissolved in 1.0 mL dioxane, followed by the addition of 4 M HCl in dioxane (300 mL). The reaction mixture was stirred at room temperature for 2 h. Concentrated to dryness and washed with dichloromethane to give 47 (143 mg, 66%) as a white powder.

LCMS: (FA) [M +1] 356; $^1$H NMR: (300 MHz, MeOD) δ 8.01 (s, 1H), 7.83 (s, 1H), 7.65 (d, $J = 43.9$ Hz, 2H), 7.44 (d, $J = 15.4$ Hz, 3H), 4.13 (s, 2H), 3.59 (s, 1H), 3.43 (dd, $J = 14.7$, 7.9 Hz, 1H), 2.84 (s, 2H), 2.16 (s, 1H), 1.84 (m, 3H), 1.29 – 1.15 (m, 4H), 0.87 (d, $J = 5.7$ Hz, 1H).
**Synthesis of methyl3-[(2R,3S)-2-(2-ethoxy-2-oxoethyl)-1-(1-naphthylcarbamoyl)piperidin-3-yl]amino]-3-oxopropanoate (49)**

A 50 mL. round bottom flask was charged with ethyl [(2R,3S)-3-amino-1-(1-naphthylcarbamoyl)piperidin-2-yl]acetate (47, 200 mg, 0.60 mmol), DIEA (0.30 mL, 2.0 mmol) and DCM (3.0 mL). To this solution methyl malonyl chloride (48, 0.80 g, 0.60 mmol) and DMAP (68.7 mg, 0.56 mmol) were added. The solution was stirred at room temperature for 18 h. LC/MS showed the reaction was complete. TLC using 50% EtOAc in hexane was developed by p-anisaldehyde stain and showed yellow product spot. 1N HCl solution was added and the mixture was extracted with EtOAc. The organic phase was washed with saturated NaHCO$_3$ solution and brine. The organic layer was dried over Na$_2$SO$_4$ and evaporated. The residue was purified by ISCO using 10% CH$_3$OH in DCM to provide 49 (100 mg, 40%) as a white solid.

LCMS: (FA) [M +1]$^+$ 456; $^1$H NMR: (300 MHz, CDCl$_3$) δ 7.70 (d, J = 7.6 Hz, 1H), 7.60 (d, J = 7.3 Hz, 1H), 7.47 (d, J = 6.2 Hz, 1H), 7.35 (d, J = 7.9 Hz, 1H), 7.11 (m, 3H), 4.98 (s, 2H), 3.69 (dd, J = 14.4, 7.2 Hz, 1H), 3.46 (d, J = 3.8 Hz, 2H), 3.36 (s, 2H), 1.61 (s, 1H), 0.98 – 0.78 (m, 6H), 0.47 (dd, J = 11.3, 4.8 Hz, 3H).
A 50 mL round bottom flask was charged with methyl3-\{\[(2R,3S)-2-(2-ethoxy-2-oxoethyl)-1-(1-naphthylcarbamoyl)piperidin-3-yl\]amino\}-3-oxopropanoate (49, 100 mg, 0.2 mmol), 5 M sodium ethoxide in ethanol and ethanol (1.0 mL), (0.175 mL). The reaction mixture was stirred at 68 °C for 8 h. The solution was cooled to room temperature, and then concentrated to give solid residue. The residue was then dissolved in a mixture of water and DCM. The organic layer was separated, dried over MgSO₄, filtered and evaporated to a white powder. No product peak was observed in LC/MS. The residue was purified by ISCO using 10% CH₃OH in DCM to provide 50 (30 mg, 30%) as a white solid. NMR showed some impurities.

\(^1\)H NMR: (300 MHz, CD₃OD) \(\delta\) 8.56 (dd, \(J = 10.3, 5.3\) Hz, 1H), 8.47 (dd, \(J = 10.5, 5.3\) Hz, 2H), 8.27 (s, 1H), 7.82 (dd, \(J = 12.8, 2.7\) Hz, 2H), 7.75 – 7.61 (m, 1H), 4.46 (s, 1H), 3.71 (d, \(J = 4.4\) Hz, 3H), 3.64 – 3.61 (m, 2H), 3.39 – 3.35 (m, 2H), 3.12 – 2.94 (m, 2H), 2.84 – 2.69 (m, 1H), 2.56 (m, 3H), 2.01 (d, \(J = 2.6\) Hz, 1H), 1.98 (s, 1H), 1.80 – 1.65 (m, 4H), 1.28 (s, 2H).
Synthesis of ethyl 2-((2R,3S)-1-(4-bromopicolinoyl)-3-((tert-butoxycarbonyl) amino)piperidin-2-yl)acetate (52)

A 50 mL round bottom flask was charged with ethyl (2R, 3S)-3-Boc-amino-2-piperidineacetate (36, 320 mg, 0.0011 mol) and DCM (15 mL). To this solution 4-bromo-2-pyridinecarboxylic acid (51, 226 mg, 0.0011 mol) and N,N-dimethylaminopyridine (13.6 mg, 0.00011 mol) were added. The reaction mixture was stirred for 5 min, then N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride was added and stirred at room temperature for overnight. LC/MS showed the reaction was complete. The reaction mixture was diluted with DCM and washed with 1N HCl (5 mL), and extracted with DCM (3 × 15 mL). The organic phase was washed with brine, dried over Na$_2$SO$_4$ and evaporated. A brown/yellowish solid was obtained. The solid was dissolved in DCM and the crude product was purified by ISCO using 30% EtOAc in hexane to provide 52 (250 mg, 48%) as a brown solid.

LCMS: (FA) [M+1] 471; $^1$H NMR (400 MHz, CDCl$_3$) δ 8.24 (d, J = 5.2 Hz, 1H), 7.81 (d, J = 1.6 Hz, 1H), 7.47 (dt, J = 10.2, 5.1 Hz, 1H), 7.20 (s, 1H), 4.47 – 4.35 (m, 2H), 4.12 – 3.90 (m, 3H), 3.52 (s, 1H), 2.92 – 2.82 (m, 2H), 2.32 (dd, J = 15.9, 4.6 Hz, 1H), 2.03 (d, J = 13.7 Hz, 1H), 1.88 – 1.60 (m, 3H), 1.55 (d, J = 13.2 Hz, 1H), 1.40 (s, 9H), 1.12 (t, J = 7.1 Hz, 3H); HRMS calculated for C$_{20}$H$_{28}$BrN$_3$O$_5$ (M + H$^+$) 470.1292, found 470.1291; [$\alpha$]$^2_5$D = -13.9
Synthesis of ethyl \((2R,3S)-3\text{-amino-1-[(4-bromopyridin-2-yl)carbonyl]piperidin-2-yl}}\)acetate hydrochloride (53)

A 50 mL round bottom flask was charged with ethyl-2-((2R,3S)-1-(4-bromopicolinoyl)-3-((tert-butoxycarbonyl)amino)piperidin -2-yl)acetate (52. 200 mg, 0.43 mmol), 4 M HCl in dioxane (400 mL) and 1.5 mL dioxane. The reaction mixture was stirred, at room temperature, for 2 h. LC/MS showed the reaction was complete. The reaction mixture was concentrated to dryness, washed with dichloromethane and dried under vacuum overnight to provide 53 (100 mg, 60%) as a solid.

LCMS: (FA) [M +1] 372; \(^1\)H NMR: (300 MHz, CD\(_3\)OD) 0.85 (d, 1H); 1.24 (t, 3H); 1.83 (d, 2H); 2.21 (d, 1H); 2.95 (d, 1H); 3.65 (d, 2H); 4.15 (t, 2H); 4.47 (s, 1H); 7.67 (d, 1H); 7.90 (s, 1H); 8.47 (d, 1H).
**Synthesis of methyl 3-\([(2R,3S)-1-\{(4-bromopyridin-2-yl)carbonyl\}-2-(2-ethoxy-2-oxoethyl)piperidin-3-yl]amino\)-3-oxopropanoate (55 and 56)**

A 50 mL round bottom flask was charged with \([(2R,3S)-3-amino-1-\{(4-bromopyridin-2-yl)carbonyl\}piperidin-2-yl}acetate (54, 140 mg, 0.38 mmol), DIEA (0.30 mL, 2.0 mmol) and DCM (3.0 mL). To this solution methyl malonyl chloride (48, 52 mg, 0.38 mmol) and DMAP (65.7 mg, 0.54 mmol) were added. The solution was stirred at room temperature for 18 h. LC/MS showed the reaction was complete. TLC using 50% EtOAc in hexane was developed by p-anisaldehyde stain showed a yellow product spot. 1N HCl solution was added and the mixture was extracted with EtOAc. The organic phase was washed with saturated NaHCO$_3$ solution, then brine, dried over Na$_2$SO$_4$ and evaporated. The residue was purified by ISCO using 10% CH$_3$OH in DCM provided monoalkylated product 55 (71 mg, 40%) and dialkylated product and 56 (23 mg, 11%) a light brown solids:

**55**: LCMS: (FA) [M +1]$^+$ 471 $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.59 (d, $J$ = 4.5 Hz, 1H), 8.33 (d, $J$ = 5.4 Hz, 1H), 7.83 (t, $J$ = 3.6 Hz, 1H), 7.52 (dt, $J$ = 6.2, 3.1 Hz, 1H), 4.55 – 4.41 (m, 2H), 4.07 (m, 1H), 3.97 (m, 1H), 3.82 (d, $J$ = 2.8 Hz, 1H), 3.70 (m, 3H), 3.33 (d, $J$ = 7.8 Hz, 2H), 2.92 (m,
2H), 2.37 (dd, $J = 16.0$, 4.7 Hz, 1H), 2.16 (d, $J = 12.0$ Hz, 1H), 1.78 – 1.70 (m, 2H), 1.61 (t, $J = 10.4$ Hz, 1H), 1.15 (t, $J = 7.1$ Hz, 3H).

$^{13}$C NMR (400 MHz, CDCl$_3$) $\delta$ 169.26, 168.28, 167.10, 164.39, 154.54, 147.57, 133.89, 128.16, 127.81, 77.03, 76.72, 76.40, 60.61, 53.53, 52.05, 48.09, 42.43, 36.81, 34.21, 22.58, 19.63, 13.64.

HRMS calculated for C$_{19}$H$_{25}$BrN$_3$O$_6$ (M + H$^+$) 470.0927, found 470.0924; [a]$^{25}$D = -10.3

56: LCMS: (FA) [M +1]$^+$ 571

$^1$H NMR (400 MHz, CDCl$_3$): 1.18 (t, 3H); 1.74 (d, 2H); 1.91 (t, 2H); 2.67-2.60 (dd, 1H); 2.78 (d, 1H); 2.96-2.88 (m, 3H); 3.72 (s, 6H); 4.01 (t, 2H); 4.08 (m, 1H); 4.16 (t, 2H); 4.66 (d, 2H); 7.45 (dd, 1H); 7.80 (s, 1H); 8.23 (d, 1H).
Synthesis of methyl 4aS, 9aR)-1-[(4-bromopyridin-2-yl)carbonyl]-6,8-dioxodecahydro-1H-pyrido[3,2-b]azepine-7-carboxylate (57)

To a solution of methyl 3-\{[(2R,3S)-1-[(4-bromopyridin-2-yl)carbonyl]-2-(2-ethoxy-2-oxoethyl)piperidin-3-yl]amino\}-3-oxopropanoate (55, 80 mg, 0.2 mmol) in ethanol (1.0 mL), was added 0.5 M sodium ethoxide in ethanol (0.136 mL). The reaction mixture was stirred, at 68 °C, for 2 h. The solution was allowed to cool to room temperature, and then concentrated to give solid residue. The residue was then dissolved in a mixture of water and DCM. The organic layer was separated, dried over MgSO₄, filtered and evaporated, provided white powder. No product peak was observed in LC/MS. The residue was purified by ISCO using 10% CH₃OH in DCM to provide 57 (30 mg, 30%) as a white solid.

LCMS: (FA) [M + Na]⁺ 445; ¹H NMR: (300 MHz, DMSO) δ 8.88 (s, 1H), 8.53 (d, J = 5.2 Hz, 1H), 8.16 (d, J = 1.6 Hz, 1H), 7.89 (dd, J = 5.1, 1.7 Hz, 1H), 3.53 (s, 4H), 3.28 (d, J = 5.7 Hz, 3H), 3.08 (m, 2H), 2.82 – 2.75 (m, 1H), 1.61 – 1.44 (m, 4H).
Synthesis of ethyl 2-((2R,3S)-3-(3-(4-chlorophenyl)ureido)-1-(napthalen-1-ylcarbamoyl) piperidin-2-yl) (59).

A 50 mL round bottom flask was charged with ethyl 2-((2R,3S)-3-amino-1-(napthalen-1-ylcarbamoyl)piperidin-2-yl)acetate (47, 75 mg, 0.21 mmol), DMF (3 mL) and triethylamine (45 mL, 0.32 mmol). To this solution 4-chlorophenyl isocyanate 58 (32 mg, 0.21 mmol) was added and the reaction mixture was stirred at room temperature for overnight. LC/MS and TLC showed the reaction was complete. The reaction mixture was evaporated to remove DMF by co-evaporation with toluene. The residue was diluted with EtOAc (5 mL), and water (5 mL) and extracted. The organic phase was washed with brine, dried (Na$_2$SO$_4$) and evaporated. The solid was dissolved in DCM the crude product was purified by ISCO using 60% EtOAc in hexane to provide 59 (40 mg, 71%) as a white solid.

LCMS: FA [M + 1]$^+$ 509; $^1$H NMR: (400 MHz, CDCl$_3$) $\delta$ 8.47 (s, 1H), 7.70 (s, 2H), 7.53 (d, J = 7.7 Hz, 2H), 7.32 – 7.27 (m, 3H), 7.16 (s, 1H), 7.15 – 7.12 (m, 2H), 7.02 (d, J = 8.7 Hz, 1H), 6.98 – 6.93 (m, 2H), 6.90 – 6.83 (m, 2H), 6.72 (d, J = 8.8 Hz, 1H), 4.54 (d, J = 10.8 Hz, 1H), 4.14 (m, 2H), 4.10 – 4.01 (m, 2H), 2.93 (dd, J = 17.6, 11.2 Hz, 2H), 2.71 – 2.58 (m, 2H), 2.43 (d, J = 17.6 Hz, 1H), 1.72 – 1.64 (m, 4H), 1.20 (d, J = 3.6 Hz, 3H).
$^{13}$C NMR: (400 MHz, CDCl$_3$) $\delta$ 172.22, 155.89, 155.05, 136.99, 136.83, 133.20, 132.60, 127.59, 127.55, 127.38, 127.33, 124.82, 120.51, 119.99, 119.92, 119.82, 119.34, 118.61, 76.31, 76.20, 76.00, 75.68, 60.90, 53.82, 47.00, 36.54, 33.48, 23.69, 18.73, 13.18, 13.07.

(5aR,9aS)-3-(4-chlorophenyl)-N-(naphthalen-1-yl)-2,4-dioxooctahydro-1H-pyrido[3,2-d][1,3]diazepine-6(2H)-carboxamide (60)

To a solution of 59 (20 mg, 0.04 mmol) in methanol (1.0 mL), was added 0.5 M sodium ethoxide in ethanol (102 mL). The reaction mixture was stirred, at 68 °C for 2 h. The solution was allowed to cool to room temperature, acidified with 1 N HCl and then concentrated to give solid residue. The residue was then dissolved in a mixture of water and EtOAc. The organic layer was separated, dried over MgSO$_4$, filtered and evaporated. The crude product was purified by ISCO flash chromatography using 50% EtOAc in hexane to provide 60 (3 mg, 20% yield) as a white powder.

LCMS: $^{1}$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.08 (t, $J$ = 7.9 Hz, 1H), 7.94 – 7.82 (m, 2H), 7.60 (dt, $J$ = 16.2, 7.4 Hz, 2H), 7.53 – 7.48 (m, 2H), 7.39 (dd, $J$ = 16.6, 8.1 Hz, 2H), 7.07 (d,
J = 8.4 Hz, 1H), 6.87 (d, J = 26.9 Hz, 1H), 6.55 (s, 1H), 4.50 (d, J = 9.1 Hz, 1H), 4.32 (d, J = 13.5 Hz, 1H), 4.20 – 4.08 (m, 2H), 3.87 – 3.77 (m, 1H), 3.14 (dd, J = 17.0, 7.2 Hz, 1H), 3.05 – 2.93 (m, 2H), 2.66 (m, 2H), 1.79 (d, J = 13.5 Hz, 1H), 1.67 – 1.61 (m, 2H). HRMS calculated for C_{25}H_{24}N_4O_3Cl (M + H^+) 463.1537, found 463.1537. [α]_D^{25} = -10.9

**Ethyl2-((2R,3S)-1-(4-bromopicolinoyl)-3-(3-(4-chlorophenyl)ureido)piperidin-2-yl)acetate (61)**

A 50 mL round bottom flask was charged with 53 (40 mg, 0.08mmol), DMF (2 mL) and triethylamine (25 mL, 0.20 mmol). To this solution 4-chlorophenyl isocyanate 58 (17 mg, 0.11 mmol) was added and the reaction mixture was stirred at room temperature for overnight. LC/MS and TLC showed the reaction was complete. The reaction mixture was evaporated to remove DMF by co-evaporation with toluene. The residue was diluted with EtOAc (5 mL), and water (5 mL) and extracted. The organic phase was washed with brine, dried (Na_2SO_4) and evaporated. The solid was dissolved in DCM the crude product was purified by ISCO using 0-60% EtOAc in hexane to provide 61 (30 mg, 65%) as an oil.

LCMS: AA [M + 1]^+ 525; ^1H NMR: (400 MHz, CDCl_3) δ 7.93 (d, J = 5.3 Hz, 1H), 7.71 (d, J = 1.4 Hz, 1H), 7.32 – 7.28 (m, 2H), 7.23 (s, 1H), 7.17 (s, 1H), 7.12 (d, J = 4.5 Hz, 1H), 4.49 (d, J =
13.9 Hz, 1H), 4.36 – 4.30 (m, 1H), 4.05 (dt, J = 6.9, 1.6 Hz, 2H), 3.96 (tt, J = 7.1, 5.1 Hz, 2H), 3.78 (s, 1H), 2.89 – 2.84 (m, 2H), 2.40 (dd, J = 15.7, 5.2 Hz, 1H), 1.76 (d, J = 4.4 Hz, 2H), 1.14 – 1.11 (m, 3H).

$^{13}$C NMR: (101 MHz, CDCl$_3$) $\delta$ 183.91, 170.21, 168.42, 155.27, 148.76, 138.31, 134.62, 129.35, 128.47, 128.26, 127.87, 120.91, 120.66, 61.21, 55.38, 48.33, 37.42, 34.62, 29.97, 28.39, 24.16, 21.35, 20.30, 14.29. HRMS calculated for C$_{22}$H$_{25}$N$_4$O$_4$Cl Br (M + H$^+$) 523.0743, found 523.0748; $[\alpha]_{D}^{25} = -10.3$

1.4. RESULTS AND DISCUSSIONS

A molecular docking analysis was performed to define the structural features of the different binding interactions of the new derivatives. The analysis was developed by using the published crystal structure of the P-selectin receptor in complex with the compound 3 (Fig. 1.2) as template. The analysis was aimed at rationalizing the effect of the different substituents at 8-position of the pyrazoloquinolinone on the P-selectin binding. Based on the molecular modeling analysis we proposed to synthesis our target pyrazoloquinolinone molecules. To study a wide variety of pyrazoloquinolinone derivatives, we focused to explore strategies using cross-coupling methodologies that would allow us many degrees of flexibility in introducing a large diversity of functionalities for medicinally interesting drug-like pyrazoloquinolinone derivatives. Compound 13 is the key intermediate in our synthesis. A diverse set of pyrazoloquinolinone derivatives could be readily elaborated through various palladium-catalyzed cross-coupling reactions at the C-8 position as shown in (Scheme 1.4).
Method A: (Sonogashira): (PPh$_3$)$_2$PdCl$_2$, CuI, iPr$_2$NEt, 1-chloro-3-ethynylbenzene, MeCN, 80 °C, 2 h. Method B. (Suzuki): Pd(dppf)Cl$_2$, K$_2$CO$_3$, benzo[b]thiophen-2-ylboronic acid, DMF/H$_2$O, MW 110 °C, 30 min. Method C. (Carboalkoxylation): CO, Pd(OAc)$_2$, dppf, TEA, EtOH, DMF, 75 °C. Method D. (Buchwald): Pd(OAc)$_2$, XanPhos, Cs$_2$CO$_3$, 4-chloroaniline, dioxane, MW 100 °C, 30 min

**Scheme 1.4** Diverse analogues using various palladium-catalyzed reactions

Introduction of a bulky, rigid group at 8-position of quinoline ring established our first strategy of hydrophobic interaction with the H$_1$ hydrophobic pocket of P-selectin receptor (Figure 1.6). We were interested in utilizing the iodo handle of pyrazoloquinolinones core to make a new C-C bond via Suzuki reaction,$^{113,114}$ carboalkoxylation$^{115}$ C-N bond by Buchwald-Hartwig$^{116}$ and arylacetylenes by the Sonogashira reaction$^{117}$. The preparation of 8-(benzofuran-2-yl)-5-benzyl-2-phenyl-2H-pyrazolo[4,3-c]quinolin-3(5H)-one 17 was accomplished by coupling of iodo analogues.
As the key step in our synthesis, the synthesis of iodo pyrazoloquinolinones 13 were efficiently prepared by microwave mediated chemistry in short period of time. All of the reactions were monitored by thin layer chromatography, LC/MS and the products were purified by column chromatography.

Scheme 1.5 Microwave Assisted Cyclization of Dihydroquinoline

Nucleophilic reaction of 4-idoaniline and diethylethoxymethylene malonate irradiated under microwave at 120 °C for 20 min in toluene provided enamines 7. 1,4-Dihydro quinoline 8 was obtained thermally by Gould-Jacobs cyclization according to a literature procedure (Scheme 1.5). 118 During heating for longer period of time in diphenyl ether at high temperature (210 °C) the unwanted side product was obtained resulting in low yield of the final product (35%). In our synthesis, microwave assisted cyclization at 210 °C in the same solvent for 30 min provided cleaner and better yield of desired product (35-64%).

Chlorination of ethyl 6-iodo-4-oxo-1,4-dihydroquinoline-3-carboxylate was achived by heating with phosphoryl trichloride at 100 °C for 2h. 119 The desired compound 9 was collected by filtration followed by drying in a vacuum oven. LC/MS analysis showed the presence of the
starting quinolinone precursor. The chloro derivative formed the starting ethyl 6-iodo-4-oxo-1,4-dihydroquinoline-3-carboxylate 9 was not stable due to the hydrolysis of the carbon-chlorine

**Scheme 1.6** Chlorination of Dihydroquinoline

bond which gave starting material. Chlorination of ethyl 6-iodo-4-oxo-1,4-dihydroquinoline-3-carboxylate 8 was carried out in SOCl\(_2\) at reflux temperature for 2h. The reaction provided 100% conversion to chlorinated product (Scheme 1.6).

**Scheme 1.7** Microwave assisted Nucleophilic Sustitution Followed by Cyclization
Pyrazoloquinolinones were formed by nucleophilic substitution of the chlorine of 9 by the primary amino group of aromatic hydrazine derivative 10 followed by cyclization. Cyclizations were previously carried out with conventional heating (130-140 °C) for longer periods of time 2h in DMF, or xylene. In our synthesis, we applied a microwave accelerated protocol to reduce the reaction time and better yield. Pyrazoloquinolinones were synthesized under microwave irradiation at 130 °C for 45 min in DMF (Scheme 1.7).

![Scheme 1.8 N-alkylation of pyrazoloquinolinone](image)

Treatment of substituted pyrazoloquinolinone 11 with NaH followed by benzyl bromide 12 provided benzylated compound. N-alkylation yield was improved by using a milder condition where cesium carbonate was used as a base at room temperature (Scheme 1.8). The iodo intermediate 13 was elaborated through various palladium catalyzed coupling reactions at the C8 position.
In the first generation diversification step, commercially available boronic acids were used for Suzuki coupling. The carbon-iodine bond at the C-8 position 8-iodo-2H-pyrazolo[4,3-c]quinolin-3(5H)-one 13 generally survived the use of high temperatures and basic conditions. The Suzuki-Miyaura coupling of 13 with various arylboronic acids proceeded smoothly to give the desired products. The commercially available boronic acids were chosen because they contain heterocycles and polar functionality that would incorporate drug-like moieties in the resulting coupled products. Sulfur and oxygen fused heterocycle containing arylboronic acids were chosen because the resulting organothiophene and organofuran Suzuki-Miyaura coupling products should be of considerable interest for various applications in industry and medicine. Subsequent arylation in the quinoline 8-position proceeded in good yield with arylboronic acid using Pd Cl₂(dppf) catalyst and potassium carbonate under microwave at 110 °C for 30 min in DMF/H₂O (Scheme 1.9). This approach provided 15 and 17. The cross-coupling methodologies allow us many degrees of flexibility in introducing a large diversity of functionalities for medicinally interesting drug-like benzo-[b]thiophene and benzo[b]furans derivatives.
Scheme 1.9 Microwave Assisted Suzuki Reaction

The terminal alkyne 18 was commercially available halogen substituted aryl acetylene. Under our reaction conditions, microwave irradiation has been shown not only to reduce the reaction times, but often provides higher yields of the desired alkyne products 19 when compared to conventional heating methods.¹¹⁷

Scheme 1.10 Microwave Assisted Sonogashira cross-coupling approach
Alkyne substituted pyrazolo[4,3-c]quinolin-3(5H)-one 19 was prepared by the palladium-copper catalyzed reaction of iodopyrazoloquinolinones 13 with 1-chloro-3-ethynylbenzene. The alkyne, 1-chloro-3-ethynylbenzene 18, was chosen as a spacer to further expand the distance between the aryl alkyne C8 carbon of pyrazoloquinoline portion of the molecule. The aryl-alkyne coupling reaction is a highly effective method for introducing an alkynyl moiety into an organic molecule. Pd-catalyzed Sonogashira coupling reactions were performed under microwave irradiation (Scheme 1.10).\textsuperscript{117}

The pharmaceutical drugs such as raloxifene,\textsuperscript{121} and zileuton,\textsuperscript{122} have benzothiophene substructure. Because of the promising pharmacological properties, a benzothiophene-containing molecule was identified as a potential lead molecule for an anti-cancer target. The requisite iodo precursor and an highly substituted alkyne amine such as benzothiaphene amine 22 can be used for amination of Buchwald coupling.\textsuperscript{116} Keeping this in mind, we synthesized substituted benzothiaphene amine 22 by the palladium/copper catalyzed Sonogashira cross-coupling\textsuperscript{117} of the 3-bromobenzo[b]thiophene 20 with terminal alkynes 21 at room temperature with 80% yield (Scheme 1.11).

\begin{center}
\includegraphics[width=0.8\textwidth]{scheme11.png}
\end{center}

\textbf{Scheme 1.11} Formation of substituted benzothiaphene amine
The ester functionality has proved to be an important moiety in pharmacologically important derivatives and can be obtained by palladium-catalyzed reactions. Carboalkoxylation of the 8-iodo-2H-pyrazolo[4,3-c]quinolin-3(5H)-one 13 using one atmosphere of carbon monoxide and ethanol in the presence of catalytic amounts of Pd(OAc)$_2$ and dppf ligand did not provide the ester-containing 2H-pyrazolo[4,3-c]quinolin-3(5H)-one. Unfortunately, compound 23 did not form (Scheme 1.12). The reason may be because bidentate phosphine ligands and 10-bis(diphenylphosphino)ferrocene (dppf, L1), were used instead of bis(diphenylphosphino)methane (dppm, L2), or 1,4bis(diphenylphosphino)butane (dppb, L3), which can stabilize the anticipated cationic palladium(II) intermediate.

![Scheme 1.12 Carboalkoxylation of the 8-iodo-2H-pyrazolo[4,3-c]quinolin-3(5H)-one](image)

Because of this is disappointing result, we decided to investigate palladium-catalyzed C-N bond-forming reactions, which have evolved into a highly versatile and synthetically attractive technique for targeting pharmaceutically useful intermediates. Additional palladium-catalyzed cross-coupling reactions such as the Buchwald reaction at C-8 of derivative 13 was performed. Standard Buchwald amination conditions Pd(OAc)$_2$, XantPhos as the ligand, Cs$_2$CO$_3$, dioxane on
13 did not affect the desired transformation. However by switching to NaO\textsuperscript{t}Bu as the base, the use of Pd(OAc)\textsubscript{2}, XantPhos, in toluene under microwave condition irradiated at 100 °C for 30 min provided messy reaction product. Generally, the rates for amination of aryl iodide are faster than those for amination of aryl bromide and chlorides. In our case, the result was opposite, possibly due to iodide reacting with Pd(II) intermediate and forming an ate complex.\textsuperscript{125}

![Scheme 1.13 Buchwald Amination Approach](image)

Thiophene is the simplest aromatic compound containing a sulfur atom, and it shares some similar chemical properties with benzene due to the pi electron cloud.\textsuperscript{123} The lone electron pairs on sulfur in the delocalized pi electron system do not exhibit the properties of thioethers but instead, aromaticity. We decided to couple thiophene boronic acid with ester functionality at the
C-8 position of the pyrazolo[4,3-c]quinolin-3(5H)-one 13 by the Suzuki coupling method (Scheme 1.14). The starting compound, [5-(methoxycarbonyl)-2-thienyl]boronic acid 28, was prepared as described previously. Subsequent arylation in the quinoline 8-position proceeded in good yield with arylboronic acid 28 using PdCl$_2$(dppf) catalyst and potassium carbonate under microwave at 110 °C for 30 min in DMF/H$_2$O to provide 29 (Scheme 1.14).

![Scheme 1.14 Microwave Assisted Arylation](image)

Compound 30 was prepared as previously described. The lipophilic aryl ether spacer at C8 carbon of pyrazoloquinoline portion of the molecule can be introduced by Suzuki coupling. Chan Lam coupling allows aryl carbon-heteroatom bond formation via an oxidative coupling of arylboronic acids, with N-H or O-H containing compounds in air. The reaction of phenylboronic acid with 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenol in presence of a stoichiometric amount of copper(II) and a base provided 57% of 4,4,5,5-tetramethyl-2-(3-phenoxyphenyl)-1,3,2-dioxaborolane. Suzuki coupling of 13 with arylboronic acids 30 produced the aryl analogues 31 as shown in Scheme 1.15.
Scheme 1.15 Microwave Assisted Suzuki Approach

Part of our current interest was directed toward conformationally restricted structures containing an ester. We thus focused our efforts on a series of rigid structures. To explore the first strategy on the basis of molecular modeling we synthesized intermediate 36, a conformationally restricted basic amino acid with absolute configuration to prepare our target compounds. An intramolecular cyclization route was applied to the stereospecific synthesis of amino-piperidine analogues 36 (Scheme 1.3). The procedure allows the introduction of two asymmetric centers from optically pure starting materials with no racemization. The compound 36, ethyl (2R, 3S)-3-Boc-amino-2-piperidineacetate was synthesized from Boc-L-Orn(Z)-OH as described by Garcia-Lopez et. al.114
Conversion of trans-piperidine 36 to the acylated piperidine methyl ester 38 was accomplished by coupling with the substituted acyl chloride 37. The N-acylated product was obtained by the reaction of methyl 4-chloro-4-oxobutanoate with compound 36 in the presence of dimethylaminopyridine and diisopropylethylamine\textsuperscript{142} to provide 80\% of trans (2R, 3S) product 38 (Scheme 1.16).

**Scheme 1.16** Formation of Acylated Piperidine Methyl Ester

**Scheme 1.17** Intramolecular Cyclization of Piperidine Methyl Ester
Substituted piperidine 38 was cyclized under base catalyzed cyclization with sodium ethoxide provided \((1S,10aR)-\text{methyl} 1-(\text{tert-butoxycarbonylamino})-6,9\text{-dioxodecahydropyrido [1,2-a]}\text{azepine-8-carboxylate 39 in 69\% yield (Scheme 1.17).}

**Scheme 1.18** Formation of Azepine Derivatives by Reductive Amination

Introduction of a bulky, rigid, aromatic side chains at C1-amino position of azepine ring could adopt more appropriate orientations to interact with the hydrophobic pocket of the receptor. The synthesis of \((1S,10aS)-\text{methyl} 1-(\text{benzo[b]}\text{thiophen-2-ylmethylamino})-6,9-
dioxodecahydropyrido[1,2-a]azepine-8-carboxylate 42 was accomplished by the coupling of amino analogues of azepine 6,9-dione with benzothiophene-2-carboxaldehyde 41. This cross-coupling strategy provides rapid generation of diverse compounds. We were interested in expanding the amino handle of azepine 6,9-dione core by making a C-N bond via reductive amination\textsuperscript{116} with different aldehydes. Deprotection of Boc group by 4 M HCl in dioxane\textsuperscript{117} at room temperature for 2 h provide the white solid of 40. The synthesis of (1S,10aS)-methyl 1-(benzo[b]thiophen-2-ylmethylamino)-6,9-dioxodecahydropyrido[1,2-a]azepine-8-carboxylate 42 was accomplished by the coupling of amino analogues of azepine 6,9-dione with benzothiophene-2-carboxaldehyde (Scheme 1.18). This cross-coupling strategy provides rapid generation of diverse compounds.

Introduction of polar groups such as urea could lead to good inhibitor of P-selectin\textsuperscript{119,120}. The synthesis of non desired compound 44 from the starting diamine was accomplished by reaction with naphthyl isocyanate 43 (Scheme 1.19). During coupling double acylation occurred, to provide mixture of mono and di-acylated products as a 4:1 ratio.

\textbf{Scheme 1.19} Formation of Urea Derivatives
With the identification of different fused cores via cyclization we turned our attention to second strategy. Our hypothesis is that introduction of urea functionality at N-1 position to increase strong hydrogen bond interactions with Lys111 and Arg85 (Figure 1.13). The following route involved the reaction of the 2,3-trans-3-amino-2-piperidineacetic acid derivative 36 with the corresponding isocyanate 45 resulting in the N-1 substituted urea piperidines 46. Then N-Boc deprotection and alkylation with methyl 3-chloro-3-oxopropanoate, using dimethylamino pyridine (DMAP) provide the mono and di-alkylated product. The mixture was purified and isolated by flash chromatography. Base catalyzed cyclization using 5.0 M sodium ethoxide in

**Scheme 1.20** Synthetic Route of Azepine Urea Analogues
Figure 1.22 Overlay pose of fused diazepine (green) with quinolinic acid (red).

ethanol gave the resulting N-substituted urea pyrido[3,2-b]azepine-derivative 50 (Scheme 1.20).

Elaboration of a second strategy, introduction of carboxamide functionality in the same ring system allowed hydrogen bond interactions with the D₁ pocket of the P-selectin receptor. Carboxamides are neutral, stable, and have both hydrogen bond donating and accepting
properties necessary for biological molecule recognition. The intermediate 36 is an excellent key intermediate for the simple chemical modification of the amino piperidine skeleton to produce a carboxamide moiety at N-1 position.

Scheme 1.21  Synthetic Route of Azepine Amide Analogues

The synthesis of the target carboxamide 57 was efficiently performed from the key intermediate amino piperidine 36 via the EDC coupling method. Coupling of this secondary amine with 4-bromo picolinic acid 51 using EDC as a coupling reagent in the presence of DMAP gave carboxamide intermediate 52. Removal of the Boc group was performed using 4 M HCl in 1,4-dioxane, and subsequent acylation followed by HPLC purification provided β-keto ester 55. The seven membered constrained carboxamide analogue 57 was obtained via base catalyzed cyclization (Scheme 1.21).
Our third strategy focused on the modification of fused core to get better interaction by introducing substitution at N-3 position. Substitution at N-3 position allowed possible interactions with the $S_1$ pocket. The construction of the pyrido[3,2-d][1,3]diazepine-2,4(3H,5H)-dione skeleton was performed using a similar synthetic pathway as that depicted in Scheme 1.22. The reaction of ethyl 2-(2R,3S)-3-amino-1-(naphthalen-1ylcarbamoyl piperidin-2-yl)acetate 47 with 1-chloro-4-isocyanatobenzene 58 gave the corresponding N-3 substituted carbamoylpiperidine derivative. Base-catalyzed cyclization of compound 59 provided 60 (Scheme 1.22).

![Scheme 1.22](image_url)  
**Scheme 1.22** Synthetic Route of Diazepine Urea Analogue.

### 1.5. CONCLUSIONS

Small molecule ligands as P-selectin inhibitor provide a novel and effective approach to attenuate cancer growth and metastasis. Our research has focused on design, synthesis and identification of potent small molecules to explore the role of the spatial orientation of the molecules in the bioactive conformation. This thesis describes research directed towards the development of compounds with anti-metastatic properties that block sialic acid-mediated cell
adhesion. The main focus was to synthesize various compounds on the basis of structure-based model, study the interactions and generation of SAR information. Sugar based sLe\(^\alpha\) (I) is the physiological ligands of the selectins which served as the lead structure in the search for selectin antagonists.

Different non-carbohydrate small and constrained compounds were synthesized as a inhibitors of P-selectin. First, we synthesized pyrazolo[4,3-c]8-substituted quinolin-3-one tricycle, which is a modification of quinolin-skeleton that has different functionality at 8-position of the pyrazoloquinoline ring. To support our hypothesis, several compounds with different functionality at 8 position of pyrazoloquinoline ring were synthesised. The effects of varying the substituents at the C-8 position of the pyrazoloquinolinone ring were studied. Synthetic protocols for the efficient introduction of C-8 substituents were developed and used to prepare a diverse set of C-8 substituted pyrazoloquinolinone analogues.

The crystal structure of P-selectin lectin/EGF domains (1G1Q) was used for docking studies to get binding conformations and their alignment to the active site of the receptor (Figure 1.2 & 1.3). The ligand-based modeling with P-selectin 1G1Q study may be helpful in development and optimization of new class of compounds with anti-cancer activity. The mechanism of interactions of compound 3,13 and 60 with the receptor showed in the Figure 1.23
In summary, the research described the use of a published X-ray structure to identify potential compounds via docking. Through the comparison our first strategy to fill hydrophobic pocket, the second strategy to get strong hydrogen bond interactions and the third strategy interactions with the water exposed regions of the receptor were described in Table 1.
<table>
<thead>
<tr>
<th>#</th>
<th>Structure</th>
<th>Yield (%)</th>
<th>Modeling results</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td><img src="image" alt="Structure" /></td>
<td>51</td>
<td>Arg85 able to interact through hydrogen bonding with the 3-carbonyl oxygen. Substitution of the ligand with a benzyl group at N5 position diminishes the formation of hydrogen bond with Lys111. C9, C8, N1 are surrounded by hydrophobic pocket. Our hypothesis on the basis of comparison of compound I with a series of pyrazoloquinolinone is that the pharmalogical profiles of these series may be correlated to the nature of the substituents at C8 position of the A ring.</td>
</tr>
<tr>
<td>15</td>
<td><img src="image" alt="Structure" /></td>
<td>50</td>
<td>Bulky groups thought to interact with the amino acids residues in the hydrophobic pocket of the receptor.</td>
</tr>
<tr>
<td>17</td>
<td><img src="image" alt="Structure" /></td>
<td>55</td>
<td>Same trend observed in the 3D superimposition of benzofuran like benzothiaphene.</td>
</tr>
<tr>
<td>#</td>
<td>Structure</td>
<td>Yield (%)</td>
<td>Modeling results</td>
</tr>
<tr>
<td>----</td>
<td>-----------</td>
<td>-----------</td>
<td>------------------</td>
</tr>
<tr>
<td>19</td>
<td><img src="image" alt="Structure 19" /></td>
<td>50</td>
<td>3D-Superimposition comparison of 19 and 3, demonstrated that with the additional phenyl ring, alkyne as a spacer in 19 and pyrazoloquinolone 4 aligned quite well.</td>
</tr>
<tr>
<td>23</td>
<td><img src="image" alt="Structure 23" /></td>
<td>NP</td>
<td>3D-alignment showed the small substituent on the 8-position of ring A was not favored for interactions with the hydrophobic pocket</td>
</tr>
<tr>
<td>25</td>
<td><img src="image" alt="Structure 25" /></td>
<td>NP</td>
<td>Introduction of the heteroatom ‘N’ into the substituent of the additional aryl ring and ring A, to give compounds 25. This modification showed better interactions of the heteroatom and the ortho substituted aryl with Glu80, Pro81, Asn 84</td>
</tr>
<tr>
<td>29</td>
<td><img src="image" alt="Structure 29" /></td>
<td>42</td>
<td>3D-superimposition of 29 and 3, showed that the thiaphene ester 29 align well as phenyl alkyne 19</td>
</tr>
<tr>
<td>#</td>
<td>Structure</td>
<td>Yield (%)</td>
<td>Modeling results</td>
</tr>
<tr>
<td>----</td>
<td>-----------</td>
<td>-----------</td>
<td>------------------</td>
</tr>
<tr>
<td>31</td>
<td><img src="image1.png" alt="Structure 31" /></td>
<td>40</td>
<td>The flexibility of this 8-position on pyrazoloquinolinone ring limited to the size of the substitution. For example, compound 31 extends the phenyl ring away from the quinoline ring A. 3D-Superimposition comparison of 31 and 3, demonstrated that with the additional phenyl ring ether does not show good alignment.</td>
</tr>
<tr>
<td>42</td>
<td><img src="image2.png" alt="Structure 42" /></td>
<td>36</td>
<td>The orientation of the lone pair of electrons of the carbonyl oxygen of the ester function is not interacted with D1 pocket. But the ring carbonyl oxygen may interact with D1 site (Figure 1.12).</td>
</tr>
<tr>
<td>44</td>
<td><img src="image3.png" alt="Structure 44" /></td>
<td>70</td>
<td>Same trend shown in the 3D overlay of compound 42</td>
</tr>
</tbody>
</table>
It has been recognized that small structural modifications in the same chemical family could lead to ligands which display different interactions with the receptor.

Introduction of carboxamide functionality in the same ring system allowed hydrogen bond interactions with the D$_1$ pocket of the P-selectin receptor like compound 60.

Alignment of 60 overlaid with template is shown in Fig. (13), wherein the carbonyl oxygen of the seven membered ring and the carbonyl oxygen of the urea bond reinforced the receptor binding by means of a 3-centered hydrogen bond with arginine85. There is a potential interaction between carbonyl oxygen of substituted urea with the lysine residue of D$_1$ pocket by hydrogen bond formation. The fused six membered ring may interact with hydrophobic region and 4-chlorophenyl exposed in the solvent site.

Table 1.3 Unified modeling data of compounds
1.6. REFERENCES


42. Erbe, D. V.; Wolitzky, B. A.; Presta, L. G.; Norton, C. R.; Ramos, R. J.; Burns, D. K.; Rumberger, J. M.; Rao, B. N.; Foxall, C.; Brandley, B. K.; et al., Identification of an E-


84. http://www.pdb.org/pdb/home/home


Chapter 2:
Microwave Assisted Radio-Fluorodenitration and Pharmacological Studies
of (4-[^18F]-Fluorophenyl)-triphenylphosphonium Nitrate (^{18F}-PTPP)
as a Cardiac Positron Emission Tomography Tracer
CHAPTER 2

2.1. GENERAL INTRODUCTION

The unique chemical characteristics of fluorine make it useful in both the chemical and pharmaceutical industries. High electronegativity (3.98), small size (0.5 Å), and high-energy bonding with carbon (481 kJ/mol in CH$_3$-F), make fluoro-organic derivatives important. Presence of fluorine in an organic compound, affects the absorption, distribution, metabolism and excretion (ADME) properties. Compounds containing fluorine have many applications in the drug industry and find utility as anesthetics, anti-depression, anti-cancer and anti-inflammatory agents.$^{1-3}$ An isostere of hydrogen is fluorine (van der Waals radius 1.2 Å for hydrogen and 1.35 Å for fluorine respectively). Introduction of fluorine in a drug molecule can change its pharmacokinetics, pharmacodynamics and toxicology properties. It also may improve the bioavailability and lipophilicity properties of a drug molecule.$^{1,2}$ Carbon-fluorine bonds possess an energy of 130 kcal/mol, and this makes fluorine containing molecules more stable \textit{in vitro} and \textit{in vivo}.$^{4-6}$

2.1.1 Positron Emission Tomography

PET is a noninvasive diagnosis technique for imaging biochemical and physiological processes in living organisms. PET has become a powerful clinical and scientific imaging modality in the fields of oncology, cardiology, and neurology to assess metabolism and to evaluate cancer, damaged heart tissue, and brain disorders. It is a nuclear medicine scanning procedure that uses short-lived positron-emitting radioactive isotopes to image the body’s metabolic activity, to the evaluation and treatment of a variety of diseases.$^{1-3}$ This leads to
advances in instrumentation and synthetic chemistry. It has proven to be powerful imaging tool because of its broad scope and sensitivity. Furthermore, it is a non-invasive, quantitative, nuclear medicine imaging technique that is used to determine the quantitative distribution of a tracer in vivo. The distribution of a specific tracer molecule can be monitored if the molecule contains a radio-labeled positron emitting nuclide.

<table>
<thead>
<tr>
<th>PET Nuclide</th>
<th>Nuclear Reaction</th>
<th>Half-life (min)</th>
<th>Max. Energy (MeV)</th>
<th>Max. Range (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-Oxygen</td>
<td>$^{14}\text{N}(d, n)^{15}\text{O}$</td>
<td>2.05</td>
<td>1.70</td>
<td>8.0</td>
</tr>
<tr>
<td>13-Nitrogen</td>
<td>$^{16}\text{O}(p, \alpha)^{13}\text{N}$</td>
<td>9.98</td>
<td>1.19</td>
<td>5.4</td>
</tr>
<tr>
<td>11-Carbon</td>
<td>$^{14}\text{N}(p, \alpha)^{11}\text{C}$</td>
<td>20.4</td>
<td>0.96</td>
<td>4.1</td>
</tr>
<tr>
<td>18-Fluorine</td>
<td>$^{18}\text{O}(p, n)^{18}\text{F}$</td>
<td>109.8</td>
<td>0.69</td>
<td>2.4</td>
</tr>
</tbody>
</table>

$^{18}\text{O}$ Ne(d, $\alpha$)$^{18}\text{F}$

**Table 2.1** Physical properties of $^{18}\text{fluorine}$, $^{11}\text{carbon}$, $^{13}\text{nitrogen}$ and $^{15}\text{oxygen}$
PET radioisotopes can be prepared by the use of a particle accelerator, cyclotron, or nuclear reactor. Commonly used positron imaging radioisotopes are 18-fluorine ($t_{1/2} = 109.7$ min), 11-carbon ($t_{1/2} = 20.4$ min), 13-nitrogen ($t_{1/2} = 9.96$ min), and 15-oxygen ($t_{1/2} = 2.1$ min) (Table 2.1 for physical properties) prepared by a number of nuclear reactions.\(^7\)\(^-\)\(^9\) Introduction of 11-carbon, 13-nitrogen, 15-oxygen in a bio-molecule do not noticeably change the properties of the radio-labeled molecule. 18-fluorine containing bio-molecules have unique chemical characteristics such as

- moderate half-life of 109.8 min permits longer synthetic sequences and increased time for purification and radio-analysis.
- due to the presence of low energy $\beta$-particles (max. 0.635 MeV) it allows highest resolution images with PET
- introduction of 18F into biologically active molecules can be easily obtained by electrophilic and nucleophilic reactions.
- its small size (0.5 Å), high electronegativity (3.98), and high-energy C-F bond (481 kJ/mol in CH$_3$-F), can have improved pharmacological properties.\(^8\)
- fluorine also increases lipophilicity of a molecule and changes the rates of transport and absorption \textit{in vivo}.\(^9\)

Therefore, introduction of fluorine into drug molecules may have effects on pharmacological properties. 18-fluorine has become versatile positron-emitting radioisotope for labelling biomolecules.
18-fluorine was reported as early as 1936\textsuperscript{10}, but was not utilized as an imaging agent until after the development of modern scanning technique of images. Scanners measure the radiation from the body and reconstruct tomographic information through the camera. The slow advancement of PET until the development of modalities such as X-ray computed tomography (CT) and magnetic resonance imaging (MRI).

PET is the leading non-invasive, quantitative nuclear imaging modality that takes advantage of positron decay to offer \textit{in vivo} imaging. The nuclide decays from a proton into a neutron and releases energy, which results in positron emission. The energy is different for each isotope and is listed in Table 2.1. The emitted positron from the radioactive atom is attracted by an electron and collides. The positron electron pair undergoes annihilation and emits two gamma photons. Each of these photons has energy of 511 keV and is emitted at 180° to each other.

The basis of PET depends on the production of gamma rays. Gamma rays are energetic, high penetration rays. These high energy photons travel through the body and are detected by dense scintillating crystals coupled to photomultiplier tubes. The acquisition of a PET image depends on the simultaneous detection of these two gamma particles.\textsuperscript{12}

\textsuperscript{18}F labeled glucose analogues have been synthesized and their application played an important role for the advancement of PET.\textsuperscript{8} The two most widely used \textsuperscript{18}F-labelled PET radiopharmaceuticals are \textsuperscript{[\textsuperscript{18}F]} 2-fluoro-2-deoxy-D-glucose (\textsuperscript{18}F-FDG) and \textsuperscript{18}F-6-fluoro-L-DOPA \textsuperscript{18}F-FDG in medical field (Figure 2.2).\textsuperscript{13,14}
The use of a cyclotron is needed for the synthesis of all radiotracers in PET. PET has an advantage over other molecular imaging modalities because all common positron emitting isotopes are oxygen, carbon, and nitrogen, which are common building blocks in organic chemistry. These isotopes allow for the synthesis of almost any organic compound as a potential PET tracer. The common reaction for the production of $^{18}$F is the single irradiation of $^{18}$O-H$_2$O using proton energy 10-17 MeV loaded in a column. Aqueous fluoride is unreactive, so $^{18}$F extracted from the column by creating an azeotrope solution with an organic solvent such as acetonitrile resulting into anhydrous and reactive $^{18}$F.$^{15}$ A counter cation potassium used in the form of K$_2$CO$_3$. The potassium form complexes with a cryptand such as Kryptofix 222 to increase the nucleophilicity of the fluoride ion. The radiochemical reaction carried out in polar aprotic solvents such as DMSO or CH$_3$CN.$^{16,17}$

Due to the short half-life of PET radionuclide, there are challenges for synthetic chemist to develop synthetic routes and to obtain PET radiopharmaceuticals with high specific activity,
reasonable radiochemical yield (RCY) and high radiochemical purity (RCP) in a short period of time.

2.1.2 Common Synthetic Route for Fluorine-18 in PET

Fluorine substituted aromatic rings are common in bio-organic molecules. \(^{18}\)F chemistry has been widely developed and offers a wide range of methods and applications. \(^{18}\) Balz-Schiemann or Wallach reaction have been used to introduce \(^{18}\)F in a targeted position of a molecule by the displacement of an aryl amino group. \(^{19}\) In the Balz-Schiemann reaction, \(^{18}\)F is introduced via thermal decomposition of an aryl diazonium salt resulting into a low RCY and low specific activity due to exchange with \(^{19}\)F-fluoride (Figure 2.1A). \(^{19}\) The Wallach reaction has same disadvantages where \(^{18}\)F is introduced by the thermal decomposition of a triazene precursor (Figure 2.1B). \(^{19}\) Nucleophilic fluorination has become a widely used method in \(^{18}\)F chemistry due to its ease of preparation and use. Introduction of \(^{18}\)F onto an aryl position is nucleophilic aromatic substitution, where leaving group is displaced by a fluoride ion. \(^{19}\) The aromatic ring is activated by an electron withdrawing group usually ortho or para to a good leaving group. Common leaving groups are halogens, nitro groups, and trimethylammonium salts (Figure 2.1C). \(^{18,19}\)

Because of low specific activities and formation of unwanted \(^{18}\)F labeled side products made electrophilic \(^{18}\)F-fluorination is less favoured to introduce \(^{18}\)F in a molecule. \(^{14}\) The \([^{18}\text{F}]\text{F}_2\) is a common and reactive electrophile used as a fluorinating agent in electrophilic fluorination (Figure 2.1D). \(^{14}\) Fluoride ion is a nucleophile in organic solvents, and formation of side products resulting from water as a HO\(^-\), RO\(^-\) from ROH does not occur. Fluorine and nitro groups are
labile neutral groups, nucleophilic substitution requires labile groups.\textsuperscript{20} This observation prompted us to design the synthesis of defluoronitrilation.

**Scheme 2.1.** A: Balz-Schiemann reaction; B: Wallach reaction; C: Nucleophilic fluorination; D: Electrophilic fluorination with \([^{18}\text{F}]\text{F}_2\)
2.1.3 Atherosclerosis

Coronary artery disease (CAD) is the most common type of cardiovascular disease. It's the leading cause of death in the United States for both men and women. CAD is a condition in which plaque builds up inside the coronary arteries. These arteries supply blood to heart muscle. When plaque builds up in the arteries, the condition is called atherosclerosis. CAD is the result of reduced blood supply to the heart muscle (myocardium) and can be diagnosed with myocardium perfusion imaging (MPI). MPI is a well-established technique for the diagnosis and prognosis of CAD after tracer uptake by the heart. Because of the reduction of blood flow in multiple large and small coronary arteries, MPI is not sensitive enough to detect uniform changes in blood flow. Quantitative detection of MBF can be done most accurately and non-invasively at the early stages of the disease using positron emission tomography (PET). In PET, a radioactive molecule, called a tracer, is injected to the patient and is imaged using a special camera. The images are then analyzed to quantify myocardium blood flow (MBF). The muscle tissue in the heart pumps blood and circulate blood through the entire body via a circulatory system. The unit of MBF is the rate at which blood perfuses the myocardial tissue, which is measured in units of mL/min of blood/g of tissue. Therefore, measurement of MBF with PET is a crucial diagnostic, and it can be developed into a prognostic tool for early detection of disease and treatment.

Early diagnosis and treatment are key to reducing mortality rate. Nuclear cardiology uses an injection of a small dose of a radioactive tracer, or radiopharmaceutical, to image the heart. Conventional nuclear medicine uses single photon emitting radionuclides. PET imaging is a powerful, quantitative noninvasive imaging modality that has been used to assess cardiovascular biology and physiology. Recently, PET imaging has became a popular technique to evaluate
myocardial blood flow and resolution can be improved by the use of positron-emitting radionuclides.\textsuperscript{28} Positron emission tomography of the heart allows the study and quantification of various aspects of heart tissue function. Cardiac positron emission tomography is a noninvasive, imaging procedure and is used to assess perfusion and metabolic activity in the heart with high spatial resolution.

While PET is an excellent modality for measuring MBF, the presence of fluorine in a molecule can change a dramatic effect on biological activity and can lead to increases in fat solubility and bioavailability.\textsuperscript{29} The introduction of $^{18}$F into a bioactive molecule for application in PET is important. Fluorine increases lipophilicity of molecules, enhances its absorption into biological membranes and half-life of $^{18}$F is 110 min. Therefore synthesis of a radioligand has to be quick with a good chemical and radiochemical yield.

2.1.4 Microwave Technology

The application of microwave technology has been known in chemistry since the late 1970’s. It’s application for synthesis is becoming an important tool in pharmaceutical and academic research.\textsuperscript{30,31} The first application of microwave radiation in organic synthesis was as a non-conventional energy source in 1986. Relatively slow uptake of this technology into the chemistry laboratory has been due to its initial lack of controllability and reproducibility. In 1990’s the use of microwave irradiation in organic synthesis has been popular in scientific community and the number of publications increased significantly.\textsuperscript{32-35}

Microwave Theory

There are two mechanisms associated with microwave irradiation based on temperature increase by dielectric heating: ionic conduction and dipole rotation.\textsuperscript{4} When a molecule is
irradiated with microwaves, that possess a dipole moment. The frequency of microwave radiation region is low so that the dipoles and ions have time to align itself with the applied electric field by rotation.\textsuperscript{4} The frequency of microwave radiation region is not low enough to follow the field, so the dipole-ion field is forced to realign itself. In this process energy is lost as heat through molecular friction and collisions, resulting into dielectric heating.\textsuperscript{4} In ionic conduction, the ions move with the applied electric field resulting into the kinetic energy due to the increased collision rate which converted into heat. The ionic conduction mechanism plays an important role in heat generation by microwave irradiation.

The rate of microwaves assisted reactions are determined by the Arrhenius equation:

\[
k = Ae^{-\frac{E_a}{RT}}
\]

- \(k\) is the rate of chemical reactions
- \(A\) is pre-exponential factor which describes the molecular mobility and depends on
- the frequency of vibrations of the molecules at the reaction interface
- \(E_a\) is activation energy
- \(R\) is universal gas constant (8.314 J K\(^{-1}\) mol\(^{-1}\))
- \(T\) is the absolute temperature that controls the kinetics of the reaction.

The energy transfer to the molecules occurs very fast such as less than a nano second (10\(^{-9}\) S). As a result, high temperature of the molecules created which is a function of microwave power input. So, the high power microwave has higher intensity, higher consistent temperature to a chemical reaction. The kinetics of microwave reactions is determined by the temperature.
The electromagnetic waves contain electric and magnetic field components. The electric field components of the microwaves are applied on charged particles, as a result of which the charged particles start to migrate or rotate. Microwaves are electromagnetic radiation, is located in between infrared radiation and radio waves. Microwaves have wavelengths of 10 mm–1 m.

<table>
<thead>
<tr>
<th>X-ray</th>
<th>Ultraviolet</th>
<th>Infrared</th>
<th>Microwave</th>
<th>Radio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wavelength in (m)</td>
<td></td>
</tr>
<tr>
<td>$10^{-12}$</td>
<td>$10^{-9}$</td>
<td>$10^{-6}$</td>
<td>$10^{-3}$</td>
<td>1</td>
</tr>
<tr>
<td>Frequency in (Hz)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{21}$</td>
<td>$10^{18}$</td>
<td>$10^{15}$</td>
<td>$10^{12}$</td>
<td>$10^{9}$</td>
</tr>
<tr>
<td>Energy in (eV)</td>
<td>2.45GHz</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^{6}$</td>
<td>$10^{3}$</td>
<td>1</td>
<td>$10^{9}$</td>
<td>$10^{6}$</td>
</tr>
</tbody>
</table>

**Figure 2.2** The electromagnetic spectrum.

corresponding to frequencies between 0.3 and 300 GHz. To avoid interference with telecommunication frequencies most domestic ovens and chemical reactors are operate at a
frequency of 2.45 GHz with corresponding 0.0016 eV energy, which are calculated by Planck-Einstein equation:

$$E = h\nu$$

E is the kinetic energy,

$$\nu$$ is the frequency

h is the Planck's constant ($4.135667516 \times 10^{-15}$ eV/sec )

The energy of a microwave photon is 0.037 kcal/mol. This much energy is not enough to break chemical bonds in compare to the energy needed to break hydrogen bonds from 0.908 to 10.038 kcal/mol).\(^3\)\(^,\)\(^10\) Microwaves cannot induce reactivity in a chemical reactions by direct absorption of electromagnetic energy, as compare to ultraviolet and visible radiation (photochemistry).

A substance’s ability to convert electromagnetic energy into thermal energy is dependent on the dielectric properties.\(^16\) The dielectric constant can be calculated by the equation:

$$\tan \delta = \varepsilon'' / \varepsilon'$$

- \(\tan \delta\), is the loss angle
- \(\varepsilon''\) is the loss factor which quantifies the efficiency of the absorbed electromagnetic energy is converted into heat
- \(\varepsilon'\) is the dielectric constant which represents the ability of molecules to be polarized by an electric field.
A reaction condition with a larger \( \tan \delta \) value the greater is the coupling with microwaves. High dielectric solvents such as water, methanol, acetone, DMF, ethyl acetate, etc. are preferred solvents for microwave irradiated reaction. Low dielectric solvents such as hexane, toluene, etc. do not heat rapidly under microwave irradiation.

The use of microwave became popular because of three reasons:

1. microwave heating equipment was available on the market for synthetic organic chemists;

2. reduces reaction time which leads to products with fewer impurities;

3. allows reaction optimization in a short period of time with higher yield. Microwave-assisted organic synthesis (MAOS) has a potential in four phases of the drug discovery process: lead generation, hit to lead efforts, lead optimization and drug development. In this fast-paced, time sensitive industry MAOS has become a useful tool in drug discovery.\(^{32-35}\)

2.1.5 Microwave Assisted \(^{18}\)F Radio-labeling

The chemical reaction by which a radio-nuclide is introduced into a target molecule to get radio-tracer is called radio-labeling. Many synthetic routes have been developed to prepare PET radiotracers during the last decades. Incorporation of microwave technology into radio-labeling has been shown to be more useful than thermal heating in the case of short-lived radioisotopes.

Fluorine-18 labeling has been widely used in the radio-synthesis of PET imaging agents because of its attractive physical and chemical properties. Since the \(^{18}\)F-radionuclides have
advantages over others for this field of chemistry it would be beneficial to find new synthetic strategies for their synthesis. It has been shown that the use of microwave technology has improved the radiochemical yield of the synthesis of $[^{18}\text{F}]$ 2-fluoro-2-deoxyglucose ($^{18}\text{FDG}$), the most commonly used radio-tracer used for monitoring glucose metabolism.$^{13,38,39}$

Because of natural occurrence of aromatic rings in bioorganic molecules nucleophilic aromatic substitution is an important reaction for synthesis of $^{18}\text{F}$ aryl fluorides. A primary goal is to develop a rapid radiolabeling process of $^{18}\text{F}$ tracers with an acceptable radiochemical yield (RCY). F-18 labeling of aromatic rings requires harsh reaction conditions and long reaction times. This is due to the weak nucleophilic properties of fluoride ion when reacting with a homoaromatic ring and this causes low RCY and undesired side products.

Microwave assisted reactions have been developed in chemistry, to increase the rate of reactions and improve product yield. The implementation of microwave techniques into PET radiochemistry illustrates remote-controlled methods for radio-labelling with the radio-isotopes. Microwave assisted radiolabeling provides advantages such as short reaction time, high RCY and high selectivity. Through the use of microwave technology many radiosynthetic processes problems like low reaction yields or extended reaction times have been overcome.

There are two ways fluorine-18 labeled radiopharmaceuticals can be synthesized. They may be prepared by nucleophilic substitution with $[^{18}\text{F}]\text{F}^-$ or electrophilic substitution using $[^{18}\text{F}]\text{F}_2$. The rate of nucleophilic $[^{18}\text{F}]$fluorination reactions depends on the type of the leaving groups present in the substrate molecule. In the literature, fluorine-18 nucleophilic aromatic substitution with a nitro leaving group are most common. Examples include in $[^{18}\text{F}]$altanserin$^{41,42}$ and $[^{18}\text{F}]$setoperone),$^{43}$ quaternary ammonium,$^{44}$ and some halides.$^{45}$
labeling, an electron-withdrawing group ortho or para to the leaving group is preferable for reaction to occur. This is due to the reduced electron density at the target carbon by resonance stabilization. The activating groups include nitro, carbonyl, and cyano substituents. Only a few halogenated compounds in highly activated aromatic rings with electron-withdrawing substituents have been studied for $[^{18}\text{F}]$ labeling. The application of microwave fluoro-denitrations and nitro-dehalogenations have been investigated in detail and an optimized procedure for microwave assisted fluoro-denitration has been reported.

2.1.6 Background of Our Research

In 1999 a group of scientists published a paper on $[^{11}\text{C}]$methyltriphenyl phosphonium cation tracer on brain tumor. The PET imaging studies with the $[^{11}\text{C}]$methyltriphenylphosphonium cation tracer on animal brain tumor showed that the uptake ratio of cation tracer by tumor to normal brain was 48:1 and a prolonged retention time. In addition, animal cardiac PET studies with the same radiotracer show heart to lung and heart to blood ratios of 14:1 and 46:1 and prolonged retention times.

In 2004 another group of scientists synthesized 3-$[^{18}\text{F}]$-fluoropropyl triphenylphosphonium and 4-$[^{18}\text{F}]$-fluorobenzyltriphenylphosphonium cations from $[^{18}\text{F}]$-fluoride. In vivo results showed that 4-$[^{18}\text{F}]$-fluorobenzyltriphenyl phosphonium cation had 15:1 heart to lung ratio and rapid clearance from blood. We decided to replace the $[^{11}\text{C}]$ radioisotope with $[^{18}\text{F}]$ could provide longer half-life with the high resolution for in vivo study. On the basis of these findings our research aimed to develop $[^{18}\text{F}]$-triphenylphosphonium tracer for cardiac blood flow imaging by PET.
2.1.7 Goal of Our Research

Several PET tracers have been used for diagnosing CAD with rubidium-82, $[^{13}\text{N}]$ammonia and $[^{15}\text{O}]$water. But, these nuclides have limitations such as short half-life, needed on site cyclotron and required fast imaging collection. However, $^{18}\text{F}$ is a lowest energy positron emitter with the highest resolution, longer half-life of the molecule, which increases its absorption into biological membranes. It has been known that lipophilic cations such as the tetraphenylphosphonium (TPP$^+$), can penetrate the plasma and mitochondrial membranes and concentrate in mitochondria, because of the negative inner transmembrane potential ($\Delta\psi_m$).

Therefore, the radiosynthesis and evaluation of an analog of TPP$^+$ as a PET imaging tracer may provide a powerful diagnostic tool for early detection of cardiac diseases.

The introduction of $^{18}\text{F}$ into bioactive molecules for application in PET is important. The physical half-life of $^{18}\text{F}$ is 110 min, therefore radiosynthesis of radioligand needs to be fast with high RCY and RCP. To drive our myocardium perfusion imaging (MPI) project forward the goal of our research has been focused on delivering $^{18}\text{F}$-PTPP tracer with high RCP and high RCY within a short period of time for in vivo study. We decided to develop a synthetic route for the synthesis of (4-$^{18}\text{F}$]-fluorophenyl triphenyl phosphonim nitrate) by microwave accelerated nucleophilic aromatic substitution. This research describes synthesis, purification, formulation, characterization and preliminary in vivo results of $^{18}\text{F}$-PTPP.
2.2 EXPERIMENTAL

2.2.1 General Methods and Materials

All reagents used in synthesis were commercial products and were used without further purification unless otherwise indicated. All solvents used were ACS or HPLC grade. High-performance liquid chromatography (HPLC) analysis and purifications were performed on a Varian/PrepStar Model SD-1 at 220 and 254 nm. Water used in the preparation of the mobile phases was purified using a Millipore/Milli-Q Gradient A10 system. Flash chromatography was conducted using silica gel 230-400 mesh (60 Å, Merck).

For radiolabeling studies, $^{18}$F was obtained from PETNET Pharmaceutical Services, Cummings Park, Woburn, MA, and the synthesis was carried out in Wheaton vials using a customized robotic synthesis platform. Biodistribution studies were measured in an autogamma counter (Wallac Wizard 1480), while cardiac imaging was carried out using a microPET camera (Focus220, CTI Molecular Imaging, Inc. Knoxville, TN).

2.2.2 Synthesis

*Synthesis of 4-nitrophenyl triphenylphosphonium iodide (63):*

The compound 63 was prepared as a precursor for radiosynthesis of $^{18}$FTPP by the
method reported previously. A mixture of 4-nitroaniline (1.4 g, 10 mmol) and sodium nitrite (0.550 g, 10 mmol) was dissolved in concentrated HCl (10 mL) and water (10 mL) at 0 °C with stirring. The reaction mixture was stirred for 10 min. A solution of sodium acetate (2.8 g) in water (10 mL) was added to the cold solution. The reaction mixture was warmed at room temperature. A solution of triphenylphosphine (2.8 g) in ethyl acetate (50 mL) was added dropwise with stirring. The reaction mixture was stirred at room temperature for 1 h. Then 1 M HCl was added until an acidic pH 3-4 was obtained, and the product was extracted with EtOAc (3 x 10 mL). To the combined aqueous phase, an aqueous solution of sodium iodide was added and a precipitate formed. The product was isolated by filtration, dried, and collected giving (4-nitrophenyl)triphenylphosphonium iodide (63, 2.9 g, 74%). Melting point 226-228 °C (lit. 228.5 °C).

Synthesis of 4-nitrophenyl)triphenylphosphonium nitrate (64):

4-nitrophenyltriphenylphosphonium iodide (63) (1.0 g, 2.0 mmol) was dissolved in ethanol (10 mL). An aqueous silver nitrate (0.6 g) solution was added with stirring. The insoluble silver iodide precipitate was removed by filtration and the filtrate was evaporated to dryness. The residue was purified by ISCO using 10% CH₃OH in CH₂Cl₂ to provide 64, (0.45 g, 50%) as a brown solid as a monohydrate. Melting point 211-214 °C; ¹H NMR (300 MHz, CDCl₃) δ 8.49
(dd, J = 8.3, 2.5 Hz, 2H), 7.91 (m, 2H), 7.4-7.7 (m, 15H), 1.63 (s, 1H, H2O). Elemental analysis: C24H21N2O6P, calcd. C, 62.07; H, 4.56; Found: C, 62.57; H, 4.61.

Synthesis of [19F]-4-fluorophenyltriphenylphosphonium nitrate (65) ([19F]-PTPP):

\[
\begin{align*}
\text{NO}_3^- & \quad K_{222}/KF, \text{DMSO} \\
\text{MW} 100 \text{ W}, 120 \text{ s} & \quad \text{15%} \\
\begin{array}{c}
\text{64} \\
\downarrow \\
\text{65}
\end{array}
\end{align*}
\]

A CEM microwave vial was charged with potassium fluoride (40 mg, 0.69 mmole), Kryptofix2.2.2 (4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8] hexacosane) (200 mg, 0.54 mmole) and (4-nitrophenyl)triphenylphosphonium nitrate (64, 153 mg, 0.22 mmole) and DMSO (5 mL). The reaction mixture was purged with argon. The mixture was irradiated in microwave at 120 °C for 10 min. After cooling, the mixture was mixed with water (25 mL) passed through a C18 column and washed with diethyl ether (10 mL). The organic layer was dried over anhydrous sodium sulfate and purified by ISCO using CH2Cl2/EtOAc (85:15) to provide 65, (18 mg, 15%) as a light brown solid as a monohydrate. Melting point 225-228 °C; 1H NMR (300 MHz, CDCl3) δ 7.82-7.77 (m, 5H), 7.71-7.67 (m, 5H), 7.56-7.51 (m, 5H), 6.75-6.67 (m, 4H), 1.63 (s, 1H, H2O). Elemental Analysis: C24H2119FNO4P, Calcd. C, 65.90; H, 4.84; Found: C, 65.49; H, 4.44.

Radiosynthesis of [18F]-PTPP

A 350 mCi sample of aqueous [18F] was obtained from (PETNET Pharmaceutical Services, Cummings Park, Woburn, MA), which was produced by 18 MeV proton bombardment of a high
pressure \(^{18}\text{O}\)-H\(_2\text{O}\) target and applied to a previously activated MP1 anion exchange resin (Bio-Rad) cartridge. A remote control radiosynthesis system was used to place the cartridge in the elution loop. The solution used to elute the cartridge was prepared as follows: \(\text{K}_2\text{CO}_3\) (15 mg) was dissolved in deionized water (1 mL), and Kryptofix (K\(_{222}\)) (90 mg) was dissolved in anhydrous acetonitrile (4 mL); the two solutions were combined, and 1.5-1.8 mL aliquot was used for elution of the column. The radioactivity was eluted from the cartridge by the addition of 1 mL of eluent and collected into a silanized 25 mL pear-shaped flask. The eluate was then concentrated to dryness by applying a gentle stream of heated He and a low vacuum. 0.5 mL of acetonitrile was added and the solvent was evaporated by the same procedure of vacuum and heated He to remove all water from the \(^{18}\text{F}\) mixture. The dry residue containing \(^{18}\text{F}\), \(\text{K}_2\text{CO}_3\) and \(\text{K}_{222}\) was reconstituted with 0.5 mL of acetonitrile and transferred to a conical bottomed 5 mL Wheaton vial containing 2.0 mg of nitro precursor 64 dissolved in 0.5 mL of acetonitrile. The thick walled wheaton vial was heated with microwave at 120 °C for 5 min. The reaction mixture was transferred to a 25 mL pear-shaped flask and diluted with 18.5 mL of water. The crude reaction mixture was passed through a Sep Pak\textsuperscript{TM} C18 cartridge and rinsed with 5 mL of water. The cartridge was then washed with 3 mL of acetonitrile. The collected acetonitrile fraction was purified by HPLC (Phenomenex LUNA C-18 column 250/10 mm, 5 \(\mu\) particle size, 100 Å pore, 57.8% acetonitrile isocratic gradient mobile phase containing 0.1% TFA as stabilizer; for 20 min, flow rate 2.5 mL/min). The \(^{18}\text{F}\)-PTPP 66 eluted at 12-13 min and was collected as a single fraction. The solvent was evaporated in rotary evaporator. Upon drying, the residue was reconstituted with a 10% ethanol solution. The final product yield was on average 12-15 mCi, with a radiosynthesis and purification time of 53 min. Aliquots of \(^{18}\text{F}\) labeled PTPP were used
for biodistribution and PET imaging studies in rats. The synthesis, purification and formulation were completed in 53 min at an average specific radioactivity of 550-1000 Ci/mmol.

2.2.3 In vivo Evaluation

Tissue Biodistribution of (66)$^{18}$F-PTPP in Rats

For tissue biodistribution of $^{18}$F-PTPP the Sprague-Dawley (300-400 g, male) rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) After anesthesia, $^{18}$F-PTPP at a dose of about 9 µCi (range of specific activity, 550-1000 Ci/mmol) in 0.3 mL of 10% ethanol saline per rat was injected intravenously through the left femoral vein of rats. The rats ($n$) 5/each time point) were sacrificed in a CO$_2$ chamber at 30 and 120 min of post injection. Blood, heart, lung, liver, spleen, kidney, femur, muscle, and brain were collected, weighed, and counted in an autogamma counter (Wallac Wizard 1480, Perkin-Elmer Life and Analytical Sciences, Shelton, CT) for radioactivity. The tissue biodistribution of $^{18}$F-PTPP was expressed as percent injected dose per gram of tissue (%ID/g).

Time Activity Curve of (66)$^{18}$F-PTPP in Rats

For time activity curve of $^{18}$F-PTPP the Sprague-Dawley (300-400 g, male) rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) After anesthesia, $^{18}$F-PTPP at a dose of about 9 µCi (range of specific activity, 550-1000 Ci/mmol) in 0.3 mL of 10% ethanol saline per rat was injected intravenously through the left femoral vein of rats. The rats ($n$) 5/each time point) were sacrificed in a CO$_2$ chamber at 30 and 120 min of post injection. Blood, heart, lung, liver, spleen, kidney, femur, muscle, and brain were collected, weighed, and counted in an autogamma counter (Wallac Wizard 1480, Perkin-Elmer Life and Analytical Sciences, Shelton,
for radioactivity. The tissue biodistribution of $^{18}$F-PTPP was expressed as percent injected dose per gram of tissue (%ID/g). At 1, 3, 5, 7, 10, 45, and 90 min blood samples were collected from these rats by the bleeding tail veins after the injection. At each time point 3 rats were sacrificed in a CO$_2$ chamber and blood samples were counted in an autogamma counter (Wallac Wizard 1480, Perkin-Elmer Life and Analytical Sciences, Shelton, CT) for radioactivity. The blood clearance of $^{18}$F-PTPP was expressed as percent injected dose per gram (%ID/g).

Cardiac Imaging with $^{18}$F-PTPP in Rats

For cardiac imaging a microPET camera (Focus220, CTI Molecular Imaging, Inc. Knoxville, TN), was used, which provides 95 transaxial slices in 22 cm operational field of view. The Sprague-Dawley (300-400 g, male) rats were anesthetized with sodium pentobarbital (50 mg/kg, ip. After anesthesia, $^{18}$F-PTPP at a dose of about 0.7 μCi (range of specific activity, 550-1000 Ci/mmol) in 0.3 mL of 10% ethanol saline per rat was injected intravenously through the left femoral vein of rats. The first images were taken 5 min after the injection until 120 min post injection. ASIPRO software was used for image visualization. The images were calculated by OSEM2D algorithm.

2.3. RESULTS AND DISCUSSIONS

2.3.1 Chemistry

The radiosynthesis of $^{18}$F-TPP was accomplished by one-step procedure of radiofluorodenitration from 64 to 66 is shown in Scheme 2.2. The precursor 4-nitrophenyltriphenylphosphonium iodide 63 was synthesized according to the method reported by Rieke et al. (Scheme 2.1).$^{54}$ 4-nitrophenyltriphenylphosphonium nitrate 64 was synthesized to optimize microwave assisted fluorodenitration method and applied the conditions for radio-fluorodenitration (Scheme 2.2).
Scheme 2.2 Synthesis of (4-[^18]F-fluorophenyl)triphenylphosphonium nitrate

[^18]F-PTPP was synthesized by nucleophilic substitution reaction of 4-nitrophenyltriphenylphosphonium nitrate 64 and ^[18]Fluoride in the presence of potassium carbonate and phase transfer agent Kryptofix 222 in acetonitrile. This solution was irradiated in

Scheme 2.3 Synthesis of (4-[^18]F--fluorophenyl)triphenylphosphonium nitrate
the microwave cavity at 120 °C for 5 min. The resulting mixture was filtered through SEP PAK™ to remove the unreacted triphenylphosphine and free $^{18}\text{F}$ prior to preparative HPLC separation. A 57.8% acetonitrile isocratic gradient was used for HPLC purification. The appropriate fractions were collected, the solvent was evaporated, and analyzed for radiochemical yield and purity. The radioactivity of the final product was approximately 15-18 mCi with >65% radio chemical yield (RCY) starting with 350mCi $^{18}\text{F}$-fluoride. The synthesis, purification and formulation were completed in 53 min with >99% radio chemical purity (RCP), as determined by analytical HPLC. The residue was dissolved in normal saline.

2.3.2 Biology

*Biodistribution Study of $^{18}\text{F}$-PTPP in Spraque-Dawley (SD) Rats*

Biodistribution studies were performed on SD rats with $^{18}\text{F}$-PTPP. The animal cardiac PET results after 30 min injection in Table 2.2 showed that high uptake of radiotracer $^{18}\text{F}$-PTPP from the blood to myocardium (2.315% injected dose per g of tissue weight (ID/g); with 86:1 ratio). The blood has 0.027% ID/g means complete uptake of tracer by different organs. The tracer also distributed to liver and kidney tissue, 0.649% and 2.735% ID/g, respectively.
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<td>Brain</td>
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**Table 2.2** Biodistribution of $^{18}$F-PTPP in Spraque-Dawley Rats

The $^{18}$F-PTPP radiotracers exhibit intense uptake by myocardium and is stable at least for 50 min time which should be more than sufficient to obtain clear images of the myocardium using PET imaging technology. In addition, Low uptake of the radiotracer in lung tissue suggests an excellent signal to noise ratio in future imaging.
**CIA : Biology**

**Biodistribution of 18F-PhenTPP in rats**

All values taken from biodistribution in SD rat at 60 minutes post-injection

Figure 2.3: Blood clearance curve of $^{18}$F-PTPP
PET imaging studies were carried out on SD rats. At one min post-injection, PET images showed the radiotracer $^{18}$F-PTPP was in blood stream. At 30 min post-injection there was complete uptake of radiotracer. At 60 min post-injection rapid clearance from the liver occurred and is depicted in Figure 2.5.

**Figure 2.4:** Time-activity curves $^{18}$F-PTPP 66

**Imaging Studies in SD Rats**
This time activity curve supports these results, that heart tissue has a prolonged retention time for 50 min of $^{18}\text{F}$-PTPP. In contrast, levels in the liver started to decrease after 8 min. (Figure 2.6). These findings are consistent with the biodistribution results. It was also noted that, some in vivo bone uptake of $^{18}\text{F}$ could be detected in images after 30 min post injection.

2.4. CONCLUSIONS

The radiosyntheses of (4-$^{18}\text{F}$-fluorophenyl)triphenylphosphonium nitrate was developed by a microwave assisted radio-fluorodenitration with modest radiochemical yields and specific radioactivities. It also allowed the reduction of the reaction time to only 5 min of irradiation. Finally this work gives the bases for a possible microwave assisted radio-fluorodenitration of additional $^{18}\text{F}$-radiatoracers. The excellent heart-to-blood ratio of $^{18}\text{F}$-FTPP suggests that this
radiotracer may have potential as a myocardium perfusion imaging agent to detect mitochondrial
damage or myocardial blood flow. *In vivo* results of $^{18}$F-PTPP 66 demonstrated high
myocardium uptake of the radiotracer by 30 min post-injection and prolonged retention time
over 50 min. However, hepatic clearance of the radiotracer was rapid and did not allow for high
quality images of the heart beyond 50 min. This work suggests that this radiopharmaceutical may
have potential as a MPI agent for characterizing mitochondrial damage or myocardial blood
flow.
2.5. REFERENCES


51. Ravert, H. T.; Madar, I.; Dannals, R. F., Radiosynthesis of 3-[18F]fluoropropanyld


1.7. APPENDIX

Ethyl 6-iodo-4-oxo-1,4-dihydroquinoline-3-carboxylate (8)
$^1$H NMR (400 MHz, CDCl$_3$)
Ethyl 4-chloro-6-iodoquinoline-3-carboxylate (9)
1H NMR (400 MHz, CDCl3)
5-benzyl-8-iodo-2-phenyl-2H-pyrazolo[4,3-c]quinolin-3(5H)-one (13)

LC/MS and $^1$H NMR (400 MHz, DMSO)
**Elemental Composition Report**

**Single Mass Analysis**  
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 150.0  
Element prediction: Off  
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions  
68 formula(e) evaluated with 2 results within limits (all results up to 1000) for each mass  
Elements Used:  
C: 0-150  H: 0-250  N: 2-4  O: 0-1  I: 0-1

Sara Sadler, AFR-NEU-55  
University of Illinois, SCS, Mass Spectrometry Lab  
Q-TOF Q-TOF 3547132 (2.293) AM (Cen,3, 80.00, Ar,15000,0.716,46,0.70,LS 3), Sm (SG,2x3.00)  
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8-(1-benzothiophene-2-yl)-5-benzyl-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (15)

LC/MS and $^1$H NMR (400 MHz, DMSO)
## Elemental Composition Report

### Single Mass Analysis
- Tolerance = 10.0 PPM / DBE: min = -1.5, max = 150.0
- Element prediction: Off
- Number of isotope peaks used for i-FIT = 3

### Monoisotopic Mass, Even Electron Ions
- 293 formula(e) evaluated with 2 results within limits (all results up to 10000 for each mass)

#### Elements Used:
- C: 0.150
- H: 0.260
- N: 0.5
- O: 0.6
- S: 1.1

Sadrz, Sara, AFF-NEU-01
University of Illinois, SCS, Mass Spectrometry Lab
Qtof_3483.95 (3.507) AM (Cen.1, 80.00, Ar:15000, 0.7164 0.7015 0.9457 0.5416); Sm (S0, 2x3.00); Cm (4950)
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176
8-(1-benzofuran-2-yl)-5-benzyl-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (17)

LC/MS and $^1$H NMR (400 MHz, DMSO)
**Elemental Composition Report**

**Single Mass Analysis**
- **Tolerance**: ±10.0 PPM
- **DBE**: min = -1.5, max = 150.0
- **Element prediction**: Off
- **Number of isotope peaks used for i-FIT**: 3

**Monoisotopic Mass, Even Electron ion**
241 formula(e) evaluated with 2 results within limits (all results up to 1000) for each mass

Elements Used:
- C: 0-150
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- N: 0-5
- O: 0-6

Badger, Sara, AFR-NEU-03, University of Illinois, SCS, Mass Spectrometry Lab

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5-benzyl-8-[(3-chlorophenyl)ethynyl]-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (19)

LC/MS and $^1$H NMR (400 MHz)
methyl 5-(5-benzyl-3-oxo-2-phenyl-3,5-dihydro-2H-pyrazolo[4,3-c]quinolin-8-yl)thiophene-2-carboxylate (29)

LC/MS and $^1$H NMR (400 MHz)
Elemental Composition Report

Single Mass Analysis
Tolerance = 10.0 PPM / DEE: min = -1.5, max = 150.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
104 formula(s) evaluated with 2 results within limits (all results up to 1000) for each mass
Elements Used:
C: 0-150  H: 0-250  N: 2-4  O: 2-4  S: 0-1
Sara Sadler, AFR/NEU-23
University of Illinois, SCS, Mass Spectrometry Lab
C10H4O4S (234.0284) AM (Con, 3, 90.00, Ar, 15000.0, 716.46, 70.0 LS 3); Sm (SG, 243.00)

Top MS ES+
1: 730.0000
2: 730.0000

Minimum:
Maximum:

Mass  Calc. Mass  mDa  PPM  DEE  i-FIT  Formula
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492.1348  3.6  7.3  25.5  11.2  C32 H18 N3 O3
4,4,5,5-tetramethyl-2-(3-phenoxyphenyl)-1,3,2-dioxaborolane (30) LC/MS and $^1$H NMR (400 MHz)
4,4,5,5-tetramethyl-2-(3-phenoxyphenyl)-1,3,2-dioxaborolane (31)

LC/MS and $^1$H NMR (400 MHz)
methyl 4-((2R,3S)-3-(tert-butoxycarbonylamino)-2-(2-methoxy-2-oxoethyl)piperidin-1-yl)-4-oxobutanoate (38)

LC/MS and $^1$H NMR (400 MHz)
**Elemental Composition Report**

**Single Mass Analysis**
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 150.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
257 formulae evaluated with 3 results within limits (all results up to 1000) for each mass
Elements Used:
C: 0-150  H: 0-250  N: 0-5  O: 0-7

Sadler, Sara, AFR-NEU-78  University of Illinois, SCS, Mass Spectrometry Lab
Qtd_54632.35 (2.507) AM (Cen, 3, 80.00, Ar: 15000.0, 716.46, 6.70, LS 3), Sm (SG, 2x3.00), Cen (35.36)
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(1S,10aR)-methyl 1-(tert-butoxycarbonylamino)-6,9 dioxodeca hydro.pyrido[1,2-a] azepine-8-carboxylate (39)

LC/MS and $^1$H NMR (400 MHz, DMSO)
(1S,10aR)-methyl 1-amino-6,9-dioxodecahydropyrido[1,2-a]azepine-8-carboxylatemethyl (40)

$^1$H NMR (400 MHz)
(1S,10aR)-methyl 1-((benzo[b]thiophen-2-ylmethyl)amino)-6,9-dioxodecahydro pyrido[1,2-a]azepine-8-carboxylate (42)

LC/MS and $^1$H NMR (400 MHz)
(1S,10aR)-methyl 1-(3-(naphthalen-1-yl)-1-(naphthalen-1-yl)carbamoyl)ureido)-6,9-dioxodecahydropyrido[1,2-a]azepine-8-carboxylate (44)

LC/MS and $^1$H NMR (400 MHz)
of ethyl 2-((2R,3S)-3-(tert-butoxycarbonylamino)-1-(naphthalen-1-ylcarbomoyl) piperidin-2-yl)acetate (46)

LC/MS and $^1$H NMR (400 MHz)
Elemental Composition Report

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 150.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
93 formula(s) evaluated with 1 results within limits (all results up to 1000 for each mass)
Elements Used:
C: 0-150  H: 0-250  N: 2-4  O: 4-6  I: 0-1

Sara Sadler, AFR-NEU-82  University of Illinois, SCS, Mass Spectrometry Lab
Q-Tof QE21 1.721 AM (Gan, 3, 80.00, Ar, 15000, 0.716, 0.70, LS 3); Sf (Sc, 2x0.00)

100%

Minimum:
Maximum:

Mass  Calc. Mass  mDa  PPM  DBE  i-FIT  Formula
456.2356  456.2458  0.0  1.6  10.5  0.5  C25 H34 N3 O5
ethyl 2-((2R,3S)-3-amino-1-(naphthalen-1-ylcarbamoyl)piperidin-2-yl)acetate hydrochloride (47)

LC/MS and $^1$H NMR (400 MHz)
methyl3-[(2R,3S)-2-(2-ethoxy-2-oxoethyl)-1-(1-naphthylcarbamoyl)piperidin-3-yl]amino]-3-oxopropanoate (49)

LC/MS and $^1$H NMR (400 MHz)
(4aS,9aR)-methyl 1-(naphthalen-1-ylcarbamoyl)-6,8-dioxodecahydro-1H-pyrido[3,2-b]azepine-7-carboxylate (50)

$^1$H NMR (400 MHz)
ethyl 2-((2R,3S)-1-(4-bromopicolinoyl)-3-((tert-butoxycarbonyl)amino)piperidin-2-yl)acetate (52)

LC/MS and $^1$H NMR (400 MHz)
**Elemental Composition Report**

**Single Mass Analysis**
- **Tolerance**: 10.0 PPM
- **DBE**: min = -1.5, max = 150.0
- **Element prediction**: ON
- **Number of isotopic peaks used for i-FIT**: 3

Monoisotopic Mass, Even Electron Ions
- 203 formula(e) evaluated with 2 results within limits (all results up to 1000) for each mass
- **Elements Used**: C: 0-100, H: 0-250, N: 0-5, O: 0-6, Br: 1-1

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ethyl \{(2R,3S)-3-amino-1-\[(4-bromopyridin-2-yl)carbonyl\]piperidin-2-yl\}acetate hydrochloride (53)

LC/MS
methyl 3-\{[(2R,3S)-1-[(4-bromopyridin-2-yl)carbonyl]-2-(2-ethoxy-2-oxoethyl) piperidin-3-yl]amino]-3-oxopropanoate (55)

LC/MS and $^1$H NMR (400 MHz)
methyl 4aS, 9aR)-1-[(4-bromopyridin-2-yl)carbonyl]-6,8-dioxodecahydro-1H-pyrido[3,2-b]azepine-7-carboxylate (57)

LC/MS and $^1$H NMR (400 MHz)
ethyl 2-((2R,3S)-3-(3-(4-chlorophenyl)ureido)-1-(naphthalen-1-ylcarbamoyl) piperidin-2-yl) (59)

LC/MS and $^1$H NMR (400 MHz)