Design, Synthesis and Analysis of Small Molecule Heterocyclic Aromatic-Based CXCR4 Modulators

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DESIGN, SYNTHESIS AND ANALYSIS OF SMALL MOLECULE HETEROCYCLIC AROMATIC-BASED CXCR4 MODULATORS

by

THERESA DENISE GAINES

Under the Direction of Suazette Mooring, PhD

ABSTRACT

CXCR4 is a chemokine receptor that has been linked to several disease related pathways including: HIV-1 proliferation, autoimmune disorders, inflammatory disease and cancer metastasis. The interaction of the C-X-C chemokine receptor type 4 (CXCR4) with C-X-C chemokine ligand 12 (CXCL12) plays a key role in triggering these disease related pathways. Various antagonists for these receptors have been synthesized and tested, but many are not useful clinically either because of toxicity or poor pharmacokinetics. Some of the most extensive CXCR4 antagonist libraries stem from a class of compounds, p-xylyl-enediamines, which all feature a benzene ring as the core of the compound. This work focuses on the design and synthesis of a new class of compounds that show potential as CXCR4 antagonists by using heterocyclic aromatic
rings (2,6-pyridine, 2,5-furan, 2,5-pyrazine and 3,4-thiophene) as the core of the scaffold. After synthesis, these analogues were probed through a variety of assays and techniques by our collaborators in the Shim lab at Emory University including: preliminary binding assays, Matrigel invasion assays, carrageenan mouse paw edema tests, and in silico analysis. In silico analysis also probed 2,5-thiophene-based analogues previously synthesized. This work has produced the beginnings of a new library of CXCR4 antagonists and has identified fifteen hit compounds that are promising leads for further testing and modification.

INDEX WORDS: CXCR4, CXCL12, Chemokine, Chemokine receptor, Inflammation, Irritable Bowel Disease, Rheumatoid arthritis, Cancer, Cancer metastasis, SAR, Structure activity relationship, Heterocycle, Small molecule, Synthesis, Drug design, Antagonist, Modulator, Pyridine, Pyrazine, Furan, Thiophene, Reductive amination, Effective concentration, Matrigel invasion assay, Carrageenan mouse paw edema, Molecular modeling, Docking, It1t, 3ODU.
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THERESA DENISE GAINES

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Chemistry in the College of Arts and Sciences

Georgia State University

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1 INTRODUCTION

This work is centered around synthesizing and analyzing small molecules to disrupt the interaction between CXCR4, a protein receptor, and CXCL12, a ligand. This interaction triggers many pathways in the body—including cancer cell metastasis and inflammation. The precedence in mitigating these diseases will be explained in two parts below.

1.1 Cancer

A formidable foe and wily enemy, cancer has been a troubling adversary to a many people from all socio-economic and racial backgrounds. Researchers, doctors, grocers, artists, students, entrepreneurs, sons and daughters—none are immune to cancer. For the year 2014, cancer was estimated to have claimed the lives of 585,720 American citizens, which is 1.3 times the population of Atlanta, Georgia. Furthermore, an estimated 1.6 million people were diagnosed with cancer in 2014 in the United States. According to the CDC, 7.6 million people—just over the population of Hong Kong—worldwide die from cancer. As intimidating as cancer is in the world today—as the second leading cause of death, cancer has been a known disease for centuries, with evidence of tumors in the bones of mummies from 1600 BC and descriptions of tumor removals in ancient Egyptian inscriptions.

Cancer is a broad and multi-faceted disease stemming from numerous mutations from the host’s DNA genome. Each mutation has the possibility of giving the cancer cell a method of escaping the body’s immune system or an advantage in order to gain more resources from the surrounding area to thrive. Because of this, cancer is always different from person to person, and possibly even from tumor to tumor in a single host’s body. This makes the treatment of cancer
difficult—not only are there multiple mutations that can cause a tumor to thrive in a person’s body, but breast cancer cells from two individuals might not respond to the same medicines or treatments.

Cancer occurs when healthy cells fall out of the healthy cell cycle—which includes cell death after a certain number of divisions. Cancerous cells continue to grow and divide and do not undergo programmed cell death. Mutated DNA causes these deviations from the healthy cell cycle. In ordinary cell replication, mutations are not extremely rare; however, organisms contain enzymes and proteins that check DNA for mutations as well as special enzymes capable of fixing any mutated strands by replacing the mutated parts before the cell division is completed and normal daughter cells are still formed. DNA mutation can become the devastating disease we know as cancer when mutations slip by the body’s processes that aim to prevent the mutations during cell division. Mutations can be triggered and caused by a number of factors. Mutated DNA can be given from parents to a child, or environmental factors can cause mutations in a person’s DNA.

Fortunately, cancer survival rates have increased for the past thirty years. In 1975, newly diagnosed cancer patients had a 48.9% survival rate over a five-year. In 2010, the five-year survival rate rose to 68.3%. This increased survival rate can be attributed to advances in medicine and novel strategies to combat cancer.

X-rays were discovered in 1896 and quickly became routine in cancer treatment and diagnosis in only three years. New technologies such as ultrasound, computed tomography, magnetic resonance imaging and positron emission tomography have all played an important role in cancer diagnosis and treatment—providing lower risk and non-invasive methods of analyzing tumors. Better diagnostic tools as well as new methodologies to destroy or remove them via lasers have improved surgery procedures. Chemotherapy agents have been synthesized and research over the years has been effective in reducing side effects. Hormonal and immunotherapy are also paving
the way for potential anti-cancer vaccines and drugs that try to reprogram a cancer cells to stop growing and undergo cell death. These new technologies and innovations have changed the fight against cancer for the better. For most cancer patients, multiple treatment strategies are used to target the cancer. This is because cancer is able to proliferate through multiple pathways.

1.1.1 Hallmarks of Cancer

Robert Weinberg and Douglas Hanahan proposed the ‘Hallmarks of Cancer,’ which are the fundamental, distinguishing characteristics that transform normal cells into cancerous cells. Cancer is different for every person and classification by treatment would prove to be difficult. Cancers can grow in different parts of the body and sometimes can metastasize to travel to other parts of the body. The hallmarks are defined as ‘acquired functional capabilities that allow cancer cells to survive, proliferate and disseminate.’ Cancerous cells can acquire these hallmarks in different ways at different times and in different orders. The original six hallmarks of cancer are: the ability to sustain proliferative signaling, the ability to evade growth suppressors, the ability to activate invasion and metastasis, the ability to enable replicative immortality, the ability to induce angiogenesis or new blood vessel generation and the ability to resist cell death. Four new characteristics have recently been added to the list. There are two abilities—the ability to deregulate cellular energetics and the ability to avoid immune destruction. The final two have been called ‘enabling characteristics’ which are tumor-promoting inflammation and genome instability and mutation. The original six hallmarks of cancer are below in Figure 1.
Various cancers can possess various hallmarks, or combination of hallmarks. As hallmarks are mutated abilities to give cancer extra functionality, a common strategy to treat cancer is to address the various hallmarks. When undergoing chemotherapy, cancer patients are prescribed several drugs—each often with a different function—to attack specific aspects or hallmarks of cancer. Chemotherapy can be combined with other cancer treatment strategies such as radiation or surgery to achieve a higher success rate of eliminating the cancer.

1.2 Inflammation

Irritable Bowel Disease (IBD) is quickly becoming a global concern; however, the incidences of IBD vary by location. An estimated 1.2 million Americans\textsuperscript{6} have been diagnosed...
with IBD, and between 2.5 and 3 million people in Europe. Another concerning feature is that as nations become industrialized, incidences of IBD increase.\textsuperscript{7} Even though 0.5% of the western world has been diagnosed with IBD,\textsuperscript{7} incidences are expected to rise exponentially over the next ten years.\textsuperscript{8} IBD is a low mortality disease; therefore, even with a steady incidence rate, the amount of people affected is compounded.

Higher rates of IBD lead to higher costs to treat this disease. Immunosuppressive treatment plans can cost upwards of $25,000 annually.\textsuperscript{8} Innovation is needed to prepare for a future where increasing members of our population need to be treated for this disease and lower the cost of said treatments.

IBD is comprised of several different diseases—including Crohn’s disease (CD) and ulcerative colitis (UC)—which are environmentally triggered and appear in genetically susceptible individuals. The trigger causes an unusual immune response to the individual’s intestinal flora resulting in gastrointestinal inflammation.\textsuperscript{9-10} Individuals with IBD have exhibited altered chemokines and receptors in their epithelial cells, which could disrupt the body’s recognition of their gut bacteria. Not only are individuals with IBD, unable to recognize enteric microbiota, but it is also possible that they are unable to regulate the amount of pro-inflammatory chemokines that are sent as the immune response.\textsuperscript{11-12}

Rheumatoid Arthritis (RA) is an auto-inflammatory disease that affects 1.5 million people in the United States with about 5-500 new diagnoses per 100,000 people each year.\textsuperscript{13-14} RA symptoms usually appear in women between the ages of 40 and 50 and a bit later for men.\textsuperscript{15} Men and women from diverse ethnicities and races can all suffer from RA.\textsuperscript{16} Even though men and women can both be diagnosed with RA, three times as many women are living with RA than men.\textsuperscript{17}
There is no one test to diagnose RA as the disease seems to both have a genetic component and environmental triggers.\textsuperscript{14} Because of this, RA is diagnosed by the symptoms displayed.\textsuperscript{18} RA is characterized as inflammation in the joints caused by the immune system attacking the joint tissue. The inflammatory response causes the synovium to become thick, and leads to swelling of the joints. As this continues, the inflamed synovium degrades the cartilage and bone and weakens the surrounding muscles, tendons and ligaments.\textsuperscript{18} This inflammatory response occurs in the joints primarily; however, organs can be inflamed as a symptom of RA.\textsuperscript{19} There is no cure for RA, however, management and treatment are more successful when it is treated early and aggressively.\textsuperscript{20}
2 DESIGN AND SYNTHESIS OF SMALL MOLECULE HETEROCYCLIC AROMATIC-BASED CXCR4 MODULATORS

2.1 Background

CXCR4 is a chemokine receptor that has been linked to various disease pathways including: HIV-1 proliferation, autoimmune disorders, inflammatory disease and cancer metastasis.\(^{21-22}\) The interaction of the C-X-C chemokine receptor type 4 (CXCR4) with C-X-C chemokine ligand 12 (CXCL12) plays an important role in cancer metastasis and inflammation.\(^{23}\) Various antagonists for these receptors have been synthesized and tested, but many are not useful clinically either because of toxicity or poor pharmacokinetics.\(^{22,24}\) This work focuses on the design and synthesis of a new class of compounds that show potential as CXCR4 antagonists by using aromatic heterocyclic rings as the core of the compound.

2.1.1 Chemokines and CXCR4

Cytokines are small proteins (5-20 kDa) involved cell signaling. A large variety of cells (endothelial cells, macrophages, T cells, B cells) produce these proteins for different functional purposes. Chemokines are cytokines that chemoattract cells to areas of inflammation by binding to G protein coupled receptors (GPCRs), chemokine receptors, and creating a chemical gradient.\(^{25-27}\) Chemokines can belong to four different groups, C, CC, CXC, and CX3C. They are named based on the location of the first two cystine residues on the N-terminus.\(^{28}\)
Most chemokines can bind to more than one chemokine receptor and receptors to more than one ligand. These chemokines are referred to as promiscuous; however, six of the eighteen known chemokine receptors bind to a single chemokine ligand.\(^{29}\) CXCR4 (shown in Figure 2) is one of these non-promiscuous receptors. CXCR4 is a seven transmembrane GPCR and has been known by a few other names—CD184, LESTR\(^{30}\) and fusin.\(^{31}\) CXCR4 binds exclusively to the chemokine ligand, CXCL12 (SDF-1).\(^{32-33}\) CXCL12 has several isoforms in the body and even though the alpha isoform is the most prevalent,\(^{34}\) CXCR4 seems to bind to each isoform with similar affinities.\(^{35}\)
Because CXCR4 is involved in many drug and disease pathways like HIV-1 entry and cancer metastasis,\textsuperscript{36} it is one of the most studied chemokine receptors.\textsuperscript{37} Through knock out mice studies, it has been shown that CXCR4 is necessary for neural development and hematopoiesis, organogenesis and vascularization in embryos.\textsuperscript{38-41} CXCR4 has different functions, in line with other chemokine receptors, in adults.\textsuperscript{42} In healthy cells, the interaction between CXCR4 and CXCL12 triggers several downstream pathways: mobilization of stem cells,\textsuperscript{43} inflammatory cells and immune cells,\textsuperscript{44} cell survival,\textsuperscript{45} gene transcription,\textsuperscript{46} intracellular calcium modulation,\textsuperscript{47} and cell adhesion.\textsuperscript{48}

2.1.2 \textit{CXCR4 Mechanism of Action}

The crystal structure of CXCR4 was first reported in 2007 with the protein bound to a small molecule antagonist, IT1t.\textsuperscript{49} The crystal structure was incomplete, in that some amino acids in the N terminus were missing.\textsuperscript{50} In 2008, an NMR structure of CXCR4’s N terminus in complex with CXCL12 was reported and it was able fill in some of the missing in pieces and identify some of
the interactions between CXCL12 and CXCR4. Unfortunately, there is no crystal structure of CXCR4 and CXCL12 bound together.

CXCL12 binds to CXCR4 in a two-step process. In the first step, the beta sheets, the 50s loop (the series of amino acids connecting the last beta sheet to the alpha helix), and N loop (the series of amino acids connecting the N terminus to the first beta sheet) of CXCL12 interact with the extracellular region on the top of CXCR4. The N terminus of CXCR4 then interacts with the RFFESH loop (residues 12-17) of CXCL12. It has been suggested that this first step causes a conformational change in CXCR4 which exposes the binding pocket in the helices. This allows the N terminus of CXCL12 to interact with the residues in the binding pocket near the top of the helices of CXCR4. The crystal structure of CXCR4 reveals that the binding pocket is larger and closer to the extracellular surface compared to other GCPRs.

Because of this two-step process, small molecule antagonists may not completely block the interactions between CXCR4 and CXCL12. Since the CXCR4/CXCL12 interaction is vital for other necessary physiological processes, and ideal antagonist would block some functionality but not all function. Particularly, small molecules that block the N terminus of CXCL12 from binding in the second step would not block interactions in the first step. Key residues involved in the second binding site influence signaling and have been identified as ASP97 (ECL2), ASP187 (TMII), and GLU288 (TMVII). ASP171, ASP 87 and ASP262 have also been suggested to be important binding sites for signaling.

2.1.3 Drug and Disease Related Pathways of CXCR4

CXCR4 is a seven transmembrane GPCR, which is embedded in the cell membrane and interacts with other proteins from the cell surface. This receptor was first discovered as a co-
receptor for HIV-1 entry and after more studies were conducted, the other functional responsibilities of CXCR4 were uncovered.\textsuperscript{61-64} CXCR4 selectively binds to the chemokine ligand, CXCL12\textsuperscript{65} and this interaction plays a very important role in the body as knock out mice for both of these genes do not survive due to a number of defects in the embryo.\textsuperscript{61-62} The interaction between CXCR4 and CXCL12 triggers downstream pathways that induce inflammation; therefore, over expression of CXCR4 has also been linked to inflammatory diseases such as allergic asthma,\textsuperscript{66} IBD,\textsuperscript{67} systemic lupus erythematosus (SLE), atherosclerosis\textsuperscript{68} and rheumatoid arthritis.\textsuperscript{69} Lastly, the CXCR4/CXCL12 promotes cancer cell metastasis.\textsuperscript{70}

Since the CXCR4-CXCL12 axis plays such an important role in these disease related pathways, blocking this interaction has become a leading strategy in an attempt to reduce the progression of cancer and other inflammatory conditions.\textsuperscript{71} Several small molecule antagonists have had success in blocking this interaction and have been shown to inhibit cancer metastasis, HIV-1 entry and have anti-inflammatory activity.\textsuperscript{72-75}

\subsection{CXCR4 and Metastasis}

Migration of cells is normal in the human body.\textsuperscript{76} The immune system is comprised of cells that migrate through the body in order to facilitate healing in infected areas and routine repair and upkeep inside the body.\textsuperscript{76-78} This migration inside the body is made possible by two groups of proteins. The first group—chemokines—is comprised of small proteins, which interact with the second group of proteins called chemokine receptors.\textsuperscript{22} In particular, the interaction between the chemokine ligand, CXCL12, and the chemokine receptor, CXCR4, plays an important role in triggering pathways to facilitate physiological functions.\textsuperscript{22, 62, 79} The interaction between CXCR4 and CXCL12 orients hematopoietic stem cells (HSCs), nonhematopoietic tissue-committed stem cells (TCSCs), pre-B lymphocytes, t lymphocytes and embryonic stem cells (ESCs) to their appropriate sites in the body.\textsuperscript{76-77, 80-81}

Metastatic cells break from the primary tumor and travel through the body and create tumors in other parts of the body.\textsuperscript{76, 82} Metastatic cancer cells take advantage of the CXCR4/CXCL12 signaling interaction in order to migrate through the body in the same way that stem cells in the body migrate.\textsuperscript{70} Many tumors develop hypoxic conditions as it grows due to changes in cell vasculature.\textsuperscript{83} HIF-1 is then activated because of the hypoxia and can promote the
expression of CXCR4. It’s also possible that other growth factors contribute to upregulation of CXCR4 because of the increased surface area of the tumor.

Over expression of CXCR4 in cancer cells can lead to production of metalloproteases, which can degrade membranes, enabling the cells to enter into the blood stream. Once circulation, these cells migrate to CXCL12 rich sites in the body—i.e. lymph nodes, lung, liver and bone marrow. These cells migrate through chemotaxis, which is a system that pilots cells through the body. CXCL12, that is secreted by cells diffuses away from their source and this creates a gradient. This gradient extends from the CXCL12 rich sites and becomes more dilute as distance from the source increases. The CXCR4-rich cancer cells will then be guided through the gradient to the sites that are CXCL12-rich. CXCR4 overexpression triggers integrin activity, enabling the cancerous cells to adhere to the surrounding tissue once they migrate.

Stage IV cancers—metastatic cancers—usually yield a poor prognosis compared to other stages and CXCR4 expression on a tumor is used to determine the prognosis for many types of cancers including leukemia, breast cancer, prostate cancer, lung cancer and ovarian cancer. As seventy five percent of all cancers over express CXCR4, it is strategic to use CXCR4 expression as marker in cancer diagnosis and prognosis.

Tissue invasion and metastasis is one of the hallmarks of cancer outlined by Weinberg and Hanahan. Slowing down metastatic cancer could help boost cancer survival rates and keep tumors localized for other treatments medicines to work more effectively. Targeting the CXCR4/CXCL12 axis to stop metastasis is an alluring tactic because the CXCR4 proteins that are expressed are less likely to mutate. The CXCR4/CXCL12 interaction is specific; therefore, if the CXCR4 was changed as response to a drug preventing it from migrating, it would likely no longer bind to
CXCL12, and still would not be able to migrate. And since the CXCL12 is produced by healthy cells, the risk of mutation of the receptor while retaining activity is low.

### 2.1.3.2 CXCR4 and Inflammation

CXCR4 and CXCL12 are expressed in healthy intestinal epithelial cells (IEC).\(^{99-101}\) Both CXCR4 and CXCL12 are needed for necessary physiological functions including electrolyte secretion,\(^ {102}\) migration, and upkeep of the epithelial mucosal membrane.\(^ {103}\) CXCR4 and CXCL12 expression, however, are both upregulated in the IEC’s of patients with IBD.\(^ {67,104}\) The presence of extra CXCL12 chemoattracts CD45RO+ T cells, which are rich with CXCR4. This interaction triggers inflammation in the intestinal membrane and disrupts the intestinal membrane’s homeostasis.\(^ {104}\) It is possible that blocking the CXCR4, CXCL12 interaction can help mitigate intestinal inflammation for patients with IBD.

In patients with Rheumatoid arthritis, CD\(^ +\) T cells, neutrophils and macrophages gather into the synovial tissue in joints and damage surrounding tissue.\(^ {105-106}\) Several studies have suggested that the overexpression of CXCR4 in the synovial tissue, recruits the T-cells to gather into those areas.\(^ {72}\) Furthermore, treatment of RA in mice with CXCR4 antagonists like AMD3100\(^ {107}\) and TN14003,\(^ {108}\) a peptide antagonist, yield a reduction in inflammation. Furthermore, treatment of RA with low-level laser irradiation (LLLI)\(^ {109}\) reduces inflammation by decreasing the expression of CXCR4 in the affected area. Overexpression of CXCR4 has also been documented in the monocytes, neutrophils and B cells of patients with lupus.\(^ {110}\) Additionally, treatment in mouse models with CXCR4 antagonists have shown reduction of symptoms.\(^ {111}\) As CXCR4 is a key factor in the inflammatory response and as existing small molecule antagonists
have shown to be effective in mitigating symptoms of inflammatory diseases, this sets a precedence for the creation of new antagonists.

2.1.4 CXCR4 Antagonists and Modulators

Since the CXCR4-CXCL12 interaction plays such an important role in these disease related pathways, blocking this interaction has become a leading strategy in an attempt to mitigate these pathways or even selectively accelerate normal physiological pathways.\textsuperscript{22} Several small molecule antagonists have had success in blocking this interaction and have been shown to inhibit cancer growth, inhibit cancer metastasis and have anti-RA activity.\textsuperscript{23, 112-114}

Many compounds that have shown potential as CXCR4 antagonists and share the common central benzene ring. This class of compounds is called p-xylyl-enediamines. There are several other categories of CXCR4 antagonists, shown in Figures 5, 6 and 7, including: cyclic pentapeptide-based antagonists, indole based antagonists, quinoline-based antagonists, pyrimidine-based antagonists and tetrahydroquinoline-based antagonists;\textsuperscript{92} however, this work focuses only on the p-xylyl-enediamines.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{tetrahydroquinoline.png}
\caption{General structure for tetrahydroquinoline-based CXCR4 antagonists.\textsuperscript{92}}
\end{figure}
Figure 6: Examples of indole-based,\textsuperscript{115} cyclic pentapeptide-based,\textsuperscript{116} quinoline-based,\textsuperscript{117} and pyrimidine-based CXCR4 antagonists.\textsuperscript{118}

![Indole-based antagonist (39)](image1)

![Cyclic pentapeptide-based antagonist (FC131)](image2)

![Quinoline-based antagonist (213)](image3)

![Pyrimidine-based antagonist (218)](image4)

Figure 7: General Structure for p-xylyl-enediamine-based CXCR4 antagonists.

2.1.4.1 AMD3100 and p-xylyl-enediamines

One of the first small molecule inhibitors of CXCR4 to show promise was AMD3100.\textsuperscript{64} The precursor to this compound was discovered as an impurity that showed anti-HIV activity by blocking CXCR4, which is one of the receptors HIV-1 uses to enter the cell. This impurity was composed of two cyclam rings connected by an aliphatic chain. Replacing the aliphatic linker with a benzene ring increased the potency of the compound—resulting in AMD3100 (Figure 8).\textsuperscript{119} Other non-aromatic ring derivatives of AMD3100 were synthesized and tested for activity, but only the compounds featuring benzene rings were active.\textsuperscript{120}
AMD3100 was the first FDA approved CXCR4 antagonist; however, even though the initial purpose for this compound was to block HIV-1 entry into the cells, this drug was not approved for this purpose.\textsuperscript{121} In addition to poor oral bioavailability,\textsuperscript{122-123} AMD3100 is cardiotoxic.\textsuperscript{122,124} The FDA approved AMD3100 for limited use in patients with multiple myeloma in order to mobilize and harvest stem cells.\textsuperscript{119,125-126}

It was speculated that the toxicity and poor oral bioavailability of AMD3100 stemmed from the bicyclam rings. To verify this, new analogues were designed and synthesized in which the bicyclams rings were replaced. These studies led to the synthesis of a potent p-xylyl-enediamines derivate, WZ811 (\textbf{Figure 9}).\textsuperscript{127} There are several other categories of CXCR4 antagonists including: cyclic pentapeptide-based antagonists, indole based antagonists, and tetrahydroquinoline-based antagonists.\textsuperscript{92} Of these classes, several modulators are in clinical trials,\textsuperscript{92} where a majority of them are peptide based.\textsuperscript{128} Many of the p-xylyl-enediamine compounds have shown CXCR4 activity but very few have gone on to clinical trials primarily due to toxicity or bioavailability issues.\textsuperscript{92} Various substituents and side chains have been analyzed in the p-xylyl-enediamine class of compounds and have been screened for CXCR4 activity. The cyclams and bicyclams are among the most studied in this group;\textsuperscript{127} however, there has not been robust structure-activity relationship (SAR) studies in which the central phenyl ring has been altered to another aromatic ring.

\textbf{2.2 Rationale for Design}

AMD3100 and other p-xylyl-enediamines are structurally specific drugs, drugs that interact at a specific site. Changes to the structure of a lead compound can affect the biological activity of such a type of drug.\textsuperscript{129} Using this information, it is possible to map out the relation
between structure of a compound and activity of a compound by making small changes to a lead compound—called Structure Activity Relationship (SAR)—and draw conclusions about the active site where the compound is interacting.

Figure 8: Progression of AMD3100 from JM1498

It was this process that lead to the development of AMD3100 from JM1498 (Figure 8). JM1657 was discovered as an HIV inhibitor through screening several commercially available cyclam rings. JM1657 was found to be an impurity from synthesizing JM1498 and was then characterized. A series of small modifications were made to the linker between the two cyclam rings and the lead out of the compound was a benzene ring, connected to the cyclam rings at the 1 and 4 position by a methylene, JM2987 (also known as AMD3100).

AMD3100 was the first CXCR4 antagonist to become FDA approved; however, it was for one time use to treat multiple myeloma—not HIV. The compound was cardio-toxic. AMD3100 was still a good lead to branch off for more SAR studies to mitigate these negative effects. This gave birth to the class of CXCR4 antagonists called p-xylyl-enediamines. The central benzene ring was preserved, and at the 1 and 4 positions, different substituents were used to replace the cyclam rings. The active compounds in this class often have benzene-based, pyridine-based or pyrimidine-based side chains replacing one or more of the cyclam rings. Two forerunners out of this class of compound (Figure 9), were WZ811 (where the cyclam rings have
been replaced with a 2-aminopyridinyl group)\textsuperscript{130} and MSX122 (where the cyclam rings have been replaced with a 2-aminopyrimidinyl group).\textsuperscript{131}

![Chemical Structures]

**Figure 9:** Progression from AMD3100 to MSX122

The p-xylyl-enediamine compounds which removed the cyclam rings have eliminated the toxicity issue due to metal chelation;\textsuperscript{130} however, only MSX-122 has shown promising oral bioavailability and has made it to clinical trials but it suspended for undisclosed reasons in 2008.\textsuperscript{92} Many of the other analogues, including WZ811 are not orally bioavailable and have a short half-life in the body.\textsuperscript{120}

In designing a new class of compounds using WZ811 and MSX122 as leads to make a more orally bioavailable analogue, the qualities that make a compound orally bioavailable must be examined. Lipinski’s rule of five\textsuperscript{132-134} states that compounds with two or more of the following traits are likely to be poorly orally bioavailable: molecular weights over 500 g/mol, more than five hydrogen bond donors, more than ten hydrogen bond acceptors and a log P value greater than 5. Naturally, there are exceptions to this rule and more detailed parameters have been outlined to account for the exceptions by Ghose et al. Ghose et al. suggests that a log P value between -0.4 and 5.6 and a molecular weight between 180 g/mol and 500 g/mol increase drug-likeliness.\textsuperscript{135} When examining WZ811 and MSX122 by each of these parameters, they fall well within the parameters of rules of five. Both leads have molecular weights above 180 g/mol and below 500 g/mol,
Hydrogen bond donors are defined as the sum of hydroxyl and amino groups. Both leads have two each and are again, well within Lipinski’s range. Hydrogen bond acceptors are defined as the sum nitrogen and oxygen atoms. WZ811 and MSX122 have four and six, respectively. The last parameter is a log P value between -0.4 and 5.6 as modified by Ghose et al. Log P is the partition coefficient which is a measure how lipophilic a molecule is and is calculated by talking the log of the solubility of the molecule in 1-octanol over the solubility of the compound in water. For WZ811 and MSX122, the calculated log P values, using Molinspiration, were 2.65 and 1.65 respectively.

Since both compounds fall within the range of these rules, it is possible that the lower log P for MSX122 contributes to its oral bioavailability. In the structure of the p-xylyl-enediamines, the common motif is the central benzene ring. In previous studies, benzene ring has been altered to aliphatic rings and aliphatic chains and both resulted in a loss of activity; however, a 2,6-pyridine analogue and a 3,5-pyridine analogue based on AMD3100 were synthesized. They were not as active as AMD3100, but they were still active. Pyridine and other heterocyclic aromatic rings such as pyrazine, furan, and thiophene have lower log P values than benzene as shown in the Table 1 below.

<table>
<thead>
<tr>
<th>Ring</th>
<th>Structure</th>
<th>Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td><img src="image" alt="Benzene" /></td>
<td>2.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyridine</td>
<td><img src="image" alt="Pyridine" /></td>
<td>0.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pyrazine</td>
<td><img src="image" alt="Pyrazine" /></td>
<td>-0.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Analogues that change the linker from benzene to other heterocyclic aromatic rings following the p-xylyl-enediamine motif may retain activity while lowering the log P value. It is these such analogues that were synthesized in this work (Figure 10). The potential of lowering the log P value combined with the frequent use of pyridines, thiophenes, and furans and pyrazines in medicine, set a precedence for this work. This work will focus particularly on antimetastatic activity and anti-inflammatory activity of these analogues since HIV-1 binds differently than CXCL12, and the analogues synthesized are generally too small to block HIV-1 as a coreceptor.
Figure 10: Progression from AMD3100 to the compounds synthesized and analyzed in this work. The 2,5-thiophene series was synthesized by Francisco Garcia.
2.3 Results and Discussion

2.3.1 Chemistry

2.3.1.1 2,6-Pyridine analogues

The 2,6-pyridine analogues (2a-x) were synthesized via a reductive amination reaction between 2,6-pyridinedicarbaldehyde (1) and a substituted amine.

\[
\begin{align*}
\text{O} & \text{N} \\
\text{O} & \text{N} \\
\end{align*}
\]

R\(^1\) = H

R\(^2\) = \text{NH} \text{X}

2a: H \quad 2f: 3-Et \quad 2k: 2-Cl \quad 2p: 4-OMe \quad 2u: 3-NO\(_2\)
2b: 2-Me \quad 2g: 4-Et \quad 2l: 3-Cl \quad 2q: 2-CF\(_3\) \quad 2v: 4-NO\(_2\)
2c: 3-Me \quad 2h: 2-F \quad 2m: 4-Cl \quad 2r: 3-CF\(_3\)
2d: 4-Me \quad 2l: 3-F \quad 2n: 2-OMe \quad 2s: 4-CF\(_3\)
2e: 2-Et \quad 2j: 4-F \quad 2o: 3-OMe \quad 2t: 4-SMe
2w: \quad 2x:
**Scheme 1**: Synthesis of 2,6-pyridine analogues.

**2.3.1.2 2,5-Furan analogues**

The 2,5-furan analogues (4a-u) were synthesized via a reductive amination reaction between 2,5-furandicarbaldehyde (3) and a substituted amine.

![Scheme 2: Synthesis of 2,5-furan analogues.](image-url)
2.3.1.3 3,4-Thiophene analogues

Synthesized by reducing the 3,4-thiophenedicarboxylic acid (5) to an alcohol using diisobutylaluminum hydride. The resulting dialcohol (6) was quenched and isolated before it was oxidized over manganese dioxide to give the dicarbaldehyde product (7).

Scheme 3: Synthesis of 3,4-thiophenedicarbaldehyde.

The 3,4-thiophene analogues (8a-j) were synthesized via a reductive amination reaction between 3,4-thiophenedicarbaldehyde (7) and a substituted amine (Scheme 4).

Scheme 4: Synthesis of 3,4-thiophene analogues.
In synthesizing the 3,4-thiophene analogues, the bis(amino) product is not the major product. In this reductive amination, when one of the aldehydes reacts with the amine, the same amine will undergo a second, intramolecular, reductive amination leading to compound 8 shown above in Scheme 4. This compound is favorable because of the formation of the five-membered ring. This percent yield of the desired analogue, compound 8, was increased by breaking up the reaction into a two-step process where the imine is isolated, and then reduced; however, the cyclized compound is still the major product.

2.3.1.4 2,5-Pyrazine analogues

The 2,5-pyrazinedicarbaldehyde starting material was synthesized through a series of reactions starting from 2,5-dimethylpyrazine. These reactions are shown below in Scheme 5. The nitrogens on the pyrazine are oxidized under mCPBA. The resulting oxidized compound is refluxed in acetic anhydride to encourage a substitution of the anhydride followed by a rearrangement to the methyl group. The resulting compound (11) is then stirred in sodium hydroxide to yield the dialcohol product (12). This compound was then oxidized using manganese dioxide to yield the starting aldehyde.

Scheme 5: Synthesis of 2,5-pyrazinedicarbaldehyde.
The 2,5-pyrazine analogues (14a-d) were synthesized via a reductive amination reaction between 2,5-prazinedicarbaldehyde (13) and a substituted amine (Scheme 6).

Scheme 6: Synthesis of 2,5-pyrazine analogues.

2.3.2 Biology

All biological assays were completed by our collaborators at Emory University in Dr. Hyunsuk Shim’s laboratory at the Winship Cancer Institute.

2.3.2.1 Binding Assay

The derivatives were tested in a semi-quantitative binding affinity assay. It is important to emphasize that this assay is used as a preliminary screen for potential CXCR4 antagonists that will warrant further testing. The MDA-MB-231 breast cancer cells are incubated with the compounds at 1 nM, 10 nM, 100 nM and 1000 nM concentrations. Next, the cells are incubated with a biotinylated peptide, TN14003 (a known CXCR4 inhibitor), followed by streptavidin-rhodamine. The fluorescence of the cells is then measured to obtain effective concentration (EC) is obtained.
EC is the lowest concentration of the compound where there is a significant reduction in fluorescence observed compared to the positive control. All compounds synthesized were screened using this binding assay. Compounds that scored at 100 nM and below were then subjected to the Matrigel invasion assay. **Figure 11** below shows examples of what the fluorescence looks like at different effective concentrations.

![Fluorescence images](image)

**Figure 11:** Reduction of inflammation observed for selected derivatives. 2i had an EC of 10 nM. 2c had an EC of 100 nM and 2r had an EC of 1000 nM.

### 2.3.2.2 Matrigel Invasion Assay

The Matrigel invasion assay is used as a functional assay to probe if the synthesized analogues can block CXCR4/CXCR12 mediated chemotaxis and invasion. This assay uses a special two chambered apparatus. MDA-MB-231 cells that have been incubated in 100 nM concentrations of the analogues are placed in the top chamber and CXCL12 is placed in the bottom
chamber as a chemoattractant. The partition between the two chambers is a Matrigel matrix that the MDA-MB-231 cells can pass through. The measurement gained in this assay is a percentage of inhibition of chemotaxis. When the assay is complete, the number of cells that migrated through the matrix is counted. The percent inhibition is the percentage of cells that were prevented from migrating compared to the negative control. The stronger the inhibitor, the fewer cells that pass through the membrane.

Only the compounds that showed promise in the binding assay (EC \( \leq \) 100 nm) were tested in the Matrigel invasion assay. The two compounds used as benchmarks were AMD3100 and WZ811. An invasion inhibition above 35% was favorable. The results for this assay have been consolidated with the results from the binding assay for each class of compound in Tables 2 through 6, where AMD3100 and WZ811 were used as benchmarks for comparison.

![Diagram](image)

<table>
<thead>
<tr>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion(^a)</th>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>aniline</td>
<td>100</td>
<td>&lt;1%</td>
<td>2m</td>
<td>4-Cl aniline</td>
<td>100</td>
<td>21%</td>
</tr>
<tr>
<td>2b</td>
<td>2-Me aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>2n</td>
<td>2-OMe aniline</td>
<td>1000</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>2c</td>
<td>3-Me aniline</td>
<td>100</td>
<td>60%</td>
<td>2o</td>
<td>3-OMe aniline</td>
<td>100</td>
<td>37%</td>
</tr>
<tr>
<td>2d</td>
<td>4-Me aniline</td>
<td>1000</td>
<td>--</td>
<td>2p</td>
<td>4-OMe aniline</td>
<td>100</td>
<td>5%</td>
</tr>
<tr>
<td>2e</td>
<td>2-Et aniline</td>
<td>1</td>
<td>59%</td>
<td>2q</td>
<td>2-CF(_3) aniline</td>
<td>&gt;1000</td>
<td>--</td>
</tr>
<tr>
<td>2f</td>
<td>3-Et aniline</td>
<td>1000</td>
<td>--</td>
<td>2r</td>
<td>3-CF(_3) aniline</td>
<td>1000</td>
<td>15%</td>
</tr>
<tr>
<td>2g</td>
<td>4-Et aniline</td>
<td>1</td>
<td>64%</td>
<td>2s</td>
<td>4-CF(_3) aniline</td>
<td>1</td>
<td>52%</td>
</tr>
</tbody>
</table>
In the pyridine analogues (Table 2), eight performed well in both assays. Six of these eight analogues contained aniline-based side chains and the other two were the morpholino analogue (2w), and the pyrrolidinyl analogue (2x) which had binding assay results of 10 nM and 1 nM respectively, and invasion assay inhibition of 63% and 58% respectively. Out of these compounds, it seems that analogues with electron withdrawing groups as side chains are not favoured; the nitro compounds did not have activity and the chloro compounds did not have favourable activity. The exception is in the trifluoromethyl family, where none of the analogues were active except for the 4-CF₃ anilino analogue (2s); where the binding assay concentration was 1 nM and the invasion inhibition was 52%. Perhaps the CF₃ groups in the para position has a favourable interaction in the pocket that wasn’t favourable for the 4-Me aniline (2d)—as they would take up nearly the same amount of space. In silico analyses would be required to gain insight into this.

The 3-Fluoro aniline analogue (2i) showed favourable activity with a binding of 10 nM and an invasion inhibition of 50%. Halogens are weakly electron withdrawing and have lone pairs that may be used for donation. This could be another reason why the chloro anilino analogues had

| 2h  | 2-F aniline | 1000 | -- | 2t | 4-SMe aniline | >1000 | -- |
| 2i  | 3-F aniline | 10   | 50% | 2u | 3-NO₂ aniline | >1000 | -- |
| 2j  | 4-F aniline | 100  | 5%  | 2v | 4-NO₂ aniline | >1000 | -- |
| 2k  | 2-Cl aniline | >1000 | -- | 2w | Morpholine | 10   | 63% |
| 2l  | 3-Cl aniline | 100  | 34% | 2x | Pyrrolidine | 1    | 58% |

AMD3100₁³¹, ₁⁴⁷ --- 1000 62%

WZ811₁²⁷ --- 10 90%

Table 2: Binding and invasion assay results for pyridine analogues synthesized. *The invasion assay concentration used for all compounds tested was 100nM.
some activity but none favourable. This could also be why this fluoro analogue had favourable activity. Of the methyl analogues, only the 3-Methyl aniline (2c) had favourable activity with an effective concentration of 100 nM and an invasion inhibition of 60%. The 2-Ethyl (2e) and 4-Ethyl (2g) aniline analogues showed promising activity with effective concentrations of 1 nM for both and invasion assay inhibitions of 59% and 64% respectively. Lastly, the 3-Methoxy aniline (2o) showed favourable activity with an effective concentration of 100 nM and an invasion inhibition of 37%. It is interesting that this analogue had some activity were the 3-Ethyl (2f) did not. It could be possible that the lone pairs on the oxygen are able to interact with residues or solvent better in the active site compared to the 3-Ethyl aniline analogue. Where the ortho and para position ethyl groups have hydrophobic interactions at those positions.

<table>
<thead>
<tr>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion⁴</th>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>Aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>4l</td>
<td>3-Cl aniline</td>
<td>&gt;1000</td>
<td>--</td>
</tr>
<tr>
<td>4b</td>
<td>2-Me aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>4m</td>
<td>4-Cl aniline</td>
<td>10</td>
<td>48%</td>
</tr>
<tr>
<td>4c</td>
<td>3-Me aniline</td>
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<td>4n</td>
<td>2-OMe aniline</td>
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<td>5%</td>
</tr>
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<td>4o</td>
<td>3-OMe aniline</td>
<td>&gt;1000</td>
<td>--</td>
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<td>2-Et aniline</td>
<td>1000</td>
<td>--</td>
<td>4p</td>
<td>4-OMe aniline</td>
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<td>--</td>
</tr>
<tr>
<td>4f</td>
<td>3-Et aniline</td>
<td>&gt;1000</td>
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<td>4q</td>
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<td>4-Et aniline</td>
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<td>4r</td>
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<td>10</td>
<td>71%</td>
<td>4s</td>
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<td>--</td>
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<td>4i</td>
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<td>4t</td>
<td>4-SMe aniline</td>
<td>&gt;1000</td>
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<td>Compound</td>
<td>Functional Group</td>
<td>Inhibition</td>
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<td>----------</td>
<td>------------------</td>
<td>------------</td>
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<td><strong>4j</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>AMD3100</td>
<td>---</td>
<td>1000</td>
<td>62%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WZ811</td>
<td>---</td>
<td>10</td>
<td>90%</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Table 3:** Binding and invasion assay results for furan analogues synthesized. *The invasion assay concentration used for all compounds tested was 100nM.

Of the 2,5-furan analogues synthesized *(Table 3)*, five analogues performed well. First was the 4-Methyl aniline *(4d)*, which had an effective concentration of 100 nM and an invasion inhibition of 75%. The 2-Fluoro *(4h)* and 4-Fluro *(4j)* aniline derivatives had effective concentrations of 10 and 100 nM respectively and invasion inhibition of 71% and 53% respectively. The 4-Chloro aniline analogue *(4m)* had an effective concentration of 10 nM and invasion inhibition of 48%. The 1-methylpiperazine analogue *(4u)* had an effective concentration of 10 nM and inhibited 82% of metastatic cells from invading—the highest percentage of any of the other compounds synthesized in this work.

It is interesting to note for the 2,5-furanoaniline-based compounds the only compounds that showed activity were compounds with ortho and para substitutions. Overall, it seems that weakly electron donating groups like methyl and weakly electron withdrawing groups like the fluorine and chlorine had the most activity. The 2-CF$_3$ analogue *(4q)* had some activity with an effective concentration of 10 nM and an invasion inhibition of 24%; however, it is not enough for it to pass on to the paw edema test. All other 2,5-furan based compounds had no significant activity.
<table>
<thead>
<tr>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion(^a)</th>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a</td>
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<td>72%</td>
<td>8f</td>
<td>4-F aniline</td>
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</tr>
<tr>
<td>8b</td>
<td>3-Me aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>8g</td>
<td>3-Cl aniline</td>
<td>&gt;1000</td>
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</tr>
<tr>
<td>8c</td>
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</tr>
<tr>
<td>8d</td>
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<td>&gt;1000</td>
<td>--</td>
<td>8i</td>
<td>3-CF(_3) aniline</td>
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<td>--</td>
</tr>
<tr>
<td>8e</td>
<td>3-F aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>8j</td>
<td>4-CF(_3) aniline</td>
<td>&gt;1000</td>
<td>--</td>
</tr>
<tr>
<td>AMD3100(^{131})</td>
<td>---</td>
<td>1000</td>
<td>62%</td>
<td>WZ811(^{127})</td>
<td>---</td>
<td>10</td>
<td>90%</td>
</tr>
</tbody>
</table>

Table 4: Binding and invasion assay results for the 3,4-Thiophene analogues synthesized. \(^a\)The invasion assay concentration used for all compounds tested was 100nM.

Of the 3,4-thiophene analogues (Table 4), only the anilino analogue (8a) showed favourable activity with an effective concentration of 10 nM and an invasion inhibition of 72%. All other compounds had an effective concentration over 1000 nM and did not show significant activity.
Table 5: Binding and invasion assay results for pyrazine analogues synthesized. *The invasion assay concentration used for all compounds tested was 100nM.

Of the 2,5-pyrazine analogues (Table 5), only the 4-Fluoro anilino analogue (14d) exhibited favorable activity with an effective concentration of 10 nM and an invasion inhibition of 76%. Since only four analogues of this series have been synthesized, there is not enough data to speculate trends.

<table>
<thead>
<tr>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion*</th>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>15a</td>
<td>aniline</td>
<td>1</td>
<td>68%</td>
<td>15n</td>
<td>2-OMe aniline</td>
<td>10</td>
<td>52%</td>
</tr>
<tr>
<td>15b</td>
<td>2-Me aniline</td>
<td>10</td>
<td>61%</td>
<td>15o</td>
<td>3-OMe aniline</td>
<td>1000</td>
<td>--</td>
</tr>
<tr>
<td>15c</td>
<td>3-Me aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>15p</td>
<td>4-OMe aniline</td>
<td>10</td>
<td>84%</td>
</tr>
<tr>
<td>15d</td>
<td>4-Me aniline</td>
<td>100</td>
<td>49%</td>
<td>15q</td>
<td>2-CF3 aniline</td>
<td>&gt;1000</td>
<td>--</td>
</tr>
<tr>
<td>15e</td>
<td>2-Et aniline</td>
<td>1000</td>
<td>--</td>
<td>15r</td>
<td>3-CF3 aniline</td>
<td>100</td>
<td>77%</td>
</tr>
<tr>
<td>15f</td>
<td>3-Et aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>15s</td>
<td>4-CF3 aniline</td>
<td>&gt;1000</td>
<td>--</td>
</tr>
<tr>
<td>15g</td>
<td>4-Et aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>15t</td>
<td>Morpholine</td>
<td>&gt;1000</td>
<td>--</td>
</tr>
<tr>
<td>15h</td>
<td>2-F aniline</td>
<td>1000</td>
<td>--</td>
<td>15u</td>
<td>Thiomorpholine</td>
<td>1</td>
<td>88%</td>
</tr>
<tr>
<td>15i</td>
<td>3-F aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>15v</td>
<td>Piperidine</td>
<td>1000</td>
<td>--</td>
</tr>
<tr>
<td>15j</td>
<td>4-F aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>15w</td>
<td>4-CF3 Benzylamine</td>
<td>1</td>
<td>97%</td>
</tr>
<tr>
<td>15k</td>
<td>2-Cl aniline</td>
<td>10</td>
<td>93%</td>
<td>15x</td>
<td>3-F Benzylamine</td>
<td>&gt;1000</td>
<td>--</td>
</tr>
<tr>
<td>15l</td>
<td>3-Cl aniline</td>
<td>100</td>
<td>51%</td>
<td>15y</td>
<td>4-Cl Benzylamine</td>
<td>&gt;1000</td>
<td>--</td>
</tr>
</tbody>
</table>
Table 6: 2,5-Thiophene analogues synthesized by Francisco Garcia\textsuperscript{148} which will be analyzed in the discussion section of this work as well as \textit{in silico}.

<table>
<thead>
<tr>
<th></th>
<th>Substituent</th>
<th>Activity</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>15m</td>
<td>4-Cl aniline</td>
<td>1</td>
<td>22%</td>
</tr>
<tr>
<td>AMD3100\textsuperscript{131,147}</td>
<td>---</td>
<td>1000</td>
<td>62%</td>
</tr>
<tr>
<td>WZ811\textsuperscript{127}</td>
<td>---</td>
<td>10</td>
<td>90%</td>
</tr>
</tbody>
</table>

A detailed summary of the 2,5-thiophene compound’s synthesis and trends can be found in Francisco Garcia’s thesis;\textsuperscript{148} however, the most direct and straightforward comparison to be made is between the 2,5-thiophene analogues and the 2-5-furan analogues. Unlike the furan aniline-based compounds which only displayed activity for weakly electron donating and weakly electron withdrawing groups, the thiophene derivatives seem to show more activity across the board. None of the thiophene fluoroaniline analogues had activity, where the furan derivatives had activity with the ortho and para fluoroaniline sidechains. The thiophene chloroaniline derivatives also had activity where only one of the furan-based chlorine substituents had activity. It is possible that the electronics of the 2,5-thiophene are such that those derivatives are able to interact better with the residues in the active site compared to the 2,5-furan. It’s also possible that the size difference between the 2,5-thiophene and the 2,5-furan contribute to the difference between the two.

Between the 3,4-thiophene and the 2,5-thiophene, it is clear that the 3,4 positioning leads to loss of activity. It’s possible that the 2,5-thiophene compounds are able to fit better into the active site because of the geometry of the analogues. This would need to be explored further \textit{in silico}.

Overall, there are no strong motifs for substituents across the different core molecules. Both the pyridine and 2,5-thiophene analogues had a variety of substituents that had activity in the
binding and Matrigel invasion assays with no clear pattern. It is worth noting that even though the 2,6-pyridine morpholino analogue (2w) had activity, the 2,5-thiophene morpholino analogue (15t) did not. Instead, the 2,5-thiophene thiomorpholino derivative (15u) had activity. Compound 4u, which also had a heterocyclic substituent, 1-methylpiperazine, follows in this trend and had significant activity. Further probing would need to be done in synthesizing more derivatives as well as in silico analysis to paint a bigger picture and complete this library.

### 2.3.2.3 In vivo carrageenan suppression

The mouse paw edema model is used as a proof-of-concept test. If these analogues are able to disrupt the CXCR4-CXCL12 interaction, then it also has an effect on inflammation. If inflammation were to be induced in the presence of these compounds, a reduction in said inflammation would be observed for potent CXCR4 antagonists. All compounds that performed well in both the binding assay and Matrigel invasion assay were submitted for the paw edema test. This test is also a way to gain insight into the toxicity of our compounds. None of the mice that were given the synthesized analogues died during this test.

In this test, the hind paws of mice are inflamed using carrageenan, and one paw is treated with a solution of the analogue (10 mg of analogue per kg), and the other is treated with a saline solution. Both the saline solution and analogue solution are delivered via injection into the paw. The paws are then measured at the end of the test using calipers to determine the percent reduction in swelling. Compounds that score 100 nM or below in the binding assay and above a 35% in the invasion assay were considered hit compounds and were submitted for further analysis in the paw edema test. Of the compounds synthesized, fifteen qualified for the mouse paw edema test (shown in Table 7).
<table>
<thead>
<tr>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Carageenan&lt;sup&gt;b,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2c</td>
<td>3-Me aniline</td>
<td>100</td>
<td>60%</td>
<td>20%</td>
</tr>
<tr>
<td>2e</td>
<td>2-Et aniline</td>
<td>1</td>
<td>59%</td>
<td>18%</td>
</tr>
<tr>
<td>2g</td>
<td>4-Et aniline</td>
<td>1</td>
<td>64%</td>
<td>42%</td>
</tr>
<tr>
<td>2i</td>
<td>3-F aniline</td>
<td>10</td>
<td>50%</td>
<td>20%</td>
</tr>
<tr>
<td>2o</td>
<td>3-OMe aniline</td>
<td>100</td>
<td>37%</td>
<td>20%</td>
</tr>
<tr>
<td>2s</td>
<td>4-CF&lt;sub&gt;3&lt;/sub&gt; aniline</td>
<td>1</td>
<td>52%</td>
<td>20%</td>
</tr>
<tr>
<td>2w</td>
<td>Morpholine</td>
<td>10</td>
<td>63%</td>
<td>39%</td>
</tr>
<tr>
<td>2x</td>
<td>Pyrrolidine</td>
<td>1</td>
<td>58%</td>
<td>--</td>
</tr>
<tr>
<td>4d</td>
<td>4-Me aniline</td>
<td>100</td>
<td>75%</td>
<td>15%</td>
</tr>
<tr>
<td>4h</td>
<td>2-F aniline</td>
<td>10</td>
<td>71%</td>
<td>--</td>
</tr>
<tr>
<td>4j</td>
<td>4-F aniline</td>
<td>100</td>
<td>53%</td>
<td>31%</td>
</tr>
<tr>
<td>4m</td>
<td>4-Cl aniline</td>
<td>10</td>
<td>48%</td>
<td>17%</td>
</tr>
<tr>
<td>4u</td>
<td>1-Methylpiperazine</td>
<td>10</td>
<td>82%</td>
<td>--</td>
</tr>
<tr>
<td>8a</td>
<td>Aniline</td>
<td>10</td>
<td>72%</td>
<td>--</td>
</tr>
<tr>
<td>14d</td>
<td>4-F aniline</td>
<td>10</td>
<td>76%</td>
<td>32%</td>
</tr>
<tr>
<td>AMD3100&lt;sup&gt;131, 147&lt;/sup&gt;</td>
<td></td>
<td>1000</td>
<td>62%</td>
<td>--</td>
</tr>
<tr>
<td>WZ811&lt;sup&gt;127&lt;/sup&gt;</td>
<td></td>
<td>10</td>
<td>90%</td>
<td>40%</td>
</tr>
</tbody>
</table>
Table 7: Assay and test results for hit compounds. \textsuperscript{a}The concentration used in the invasion assay was 100 nM. \textsuperscript{b}Mice were dosed used 10 mg of compound for every kg the mouse weighed in this assay. \textsuperscript{c}Compounds with a dash in the carrageenan studies have not yet been submitted.

WZ811 is used as the benchmark in this assay, where paw edema test, WZ811 reduced inflammation by 40% compared to the control. Two compounds, the 4-Ethyl pyridine (2g) and the morpholino pyridine (2w) reduced inflammation on par with WZ811 at 42% and 39% respectively. The para fluoro furan (4j) ands pyrazine derivative (14d) both follow close behind with inflammation reduction of 31% and 32% respectively.

The tissue slice shown in Figure 12 was taken from a mouse paw that was treated with the 3-fluoro pyridine-based analogue 2i. Even though 2i did not perform the best out of the compounds selected for the carrageenan study (20%), these tissue slices show that even though the reduction in swelling was smaller, these compounds are still mitigating the inflammatory response in the inflamed tissue—where the inflamed dermal papilla is returning to its normal curved and spiked shape compared to the inflamed shape where it becomes flat and smooth.

In light of this, it is worth noting that several compounds reduced swelling in the range of 2i. The 3-methyl pyridine derivative (2c), reduced swelling by 20%. The 3-methoxy pyridine (2o) reduced inflammation by 20%. The para trifluoromethyl pyridine analogue (2s) reduced swelling by 20%. Since tissue slices were not taken for each of these compounds, it could be a stretch to assume that all of them are showing this activity in the tissue; however, it is promising to know that some compounds that don’t reduce swelling as dramatically are restoring normal tissue shape.
Figure 12: Histological assay of compound 2i. Paw tissue sections were stained with H&E. The whole tissue slices were scanned/digitized by NanoZoomer 2.0 HT. Software NDP.view 2 was used to zoom in.

The other remaining compounds, did not have a reduction in swelling above 20% compared to the control, but some swelling reduction was observed. There is no clear relation between low effective concentration, high invasion inhibition and inflammation reduction. Several compounds that scored well in the preliminary assays showed lower edema reduction. 2e and 4d are prime examples of this, it could be possible that these compounds do not completely block CXCR4 and CXCL12 is still able to interact with the receptor to trigger inflammation. Bioavailability may be an issue as these analogues might not stay in circulation long enough to mitigate inflammation before they are flushed out by the body. Further studies would be needed to determine the
pharmacokinetic properties and discern if this is an issue. There are remaining compounds that need to be submitted for paw edema analysis; however, the data so far lays a good foundation for analysis and future plans for this work.

2.4 Summary of Analogues Designed and Synthesized

The compounds shown in Table 8 below show the hit compounds from this work and Francisco Garcia’s work. Very few of the 2,5-thiophene compounds synthesized have been submitted for the paw edema test, so it is difficult to make a complete analysis or comparison in that regard; however, out of all the five core molecules that have been synthesized, the 2,5-thiophenes have had the most active compounds with the best invasion assay inhibitions, where compounds 15k, 15p, 15u and 15w all inhibited upwards of 80% of had invasion assay results that high. In the pyridine analogues, the 3-methyl (2c), 4-ethyl (2g), and the morpholino (2w) analogues all had invasion assay results above 60% inhibition. The 2,5-furan (4d, 4h and 4u) and the 3,4-thiophene series (8a) both had derivatives with invasion assay inhibition over 70%.

Complete inhibition of invasion—and by extension, the CXCR4/CXCL12 interaction is undesirable as activity is necessary for normal cell function; however, in probing what about these 2,5-thiophene analogues enables it to block this interaction and prevent metastasis, future analogues could be used as a lead to further tweak to be a suitable antagonist.
<table>
<thead>
<tr>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion(^a)</th>
<th>Carageenan(^{b,c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2c</td>
<td>3-Me aniline</td>
<td>100</td>
<td>60%</td>
<td>20%</td>
</tr>
<tr>
<td>2e</td>
<td>2-Et aniline</td>
<td>1</td>
<td>59%</td>
<td>18%</td>
</tr>
<tr>
<td>2g</td>
<td>4-Et aniline</td>
<td>1</td>
<td>64%</td>
<td>42%</td>
</tr>
<tr>
<td>2i</td>
<td>3-F aniline</td>
<td>10</td>
<td>50%</td>
<td>20%</td>
</tr>
<tr>
<td>2o</td>
<td>3-OMe aniline</td>
<td>100</td>
<td>37%</td>
<td>20%</td>
</tr>
<tr>
<td>2s</td>
<td>4-CF(_3) aniline</td>
<td>1</td>
<td>52%</td>
<td>20%</td>
</tr>
<tr>
<td>2w</td>
<td>Morpholine</td>
<td>10</td>
<td>63%</td>
<td>39%</td>
</tr>
<tr>
<td>2x</td>
<td>Pyrrolidine</td>
<td>1</td>
<td>58%</td>
<td>--</td>
</tr>
<tr>
<td>4d</td>
<td>4-Me aniline</td>
<td>100</td>
<td>75%</td>
<td>15%</td>
</tr>
<tr>
<td>4h</td>
<td>2-F aniline</td>
<td>10</td>
<td>71%</td>
<td>--</td>
</tr>
<tr>
<td>4j</td>
<td>4-F aniline</td>
<td>100</td>
<td>53%</td>
<td>31%</td>
</tr>
<tr>
<td>4m</td>
<td>4-Cl aniline</td>
<td>10</td>
<td>48%</td>
<td>17%</td>
</tr>
<tr>
<td>4u</td>
<td>1-Methylpiperazine</td>
<td>10</td>
<td>82%</td>
<td>--</td>
</tr>
<tr>
<td>8a</td>
<td>Aniline</td>
<td>10</td>
<td>72%</td>
<td>--</td>
</tr>
<tr>
<td>14d</td>
<td>4-F aniline</td>
<td>10</td>
<td>76%</td>
<td>32%</td>
</tr>
<tr>
<td>15a(^d)</td>
<td>Aniline</td>
<td>1</td>
<td>68%</td>
<td>30%</td>
</tr>
<tr>
<td>15b(^d)</td>
<td>2-Me aniline</td>
<td>10</td>
<td>61%</td>
<td>--</td>
</tr>
<tr>
<td>15d(^d)</td>
<td>4-Me aniline</td>
<td>100</td>
<td>49%</td>
<td>--</td>
</tr>
<tr>
<td>15k(^d)</td>
<td>2-Cl aniline</td>
<td>10</td>
<td>93%</td>
<td>&gt;5%</td>
</tr>
<tr>
<td>15l(^d)</td>
<td>3-Cl aniline</td>
<td>100</td>
<td>51%</td>
<td>--</td>
</tr>
<tr>
<td>15n(^d)</td>
<td>2-OMe aniline</td>
<td>10</td>
<td>52%</td>
<td>--</td>
</tr>
</tbody>
</table>
Table 8: Assay and test results for hit compounds. 

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Inhib.</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>15p^d</td>
<td>4-OMe aniline</td>
<td>10</td>
<td>84%</td>
</tr>
<tr>
<td>15r^d</td>
<td>3-CF$_3$ aniline</td>
<td>100</td>
<td>77%</td>
</tr>
<tr>
<td>15u^d</td>
<td>Thiomorpholine</td>
<td>1</td>
<td>88%</td>
</tr>
<tr>
<td>15w^d</td>
<td>4-CF$_3$ Benzylamine</td>
<td>1</td>
<td>97%</td>
</tr>
<tr>
<td>AMD3100$^{131,147}$</td>
<td></td>
<td>1000</td>
<td>62%</td>
</tr>
<tr>
<td>WZ811$^{127}$</td>
<td>10</td>
<td>90%</td>
<td>40%</td>
</tr>
</tbody>
</table>

Table 8: Assay and test results for hit compounds. 

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc.</th>
<th>Inhib.</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>15p^d</td>
<td>4-OMe aniline</td>
<td>10</td>
<td>84%</td>
</tr>
<tr>
<td>15r^d</td>
<td>3-CF$_3$ aniline</td>
<td>100</td>
<td>77%</td>
</tr>
<tr>
<td>15u^d</td>
<td>Thiomorpholine</td>
<td>1</td>
<td>88%</td>
</tr>
<tr>
<td>15w^d</td>
<td>4-CF$_3$ Benzylamine</td>
<td>1</td>
<td>97%</td>
</tr>
<tr>
<td>AMD3100$^{131,147}$</td>
<td></td>
<td>1000</td>
<td>62%</td>
</tr>
<tr>
<td>WZ811$^{127}$</td>
<td>10</td>
<td>90%</td>
<td>40%</td>
</tr>
</tbody>
</table>

**Even though the 2,5-thiophene series seem to perform well in the binding and invasion assay, the preliminary data from the paw edema test shows that they are not reducing inflammation in vivo as well as they prevented metastasis in vitro compared to the pyridine or furan-based analogues. Where 15w and 15k, had invasion scores of 93% and 97% respectively, they only reduced inflammation by 30% and 23% respectively. Compared to 2g which had invasion assay inhibition of 64% and an inflammation reduction of 42%, it seems that performance in the invasion assay is not a good predictor for the success of the paw edema test.**

**As mentioned in the rationale for design, desirable pharmacokinetic properties tend to increase as the partition coefficient of a potential drug is lowered. Both the pyridine and furan rings have a lower log P than thiophene. If the 2,5-thiophene analogue’s log P is not low enough, it may be flushed out of the body too quickly to effectively reduce swelling in this assay. If this trend continues as more and more 2,5-thiophene analogues are submitted for the paw edema test, using substituents that lower the overall partition coefficient may yield better results.**
Similarly, perhaps the pyridine compounds were able to do relatively well in the paw edema test because of the lower partition coefficient. Even though they did not inhibit invasion of more than 60% of the metastatic cells compared to the control, perhaps their results in the paw edema assay are consistently active because of their drug-likeness due to the lowered log P. This would need to be further evaluated in pharmacokinetic tests.

The 3,4-thiophene analogues overall were not active, with only the aniline derivative (8a) showing activity. This seems to suggest, that the geometry of the 2,5 positioned five membered rings may be favorable. This could be analyzed further by synthesizing 3,4-furan-based analogues for comparison. Since the log P of pyrazine is lower than all of the other heterocyclic aromatic rings, the pyrazine, further analogue synthesis should be conducted in order to complete this section of the library; however, the activity of the 4-fluoro pyrazine (14d) is a promising first look.

Overall, analogues 2g, 2w, 4j, 14d, and 15a are the five best compounds synthesized and analyzed so far with favorable effective concentrations, invasion inhibition properties and with inflammation reduction above 30% compared to the control.
3  DOCKING ANALYSIS

3.1  Schrödinger Small Molecule Suite

To gain insight as to how these analogues are interacting with the active site, *in silico* methods used. In addition to the analogues synthesized in this work, analysis for 2,5-thiophene (15a-y) derivatives synthesized by Francisco Garcia\textsuperscript{148} are also provided in this work. Preliminary docking analysis of several of the 2,6-pyridine compounds were conducted in a previous work by Dr. Davita Camp. Previous work has identified several key residues that can block the CXCR4-CXCL12 interaction. Some of these residues include ASP97, GLU288, ASP187, PHE87, ASP171, and PHE292, where the first three are required for signaling pathways triggered by CXCL12.\textsuperscript{57-58} Docking scores and key residue interactions for hit compounds are shown in Table 9.

<table>
<thead>
<tr>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Docking Score (kcal/mol)</th>
<th>Key Residue Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2c</td>
<td>3-Me aniline</td>
<td>100</td>
<td>-7.314</td>
<td>GLU288, TRP94, CYS186</td>
</tr>
<tr>
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<td>2-Et aniline</td>
<td>1</td>
<td>-5.944</td>
<td>HID113</td>
</tr>
<tr>
<td>2g</td>
<td>4-Et aniline</td>
<td>1</td>
<td>-5.716</td>
<td>TRP94, GLU288</td>
</tr>
<tr>
<td>2i</td>
<td>3-F aniline</td>
<td>10</td>
<td>-6.822</td>
<td>ASP97\textsuperscript{a}</td>
</tr>
<tr>
<td>2o</td>
<td>3-OMe aniline</td>
<td>100</td>
<td>-6.187</td>
<td>TRP94, ASP97\textsuperscript{a}</td>
</tr>
<tr>
<td>2s</td>
<td>4-CF\textsubscript{3} aniline</td>
<td>1</td>
<td>-6.026</td>
<td>TRP94, ASP97, HID113, CYS186</td>
</tr>
<tr>
<td>2w</td>
<td>Morpholine</td>
<td>10</td>
<td>-7.173</td>
<td>ASP97, HID113, TYR116</td>
</tr>
<tr>
<td>2x</td>
<td>Pyrrolidine</td>
<td>1</td>
<td>-6.400</td>
<td>ASP97, TYR116, CYS186</td>
</tr>
</tbody>
</table>
Table 9: In silico results for hit compounds suing the Schrödinger Small Molecule Suite. aThe compound has two or more interactions with this residue.

<table>
<thead>
<tr>
<th></th>
<th>Compound</th>
<th>Score</th>
<th>Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>4d</td>
<td>4-Me aniline</td>
<td>100</td>
<td>-4.809, HID113, ARG188, GLN200</td>
</tr>
<tr>
<td>4h</td>
<td>2-F aniline</td>
<td>10</td>
<td>-4.869, TRP94</td>
</tr>
<tr>
<td>4j</td>
<td>4-F aniline</td>
<td>100</td>
<td>-5.524, TRP94, CYS186, ARG188, GLU288</td>
</tr>
<tr>
<td>4m</td>
<td>4-Cl aniline</td>
<td>10</td>
<td>-5.300, HID113, CYS186</td>
</tr>
<tr>
<td>4u</td>
<td>1-Methylpiperazine</td>
<td>10</td>
<td>-6.373, TRP94, TYR116, GLU288</td>
</tr>
<tr>
<td>8a</td>
<td>Aniline</td>
<td>10</td>
<td>-3.437, ASP97</td>
</tr>
<tr>
<td>14d</td>
<td>4-F aniline</td>
<td>10</td>
<td>-4.600, ASP97</td>
</tr>
<tr>
<td>15a b</td>
<td>Aniline</td>
<td>1</td>
<td>-4.773, ASP97</td>
</tr>
<tr>
<td>15b b</td>
<td>2-Me aniline</td>
<td>10</td>
<td>-4.354, TRP94, HID113</td>
</tr>
<tr>
<td>15d b</td>
<td>4-Me aniline</td>
<td>100</td>
<td>-4.506, TRP94, CYS186</td>
</tr>
<tr>
<td>15k b</td>
<td>2-Cl aniline</td>
<td>10</td>
<td>-4.840, TRP94, ASP97</td>
</tr>
<tr>
<td>15l b</td>
<td>3-Cl aniline</td>
<td>100</td>
<td>-5.975, ASP97</td>
</tr>
<tr>
<td>15n b</td>
<td>2-OMe aniline</td>
<td>10</td>
<td>-5.409, ASP97</td>
</tr>
<tr>
<td>15p b</td>
<td>4-OMe aniline</td>
<td>10</td>
<td>-4.479, GLU32, ASP97, ARG183</td>
</tr>
<tr>
<td>15r b</td>
<td>3-CF₃ aniline</td>
<td>100</td>
<td>-5.944, TRP94, ASP97</td>
</tr>
<tr>
<td>15u b</td>
<td>Thiomorpholine</td>
<td>1</td>
<td>-3.782, TRP94, GLU288</td>
</tr>
</tbody>
</table>

Out of all the compounds docked, the hit pyridine compounds had the lowest docking scores and out of the hit compounds the pyridine-based compounds had the best scores. The Ligand Preparation wizard in the Schrödinger program created two different ionized states for each of the pyridine analogues. The first, where the central ring was deprotonated, and a second where the nitrogen in the central pyridine was protonated (Figure 13). The protonated pyridine compounds had lower docking scores compared to the deprotonated version.
Figure 13: Compound 2i is shown here with the central pyridine protonated.

Previous studies suggest that small molecules with a positively charged center can interact with better the acid residues in the active site. None of the other heterocyclic cores had an ionization where the center was protonated; however, certain substituents lead to protonation of the connecting amine. These substituents—morpholine, thiomorpholine and pyrrolidine have a generated ionization (Figure 14) where both amines are protonated creating a positive charge in the active site. These compounds also had a low docking score.
Figure 14: Protonated 1-methyl piperazine substituent in compound 4u. Pyrrolidine, morpholine and thiomorpholine substituents also had lower docking scores when protonated.

Most of the active compounds docked have key residue interactions with ASP97, or GLU288—two of the three residues mentioned earlier that are necessary for signaling. CYS186, TRP94, and TYR116, when docked are programmed to freely rotate to allow the ligands to interact via hydrogen bonding, pi-pi interactions or pi-cation interactions. Compound 2o, shown in Figure 15, displays pi-pi interactions with aromatic residues like TRP94, TYR116 and HID113.

It is worth noting that several ligands which were not active in the binding and Matrigel assays were docked with low scores. One such example is 2h, shown in Figure 16. This compound has a docking score of -6.237 kcal/mol and has key residue interactions with TRP94, ASP97, and HID113. 2h was not active in any of the assays, with an effective concentration of 1000 nM. This
was the same, for other inactive compounds with a charged ionization state, including charged substituents like morpholine.

**Figure 15:** Compound 2o is shown with pi-pi interactions with TRP94.

**Figure 16:** Compound 2h had a low docking score and key residue interactions with TRP 94, ASP97, and HID113 *in silico*; however, *in vivo* failed to show activity.
Similarly, there were several compounds that had low effective concentrations and high invasion inhibition *in vitro*, but obtained a low docking score *in silico*. Compound 15u (shown in Figure 17) had an effective concentration of 1 nM and inhibited invasion of metastatic cells by 88%. *In silico* results yielded a docking score of -3.782 kcal/mol even though it had a key residue interactions with GLU288. Overall, compared to the activity displayed in the assays, the 2,5-thiophene analogues received higher docking scores compared to their *in vitro* activity.

**Figure 17:** Compound 15u received a high docking score of -3.782 kcal/mol, contrary to promising scores the *in vitro* results in the binding and Matrigel invasion assays.

These discrepancies show that the docking model is not perfect; however, there are a few themes that can be taken away and used to direct future compounds synthesized. Positive charged ligands are favoured in the active site. All pyridine-based and most furan-based analogues with a positive charge scored below -5.000 kcal/mol. Synthesis of these analogues with non-aromatic cyclical amines and heterocyclic as substituents that can be protonated as secondary amines could yield active compounds.
Additionally, as some analogues with aromaticity are able to have pi-pi interactions with nearby residues, the use of substituted benzylamines, which have an extra sp3 hybridized carbon linking the substituent to the core molecule, instead of substituted anilines may give more flexibility to the analogue to fit better inside the pocket to make those pi-pi interactions.

3.2 AutoDock Vina

Due to the ambiguity of the results of the docking analysis using the Schrödinger software, the docking analysis was partially repeated using AutoDock Vina\textsuperscript{149} on a select subset of the 2,6-pyridine analogues to determine if the resulting data in these programs yield trends. Ten pyridine compounds were selected; six had activity in the binding and invasion assays, the other four did not. The docking scores are displayed below in Table 10. The key residue interactions are not listed because the AutoDock Vina program does not have an interaction wizard.

\begin{table}
\begin{tabular}{llcccc}
\hline
Compd & R Group & EC (nM) & Invasion Assay & Docking Score (kcal/mol) \\
\hline
2a & Aniline & 100 & <1\% & -6.8 \\
2c & 3-Me aniline & 100 & 60\% & -7.3 \\
2e & 2-Et aniline & 1 & 59\% & -6.7 \\
2f & 3-Et aniline & 1000 & -- & -6.6 \\
2g & 4-Et aniline & 1 & 64\% & -7.1 \\
2h & 2-F aniline & 1000 & -- & -7.2 \\
2i & 3-F aniline & 100 & 50\% & -7.0 \\
2q & 2-CF\textsubscript{3} aniline & >1000 & -- & -6.3 \\
\hline
\end{tabular}
\end{table}
Table 10: *In silico* results for select compounds using AutoDock Vina.

<table>
<thead>
<tr>
<th></th>
<th>Compound</th>
<th>1</th>
<th>%</th>
</tr>
</thead>
</table>
| 2w | Morpholine   | 1 | 63%| -5.5  
| 2x | Pyrrolidine  | 1 | 58%| -5.3  

*Figure 18:* The docked pyridine compounds in AutoDock Vina. All compounds are located away from the key residues. The CXCR4 monomer is shown here in ribbon form; however, the key residues are highlighted as stick representations.

The docking in this program has no correlation between the assay results compared to *in silico* results. The morpholine and pyrrolidine analogues scored the highest; however, all the other analogues consistently scored between -6.3 and -7.3. The analogues were then visualized in AutoDock. *Figure 18* shows the compounds in relation to the key residues and active site. The docked analogues are neither interacting with the key residues nor located within the active site.

*Figure 19* shows the analogues in relation to the docking gridbox.
Figure 19: The docked pyridine compounds shown in AutoDock in the docking gridbox. All of the docked compounds are centered away from the key residues towards the top of the gridbox. The CXCR4 monomer is shown here in ribbon form; however, the key residues are highlighted as stick representations.

In response to the lack of trends in the first set of *in silico* results, the same selected pyridine analogues were docked using a biased gridbox. The gridbox was reduced in size and was re-centered on the active site of the CXCR4 monomer. Docking with a biased gridbox is investigative in nature as it may help force compounds into a favorable position in the active site that was not seen in the first gridbox was larger at the same runtime; however, the results from this set of docking analyses cannot replace the original results from the non-biased docking analysis. The results of the biased docking analysis can be found in Table 11, below.
The docking scores in the biased gridbox are markedly higher than in the non-biased gridbox with docking scores ranging from -1.7 to -6.8. Unfortunately the broadness in the range has not lead to correlation between active compounds and their assay scores. Hit compounds such as 2e, 2g, and 2w scored -1.7, -1.8 and -4.3 respectively, where inactive compounds such as 2f, 2h, and 2q scored -3.1, -5.1 and -1.9 respectively. The visualization shown in Figure 20 and Figure 21 show the biased docked compounds and the biased gridbox. Like the unbiased gridbox first used, the analogues are as far away from the active site as they can be and are clustered in the top edge of the gridbox.

### Table 11: In silico results for selected compounds using a biased gridbox in AutoDock Vina.

<table>
<thead>
<tr>
<th>Compd</th>
<th>R Group</th>
<th>EC (nM)</th>
<th>Invasion Assay</th>
<th>Docking Score (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>Aniline</td>
<td>100</td>
<td>&lt;1%</td>
<td>-6.8</td>
</tr>
<tr>
<td>2c</td>
<td>3-Me aniline</td>
<td>100</td>
<td>60%</td>
<td>-3.6</td>
</tr>
<tr>
<td>2e</td>
<td>2-Et aniline</td>
<td>1</td>
<td>59%</td>
<td>-1.7</td>
</tr>
<tr>
<td>2f</td>
<td>3-Et aniline</td>
<td>1000</td>
<td>--</td>
<td>-3.1</td>
</tr>
<tr>
<td>2g</td>
<td>4-Et aniline</td>
<td>1</td>
<td>64%</td>
<td>-1.8</td>
</tr>
<tr>
<td>2h</td>
<td>2-F aniline</td>
<td>1000</td>
<td>--</td>
<td>-5.1</td>
</tr>
<tr>
<td>2i</td>
<td>3-F aniline</td>
<td>100</td>
<td>50%</td>
<td>-3.8</td>
</tr>
<tr>
<td>2q</td>
<td>2-CF₃ aniline</td>
<td>&gt;1000</td>
<td>--</td>
<td>-1.9</td>
</tr>
<tr>
<td>2w</td>
<td>Morpholine</td>
<td>1</td>
<td>63%</td>
<td>-4.3</td>
</tr>
<tr>
<td>2x</td>
<td>Pyrrolidine</td>
<td>1</td>
<td>58%</td>
<td>-4.4</td>
</tr>
</tbody>
</table>
Figure 20: The docked pyridine compounds in AutoDock Vina using a biased gridbox. All compounds are located away from the key residues. The CXCR4 monomer is shown here in ribbon form; however, the key residues are highlighted as stick representations.

Figure 21: The docked pyridine compounds shown in AutoDock in the biased gridbox. All of the docked analogues are centered away from the key residues towards the top of the gridbox. The CXCR4 monomer is shown here in ribbon form; however, the key residues are highlighted as stick representations.
The docking completed using AutoDock Vina has not yielded any additional information highlighting how these heterocyclic analogues are interacting with the active site of CXCR4 using the parameters outlined in this work. Further analysis using another program or perhaps more exhaustiveness may give more information and insight.
4  CONCLUSIONS AND FUTURE OUTLOOK

In this work, a small library of heterocyclic-based modulators for CXCR4 mimicking p-xylyl-enediamines have been synthesized and analyzed in binding assays, Matrigel invasion assays and an *in vivo* paw edema test. Overall, the 2,6-pyridine-based compounds and the 2,5-thiophene based compounds had the best results in the assays overall; however, more 2,5-thiophene compounds need to be submitted for the paw edema test. The 3,4-thiophene analogues were largely inactive, possibly due to the geometry. The 2,5-furan analogues had several with activity; however, compared to the 2,5-thiophene compounds, they exhibit less activity.

There are several ways to extend this project going into the future. The 2,5-pyrazine library needs to be completed and analyzed. For the 2,6-pyridine and 2,5-furan compounds, extending the sidechains by using benzylamines instead of anilines may boost activity as well, potentially enabling analogues to have pi-pi interactions with nearby residues and granting them more flexibility in the binding pocket. In addition to benzylamine substituents, more cyclic non-aromatic analogues need to be synthesized. *In silico* analysis suggests that these substituents are likely to be positively charged in physiological conditions and will interact with acidic key residues. The success of the pyrrolidine, morpholine, thiomorpholine and 1-methylpiperazine compounds set a precedence to further investigate this trend.

In order to verify if the 3,4-thiophene was largely inactive because of the 3,4 positioning of the side chains, synthesis of 3,4-furan based derivatives can provide insight into if this is true. There are a few other core molecules that would contribute to this library as the 2,6-pyridine and 2,5-pyrazine analogues have shown activity. Modeled after the 2,6-pyridine analogues, synthesis
of 2,6-pyrazine analogues could possibly increase activity as pyrazine has a lower log P than pyridine. It would also provide more information as to if the analogues are able to bind better in the pocket with another nitrogen in the heterocyclic ring can increase activity as in silico analysis suggests that the pyridine analogues are protonated in the active site and are able to bind differently be because of the positioning of the cation on the ring instead of on the amine. In silico results did not suggest protonation for the 2,5-pyrazine cores; however, 2,6-pyrazine should still be explored. In the same vein, synthesizing 2,5-pyrdine analogues that match the 2,5-pyrazine would be a useful addition to the library.

For existing core molecules like the 2,5-thiophene and the 2,6-pyridine, using other amines that have shown to have exception activity in the p-xylyl-enediamine series such as the 2-aminopyridine and 2-aminopyrimidine and halogen substituted 2-aminopyridine and 2-aminopyrimidines. Design and synthesis of asymmetrical analogues of these core molecules could help increase activity by designing a small molecule that can fit better into the active site.
5 EXPERIMENTAL

5.1 Biology

All biological assays were completed by our collaborators at Emory University in Dr. Hyunsuk Shim’s laboratory at the Winship Cancer Institute.

5.1.1 Primary Binding Affinity Screening

The binding affinity assay is a competitive assay where approximately twenty thousand MDA-MB-231 breast cancer cells are incubated an 8-well slide chamber for two days in 300 μL of medium. The compounds were also incubated in separate wells at several concentrations (1, 10, 100, 1000 nM) for ten minutes at room temperature. The cells were then fixed in a chilled solution of 4% paraformaldehyde. After the cells were rehydrated in phosphate-buffered saline (PBS), the slides were prepared by incubating them with 0.05 μg/mL biotinylated TN14003 for thirty minutes at room temperature. These slides were washed three times with the PBS solution and were then incubated for thirty minutes at room temperature in streptavidin-rhodamine (1:150 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). The slides were washed again with the PBS solution and were mounted in an antifade mounting solution (Molecular Probes, Eugene, OR). A Nikon Eclipse E800 microscope was used to analyze the samples.\textsuperscript{127,150}

5.1.2 Matrigel Invasion Assay

This assay was performed using a Matrigel invasion chamber (Corning Biocoat; Bedford, MA). In the bottom chamber, a solution of CXCL12 (200ng/mL; R&D Systems, Minneapolis, MN) was added to the apparatus. 100nm of the selected compounds (or AMD3100 as a control)
were added to the MDA-MB-231. The cells were then placed in the top chamber. The apparatus was then incubated in a humidified incubator for 22 hours. The remaining cells in the top chamber were removed using a cotton swab and the invading cells in the bottom chamber were stained hematoxylin and eosin (H&E) and fixed with methanol. The rate of invasion was calculated by counting the invading (stained) cells.\textsuperscript{127,150}

5.1.3 Paw Inflammation Suppression Test

In this test, C57BL/6J does (Jackson Laboratories) are subcutaneously injected with $\lambda$-carrageenan (50 $\mu$L in 1% w/v in saline) in the right hind paw to trigger inflammation; the other hind paw is used as the non-inflammation control. The selected analogues were prepared in 10% DMSO and 90% of 45% (2-hydroxypropyl)-$\beta$-cyclodextrin (CD) in PBS. Doses of the analogues were set at 10mg/kg and the dose for TN14003 was set at 300 $\mu$g/kg. The TN14003 dose was set lower for this experiment because it was found that 300 $\mu$g/kg gave the maximum efficacy at minimum concentration in breast cancer metastasis in an animal model. The mice were dosed 30 minutes after the carrageenan injection and then once a day following the initial dose. The mice were sacrificed 74 hours after inflammation was induced and two hours after the last injection of the selected analogues. The hind paws of the mice were photographed and calipers were used to measure the thickness of the paw from front to back. To quantify the edema, the measurement from the untreated paw was subtracted from the volume of the treated paw. The inflammation suppression percentage was determined by comparing the analogue treated groups to the control group. Each analogue was tested in quintuplicate using the above procedure.\textsuperscript{127,131} Paw tissue slices were also collected and stained with H&E. Tissue slices were scanned and digitized by NanoZoomer 2.0 HT. The software NDP.view 2 was used to view the slices in detail.
5.2 Docking Studies

5.2.1 Schrodinger Small Molecule Suite

The structure of the target receptor CXCR4, PDB-ID 3ODU, was retrieved from the RCSB Protein Data Bank. Docking calculations were performed by the Schrodinger suite with default settings unless otherwise indicated. The Protein Preparation Wizard was used to prepare the CXCR4 protein for docking. CXCR4 along with its co-crystallized ligand IT1t went through a preprocess procedure with the additional options to fill in missing side chains and loops using Prime and removing waters beyond 5Å from heteroatom groups. The heterostate for the co-crystallized ligand was generated using Epik. The PROPKA feature was utilized to optimize the hydrogen bond network at a physiological pH. After hydrogen bond optimization, water molecules were removed with less than 3 H-bonds to non-waters. The protein was then energetically minimized using a default constraint of 0.3Å RMSD and OPLS 2005 force field. Ligands were prepared by the LigPrep function of the Schrodinger Suite with default parameters in the gas phase. Epik generated possible ionization states of the ligands at physiological pH. Receptor grid generation was performed by inputting the prepared receptor directly from the protein preparation wizard. Docking was performed using the Extra Precision (XP) feature of the GLIDE program. Ligands were docked flexibly in the rigid protein devoid of the IT1t ligand and were ranked based on their GLIDE docking score.
5.2.2 **Autodock Vina**

The structure of the target receptor CXCR4, PDB-ID 3ODU, was retrieved from the RCSB Protein Data Bank.\textsuperscript{50} Docking calculations were performed AutoDock Vina and default settings were used unless otherwise specified. The protein in PDB form was uploaded into the program. The B dimer of the protein was then selected and removed to create a monomer. The monomer was then exported as a macromolecule, converting it to a PDBQT file. The gridbox was then selected; the initial gridbox had the following parameters: \(\text{center}_x = 16.807, \text{center}_y = -6.232, \text{center}_z = 69.119, \text{size}_x = 28, \text{size}_y = 28, \text{size}_z = 28, \text{spacing} = 1\) angstrom. The biased gridbox had the following parameters: \(\text{center}_x = 19.717, \text{center}_y = -6.232, \text{center}_z = 69.53, \text{size}_x = 22, \text{size}_y = 20, \text{size}_z = 14, \text{spacing} = 1\) angstrom. Ligands were uploaded into Autodock as PDB files. Once inputted, charges were assigned automatically. Ligand was then exported as a PDBQT file. The PDBQT files were then coded in the configuration files labeled as ligand and receptor with an exhaustiveness of 16. The compounds were then docked in AutoDock Vina and the resulting log files were retained.
5.3 Chemistry

The $^1$H NMR (400 MHz) and $^{13}$C NMR (100MHz) spectra were recorded on a Bruker Ac 400 FT NMR spectrometer in deuterated chloroform (CDCl$_3$). All chemical shifts were reported using parts per million (ppm). Mass spectra were recorded on a JEOL spectrometer at Georgia State University Mass Spectrometry Center. Detailed syntheses for the 2,5-Thiophene analogues can be found in the thesis of Francisco Garcia.$^{148}$

5.3.1 General Procedure for the Synthesis of the 2,6-Pyridine Analogues (2).

To a solution of pyridine-2,6-dicarbaldehyde (50 mg, 0.37 mmol) in methanol (2 mL) was added the aniline derivative of choice (0.81 mmol) and ZnCl$_2$ (100 mg, 0.74 mmol). The solution was stirred for two hours at room temperature. NaBH$_3$CN (46.5mg, 0.81 mmol) was then added and the solution as stirred overnight. The crude product was purified by column chromatography.

2,6-Bis(anilinomethyl)pyridine, (2a) Product purified using a 1:1 Hexane/Ethyl Acetate solvent system and was obtained in 37% yield as an off-white semi-solid. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 4.38 (s, 4H), 6.59 (d, $J = 7.8$ Hz, 4H), 6.65 (t, $J = 7.2$ Hz, 2H), 7.06 - 7.15 (m, 6 H), 7.49 (t, $J = 7.7$ Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 158.1, 148.0, 137.3, 129.3, 119.9, 117.7, 113.1, 49.3; HRMS: m/z [M + H]$^+$ calcd for C$_{19}$H$_{19}$N$_3$: 290.1657, found: 290.1657.

2,6-Bis(2-methylanilinomethyl)pyridine, (2b) The product was obtained in a 7:1 Hexanes/Ethyl Acetate solvent system and was obtained as an orange oil in 21% yield. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 2.25 (s, 6H), 4.52 (s, 4H), 6.60 (d, $J = 7.83$ Hz, 2H), 6.68 (t, $J = 7.33$ Hz, 2H), 7.05 - 7.14 (m, 4H), 7.22 (d, $J = 7.83$ Hz, 2H), 7.60 (t, $J = 7.71$ Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 158.02, 145.84, 137.26, 130.08, 127.13, 122.27, 119.99, 117.22, 110.14, 49.22, 17.59; HRMS: m/z [M + H]$^+$ calcd for C$_{21}$H$_{23}$N$_3$: 318.1965, found: 318.1958.
2,6-Bis(3-methylanilinomethyl)pyridine, (2c) The product was purified via flash chromatography using DCM as a solvent and was obtained as an orange oil in 50% yield. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 2.26 (s, 6 H) 4.42 (s, 4 H) 6.42 - 6.52 (m, 4 H) 6.54 (d, J=7.33 Hz, 2 H) 7.06 (t, J=7.58 Hz, 2 H) 7.17 (d, J=7.58 Hz, 2 H) 7.54 (t, J=7.58 Hz, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 158.23, 148.06, 139.06, 137.26, 129.20, 119.86, 118.61, 113.95, 110.23, 49.30, 21.70. HRMS: m/z [M+ Z]$^+$ calcd for C$_{21}$H$_{23}$N$_3$: 318.1965, found: 318.1965.

2,6-Bis(4-methylanilinomethyl)pyridine, (2d) The product was purified via flash chromatography using DCM as a solvent and was obtained as a yellow-orange solid in 34% yield. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 2.24 (s, 6 H) 4.43 (s, 4 H) 6.59 (d, J=8.34 Hz, 4 H) 6.99 (d, J=8.08 Hz, 4 H) 7.19 (d, J=7.83 Hz, 2 H) 7.56 (t, J=7.71 Hz, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 158.36, 145.70, 137.22, 129.78, 126.84, 119.85, 113.27, 49.64, 20.42. HRMS: m/z [M+ Z]$^+$ calcd for C$_{21}$H$_{23}$N$_3$: 318.1965, found: 318.1962.

2,6-Bis(2-ethylanilinomethyl)pyridine, (2e) The product was purified in a 1:1 Hexanes/Ethyl Acetate solvent system and was obtained in 53% yield as a brown solid. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 1.31 (t, J = 7.45 Hz, 6H), 2.60 (q, J = 7.58 Hz, 4H), 4.52 (s, 4H), 6.61 (d, J = 7.83 Hz, 2H), 6.73 (t, J = 7.33 Hz, 2H), 7.06 - 7.14 (m, 4H), 7.18 - 7.25 (m, 2H), 7.58 (t, J = 7.71 Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 158.17, 145.32, 137.29, 127.89, 127.04, 119.98, 117.43, 110.50, 49.36, 23.98, 12.92. HRMS: m/z [M + H]$^+$ calcd for C$_{23}$H$_{27}$N$_3$: 346.2278, found: 346.2276.

2,6-Bis(3-ethylanilinomethyl)pyridine, (2f) The product was purified in a 3:1 Hexanes/Ethyl Acetate solvent system and was obtained as an orange oil in 23% yield. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 1.18 - 1.24 (m, 6H), 2.56 (d, J = 6.32 Hz, 4H), 4.46 (br. s., 4H), 6.52 (d, J = 14.65 Hz, 4H), 6.59 (br. s., 2H), 7.02 - 7.15 (m, 2H), 7.21 (d, J = 4.04 Hz, 2H), 7.51 - 7.62 (m, 1H); $^{13}$C
NMR (100 MHz, CDCl₃): δ ppm 158.15, 148.10, 145.45, 137.20, 129.19, 119.83, 117.36, 112.80, 110.34, 49.31, 29.02, 15.57; HRMS: m/z [M + H]+ calcd for C₂₃H₂₇N₃: 346.2278, found: 346.2285.

2,6-Bis(4-ethylanilinomethyl)pyridine, (2g) The product was purified in a 7:1 Hexanes/Ethyl Acetate solvent system and was obtained in 11% yield as an orange oil; ¹H NMR (400 MHz, CDCl₃): δ ppm 1.18 (t, J = 7.6 Hz, 6H), 2.50 - 2.57 (m, 4H), 4.41 (s, 4H), 6.59 (d, J = 8.3 Hz, 4H), 6.97 (d, J = 8.1 Hz, 2H), 7.01 (d, J = 8.3 Hz, 4H), 7.53 (t, J = 7.7 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃): δ ppm 158.4, 146.0, 137.3, 133.6, 128.7, 119.9, 115.3, 113.3, 49.7, 28.0, 16.02. HRMS: m/z [M + H]+ calcd for C₂₃H₂₇N₃: 346.2283, found: 346.2291.

2,6-Bis(2-fluoroanilinomethyl)pyridine, (2h) The product was purified in a 3:1 Hexanes/Ethyl Acetate solvent system and was obtained in 7% yield as an orange oil. ¹H NMR (400 MHz, CDCl₃): δ ppm 4.51 (br. s., 4H), 6.59 - 6.74 (m, 4H), 6.88 - 7.07 (m, 4H), 7.24 (d, J = 5.05 Hz, 2H), 7.61 (d, J = 6.06 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃): δ ppm 157.81, 152.93, 137.39, 136.33, 124.56, 119.85, 116.96, 114.57, 112.50, 48.90; HRMS: m/z [M + H]+ calcd for C₁₉H₁₇N₃F₂: 326.1463, found: 326.1451.

2,6-Bis(3-fluoroanilinomethyl)pyridine, (2i) Product was purified in a 3:1 Hexanes/Ethyl Acetate solvent system and was obtained in 62% yield as a brown semi-solid. ¹H NMR (400 MHz, CDCl₃): δ ppm 4.34 (s, 4H), 6.21 - 6.40 (m, 6H), 6.95 - 7.06 (m, 2H), 7.10 (d, J = 7.8 Hz, 2H), 7.51 (t, J = 7.71 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃): δ ppm 165.4, 162.9, 157.5, 149.8, 149.7, 137.4, 130.3, 120.1, 109.0, 104.2, 104.0, 99.9, 99.6, 48.9; HRMS: m/z [M + H]+ calcd for C₁₉H₁₇N₃F₂: 326.1469, found: 326.1462.

2,6-Bis(4-fluoroanilinomethyl)pyridine, (2j) Product purified using a 3:1 Hexanes/Ethyl Acetate solvent system and was obtained in 34% yield as a brown semi-solid; ¹H NMR (400 MHz, CDCl₃): δ ppm 4.33 (s, 4H), 6.51 (dd, J = 8.72, 4.17 Hz, 4H), 6.81 (t, J = 8.59 Hz, 4H), 7.12 (d, J = 7.58
Hz, 2 H), 7.52 (t, \( J = 7.71 \) Hz, 1H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) ppm 158.0, 157.2, 154.8, 137.4, 137.2, 120.0, 115.9, 115.7, 115.6, 115.5, 114.3, 113.5, 113.4, 49.9; HRMS: m/z [M + H]\(^+\) calcd for C\(_{19}\)H\(_{17}\)N\(_3\)F\(_2\): 326.1469, found: 326.1462.

**2,6-Bis(2-chloroanilinomethyl)pyridine, (2k)** The product was purified in a 3:1 Hexane/Ethyl Acetate solvent system and was obtained in 39% yield as a yellow solid. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) ppm 4.54 (d, \( J = 5.05 \) Hz, 4H), 6.61 - 6.69 (m, 4H), 7.10 (t, \( J = 7.45 \) Hz, 2H), 7.21 (d, \( J = 7.58 \) Hz, 2H), 7.28 (d, \( J = 7.58 \) Hz, 2H), 7.61 (t, \( J = 7.71 \) Hz, 1H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) ppm 157.64, 143.70, 137.42, 139.18, 137.80, 119.83, 119.49, 117.45, 111.63, 48.94. HRMS: m/z [M + H]\(^+\) calcd for C\(_{19}\)H\(_{17}\)N\(_3\)Cl\(_2\): 358.0872, found: 358.0877.

**2,6-Bis(3-chloroanilinomethyl)pyridine, (2l)** Product was purified in a 4:1 Hexanes/Ethyl Acetate solvent system and was obtained in 14% yield as a yellow semi-solid; \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) ppm 4.36 (s, 4 H), 6.46 (dd, \( J = 8.1, 1.0 \) Hz, 2 H), 6.58 (s, 2 H) 6.61 (d, \( J = 7.8 \) Hz, 2 H), 7.01 (t, \( J = 8.0 \) Hz, 2 H), 7.12 (d, \( J = 7.6 \) Hz, 2 H), 7.54 (t, \( J = 7.71 \) Hz, 1 H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) ppm 157.4, 149.0, 137.5, 135.1, 130.3, 120.1, 117.5, 112.7, 111.4, 48.9. HRMS: m/z [M + H]\(^+\) calcd for C\(_{19}\)H\(_{17}\)N\(_3\)Cl\(_2\): 358.0854, found: 358.0864.

**2,6-Bis(4-chloroanilinomethyl)pyridine, (2m)** Product was purified in a 4:1 Hexanes/Ethyl Acetate solvent system and was obtained in 15% yield as a yellow solid; mp 116-118°C. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) ppm 4.36 (br, s, 4H), 6.51 (d, \( J = 8.34 \) Hz, 4H), 7.06 (d, \( J = 8.34 \) Hz, 4H), 7.12 (d, \( J = 7.6 \) Hz, 2H), 7.54 (t, \( J = 7.7 \) Hz, 1H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta \) ppm 157.6, 146.4, 137.4, 129.1, 122.3, 120.0, 114.2, 49.2. HRMS: m/z [M + H]\(^+\) calcd for C\(_{19}\)H\(_{17}\)N\(_3\)Cl\(_2\): 358.0872, found: 358.0864.

**2,6-Bis(2-methoxyanilinomethyl)pyridine, (2n)** The product was purified in a 7:1 Hexane/Ethyl Acetate solvent system and was obtained in 51% yield as a brown solid; mp 112-114°C. \(^1\)H NMR
(400 MHz, CDCl$_3$): δ ppm 3.81 (s, 6 H), 4.43 (s, 4 H), 6.49 (d, $J = 7.58$ Hz, 2H), 6.58 - 6.64 (m, 2H), 6.70 - 6.79 (m, 4H), 7.14 (d, $J = 7.8$ Hz, 2H), 7.48 (t, $J = 7.7$ Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 158.7, 147.0, 137.9, 137.3, 121.3, 119.6, 116.7, 110.3, 109.5, 55.5, 49.4. HRMS: m/z [M + H]$^+$ calcd for C$_{21}$H$_{23}$N$_3$O$_2$: 350.1869, found: 350.1862.

**2,6-Bis(3-methoxyanilinomethyl)pyridine, (2o)** The product was purified in a 3:1 Hexanes/Ethyl Acetate solvent system and was obtained in 16% yield as an off white semisolid. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 3.76 (br. s., 6H), 4.45 (br. s., 4H), 6.23 (br. s., 2H), 6.30 (m, 4H), 7.09 (d, $J = 3.28$ Hz, 2H), 7.20 (m, 2H), 7.59 (m, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 160.79, 157.91, 149.30, 137.28, 130.00, 119.89, 106.18, 102.75, 99.03, 55.08, 49.19. HRMS: m/z [M + H]$^+$ calcd for C$_{21}$H$_{23}$N$_3$O$_2$: 350.1863, found: 350.1854.

**2,6-Bis(4-methoxyanilinomethyl)pyridine, (2p)** Product was purified in two silica columns. The fist was purified in a solution of 9:1 DCM/Methanol. The compound was then further purified in 4:5 solution of Hexanes/Ethyl Acetate. Product was obtained in 30% yield as a brown solid; mp 63-65°C. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 3.66 (s, 6 H), 4.33 (s, 4 H), 6.55 (d, $J = 8.84$ Hz, 4 H), 6.70 (d, $J = 8.84$ Hz, 4 H), 7.12 (d, $J = 7.58$ Hz, 2 H), 7.49 (t, $J = 7.7$ Hz, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 158.39, 152.25, 142.22, 137.20, 119.94, 114.90, 114.36, 55.79, 50.24; HRMS: m/z [M + H]$^+$ calcd for C$_{21}$H$_{23}$N$_3$O$_2$: 350.1869, found: 350.1865.

**2,6-Bis(2-trifluoromethylanilinomethyl)pyridine, (2q)** The product was purified in a 7:1 Hexane/Ethyl Acetate solvent system and was obtained in 19% yield as an off white solid. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 4.55 (d, $J = 5.05$ Hz, 4H), 6.63 - 6.79 (m, 4H), 7.20 (d, $J = 7.58$ Hz, 2H), 7.32 (t, $J = 7.71$ Hz, 2H), 7.47 (d, $J = 7.58$ Hz, 2H), 7.61 (t, $J = 7.71$ Hz, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 157.32, 145.14, 137.58, 133.14, 126.72, 126.67, 119.77, 116.27, 113.66, 112.26, 48.69. HRMS: m/z [M + H]$^+$ calcd for C$_{21}$H$_{17}$F$_6$N$_3$: 426.1401, found: 426.1399.
2,6-Bis(3-trifluoromethylanilinomethyl)pyridine, (2r) The product was purified in a 3:2 Hexanes/Ethyl Acetate solvent system and was obtained in 50% yield as an off white solid. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 4.46 (br. s., 4 H) 6.78 (d, J=8.08 Hz, 2 H) 6.87 (br. s., 2 H) 6.95 (d, J=7.58 Hz, 2 H) 7.19 (d, J=7.83 Hz, 2 H) 7.25 (t, J=1.00 Hz, 2 H) 7.61 (t, J=7.71 Hz, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 157.24, 148.06, 137.51, 131.75, 129.71, 125.74, 123.03, 120.20, 116.02, 114.05, 109.23, 48.80. HRMS: m/z [M + H]$^+$ calcd for C$_{21}$H$_{17}$F$_6$N$_3$: 426.1399, found: 426.1384.

2,6-Bis(4-trifluoromethylanilinomethyl)pyridine, (2s) The product was purified in a 2:1 Hexanes/Ethyl Acetate solvent system and was obtained in 61% yield as a light orange semi-solid. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 4.49 (s, 4 H) 6.65 (d, J=8.34 Hz, 4 H) 7.20 (d, J=7.83 Hz, 2 H) 7.41 (d, J=8.34 Hz, 4 H) 7.63 (t, J=1.00 Hz, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 157.15, 150.28, 137.59, 126.68, 126.32, 123.64, 120.15, 112.21, 48.46. HRMS: m/z [M + H]$^+$ calcd for C$_{21}$H$_{17}$F$_6$N$_3$: 426.1399, found: 426.1381.

2,6-Bis(4-thiomethylanilinomethyl)pyridine (2t) The product was purified in a 3:1 Hexanes/Ethyl Acetate solvent system and was obtained in 14% yield as a white solid; $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 2.41 (s, 6 H) 4.44 (br. s., 4 H) 6.61 (d, J=8.34 Hz, 4 H) 7.16 - 7.31 (m, 6 H) 7.59 (t, J=7.71 Hz, 1 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 19.02, 49.12, 113.70, 119.94, 124.61, 131.34, 137.30, 146.70, 157.80. HRMS: m/z [M + H]$^+$ calcd for C$_{21}$H$_{24}$N$_3$S$_2$: 382.1206, found: 382.1404.

2,6-Bis(3-nitroanilinomethyl)pyridine, (2u) The product was purified using a 30:1 DCM/Methanol solvent system and was obtained in 34% yield as a yellow-orange solid; mp 147-149°C. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 4.54 (d, J = 5.05 Hz, 4H), 6.96 (d, J = 8.08 Hz, 2H), 7.24 (br s, 2H), 7.31 (t, J = 8.1 Hz, 2H), 7.49 (br s, 2 H), 7.56 (d, J = 8.08 Hz, 2H), 7.65 - 7.71 (m,
H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 156.8, 148.6, 137.7, 129.8, 120.4, 119.2, 112.4, 106.6, 48.7. HRMS: m/z [M + H]$^+$ calcd for C$_{19}$H$_{17}$N$_5$O$_4$: 380.1359, found: 380.1358.

2,6-Bis(4-nitroanilinomethyl)pyridine, (2v) The product was purified using a 10:1 DCM/Methanol solvent system and was obtained in 10% yield as a yellow semi-solid; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 4.51 (d, $J = 5.1$ Hz, 4H), 6.56 (d, $J = 9.1$ Hz, 4H), 7.17 (d, $J = 7.8$ Hz, 2H), 7.64 (t, $J = 7.71$ Hz, 1H), 8.05 (d, $J = 9.09$ Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 156.1, 152.7, 137.9, 126.4, 120.5, 111.6, 48.9. HRMS: m/z [M + H]$^+$ calcd for C$_{19}$H$_{18}$N$_5$O$_4$: 380.1359, found: 380.1359.

2,6-Bis(morpholinylmethyl)pyridine (2w) The product purified in a 25:1 DCM/Methanol solvent system and was obtained was a white semi-solid in 1% yield. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 7.73 (1H t), 7.47 (2H d), 5.30 (4H s), 2.60 (8H m), 1.65 (8H m), 1.50 (4H, m); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 152.75, 137.55, 123.65, 120.96, 60.43, 63.80, 25.68, 25.11, 23.78. HRMS: m/z [M + H]$^+$ calcd for C$_{15}$H$_{23}$N$_3$O$_2$: 278.1863, found: 278.1852.

2,6-Bis(pyrrolidinylmethyl)pyridine (2x) The product was purified in a 5:1 DCM:Methanol solvent system and was obtained was an orangeish oil in 9% yield. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 7.59 (t, $J = 7.7$ Hz, 1H), 7.29 (s, 1H), 7.27 (s, 1H), 3.60 (s, 4H), 2.42-2.44 (m, 8H), 1.55-1.61 (m, 8H), 1.40-1.46 (m, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 158.71, 136.67, 121.20, 65.57, 54.99, 26.20, 24.52. HRMS: m/z [M+H]$^+$ calcd for C$_{15}$H$_{24}$N$_3$: 246.1965, found: 246.1956.
5.3.2 General Procedure for the Synthesis of the 2,5-Furan Analogues (4)

General Procedure for the Synthesis of the 2,5-furan-based analogues (4). To a solution of methanol, 50 mg (0.4029 mmol) of furan-2,5-dicarbaldehyde was combined in a dry vial with the aniline derivative of choice (0.8865 mmol) and 75.963 mg (1.2088 mmol) of sodium cyanoborohydride (NaBH$_3$CN). The solution was stirred for five minutes at room temperature before 164.578 mg (1.2088 mmol) of zinc chloride (ZnCl$_2$) was added. The solution was then stirred for two hours and purified by flash chromatography.

2,5-Bis(anilinomethyl)furan, (4a) Product was purified in a 7:1 Hexanes/Ethyl Acetate solvent system and was obtained in 38% yield as a yellow oil. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 4.27 (s, 4 H) 6.15 (s, 2 H) 6.66 (d, $J$=7.58 Hz, 4 H) 6.74 (t, $J$=7.33 Hz, 2 H) 7.18 (t, $J$=7.96 Hz, 4 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 152.11, 147.58, 129.22, 118.04, 113.18, 107.80, 41.49. HRMS: m/z [M + H]$^+$ calcd for C$_{18}$H$_{19}$ON$_2$: 279.1492, found: 279.1487.

2,5-Bis(2-methylanilinomethyl)furan, (4b) The product was purified in a 13:1 solvent system and was obtained as a yellow solid in 35% yield. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 2.14 (s, 6 H) 3.84 (br s, 2 H) 4.33 (s, 4 H) 6.17 (s, 2 H) 6.63 - 6.75 (m, 4 H) 7.00 - 7.17 (m, 4 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 152.22, 145.56, 130.14, 127.07, 122.34, 117.61, 110.13, 107.83, 41.50, 17.49. HRMS: m/z [M + H]$^+$ calcd for C$_{20}$H$_{23}$ON$_2$: 307.1805, found: 307.1792.

2,5-Bis(3-methylanilinomethyl)furan, (4c) The product was purified in a 3:1 Hexanes/Ethyl Acetate solvent system and was obtained as a light orange solid in 49% yield. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 2.27 (s, 6 H) 4.25 (s, 4 H) 6.13 (s, 2 H) 6.42 - 6.50 (m, 4 H) 6.56 (d, $J$=7.58 Hz, 2 H) 7.06 (t, $J$=1.00 Hz, 2 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 152.17, 147.64, 138.98, 129.9,
2,5-Bis(4-methylanilinomethyl)furan, (4d) The product was purified in a 3:1 Hexanes/Ethyl Acetate solvent system and was obtained as an orange solid in 49% yield. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 2.22 (s, 6 H) 4.21 (s, 4 H) 6.10 (s, 2 H) 6.56 (d, $J$=8.08 Hz, 4 H) 6.97 (d, $J$=7.83 Hz, 4 H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 152.25, 145.33, 129.68, 127.21, 113.38, 107.67, 41.82, 20.40. HRMS: m/z [M + Z]$^+$ calcd for C$_{20}$H$_{23}$ON$_2$: 307.1817, found: 307.1810.

2,5-Bis(2-ethylanilinomethyl)furan, (4e) The product was purified in a 9:1 Hexanes/Ethyl Acetate solvent system and was obtained in 44% yield as a yellow oil. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 1.24 (br d, $J$=5.56 Hz, 6 H) 2.44 - 2.56 (m, 4 H) 3.94 (br s, 2 H) 4.33 (br s, 4 H) 6.16 (br s, 2 H) 6.66 - 6.78 (m, 4 H) 7.05 - 7.16 (m, 4 H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 152.28, 144.98, 128.02, 127.92, 126.93, 117.81, 110.52, 107.79, 41.59, 23.79, 12.90. HRMS: m/z [M + H]$^+$ calcd for C$_{22}$H$_{27}$ON$_2$: 335.2118, found: 335.2103.

2,5-Bis(3-ethylanilinomethyl)furan, (4f) The product was purified in a 9:1 Hexanes/Ethyl Acetate solvent system and was obtained as an orange oil in 42% yield. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 1.21 (br d, $J$=5.31 Hz, 6 H) 2.51 - 2.63 (m, 4 H) 3.93 (br s, 2 H) 4.26 (br s, 4 H) 6.14 (br s, 2 H) 6.45 - 6.54 (m, 4 H) 6.54 - 6.65 (m, 2 H) 7.05 - 7.14 (m, 2 H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 152.20, 147.70, 145.40, 129.15, 117.78, 112.93, 110.47, 107.75, 41.57, 29.00, 15.51. HRMS: m/z [M + H]$^+$ calcd for C$_{22}$H$_{27}$ON$_2$: 335.2118, found: 335.2103.

2,5-Bis(4-ethylanilinomethyl)furan, (4g) The product was purified in a 7:1 Hexanes/Ethyl Acetate solvent system and was obtained in 27% yield as an orange oil; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 1.18 (t, $J$=7.58 Hz, 6 H) 2.54 (q, $J$=7.41 Hz, 4 H) 4.24 (s, 4 H) 6.12 (s, 2 H) 6.60 (d, $J$=8.34 Hz, 4 H) 7.01 (d, $J$=8.08 Hz, 4 H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 152.28, 145.58,
133.88, 128.52, 113.35, 107.69, 41.83, 27.93, 15.91. HRMS: m/z [M + H]^+ calcd for C_{22}H_{27}ON_2: 335.2118, found: 335.2111.

2,5-Bis(2-fluoroanilinomethyl)furan, (4h) The product was purified in a 20:1 Hexanes/Ethyl Acetate solvent system and was obtained in 40% yield as an yellow oil. ^1H NMR (400 MHz, CDCl_3): δ ppm 4.28 (br s, 4 H) 6.14 (s, 2 H) 6.59 - 6.78 (m, 4 H) 6.90 - 7.05 (m, 4 H); ^13C NMR (100 MHz, CDCl_3): δ ppm 152.84, 151.84, 150.47, 136.04, 135.93, 130.41, 126.93, 124.53, 124.50, 118.58, 117.38, 117.31, 114.90, 114.40, 112.51, 107.94, 41.02. HRMS: m/z [M + H]^+ calcd for C_{18}H_{17}ONF_2: 315.13, found: 315.1299.

2,5-Bis(3-fluoroanilinomethyl)furan, (4i) Product was purified in 9:1 Hexanes/Ethyl Acetate solvent system and was obtained in 35% yield as an orange oil. ^1H NMR (400 MHz, CDCl_3): δ ppm 4.23 (s, 4 H) 6.15 (s, 2 H) 6.34 (dd, J=11.49, 1.64 Hz, 2 H) 6.37 - 6.45 (m, 4 H) 7.05 - 7.13 (m, 2 H); ^13C NMR (100 MHz, CDCl_3): δ ppm 165.22, 162.80, 151.69, 149.35, 149.25, 130.35, 130.25, 109.00, 108.98, 108.07, 104.53, 104.31, 99.95, 99.69, 41.27. HRMS: m/z [M + H]^+ calcd for C_{18}H_{17}ONF_2: 315.1303, found: 315.1292.

2,5-Bis(4-fluoroanilinomethyl)furan, (4j) Product was purified in 3:1 Hexanes/Ethyl Acetate solvent system and was obtained in 52% yield as an orange oil. ^1H NMR (400 MHz, CDCl_3): δ ppm 4.22 (s, 4 H) 6.13 (s, 2 H) 6.53 - 6.64 (m, 4 H) 6.88 (m, J=8.70, 8.70 Hz, 4 H); ^13C NMR (100 MHz, CDCl_3): δ ppm 157.35, 155.00, 152.04, 115.77, 115.55, 114.18, 114.11, 107.90, 42.10. HRMS: m/z [M + H]^+ calcd for C_{18}H_{17}ONF_2: 315.1294, found: 315.1303.

2,5-Bis(2-chloroanilinomethyl)furan, (4k) The product was purified in a 12:1 Hexanes/Ethyl Acetate solvent system and was obtained in 6% yield. ^1H NMR (400 MHz, CDCl_3): δ ppm 4.35 (br s, 4 H) 4.67 (br s, 2 H) 6.17 (br s, 2 H) 6.63 - 6.69 (m, 2 H) 6.73 (br s, 2 H) 7.12 (br s, 2 H) 7.26 (br s, 2 H); ^13C NMR (100 MHz, CDCl_3): δ ppm 151.72, 143.42, 129.19, 127.76, 119.46,
117.89, 111.59, 107.98, 41.16. HRMS: m/z [M + H]^+ calcd for C_{18}H_{17}ON_2Cl_2: 347.0712, found: 347.0509.

2,5-Bis(3-chloroanilinomethyl)furan, (4l) Product was purified in a 7:1 Hexanes/Ethyl Acetate solvent system and was obtained in 59% yield as an orange oil. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 4.21 (4 H, s) 6.13 (2 H, s) 6.49 (2 H, d, $J$=8.34 Hz) 6.61 (2 H, br. s.) 6.69 (2 H, d, $J$=6.82 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 41.18, 108.10, 111.43, 112.79, 114.89, 117.83, 130.21, 148.69, 151.67. HRMS: m/z [M + H]^+ calcd for C_{18}H_{17}ON_2Cl_2: 347.0685.

2,5-Bis(4-chloroanilinomethyl)furan, (4m) Product was purified in a 7:1 Hexanes/Ethyl Acetate solvent system and was obtained in 71% yield as an orange oil. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 4.21 (4 H, s) 6.12 (2 H, s) 6.54 (4 H, d, $J$=8.59 Hz) 7.09 (4 H, d, $J$=8.59 Hz). $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 41.26, 107.80, 114.06, 122.32, 28.85, 146.01, 151.69. HRMS: m/z [M + H]^+ calcd for C_{18}H_{17}ON_2Cl_2: 347.0696.

2,5-Bis(2-methoxyanilinomethyl)furan, (4n) The product was purified in a 9:1 Hexanes/Ethyl Acetate solvent system and was obtained in 29% yield as a white solid; $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 7.12 (t, $J$ = 7.71 Hz, 2H), 7.06 (d, $J$ = 7.07 Hz, 2H), 6.64 - 6.72 (m, 4H), 6.17 (s, 2H), 4.33 (s, 4H), 2.14 (s, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 152.22, 145.56, 130.14, 127.07, 122.34, 117.61, 110.13, 107.83, 41.5, 17.49. HRMS: m/z [M + H]^+ calcd for C_{20}H_{22}N_2O_3Na: 361.1528, found: 361.1537.

2,5-Bis(3-methoxyanilinomethyl)furan, (4o) The product was purified in a 2:1 Hexane/Ethyl Acetate solvent system and was obtained in 30% yield as an orange oil. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 7.00 - 7.13 (m, 2H), 6.19 - 6.35 (m, 6H), 6.15 (s, 2H), 4.25 (s, 4H), 3.65 - 3.79 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 160.74, 151.99, 148.98, 129.96, 107.87, 106.23, 103.09, 99.20, 55.07, 41.45. HRMS: m/z [M + H]^+ calcd for C_{20}H_{23}N_2O_3: 339.1676, found: 339.1687.
2,5-Bis(4-methoxyanilinomethyl)furan, (4p) Product was purified in a 7:1 Hexanes/Ethyl Acetate solvent system and was obtained in 35% yield as a brown solid. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 3.74 (6 H, s) 4.22 (4 H, s) 6.13 (2 H, s) 6.63 (4 H, d, $J$=8.84 Hz) 6.78 (4 H, d, $J$=8.84 Hz); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 152.58, 152.34, 141.77, 116.42, 114.81, 107.72, 55.73, 42.52. HRMS: m/z [M + H]$^+$ calcd for C$_{20}$H$_{23}$N$_2$O$_3$: 339.1701, found: 339.1703.

2,5-Bis(2-trifluoromethylanilinomethyl)furan, (4q) The product was purified in a 13:1 Hexanes/Ethyl Acetate solvent system and was obtained in 9% yield as a yellow oil. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 4.37 (br d, $J$=4.55 Hz, 4 H) 4.72 (br s, 2 H) 6.17 (s, 2 H) 6.70 - 6.8 (m, 4 H) 7.31 - 7.41 (m, 2 H) 7.45 (br d, $J$=7.58 Hz, 2 H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 151.48, 144.97, 133.06, 126.68, 116.66, 114.10, 112.18, 108.02, 41.06. HRMS: m/z [M + H]$^+$ calcd for C$_{20}$H$_{17}$F$_6$N$_2$O: 415.1240, found: 415.1246.

2,5-Bis(3-trifluoromethylanilinomethyl)furan, (4r) The product was purified in a 3:1 Hexanes/Ethyl Acetate solvent system and was obtained in 60% yield as an orange oil. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 7.20 - 7.24 (m, 2H), 6.95 - 6.98 (m, 2H), 6.86 (br. s., 2H), 6.76 - 6.80 (m, 2H), 6.17 (s, 2H), 4.29 (s, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 151.62, 147.67, 129.73, 129.65, 117.97, 116.11, 114.46, 109.34, 108.23, 41.14. MS: m/z [M]$^+$ calcd for C$_{20}$H$_{16}$F$_6$N$_2$O: 413.1080.

2,5-Bis(4-trifluoromethylanilinomethyl)furan, (4s) The product was purified in a 3:1 Hexanes/Ethyl Acetate solvent system and was obtained in 33% yield as an orange oil. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ ppm 7.41 (d, $J$=8.08 Hz, 4H), 6.66 (d, $J$=8.34 Hz, 4H), 6.18 (s, 2H), 4.32 (s, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ ppm 151.54, 149.93, 126.64, 123.53, 119.80, 112.23, 108.23, 40.94. HRMS: m/z [M+ Z]$^+$ calcd for C$_{20}$H$_{16}$F$_6$N$_2$ONa: 437.1066, found: 437.1059.
2,5-Bis(4-thiomethylanilinomethyl)furan (4t) The product was purified in a 7:1 Hexanes/Ethyl Acetate solvent system and was obtained in 25% yield as an orange oil. \(^1\text{H NMR (400 MHz, CDCl}_3\):} \delta \text{ ppm 2.40 (s, 6 H) 4.25 (s, 4 H) 6.14 (s, 2 H) 6.60 (d, } J=8.59 \text{ Hz, 4 H) 7.20 (d, } J=8.59 \text{ Hz, 4 H).} \(^{13}\text{C NMR (100 MHz, CDCl}_3\):} \delta \text{ ppm 151.94, 146.31, 131.12, 125.17, 113.81, 107.92, 41.48, 18.87. HRMS: m/z [M + H]\(^+\) calcd for C\(_{20}\)H\(_{29}\)N\(_2\)OS\(_2\): 371.1238, found: 371.1246.}

2,5-Bis(4-methypiperazin-1-ylmethyl)furan, (4u) The product was purified in a 30:1 DCM/Methanol solvent system and was obtained as a yellow oil in 40% yield. \(^1\text{H NMR (400 MHz, CDCl}_3\):} \delta \text{ ppm 6.13 (s, 2H), 3.54 (s, 4H), 2.53 (m, } J=16.17 \text{ Hz, 16H), 2.30 (s, 6H);} \(^{13}\text{C NMR (100 MHz, CDCl}_3\):} \delta \text{ ppm 151.09, 109.48, 54.89, 54.76, 52.48, 45.84. HRMS: m/z [M + H]\(^+\) calcd for C\(_{16}\)H\(_{29}\)N\(_4\)O: 293.2336, found: 293.2323.
5.3.3 General Procedure for the Synthesis of the 3,4-Thiophene Analogues (8)

Procedure for the Synthesis of Thiophene-3,4-dimethanol (6).

The dimethanol compound was prepared according to a literature procedure\textsuperscript{151} where 500 mg of thiophene-3,4-dicarboxylic acid (2.9 mmol) was added to 10 mL of dry THF in a dry round bottom flask and was chilled to 0°C. 17.375 mL (17.375 mmol) of diisobutylaluminum hydride (139 mL in 1 M hexanes) was added to the flask and the solution was stirred at room temperature for 16 hours. The reaction was quenched with methanol and water and then 25 mL of HCl was added to break up the solid chunks in the reaction. The solution as then extracted with ethyl acetate, washed with brine and dried with MgSO\textsubscript{4}. The solution was then evaporated under reduced pressure to give an orange oil in 77% yield.

Procedure for the Synthesis of Thiophene-3,4-dialdehyde (7). The thiophene aldehyde was prepared using an adapted literature procedure\textsuperscript{152} where 2.0 g of Compound 6 (13.9 mmol) was added to a 100 mL solution of dry 1,4-dioxane. 6.0 g of dry activated MnO\textsubscript{2} (69.00 mmol) was added and the mixture was refluxed under N\textsubscript{2} for forty-five minutes at 96°C. The solution was filtered through a fritted filter funnel and was concentrated under reduced pressure to yield a light yellow solid in 90% yield. \textsuperscript{1}H NMR was used to confirm the structure with the literature characterization.

General Procedure for the Synthesis of the 3,4-bis(anilino)thiophene analogues (8). To a solution of methanol, 50 mg (0.3568 mmol) of thiophene-3,4-dicarbaldehyde was combined in a dry vial with the aniline derivative of choice (0.7849 mmol), 145.68 mg (1.070 mmol) of zinc chloride (ZnCl\textsubscript{2}). The solution was stirred at room temperature overnight. The solution was reduced to dryness and was then dissolved in ethanol, where 67.24 mg (1.070 mmol) of sodium
cyanoborohydride (NaBH₃CN) was added. The solution was then stirred overnight and purified by flash chromatography.

**3,4-Bis(anilinomethyl)thiophene, (8a)** The product was purified in a 7:1 Hexanes/Ethyl Acetate solvent system and was obtained in this reaction was an brown semisolid in 4% yield. 

$^1$H NMR (400 MHz, CDCl₃): $\delta$ ppm 7.24 (2H s), 7.18 (4H t), 6.65 (4H d), 6.44 (2H d), 4.31 (4H s); $^{13}$C NMR (100 MHz, CDCl₃): $\delta$ ppm 147.94, 138.38, 129.30, 124.23, 117.98, 113.21, 42.89. HRMS: m/z [M + H]$^+$ calcd for C₁₈H₁₉N₂S: 291.0956, found: 291.0963.

**3,4-Bis(3-methylanilinomethyl)thiophene, (8b)** The product was purified in a 20:1 Hexanes/Ethyl Acetate solvent system and was obtained as an orange solid in 26% yield. $^1$H NMR (400 MHz, CDCl₃): $\delta$ ppm 2.34 (6 H, s) 4.77 (4 H, s) 6.81 (2 H, d, $J$=7.83 Hz) 6.84 (2 H, s) 7.00 (2 H, s) 7.18 - 7.28 (4 H, m); $^{13}$C NMR (100 MHz, CDCl₃): $\delta$ ppm 21.83, 49.59, 114.71, 116.91, 118.75, 120.29, 128.74, 138.62, 140.60, 151.66. HRMS: m/z [M + H]$^+$ calcd for C₂₀H₂₃N₂S: 323.1576, found: 323.1561.

**3,4-Bis(4-methylanilinomethyl)thiophene, (8c)** The product was purified in a 20:1 Hexanes/Ethyl Acetate solvent system and was obtained as an orange solid in 19% yield. $^1$H NMR (400 MHz, CDCl₃): $\delta$ ppm 2.33 (6 H, s) 4.77 (4 H, s) 6.90 (4 H, d, $J$=8.08 Hz) 7.01 (2 H, s) 7.80 (4 H, d, $J$=8.34 Hz); $^{13}$C NMR (100 MHz, CDCl₃): $\delta$ ppm 20.82, 49.67, 114.69, 120.05, 121.71, 123.21, 129.47. HRMS: m/z [M + H]$^+$ calcd for C₂₀H₂₃N₂S: 323.1576, found: 323.1565.

**3,4-Bis(4-ethylanilinomethyl)thiophene, (8d)** The product was purified in a 15:1 Hexanes/Ethyl Acetate solvent system and as obtained as an orange oil in 3% yield. $^1$H NMR (400 MHz, CDCl₃): $\delta$ ppm 1.19 (t, $J$ = 7.45 Hz, 6 H) 2.54 (q, $J$ = 7.58 Hz, 4 H) 4.28 (s, 4 H) 6.60 (d, $J$ = 8.08 Hz, 4 H) 7.02 (d, $J$ = 8.08 Hz, 4 H) 7.23 (s, 2 H); $^{13}$C NMR (100 MHz, CDCl₃): $\delta$ ppm 147.94, 138.38,
129.30, 124.23, 117.98, 113.21, 42.89. HRMS: m/z [M + H]^+ calcd for C_{22}H_{27}N_{2}S: 351.1889, found: 351.1888.

3,4-Bis(3-fluoroanilinomethyl)thiophene, (8e) The product was purified in a 7:1 Hexanes/Ethyl Acetate solvent system and was obtained as an off-white semi-solid in 12% yield. \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ ppm 4.43 (br. s., 4 H) 6.21 - 6.66 (m, 8 H) 7.14 (d, \(J = 7.07\) Hz, 2 H); \(^13\)C NMR (100 MHz, CDCl\(_3\)): δ ppm 141.68, 137.70, 130.35, 124.53, 114.69, 107.13, 104.13, 99.97, 49.13. HRMS: m/z [M + H]^+ calcd for C\(_{18}\)H\(_{17}\)N\(_2\)S: 331.1061, found: 331.1075.

3,4-Bis(4-fluoroanilinomethyl)thiophene, (8f) The product was purified in a 6:1 Hexane/Ethyl Acetate solvent system and was obtained as a yellowish semisolid in 15% yield. \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ ppm 4.25 (s, 4 H) 6.49 - 6.61 (m, 4 H) 6.88 (t, \(J = 8.72\) Hz, 4 H) 7.23 (s, 2 H); \(^13\)C NMR (100 MHz, CDCl\(_3\)): δ ppm 157.32, 144.26, 138.22, 124.40, 115.86, 115.64, 112.16, 43.58. HRMS: m/z [M + H]^+ calcd for C\(_{18}\)H\(_{17}\)N\(_2\)S: 331.1061, found: 331.1075.

3,4-Bis(3-chloroanilinomethyl)thiophene, (8g) Product was purified in a 15:1 Hexanes/Ethyl Acetate solvent system and was obtained in 31% yield as an off white oil. \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ ppm 4.26 (4 H, s) 6.48 (2 H, dd, \(J=8.21, 1.39\) Hz) 6.60 (2 H, s) 6.69 (2 H, dd, \(J=0.80\) Hz) 7.06 (2 H, t, \(J=7.96\) Hz); \(^13\)C NMR (100 MHz, CDCl\(_3\)): δ ppm 42.60, 111.47, 112.76, 117.85, 124.53, 130.27, 135.05, 137.64, 148.95. HRMS: m/z [M + H]^+ calcd for C\(_{18}\)H\(_{17}\)N\(_2\)Cl\(_2\)S: 363.0484, found: 363.0466.

3,4-Bis(4-chloroanilinomethyl)thiophene, (8h) Product was purified in a 15:1 Hexanes/Ethyl Acetate solvent system and was obtained in 23% yield as a brown solid. \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ ppm 4.26 (s, 4 H) 6.54 (d, \(J=8.84\) Hz, 4 H) 7.11 (d, \(J=8.59\) Hz, 4 H) 7.22 (s, 2 H); \(^13\)C NMR (100 MHz, CDCl\(_3\)): δ ppm 146.39, 137.82, 129.15, 124.50, 122.67, 114.26, 42.97. HRMS: m/z [M + H]^+ calcd for C\(_{18}\)H\(_{17}\)N\(_2\)Cl\(_2\)S: 363.0408, found: 363.0466.
3,4-Bis(3-trifluoromethylanilinomethyl)thiophene, (8i) The product was purified in a 10:1 solvent system of Hexanes/Ethyl Acetate and was obtained in 20% yield as an orange oil. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 4.32 (s, 4 H) 6.74 (d, $J$=8.08 Hz, 2 H) 6.82 (br. s., 2 H) 6.89 - 7.04 (m, 2 H) 7.23 - 7.33 (m, 4 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 147.95, 137.47, 131.48, 129.76, 125.63, 125.77, 116.09, 114.50, 109.28, 42.61 HRMS: m/z [M + H]$^+$ calcd for C$_{20}$H$_{17}$F$_6$N$_2$S: 431.1011, found: 431.0994.

3,4-Bis(4-trifluoromethylanilinomethyl)thiophene, (8j) The product was purified in a 10:1 Hexanes/Ethyl Acetate solvent system and was obtained in 6% yield as a white semi-solid. $^1$H NMR (400 MHz, CDCl$_3$): δ ppm 4.33 (br. s., 4 H) 6.62 (d, $J$=8.08 Hz, 4 H) 7.25 (s, 2 H) 7.40 (d, $J$=8.34 Hz, 4 H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ ppm 150.17, 137.26, 126.73, 126.69, 126.66, 124.77, 112.21, 42.36. HRMS: m/z [M + H]$^+$ calcd for C$_{20}$H$_{17}$F$_6$N$_2$S: 431.1011, found: 431.0995.
5.3.4 General Procedure for the Synthesis of the 2,5-Pyrazine Analogues (14)

Procedure for the Synthesis of 2,5-Dimethylpyrazine-1,4-dioxide (10)

The dioxide compound was prepared from a literature procedure\textsuperscript{152} where 20.20mL (184.94 mmol) of 2,5-dimethyl pyrazine (9) was added to 50 mL of ethyl acetate. This solution was combined to a solution of \textit{m}-CPBA (70\% purity, 405.63 mmol) dissolved in 150 mL of ethyl acetate that had been washed with 150 mL of brine and dried over MgSO\textsubscript{4}. The mixture was stirred at room temperature for 24 hours and a white precipitate formed. The precipitate was then filtered and washed with 100 mL of ethyl acetate in triplicate. This compound was yielded in 90\% percent as a white solid. \textsuperscript{1}H was used to confirm structure with the literature characterization.

Procedure for the Synthesis of 2,5-Di(acetoxymethyl)pyrazine (11)

The di(acetoxymethyl) compound was prepared from a literature procedure\textsuperscript{152} where 20 g of compound 10 (142.72 mmol) was added to 100 mL of acetic anhydride and was refluxed at 158\degree C for seven hours. The solution was then stirred at room temperature for twelve hours. The resulting product was a black residue and was acquired by removing the acetic anhydride under reduced pressure. 500 mL of diethyl ether was added to the black residue and was stirred at room temperature for two hours. The solution was then filtered and the remaining residue was washed with 100 mL of diethyl ether. The filtrate was condensed under reduced pressure to yield a dark yellow residue. The residue was purified via flash column chromatography using a solution of 40\% ethyl acetate and 60\% hexanes. This yellow product was then recrystallized from ethyl acetate and hexanes to yield a white solid in 16\% yield. \textsuperscript{1}H NMR was used to confirm the structure with the literature characterization.
Procedure for the Synthesis of 2,5-Di(hydroxymethyl)pyrazine (12)

The di(hydroxymethyl) compound was prepared from a literature procedure\textsuperscript{152} where 4.0 g of compound 11 (17.84 mmol) was suspended in 100 mL of dry methanol. 0.8 g of NaOMe (14.80 mmol) was added. The solution was stirred at room temperature for three hours under N\textsubscript{2}. 105 g of NH\textsubscript{4}Cl was added to quench the reaction and the methanol was removed under reduced pressure. The remaining residue was purified by flash chromatography using a solution of 10\% methanol and 90\% chloroform to yield a white solid in 91\% yield. $^1$H NMR was used to confirm the structure with the literature characterization.

Procedure for the Synthesis of 2,5-pyrazinedicarbaldehyde (13)

The di(hydroxymethyl) compound was prepared from a literature procedure\textsuperscript{152} where 2.0 g of compound 12 (14.27 mmol) was added to a 100 mL solution of dry 1,4-dioxane. 6.0 g of dry activated MnO\textsubscript{2} (69.00 mmol) was added and the mixture was refluxed under N\textsubscript{2} for forty-five minutes at 96\degree C. The solution was filtered through a fritted filter funnel and was concentrated under reduced pressure to yield a light yellow solid in 80\% yield. $^1$H NMR was used to confirm the structure with the literature characterization.

General Procedure for the Synthesis of the 2,5-pyrazinedicarbaldehyde analogues (14)

To a solution of methanol, 32 mg of compound 13 (0.2351 mmol) was combined in a dry vial with the aniline derivative of choice (0.5172 mmol) and 96.02 mg (0.7053 mmol) of ZnCl\textsubscript{2}. The solution was stirred for two hours at room temperature before 44.32 mg (0.7053 mmol) of
NaBH₃CN was added. The solution was then stirred overnight and purified by flash chromatography.

**3,4-Bis(anilinomethyl)pyrazine, (14a)** The product was purified in a 1:1 Hexane/Ethyl Acetate solvent system and was obtained as an orange solid in 10% yield. \(^1\)H NMR (400 MHz, CDCl₃): \(\delta\) ppm 4.50 (s, 4 H) 6.68 (d, \(J=7.83\) Hz, 4 H) 6.75 (t, \(J=7.33\) Hz, 2 H) 7.19 (t, \(J=7.83\) Hz, 4 H) 8.60 (s, 2 H); \(^{13}\)C NMR (100 MHz, CDCl₃): \(\delta\) ppm 152.42, 147.42, 142.71, 129.37, 118.17, 113.18, 47.08. HRMS: m/z [M + H]⁺ calcd for C₁₈H₁₉N₄: 291.1604, found: 291.1599.

**3,4-Bis(3-methylanilinomethyl)pyrazine, (14b)** The product was purified in a 1:1 Hexane/Ethyl Acetate solvent system and was obtained as a brown solid in 4% yield. \(^1\)H NMR (400 MHz, CDCl₃): \(\delta\) ppm 8.59 (s, 2H), 7.07 (t, \(J=7.71\) Hz, 2H), 6.23 - 6.67 (m, 6H), 4.49 (s, 4H), 2.27 (s, 6H); \(^{13}\)C NMR (100 MHz, CDCl₃): \(\delta\) ppm 147.49, 146.34, 142.69, 139.18, 129.17, 119.47, 115.92, 112.25, 47.13, 21.44. HRMS: m/z [M + H]⁺ calcd for C₂₀H₂₃N₄: 319.1917, found: 319.1909.

**3,4-Bis(4-ethylanilinomethyl)pyrazine, (14b)** The product was purified in a 1:1 Hexane/Ethyl Acetate solvent system and was obtained as a brown solid in 5% yield. \(^1\)H NMR (400 MHz, CDCl₃): \(\delta\) ppm 1.18 (t, \(J=7.58\) Hz, 6 H), 2.54 (d, \(J=7.58\) Hz, 4 H), 4.48 (s, 4 H), 6.62 (d, \(J=8.08\) Hz, 4 H), 7.03 (d, \(J=8.08\) Hz, 4 H), 8.9 (s, 2 H); \(^{13}\)C NMR (100 MHz, CDCl₃): \(\delta\) ppm 152.60, 145.37, 142.72, 134.05, 128.67, 113.33, 47.45, 27.93, 15.91. HRMS: m/z [M + H]⁺ calcd for C₂₂H₂₇N₄: 347.2230, found: 347.2225.

**3,4-Bis(4-fluoroanilinomethyl)pyrazine, (14d)** The product was purified in a 1:1 Hexane/Ethyl Acetate solvent system and was obtained as a light orange solid in 5% yield. \(^1\)H NMR: \(\delta\) ppm 4.46 (br. s., 4 H) 6.55 - 6.67 (m, 4 H) 6.90 (m, \(J=8.20\), 8.20 Hz, 4 H) 8.58 (s, 2 H); \(^{13}\)C NMR: \(\delta\) ppm
157.39, 155.05, 152.31, 143.71, 142.73, 115.94, 114.09, 114.02, 47.63. HRMS: m/z [M + H]^+ calcd for \( \text{C}_{18}\text{H}_{17}\text{N}_{4}\text{F}_{2} \): 327.1416, found: 327.1409.
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91.


APPENDICES

NMR Spectra

\textit{\textsuperscript{1}H NMR Spectra}

\begin{center}
\includegraphics{TG12-1_FINAL_PROTON_COPY.ESP}
\end{center}

\begin{center}
\includegraphics{1H_NMR_Spectrum_for_Compound_2a}
\end{center}

\textsuperscript{1}H NMR Spectrum for Compound 2a
$^1$H NMR Spectrum for Compound 2b
$^1$H NMR Spectrum for Compound 2c
1H NMR Spectrum for Compound 2d
\(^1\)H NMR Spectrum for Compound 2e

![NMR Spectrum](image-url)
$^1$H NMR Spectrum for Compound 2f

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\end{array}
\]
$^1$H NMR Spectrum for Compound 2g

![NMR Spectrum Image]
$^1$H NMR Spectrum for Compound 2h
$^1$H NMR Spectrum for Compound 2i

![NMR Spectrum](TG13-1_Final_proton.esp)
\(^1\text{H NMR Spectrum for Compound 2j}\)

![Chemical structure diagram](attachment:Chemical-Structure-Diagram.png)
$^1$H NMR Spectrum for Compound 2k

\[
\begin{align*}
\text{Cl} & \quad \text{N} \\
\text{H} & \quad \text{N} \\
\text{N} & \quad \text{Cl}
\end{align*}
\]
\[ ^1 \text{H NMR Spectrum for Compound 2l} \]
$^1$H NMR Spectrum for Compound 2m
$^1$H NMR Spectrum for Compound 2n

\[
\begin{array}{c}
\text{OMe} \\
\text{MeO}
\end{array}
\]
$^1$H NMR Spectrum for Compound 2o

![NMR Spectrum](image_url)
$^1$H NMR Spectrum for Compound 2p

![H NMR Spectrum](image-url)
$^1$H NMR Spectrum for Compound 2q

![NMR Spectrum Image]

The spectrum shows multiple peaks at different chemical shifts, indicating the presence of various protons in the compound.

The structure of Compound 2q is shown below:

![Compound Structure Image]
$^1$H NMR Spectrum for Compound 2r

![NMR Spectrum Image]
$^1$H NMR Spectrum for Compound 2s
$^1$H NMR Spectrum for Compound 2t

\[ \text{MeS} \quad \text{MeS} \]
$^1$H NMR Spectrum for Compound 2u

![H NMR Spectrum for Compound 2u](image-url)
\(^\text{1H}\) NMR Spectrum for Compound 2v

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{H} \quad \text{N} \\
\text{H} & \quad \text{N} \quad \text{H} \\
& \quad \text{O}_2\text{N}
\end{align*}
\]
$^1$H NMR Spectrum for Compound 2w
$^1$H NMR Spectrum for Compound 2x
\^1H NMR Spectrum for Compound 4a
$^1$H NMR Spectrum for Compound 4b

\[
\text{Structure of Compound 4b}
\]
$^1$H NMR Spectrum for Compound 4c

![NMR Spectrum Image]
$^1$H NMR Spectrum for Compound 4d

\[ \text{Chemical Shift (ppm)} \]

\[
\begin{array}{cccccccc}
6.98 & 4.00 & 4.20 & 1.92 & 6.57 & 6.55 & 6.10 & 4.21 & 2.22 \\
\end{array}
\]
$^1$H NMR Spectrum for Compound 4e

\[
\begin{align*}
\text{HN} & - \text{O} - \text{HN} \\
\text{N} & - \text{C} - \text{N} \\
\text{N} & - \text{C} - \text{N}
\end{align*}
\]
\( ^1H \) NMR Spectrum for Compound 4f

\[
\begin{align*}
\text{Chemical Shift (ppm)} & \quad \text{Normalized Intensity} \\
2.00 & \quad 8.00 \\
5.93 & \quad 8.00 \\
1.92 & \quad 8.00 \\
4.03 & \quad 8.00 \\
4.02 & \quad 8.00 \\
6.11 & \quad 8.00
\end{align*}
\]
$^1$H NMR Spectrum for Compound 4g
$^1$H NMR Spectrum for Compound 4h

![Chemical structure of compound 4h]
$^1$H NMR Spectrum for Compound 4i

\[ \text{Chemical Shift (ppm)} \]

Normalized Intensity:
- 3.84
- 1.80
- 6.21
- 2.00

M02(s), M03(dd), M04(m), M05(m), M01(s)

Chemical Shifts:
- 7.11
- 7.09
- 7.08
- 7.05
- 6.43
- 6.41
- 6.38
- 6.35
- 6.15
- 4.23
$^1$H NMR Spectrum for Compound 4j

![NMR Spectrum Image]
$^1$H NMR Spectrum for Compound 4k
$^1$H NMR Spectrum for Compound 4l
$^1$H NMR Spectrum for Compound 4m

\[
\text{Chemical Shift (ppm)}: 7.10, 7.08, 6.55, 6.12, 4.21
\]

\[
\text{Normalized Intensity: } 4.44, 2.03, 4.81, 4.00
\]
$^1$H NMR Spectrum for Compound 4n

![NMR Spectrum Image]
$^1$H NMR Spectrum for Compound 4o

![H NMR Spectrum](image-url)
$^1$H NMR Spectrum for Compound 4p

MeO \[\begin{array}{c}
\text{H} \\
\text{N} \\
\text{N} \\
\text{H} \\
\text{N} \\
\text{O} \end{array} \]
\(^1\)H NMR Spectrum for Compound 4q

![Chemical structure of Compound 4q](image-url)
\[ ^1\text{H NMR Spectrum for Compound 4r} \]

\[
\begin{align*}
\text{F}_3\text{C} & \quad \text{N} \\
& \quad \text{O} \\
& \quad \text{N} \\
& \quad \text{CF}_3
\end{align*}
\]
\(^1\)H NMR Spectrum for Compound 4s
$^1$H NMR Spectrum for Compound 4t
$^1$H NMR Spectrum for Compound 4u

![H NMR Spectrum](image-url)
$^1$H NMR Spectrum for Compound \textit{8a}
$^1$H NMR Spectrum for Compound 8b
$^1$H NMR Spectrum for Compound 8c
1H NMR Spectrum for Compound 8d

![NMR Spectrum Diagram]
$^1$H NMR Spectrum for Compound 8e
$^1$H NMR Spectrum for Compound 8f
\(^1\)H NMR Spectrum for Compound 8g
$^1$H NMR Spectrum for Compound 8h
$^1$H NMR Spectrum for Compound 8i
\(^1\)H NMR Spectrum for Compound **8j**

![H NMR Spectrum](image)

**Chemical Shift (ppm)**

- M01(d): 7.41
- M03(br. s.): 7.39
- M02(d): 7.25
- M04(s): 6.63
- 3.94
- 3.63
- 3.54
- 3.00
- 2.00
- 3.54

**Normalized Intensity**

- 3.94
- 3.63
- 2.00
- 3.54

**Compound Structure**

![Compound Structure](image)
$^1$H NMR Spectrum for Compound 14a

\[
\text{\begin{tikzpicture}
  \draw (0,0) -- (0,1); \draw (1,0) -- (1,1);
  \draw (0.5,0.5) -- (1.5,0.5);
  \node at (0.75,0.75) {\text{NH}};
  \node at (0.75,0.25) {\text{N}};
  \node at (0.25,0.75) {\text{NH}};
  \node at (0.25,0.25) {\text{N}};
  \node at (0.25,0.25) {\text{N}};
  \node at (0.75,0.75) {\text{N}};
\end{tikzpicture}}
\]
$^1$H NMR Spectrum for Compound 14b
\(^1\)H NMR Spectrum for Compound 14c

\[
\text{\includegraphics[width=\textwidth]{compound14c.png}}
\]
$^1$H NMR Spectrum for Compound 14d
$^{13}$C NMR Spectra

$^{13}$C NMR Spectrum for Compound 2a

![Chemical Structure](image)

Normalized Intensity

- 158.13
- 147.96
- 137.28
- 129.31
- 119.91
- 113.13
- 77.41
- 77.09
- 76.77
- 49.29
- 29.75
\[ ^{13}C \text{ NMR Spectrum for Compound 2b} \]

\[
\begin{align*}
-158.02 & \\
-145.84 & \\
-137.28 & \\
-130.08 & \\
-127.13 & \\
-122.27 & \\
-119.99 & \\
-117.22 & \\
-110.14 & \\
-77.33 & \\
-77.02 & \\
-75.70 & \\
-49.22 & \\
-17.59 & 
\end{align*}
\]
$^{13}$C NMR Spectrum for Compound $2c$

![Chemical Structure](image)
$^{13}$C NMR Spectrum for Compound 2d
$^{13}$C NMR Spectrum for Compound 2e

![Chemical Structure](image)
$^{13}$C NMR Spectrum for Compound 2f
$^{13}$C NMR Spectrum for Compound 2g
$^{13}$C NMR Spectrum for Compound $2h$

![C NMR Spectrum](image.png)
$^{13}$C NMR Spectrum for Compound 2i
$^{13}$C NMR Spectrum for Compound 2j
$^{13}$C NMR Spectrum for Compound 2k

\[
\begin{array}{c}
\text{Cl} \\
\text{N} \\
\text{H} \\
\text{N} \\
\text{Cl}
\end{array}
\]
$^{13}$C NMR Spectrum for Compound 2l

![Diagram of compound 2l]
$^{13}$C NMR Spectrum for Compound 2m

\[ \text{\textbf{Chemical Shift (ppm):}} \]

\[ \begin{align*}
157.64 & \quad 146.43 & \quad 137.38 & \quad 129.12 & \quad 122.29 & \quad 120.02 & \quad 114.16 & \quad 77.34 & \quad 77.03 & \quad 76.71 & \quad 49.21
\end{align*} \]
$^{13}$C NMR Spectrum for Compound 2n

![Chemical Structure](image)
$^{13}$C NMR Spectrum for Compound 2o

\[
\begin{align*}
\text{OMe} & \quad \text{OMe} \\
\end{align*}
\]
$^{13}$C NMR Spectrum for Compound 2p

MeO

\[
\begin{align*}
\text{H} & \quad \text{N} & \quad \text{N} & \quad \text{N} & \quad \text{H} \\
\text{O} & \quad \text{Me} & \quad \text{Me} & \quad \text{Me} & \quad \text{Me}
\end{align*}
\]
$^{13}\text{C}$ NMR Spectrum for Compound $2q$

![Carbon-13 NMR Spectrum](TG90_Carbon.esp)
$^{13}$C NMR Spectrum for Compound 2r

\[
\begin{align*}
\text{CF}_3 & \quad \text{N} \quad \text{N} \\
\text{H} & \quad \text{N} \quad \text{N} \\
\text{CF}_3 & \quad \text{H}
\end{align*}
\]
$\text{C NMR Spectrum for Compound 2s}$

$\text{F}_3\text{C}$

$\text{NH}$

$\text{N}$

$\text{NH}$

$\text{CF}_3$
$^{13}$C NMR Spectrum for Compound 2t

![Chemical Structure Image]

```latex
\text{MeS} \quad \begin{array}{c} N \quad \begin{array}{c} H \quad \begin{array}{c} N \quad \begin{array}{c} H \quad \begin{array}{c} N \quad \begin{array}{c} \text{H} \quad \begin{array}{c} \text{SMe} \end{array} \end{array} \end{array} \end{array} \end{array} \end{array} \end{array}
```
$^{13}$C NMR Spectrum for Compound 2u

![NMR spectrum diagram]
$^{13}$C NMR Spectrum for Compound 2v

\[
\text{O}_2\text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{O}_2
\]
$^{13}$C NMR Spectrum for Compound 2w

\[
\begin{array}{c}
\text{O} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{O} \\
\end{array}
\]
$^{13}$C NMR Spectrum for Compound 2x

\[ \text{Diagram of compound} \]
$^{13}$C NMR Spectrum for Compound 4a

\[
\text{\begin{picture}(100,100)
\end{picture}}\]
$^{13}$C NMR Spectrum for Compound 4b

![Chemical Structure Image]

152.22
145.56
130.14
127.07
122.34
117.61
110.13
107.83
77.34
77.02
76.71
41.50
17.49
\(^{13}\)C NMR Spectrum for Compound 4c
$^{13}$C NMR Spectrum for Compound 4d
$^{13}$C NMR Spectrum for Compound 4e
$^{13}$C NMR Spectrum for Compound 4f
\(^{13}\)C NMR Spectrum for Compound 4g
$^{13}$C NMR Spectrum for Compound 4h

\[\text{Diagram of Compound 4h}\]
$^{13}$C NMR Spectrum for Compound 4i

![Chemical structure of Compound 4i]
$^{13}$C NMR Spectrum for Compound 4j

\[
\text{F} - \text{N} - \text{O} - \text{N} - \text{F}
\]
$^{13}$C NMR Spectrum for Compound $4k$

![Chemical structure diagram]
$^{13}$C NMR Spectrum for Compound 41
$^{13}$C NMR Spectrum for Compound 4m
$^{13}$C NMR Spectrum for Compound 4n

![C NMR Spectrum for Compound 4n](image)
$^{13}$C NMR Spectrum for Compound 4o

![Chemical Structure of Compound 4o]
$^{13}$C NMR Spectrum for Compound 4p

![Chemical Structure of Compound 4p](image)
$^{13}$C NMR Spectrum for Compound 4q
$^{13}$C NMR Spectrum for Compound $4r$

![Chemical Shift Graph]

Normalized Intensity

Normalized Intensity

Normalized Intensity

Normalized Intensity

Normalized Intensity

Normalized Intensity

Normalized Intensity

Normalized Intensity

Chemical Shift (ppm)

0

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1.0

151.62

147.67

129.73

129.65

117.97

116.11

114.46

108.23

77.75

77.71

76.71

41.15

220 200 180 160 140 120 100 80 60 40 20 0 -20

Chemical Shift (ppm)
$^{13}$C NMR Spectrum for Compound 4s
$^{13}$C NMR Spectrum for Compound 4t

![Chemical Structure of Compound 4t](image)
$^{13}$C NMR Spectrum for Compound 4u
$^{13}$C NMR Spectrum for Compound 8a
$^{13}$C NMR Spectrum for Compound 8b
$^{13}$C NMR Spectrum for Compound 8c
<table>
<thead>
<tr>
<th>Chemical Shift (ppm)</th>
<th>Normalized Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.41</td>
<td>3.94</td>
</tr>
<tr>
<td>7.39</td>
<td>3.63</td>
</tr>
<tr>
<td>7.25</td>
<td>2.00</td>
</tr>
<tr>
<td>6.63</td>
<td>3.54</td>
</tr>
<tr>
<td>6.61</td>
<td></td>
</tr>
<tr>
<td>4.33</td>
<td></td>
</tr>
</tbody>
</table>

\( ^{13} \text{C NMR Spectrum for Compound 8d} \)
$^{13}$C NMR Spectrum for Compound 8e

![NMR Spectrum](image)
$^{13}$C NMR Spectrum for Compound 8f
$^{13}$C NMR Spectrum for Compound 8g
$^{13}$C NMR Spectrum for Compound 8h

![Chemical Structure](image-url)
$^{13}$C NMR Spectrum for Compound 8i

![](image)
$^{13}$C NMR Spectrum for Compound 8j
$^{13}$C NMR Spectrum for Compound 14a

\[
\begin{array}{c}
\text{NH} \\
\text{N=}\text{N} \\
\text{NH} \\
\end{array}
\]
$^{13}$C NMR Spectrum for Compound 14b

![Chemical Structure](image)
$^{13}$C NMR Spectrum for Compound 14c

\[
\begin{array}{c}
\text{Chemical Shift (ppm)}
\end{array}
\]

\[
\begin{array}{c}
\text{Normalized Intensity}
\end{array}
\]

\[
\begin{array}{c}
\text{TMS}
\end{array}
\]

\[
\begin{array}{c}
152.60 \\
145.37 \\
142.73 \\
134.05 \\
128.67 \\
113.33 \\
77.34 \\
77.02 \\
76.70 \\
47.45 \\
27.93 \\
15.91
\end{array}
\]
$^{13}$C NMR Spectrum for Compound 14d
Mass Spectra for compound 2a in MeOH 100x dilution

THERESA_TG12-1_MOORING-ACCU_07012013_ESI-POS02 64 (1.193) AM (Top,2, Ar,5000.0,0.00,1.00); Sm (SG, 2x3.00); Cr 1.15e4

mass spectra

mass spectra for compound 2a

100 150 200 250 300 350 400 450 500 550 600

0 100 200 300 400 500 600

m/z

93.1245 183.1013 197.1125 286.1455 291.1728 292.1725 379.2231
Mass Spectra for compound 2b
Mass Spectra for compound 2c
Mass Spectra for compound 2d
Mass Spectra for compound 2e
Mass Spectra for compound 2f
Mass Spectra for compound 2g
Mass Spectra for compound 2h
Mass Spectra for compound 2i

THERESA TG13-1_MOORING-ACCU_07012013_ESI-POS01 54 (1.006) AM (Cen,2, 80.00, Ar,5000.0,0.00,1.00); Sm (SG, 2x3
6.93e3

152.1014 195.0959 215.0945 238.0572
326.1462 327.1527 328.1463 376.1253

m/z

0-1000

50-100

0-100

0-100

0-100

0-100

Mass Spectra for compound 2i
Mass Spectra for compound 2j

THERESA_GG7_HR_ESI_POS_MOORING_03152013_1 181 (3.610) AM (Cen,2, 80.00, Ar,5000.0,556.28,0.70); Cm (177:1) 1.70e4

m/z
300 320 340 360 380 400 420 440 460 480 500 520 540 560 580

F
N
H
N
H
F

in MeOH+0.1%HCOOH 556.2771
Mass Spectra for compound 2k
Mass Spectra for compound 2l
Mass Spectra for compound 2m
Mass Spectra for compound \(2n\)
Mass Spectra for compound 2o
Mass Spectra for compound 2p

in MeOH 100x dilution
Mass Spectra for compound 2q
Mass Spectra for compound 2r
Mass Spectra for compound 2s
Mass Spectra for compound 2t
Mass Spectra for compound 2u

1.60e4

THERESA_TG19_MOORING-ACCU_01142014_ESI-POS01 17 (0.317) AM (Cen,2, 80.00, Ar,5000.0,0.00,1.00); S

Mass Spectra for compound 2u
Mass Spectra for compound 2v
Mass Spectra for compound 2w
Mass Spectra for compound 2x
Mass Spectra for compound 4a
Mass Spectra for compound 4b
Mass Spectra for compound 4c

in ACN+0.1%HCOOH 556.2771 as ITSD

THERESA_TG54_HR_ESIPOS_MOORING_062314 300 (5.578) AM (Cen,4, 80.00, Ar,6000.0,556.28,0.80)

2.29e3

307.1817
319.1493
321.1647
329.1757
426.2225
556.2771
578.2488
Mass Spectra for compound 4d
Mass Spectra for compound 4e
Mass Spectra for compound 4f
Mass Spectra for compound 4g
Mass Spectra for compound 4h
Mass Spectra for compound 4i
Mass Spectra for compound 4j
Mass Spectra for compound 4k
Mass Spectra for compound 4l
Mass Spectra for compound 4m
Mass Spectra for compound 4n
Mass Spectra for compound 4o
Mass Spectra for compound 4p
Mass Spectra for compound 4q
Mass Spectra for compound 4r
Mass Spectra for compound 4s
Mass Spectra for compound 4t
Mass Spectra for compound 4u
Mass Spectra for compound 8a
Mass spectra for compound 8b
Mass spectra for compound 8c
Mass Spectra for compound 8d
Mass Spectra for compound 8e
Mass Spectra for compound 8f

Theresa_TG8_ESIPOS_Mooring_04232014_2 #75  RT: 1.01  AV: 1  NL: 2.82E8
T: FTMS + p ESI Full ms [150.00-2000.00]

331.1077
z=1

220.0592
z=1

Mass Spectra for compound 8f
Mass Spectra for compound 8g
Mass Spectra for compound 8h

TGraines_TG69_ESIPOS_Mooring_100182014 #162  RT: 2.29  AV: 1  NL: 1.24E7
T: FTMS + p ESI Full ms [100.00-1000.00]
Mass Spectra for compound 8i
Mass Spectra for compound 8j
Mass Spectra for compound 14a
Mass Spectra for compound 14b
Mass Spectra for compound $14c$

Theresa_TG43_ESIPOS_Mooring_06112014 #104-234 RT: 1.48-3.27 AV: 131 NL: 6.65E7
T: FTMS + p ESI Full ms [200.00-2000.00]

m/z

Relative Abundance

347.2225
291.1601

200 400 600 800 1000 1200 1400 1600 1800 2000

m/z
Mass Spectra for compound 14d