Analysis and Synthesis of Novel Inhibitors of MMPs and other Biological Targets

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Abstract

Matrix metalloproteinases (MMPs) are a group of complex enzymes known for their role in ECM turnover and homeostasis. These proteinases additionally participate in many other biological processes, but when deregulated contribute to different pathological processes and diseases such as multiple sclerosis and rheumatoid arthritis. Therefore MMPs require tight regulation of their activity. The focus of this project was to gain an understanding of MMPs and to synthesize different novel inhibitors aimed at MMPs and other biological targets.
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Chapter 1: Introduction

Homeostasis is necessary for ideal physiology in any cell. It is not only limited to the intracellular environment of cells, but also applies to a cell’s extracellular environment. The extracellular environment, known as the extracellular matrix (ECM), is assembled when surrounding cells secrete various proteins and polysaccharides (Stamenkovic, 2003). The result is an organized meshwork of molecules that provides both structural and chemical support to cells. Among the molecules that compose and moderate the ECM are ECM proteinases (Sternlicht & Werb, 2001). These molecules are responsible for degrading extracellular proteins, thereby contributing to cell-matrix interaction, physiological and pathological processes, and matrix homeostasis (Stamenkovic, 2003). One such group of proteinases is matrix metalloproteinases (MMPs). Their name matrix metalloproteinase stems from their ability to degrade proteins of the ECM and their dependency on metal ions for catalytic activity (Nagase & Woessner, 1999). All MMPs are synthesized and secreted in their latent proenzyme form and require activation in order to be catalytically active. Once active, MMPs have many beneficial functions including, but not limited to, embryonic development, organ morphogenesis, nerve growth, wound healing, angiogenesis, and apoptosis (Supuran & Winum, 2009). Due to the diverse roles of MMPs, regulation of their activity and expression is essential in order to maintain homeostasis. When not controlled, MMPs contribute to the pathogenesis of various diseases, such as cancer, arthritis, and multiple sclerosis (Nagase & Woessner, 1999). Therefore, their activity is closely regulated, both transcriptionally and post-transcriptionally, with regulation starting at gene expression and extending to their protein level inhibition (Martel-Pelletier et al., 2001).

The involvement of MMPs in pathological processes justifies the impetus for finding viable inhibitors that can specifically target individual or multiple MMPs, in hopes
of mitigating the effects of MMPs in disease pathogenesis (Rodríguez et al., 2010). A focus on MMP inhibition not only, helps in defining how MMPs can be controlled, but the elucidation of their inhibition helps in understanding their roles in various diseases. Consequently, it is of particular value to obtain this information because it details the different features of MMP activity. The goal of this project was to synthesize small inhibitors to test against MMPs and different biological targets. The following chapters detail the background research, experimental procedures, and results during the course of the project.

2.1 Overview of Proteinases

Proteinases are highly characterized enzymes that are classified based on different parameters such as their structure, function, etc. (Blobel & Overall, 2007). These enzymes are first classified based on their position of proteolytic cleavage, with endopeptidases targeting nonterminal peptide bonds and exopeptidases targeting N-terminal or C-terminal peptide bonds (Lopez-Otín & Bond, 2008). In regards to their catalytic mechanism, mammalian endopeptidases are next separated into five classes of aspartic, cysteine, serine, threonine, and metalloproteinases (Cawston & Wilson, 2006). However, despite the contribution of all five classes of proteases to the degradation of the ECM, it is believed that most of the proteolytic activity can be attributed to metalloproteinases (Wojtowicz-Praga et al., 1997). The metalloproteinase class, also known as the metzincin superfamily, is further split into five distinct families (e.g. adamlysins, astacins, matrix metalloproteinases, serralysins, and pappalysins) based on their amino acid sequence (Cawston & Wilson, 2006) (Refer to Figure 1). Furthermore, within the metzincin superfamily, MMPs are of particular interest in drug design due to their dual involvement in both normal and diseased states (Supuran & Winum, 2009).
To fully comprehend MMPs involvement in various diseases, the normal behavior of MMPs must first be understood. The following sections present background information on the characteristics essential to the understanding of MMPs. We will first broadly view MMPs before the focus shifts to the effect their structure and function have on physiological activity. Next, their regulation will be discussed, followed by a look at their roles in specific diseases.

### 2.2 Matrix Metalloproteinases

Human matrix metalloproteinases comprise a family of 27 zinc endopeptidases (Supuran & Winum, 2009). MMPs, or matrixins, are commonly categorized according to three criteria: substrate specificity, domain, and sequence (Sternlicht & Werb, 2001).
Thus, MMPs can be subdivided into five smaller groups based on the aforementioned criteria (Cawston & Wilson, 2006). However, before a protease can be considered an MMP, it must first meet specific features common to MMPs. Classified MMPs all share features such as structural domains, synthesis methods, activation mechanisms, specific inhibitors, and the requirement of zinc for catalytic activity (Parks, 1999). The following sections detail each of the common features and subsequent categorization of MMPs.

2.2.1 Structure and Function

MMPs have well-defined structures that distinguish them from other protease classes and one another as well (Martel-Pelletier et al., 2001). The modular structure of an MMP is comprised of several distinct domains that influence the enzymes complexity (Overall, 2002). Accordingly, classified MMPs are often divided into 8 distinct structural groups based on their modular composition (Sternlicht & Werb, 2001) (See Figure 2).

The MMP family contains three principal domains, the pre-peptide domain, propeptide domain, and zinc dependent catalytic domain, which all remain invariable among the family members. In addition to their principal domains, MMPs may include ancillary domains, such as a hemopexin or a transmembrane domain that further enhance their structure. (Nagase et al., 2006)

Similar to other proteins involved in the secretory pathway, MMPs contain an N-terminal signal or pre-peptide that directs the enzyme out of the cell—the sequence is excised before secretion. The propeptide domain following the signal sequence is the region of the protein responsible for the latency of the enzyme. This ≈ 80-residue domain exhibits a cysteine residue that chelates the zinc ion in the catalytic domain creating a “cysteine switch”. (Sternlicht & Werb, 2001)
At the other end of the cysteine-zinc complex lies the zinc dependent catalytic domain (Nagase & Woessner, 1999). This zinc-binding region, singlehandedly, guides substrate specificities and interactions, contains auxiliary metal ions (e.g. calcium) to facilitate stability, and maintains a methionine residue unique to the whole metzincin family (Sternlicht & Werb, 2001). In the majority of MMPs, with the exception of MMP7

Figure 2: Structure and Composition of MMPs. Acquired from Sternlicht & Werb, 2001.
and MMP26, the catalytic domain is also attached to the C-terminal domain through a variable hinge region that lends flexibility and specificity to the protein (Sternlicht & Werb, 2001). The C-terminal domain is mainly just the hemopexin domain, but some MMPs have additional domains attached as well. The ≈ 210-residue hemopexin domain, composed of four repeat units, is largely recognized for substrate binding, but also contributes to activation among other roles. (Martel-Pelletier et al., 2001)

The variability between MMPs first becomes noticeable in the catalytic domain beginning with a furin-susceptible site (Cawston & Wilson, 2006). MMP 11 and MMP 17 both contain the furin site and as a result are capable of intracellular activation along with all the other MMPs with this site (Pei & Weiss, 1995). Other N-terminal auxiliary domains include the vitronectin-like domain found in MMP21 and the fibronectin-like domain unique to MMP2 and MMP9 for binding collagen, elastin, and gelatin (Klein & Bischoff, 2011; Sternlicht & Werb, 2001). As previously mentioned, some MMPs additionally have other C-terminal domains alongside the hemopexin domain (Nagase et al., 2006). Additional C-terminal domains include a transmembrane domain, GPI domain, and an immunoglobulin like domain (Sternlicht & Werb, 2001).

Each domain found in an MMP contributes to the overall structure and function of the protein. Together the linked domains create a structural and functional synergy. For example, all MMPs require zinc and have optimal activity at a neutral pH (Cawston & Wilson, 2006). Hence, the specialized domains of MMPs notably support substrate specificity, localization, and other properties necessary to their normal function.

2.3 Regulation of MMPs

While MMPs play a major role in the regulation of cellular behavior, these multifaceted enzymes need to be closely regulated as well. Controlling MMP activity is crucial to their normal functioning because without proper regulation pathological behavior arises (Mott & Werb, 2004). Therefore, MMPs are regulated at many levels,
beginning with their transcriptional expression and extending to their post-transcriptional proteolytic activity (Stamenkovic, 2003).

2.3.1 Transcriptional

At any level, the regulation of MMPs can best be described as a balance between stimulation and suppression. The first step of MMP regulation is control of gene expression (Chakraborti et al., 2003). MMPs are normally present in basal amounts in cells, however many different factors and signaling pathways aid in the production of additional MMPs for cell function (Nagase & Woessner, 1999). Inducers such as cytokines, growth factors, chemical agents, cellular stress, etc., can all be stimulators that are capable of interacting, either directly or indirectly, with activators within MMP gene promoter regions. For instance, most MMPs, with the exception of MMP 2 and MMP11, contain an activator protein-1 (AP-1) binding site that primarily interacts with different inducers leading to gene expression (Martel-Pelletier et al., 2001).

Although the aforementioned effectors act as stimulators, it is still possible for these factors and others to act as suppressors as well (Sternlicht & Werb, 2001). Thus, specific signaling pathways or factors may vary in the affect they have on certain MMP genes. One example is transforming growth factor beta (TGF beta), which induces MMP13 transcription, but inhibits the expression of both MMP1 and MMP3 (Sternlicht & Werb, 2001). Another example can be seen with UV-B, where UV-B radiation specifically stimulates MMP 1, MMP 3, and MMP 9 in human dermal fibroblasts (Martel-Pelletier et al., 2001).

Regulation of MMP gene expression is closely controlled due to its variability and complexity (Nagase & Woessner, 1999). Gene expression, for example, can be influenced by soluble regulatory factors or cell-cell and cell-matrix interactions can induce expression (Nagase & Woessner, 1999). MMPs may also be chiefly localized to
a specific area of the cell or it is also possible for an MMP to only be expressed in one cell type. As a result of the variable and complex nature of MMP regulation, each protease is unique and there is no single regulatory pathway that can be applied to every MMP alike. Accordingly, tight regulation helps account for the varying features between different MMPs. (Sternlicht & Werb, 2001)

2.3.2 Post-transcriptional

Following transcription, MMPs are further closely regulated in order to maintain homeostasis. MMP post-transcriptional regulation focuses on MMPs proteolytic behavior, which involves their secretion and activation, localization, inhibition, and other proteolytic processes (Cawston & Wilson, 2006). The subsequent sections address and describe the different ways MMPs are controlled at the protein level.

Secretion and Activation

Once MMPs are translated, they are secreted and require activation before they are capable of degradation (Stamenkovic, 2003). MMPs are first synthesized, within the cell, as inactive zymogens before their signal sequences relay for the enzymes to be secreted from the cell. MMP secretion can be either constitutive or in some cases regulated, which involves other factors for secretory control. An example of an MMP whose secretion is regulated is MMP12, with both plasmin and thrombin prompting secretion. (Sternlicht & Werb, 2001)

After secretion, the endopeptidases are localized within the extracellular space as latent zymogens (Martel-Pelletier et al., 2001). As stated earlier, a “cysteine switch”, within the N-terminal domain, is responsible for conserving the latency of MMPs. In the propeptide domain, a cysteine residue interacts with the zinc ion in the catalytic domain by chelating it and restricting the enzyme to its latent form (Sternlicht & Werb, 2001). When secreted from the cell, MMPs undergo a two-step activation where the cysteine switch is disturbed and successively the propeptide sequence is cleaved thereby
transitioning an MMP from latent to active form. Mechanistically, the thiol group is dispelled and replaced by water allowing the uninhibited zinc ion and water molecule to sequester various substrates. (Van Wart & Birkedal-Hansen, 1990)

While most MMPs are activated outside the cell by other MMPs or proteases (Figure number), some MMPs have ancillary sites that allow intracellular activation. For example, MMP28 contains a furin-susceptible cleavage site and is activated before secretion. (Visse & Nagase, 2003)

**Inhibition**

At the protein level, control of MMPs is essential in order to limit their expression and their irreversible degradation. Furthermore, both specific inhibitors and nonspecific inhibitors are responsible for suppressing active MMPs. (Loffek et al., 2011)

A family of four homologous inhibitors, tissue inhibitor of metalloproteinases (TIMPs 1-4), is known as the major specific, endogenous inhibitor of MMPs (Supuran & Winum, 2009). All active MMPs are inhibited reversibly by TIMPs in a 1:1 ratio (Sternlicht & Werb, 2001). TIMPs are 184-194 residue proteins from different genes that share greater than 35% of sequence homology (Nagase et al., 2006; Martel- Pelletier et al, 2001). Structurally, TIMPs have similar features; all four require their N-terminal domain for MMP inhibition and contain 12 conserved cysteine residues (Supuran & Winum, 2009). According to Murphy and Willenbrock (1994) shortened TIMPs are capable of maintaining inhibitory activity as long as their N-terminal domain is preserved. TIMPs also share the same inhibitory mechanism. The N-terminal amino group and carbonyl group from the first cysteine residue bidentately chelate the zinc ion of an MMP when the ion is wedged within the active site (Nagase et al., 2006). However, despite their structural and functional similarities, TIMPs inhibitory capacities differ among MMPs. For example, TIMP3 inhibits MMP9 better than the remaining TIMPs while TIMP2 and TIMP3 can inhibit MT-MMPs, unlike TIMP1 (Sternlicht & Werb,
Like MMPs, TIMPs are multifaceted and additionally contribute to other biological processes such as angiogenesis, cell division, etc. (Nagase & Woessner, 1999).

TIMPs are not the only endogenous inhibitors, but are joined by a non-specific inhibitor α₂-macroglobulin (Chakraborti et al., 2003). Not only does α₂-macroglobulin inhibit MMPs, but is able to inhibit all proteases. The large ≈ 720 kDa protein irreversibly inhibits MMPs by ensnaring the enzyme, however the effectiveness of the inhibitor is questioned due to its size and inability to penetrate through tissue (Nagase et al., 2006). Other nonspecific inhibitors of MMPs, such as synthetic inhibitors, are also common. Work with these synthesized suppressors tends to focus on designing inhibitors aimed at specific diseases and targeting MMPs allosterically (Fisher & Mobashery, 2006).

Despite their differences, the goal of all MMP inhibitors is to control the overexpression of MMPs and maintain a balanced level of matrix turnover (Wojtowicz-Praga et al., 1997). For, only when there is an imbalance and little or no control over the activity of MMPs do malignancies arise.

**Other Post-transcriptional Regulation**

MMP post-transcriptional regulation is not only limited to secretion, activation, and inhibition, but MMPs also have additional proteolytic processes that are controlled (Cawston & Wilson, 2006). Localization is one way that the enzymes are further modulated. Secreted MMPs are directed to certain areas of the cell, such as membranes, receptors or the ECM (Figure 3), allowing for improved activation and closer proximity to substrates (Sternlicht & Werb, 2001). Enzyme clearance also serves as another way MMPs are moderated. An example of this can be seen when the previously mentioned nonspecific inhibitor, α₂-macroglobulin complexes with an MMP. The enzyme-inhibitor complex undergoes a conformational change and the two are effectively released from the cell. (Nagase et al., 2006).
2.4 MMPs in Disease

MMPs participate in many biological processes, but when deregulated these enzymes are also involved in a variety of diseases such as osteoarthritis, rheumatoid arthritis, and multiple sclerosis (Klein & Bischoff, 2011). Due to the variability of diseases MMPs are associated with, in the upcoming sections MMPs will be classified based on their roles in several non-malignant and malignant diseases.
2.4.1 MMPs roles in non-malignant diseases

Non-malignant diseases may be considered non-invasive compared to other metastatic diseases; however, they may still be chronic or equally debilitating to one’s health. Rheumatoid arthritis (RA) is a chronic autoimmune disease that involves the overexpression of MMP2 and MMP9 in synovial fluid (Cawston & Wilson, 2006). Marked by cartilage breakdown and joint inflammation, RA is seen in 1% of the global population with greater incidence in women (Andrews et al., 2012). MMP3 and MMP10, also known as stromelysins, are seen in articular cartilage and soft tissue of RA patients as well. The role of these several MMPs in RA is seen with the increase in matrix degradation (Zhang et al., 2004). The MMPs proliferate within the synovial joints and synovial tissue performing catabolic activity and progressively destroying both cartilage and bone (Cawston & Wilson, 2006). It is also possible for RA to be malignant, but this form of the disease is rare and is characterized by accelerated joint and articular damage.

Osteoarthritis (OA) is another disease that involves the proliferation of MMPs and is marked by destruction of cartilage and increased inflammation (Wang et al., 2011). Cawston (2006) claims that a major difference between RA and OA is that OA is collectively marked by matrix degradation and matrix synthesis where RA is only affected by matrix degradation. MMPs known to affect OA are the abovementioned stromelysins and MMP13 (Klein & Bischoff, 2011).

Gastrointestinal diseases, like Crohn’s disease and celiac disease, have even been seen to show signs of uncontrolled MMPs. Each of these two respective diseases is characterized by intestinal discomfort and inflammation and research has shown that MMP1, MMP3, MMP12, and MMP13 are all involved in these diseases (Schuppan et al., 2009). Periodontal diseases are another area that shows the appearance of poorly controlled MMPs with MMP 1, MMP8, MMP9, MMP13, and MMP14 all linked to oral conditions (Sorsa et al., 2004).
2.4.2 MMPs roles in malignant diseases

With so many levels to cancer research, it comes as no surprise that MMPs play a role in different forms of cancer as well. To date most MMPs contribute to cancer in some way, whether an MMP is responsible for tumor growth or like MMP 7, made by cancerous cells (Klein & Bischoff, 2011). Various MMPs have been specifically associated to one or multiple types, for example, MMP13 is linked to tumor progression in breast cancer, the gelatinases, MMP 2 and MMP9, are seen in a range of cancers including skin and gastric cancer (Klein & Bischoff, 2011), and MMP2 is also present in prostate cancer (Kleiner & Stetler-Stevenson, 1999).

Neuroinflammation is another area where MMPs have been noted as important (Rosenberg, 2002a). It is understood that MMPs help disrupt the blood brain barrier by heightening the rate and manifestation of inflammation (Rosenberg, 2002b). Again, similar to MMPs roles in other diseases, it is the increased levels of MMPs that affect different neurodegenerative diseases. With the demyelinating disease multiple sclerosis (MS), MMPs are specifically secreted from leukocytes to spread into the CNS and target the myelin sheath (Rosenberg, 2002b). It was even discovered that the raised levels of primarily MMP9, though MMP2 as well, are directly correlated to the appearance of MS (Rosenberg, 2002b).

Chapter Summary

MMPs are complex and tightly modulated enzymes that are considered major contributors to the turnover and composition of the ECM (Stamenkovic, 2003). Understanding the physiological and pathological affects MMPs have on the ECM is not only important, but helps with the design of possible potent inhibitors (Supuran & Winum, 2009). The focus and objective of this project was peptide synthesis of small inhibitors that would be used to test against MMPs and different biological targets to measure biological activity, potency, etc. The research conducted above aided in the
comprehensive analysis by elucidating how MMPs function, the effect of inhibitors on MMPs, and the dynamic of the enzyme-inhibitor complex.
Chapter 3: Experimental

The following methods were all general procedures adapted from Goodman et al. and modified for the purpose of the syntheses shown. All UHPLC-MS were obtained using an Ultimate 3000. All $^1$H and $^{13}$C NMR spectra were measured with a Bruker AVANCE III HD 500 MHz system. Chemical shifts are presented in ppm relative to the internal standard tetramethylsilane found at 0.00 ppm. Flash chromatography was run with a silica gel column and observed on a Teledyne Isco CombiFlash Rf+.
Compound 2: 3-amino-3-phenylpropanoic acid or Beta-phenylalanine

In a 250 mL round bottom flask equipped with a condenser, compound 1 (9.70 mL, 9.6 mmol) was dissolved in ethanol (125 mL) followed by the addition of malonic acid (9.98 g, 9.6 mmol) and ammonium acetate (14.85 g, 9.6 mmol). The mixture was stirred and allowed to reflux for 24 hr. The mixture was cooled, vacuum filtrated and washed with 50/50 ethanol and diethyl ether. The final white solid obtained was used without further purification to yield 2 (6.08 g, 74%). $^1$H NMR (D2O, 500 MHz) δ 2.79 (2 x dd RE, $J = 16.2, 16.2, 8.1, 6.5$ Hz, 2H), 4.58 (dd, $J = 8.0, 6.7$ Hz, 1H), 7.40 (m, 5H).
Compound 3: 3-amino-3-phenylpropanoic acid methyl ester or Beta-phenylalanine methyl ester

In a 250 mL three-neck round bottom flask outfitted with a condenser and gas chamber, solid 2 (5.00 g, 1.0 eq.) was suspended in methanol (200 mL). The suspension was cooled to -5 °C with an ice bath followed by the addition of thionyl chloride (5.4 mL, 2.5 eq.) dropwise over a period of 10 minutes. The reaction mixture was stirred and allowed to reflux for 4 hours. The reaction was monitored by UHPLC-MS before quenching with water. The solvent was removed under vacuum. The white slurry was refrigerated overnight at -20 °C. The crystallized product was resuspended in ethyl acetate, vacuum filtrated, and dried by high vacuum to produce 3 (5.31 g, 99%) as a crystal white solid. 1H NMR (D2O, 500 MHz) δ 3.11 (2 x dd RE, J = 16.9, 16.9, 7.5, 7.0 Hz, 2H), 3.60 (s, 3H), 4.74 (t, J = 7.3 Hz, 1H), 7.40 (m, 5H).
Compound 4: methyl 3-[3-(benzyloxy)-2-[(tertbutoxycarbonyl) amino]propanamido]-3-phenylpropanoate

To a 100 mL round bottom flask, compound 3 (0.54 g, 1.0 eq.) and Boc-Ser(Bzl)-OH (0.89 g, 1.0 eq.) were dissolved in DMF (42 mL). The solution was cooled to -10 °C in an ice bath before solid hydroxybenzotriazole (HOBt) (0.46 g, 1.0 eq.) was first introduced followed by the addition of solid tetramethyluronium tetrafluoroborate (TBTU) (1.93 g, 2.0 eq.). The reaction mixture was allowed to stir and neutralized with 4-methylmorpholine (1.3 mL, 4.0 eq.). The solution was left overnight at room temperature with constant stirring. The solvent was removed under vacuum and the orange-red colored crude oil was partitioned between ethyl acetate and 5% citric acid. The aqueous phase was further washed with ethyl acetate and the organic phases were collected and washed with 5% sodium bicarbonate and saturated NaCl. The washed organic layer was dried over Na$_2$SO$_4$ and solvent was removed under vacuum. The resulting viscous yellow oil obtained was purified by normal phase chromatography on silica gel (0-100% ethyl acetate in cyclohexane) to yield 4 (0.78 g, 57%) as a white solid. $^1$H NMR (CDCl$_3$, 500 MHz) δ 1.40 (s, 9H), 1.50 (s, 3H), 2.80 (2 x dd RE, 2H), 7.30 (m, 10H). Not all signals were seen with $^1$H NMR, however the mass was seen with UHPLC-MS.
**Compound 5: methyl 3-[2-amino-3-(benzyloxy)propanamido]-3-phenylpropanoate**

A 3M HCl-dioxane solution (22 mL) was prepared and added to a 50 mL round bottom flask with 4 (1.0 g, 2.2 mmol). The solution was stirred at room temperature and monitored with TLC (1:1 ethyl acetate/cyclohexane). After 3 hours, more dioxane solution (10 mL) was added. The reaction was stopped after 5 hours and poured into cooled saturated sodium carbonate solution (40 mL) and left overnight with constant stirring. Solvent was removed and the white slurry was extracted with dichloromethane. The organic layer was washed with 5% sodium bicarbonate and saturated NaCl and dried over Na$_2$SO$_4$. Solvent was removed under vacuum yielding 5 (0.72 g, 92%). The crude yellow oil was used without additional purification. $^1$H NMR (CDCl$_3$, 500 MHz) 2.80 (2 x dd RE, 2H), 7.30 (m, 10H), 8.30 (dd, 1 H). C-DEPT was used to help clarify the results of $^1$H NMR.
Compound 6: methyl 3-[3-(benzyloxy)-2-(5-phenylpentanamido) propanamido]-3-phenylpropanoate

To a 25 mL three-neck round bottom flask, 5 (100 mg, 0.3 mmol, 1.0 eq.) and 5-phenylvaleric acid (71 mg, 0.4 mmol, 1.3 eq.) were added and dissolved in DMF (18 mL). The mixture was cooled to -10°C with an ice bath when HOBt (50 mg, 0.3 mmol, 1.0 eq.) followed by TBTU (190 mg, 0.6 mmol, 2.0 eq.) were each added as solid. The reaction mixture was allowed to stir and neutralized with 4-methylmorpholine (0.13 mL, 1.2 mmol, 4.0 eq.). The reaction continued to stir at room temperature and was monitored by TLC (1:1 ethyl acetate/ cyclohexane) and left overnight. UHPLC-MS was used to determine the end of the reaction and the solvent was removed under vacuum.

The product was extracted with ethyl acetate and 5% citric acid. The aqueous phase was further washed with ethyl acetate and the organic phases were collected and washed with 5% sodium bicarbonate and saturated NaCl. The washed organic layer was dried over Na₂SO₄ and solvent was removed under vacuum. The resulting white residue obtained was purified by normal phase chromatography on silica gel (0-100% ethyl acetate in cyclohexane) to produce 6 (100 mg, 69%) as a white solid. ¹H NMR (CDCl₃, 500 MHz) was measured, however it was difficult to specifically elucidate which signal belonged to each H atom.
Compound 7: 3-[3-(benzyloxy)-2-(5-phenylpentanamido) propanamido]-3-phenylpropanoic acid

Compound 6 (70 mg, 0.14 mmol) was dissolved in dioxane (13.5 mL) and allowed to stir at room temperature. The solution received the addition of NaOH (0.2 mL) and the reaction was monitored by UHPLC-MS. After 3 hours, the solution was heated to 60°C and 10% KOH (4 mL) was introduced. The solution was left overnight at room temperature. KOH (2 mL) was added to the solution before solvent was removed by vacuum. The obtained product was purified by chromatography on silica gel (0-80% methanol in dichloromethane). Results of ¹H-NMR concluded that no purified 7 was yielded.
Chapter 4: Results and Discussion
The intermediates and products of the reaction scheme described above can be found in the figure below.

Figure 4: Reaction scheme for synthesis of inhibitors. Adapted from Goodman et al.

The beta-amino acid was prepared by treating benzaldehyde with malonic acid and ammonium acetate to produce Compound 1 as a pure white solid with a final yield of 74%. The results of $^1$H NMR for beta phenylalanine (TAI-01-8/13) were as expected and showed the appearance of three signals used to identify the compound. The spectrum displayed a 5H aromatic multiplet at 7.40 ppm and a 1H doublet of doublets at 4.58 ppm. A signal accounting for the two distinct protons on C2 was found with a roof effect at 2.79 ppm to give a 1H doublet of doublets at 2.83 ppm (J=16.2, 8.1) and another at 2.75 ppm (J= 16.2, 6.5). The two protons were found to be diastereotopic to one another making it hard to elucidate which proton belonged to each signal. However, all protons within the compound were accounted for with the exception of three protic protons, two from the amino group and one from the hydroxyl group, which were invisible due to their exchange with deuterium.

The product was used without further purification and the conversion of 1 to an ester was conducted using methanol and thionyl chloride. The formation of 2 was monitored by UHPLC-MS, which indicated the desired mass after 3 hours. In the $^1$H
NMR spectrum of 2 (TAI-01-16/11), the appearance of a new singlet with an intensity of 3 at 3.60 ppm was indicative of the formation of the ester in excellent yield (99%). The appearance of the other signals was consistent with 1, with two doublet of doublets for the two C2 protons at 3.11 ppm and a 5H multiplet at 7.40 ppm. Another notable change to the spectrum was the loss of the 1H doublet of doublets substituted for a 1H triplet at 4.74 ppm (J=7.3 Hz).

The first coupling could be achieved with Compound 2 by using TBTU along with HOBt to suppress racemization. The UHPLC-MS analysis for compound 3 displayed the desired mass, but initially 3 contained impurities. Following flash chromatography, 3 remained in good yield (57%) and a diastereomeric mixture was observed. The 1H NMR spectrum (TAI-01-15P/14) was difficult to interpret, as not all signals were observable. The elucidation of 3 was not only based on 1H NMR, but UHPLC-MS analysis was also used to verify the formation of 3 since a peak was seen corresponding to the expected mass.

The diastereomeric nature of the compound made the elucidation of the structure more challenging, although some signals were found. The two doublet of doublets appeared with a roof effect at 2.80 ppm revealing that the beta-amino ester remained. A singlet, intensified with solvent, could also be viewed at 1.40 ppm and a 10H multiplet appeared at 7.30 ppm representing the presence of a tertiary butyl group and two aromatic groups, respectively.

Removal of the Boc group from 3 led to deprotected 4. The formation of 4 was justified by the disappearance of a singlet at ≈1.40 ppm. The disappearance of the said signal is indicative of the loss of the tertiary butyl group. The 1H NMR spectrum for 4 (TAI-01-21/11) was more challenging to elucidate, compared to 3, and as a result 13C NMR, C-DEPT NMR, and UHPLC-MS were used to facilitate with the determination of the structure.
In the carbon NMR spectrum, many signals were found that were missing from the $^1$H NMR spectrum; however, a challenge still remained with distinguishing which carbons belonged to each signal. The $^{13}$C-NMR results seemed to be very consistent with the expected spectrum, however some signals remained undetectable which could be due to signal broadening or other factors.

Product 4 was synthesized as a diastereomeric mixture in high yield (92%) and used without any purification. A second coupling reaction could be conducted with 4 and 5-phenylvaleric acid, following the same conditions as the previous coupling with 2, to create 5 (Figure 5).

![Figure 5: Structure of 3-[3-(benzyloxy)-2-(5-phenylpentanamido)propanamido]-3-phenylpropanoic acid](image)

The results of the NMR measurements (TAI-01-24/10) were unclear and therefore, UHPLC-MS analysis had to be used to confirm the synthesis of 5. An attempt directed at synthesizing another compound in the reaction scheme was conducted, however the final NMR data showed neither the disappearance of starting material 5 or new product.
Chapter 5: Conclusion

MMPs are complex, multifunctional enzymes, e.g. their roles in ECM turnover and other cellular processes, their various substrates, their predominantly conserved specificities, their extensive expression, etc., making the criteria and design of inhibitors extremely challenging (Loffek et al., 2011). At the present time, a vast amount of research is available for MMPs and more elucidations continue to be made. With respect to MMP inhibition, previous studies have used different approaches. Some groups have focused on exosites along with allosterically targeting the various enzymes, some groups work aims to enhance current MMP inhibitory mechanisms (i.e. TIMPs), while other groups look into the effects of signaling pathways and transcription factors (Wojtowicz-Praga, 1997; Murphy & Nagase, 2008). Another important aspect of inhibitor design, that researchers also have to consider, is whether or not inhibitors should be synthesized to be enzyme and disease specific or common to multiple, if not all, MMPs (Nagase et al., 2006).

Researchers also face the challenge of controlling the negative effects inhibition can have on physiological MMP activity and other proteases, however so far the effects of inhibitors have been reversible. Although valid inhibitors have been synthesized and tested, imperfections with these inhibitors still pose as a setback for their effectiveness, commercial uses, etc. (Wojtowicz-Praga, 1997)

This study proved that not only the design, but also the synthesis of inhibitors is challenging. The work with these small, novel molecules is still not complete. More compounds need to be synthesized and altogether measures, such as differential MMP binding affinities and kinetics, need to be gathered for each compound. From the results of the overall synthesis, the final compound 7 was not obtained due to time constraints—however, various measures, such as running additional trials, changing the amount of solvent, or trying different solvents, are all techniques that could be used to improve the yield.
Many questions still remain unanswered related to inhibitor design, for example should all levels of regulation be considered as targets or should MMP inhibitors be paired with other treatments for disease (Chakraborti et al., 2003; Wojtowicz-Praga, 1997). At this point and time these inquiries and others continue to be studied, but at the rate that MMP research continues to advance, should be elucidated in no time. With the many physiological and pathological processes MMPs participate in, future hopes for MMP research will remain to be how each inhibitor can effectually regulate MMP behavior.
References


Appendices

Appendix A: NMR Data