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Design, Synthesis, and Evaluation of a Novel Serine Protease Inhibitor Based on Monocyclic β-Lactams

An Honors Thesis

Presented to
The Faculty of the Department of Chemistry and Physics
University of New England

In Fulfillment of the
Requirements for the Degree of
Bachelor of Science
With Honors

by
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3/0/00
Advisor: Prof. William P. Malachowski

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Introduction

Serine proteases are a large family of proteolytic enzymes which utilize a serine residue as a reactive nucleophile in the active site. Some of the more common serine proteases are chymotrypsin, trypsin, thrombin, human leukocyte elastase (HLE), human cathepsin G, and human cytomegalovirus protease (HCMV protease). The method of catalysis of the prototypical serine protease, chymotrypsin, is shown in figure 1.

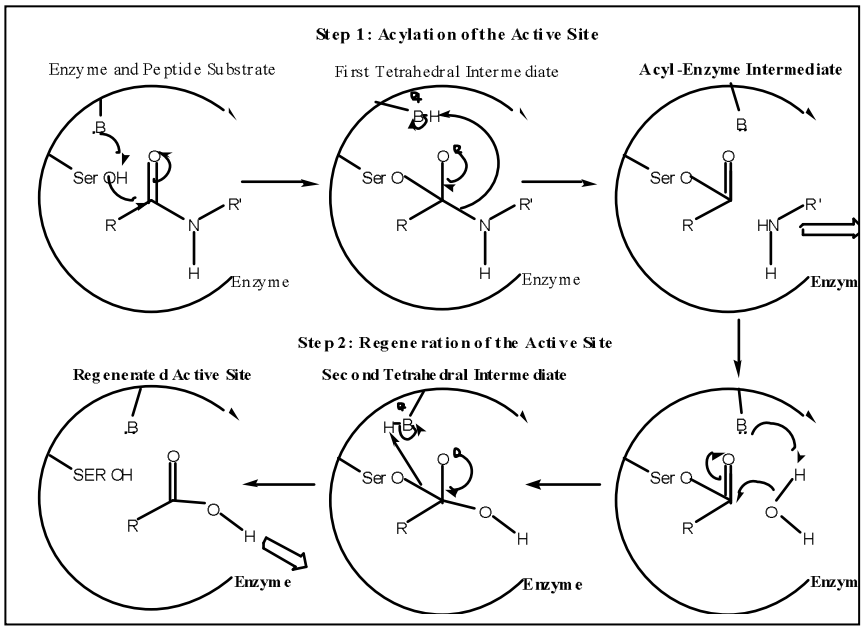


Figure 1[10]**:** Catalytic Method of Chymotrypsin

Unregulated serine protease activity has been implicated in several common ailments, including rheumatoid arthritis, emphysema, pancreatitis, atherosclerosis, cystic fibrosis, and Alzheimer's disease. Since serine proteases are responsible for the onset of such a wide range of diseases, there is an equivalent need for a large library of synthetic serine protease inhibitors to selectively counteract these conditions. To date, several serine protease inhibitors have been developed with reactive functionality,[1] such as aldehydes, azapeptides, boronic acids, chloromethyl ketones, and chloropyrones to disrupt the enzyme's proteolytic activity by trapping the reactive serine nucleophile. A common structural motif in many of these inhibitors has been the incorporation of reactive functionality into a peptide backbone. These molecules compete with natural substrate for binding sites in the enzyme's active site, and utilize the reactive functionality to counteract enzyme activity by trapping the serine alcohol. In addition to these molecules, compounds which have incorporated the monocyclic β-lactam into their structure have also been reported to inhibit some serine proteases. To date, monocyclic β-lactam derivatives have been reported as inhibitors of HLE,[2,3,4] human cathepsin G,[3] β-lactamase,[5]

bacterial leader peptidase,[6] and HCMV protease.[7] All of these reported monocyclic β-lactam inhibitors take advantage of the reactivity of the β-lactam ring, however, none of these previously synthesized inhibitors attempt to deliberately mimic the natural substrates of serine proteases.

The inhibitors proposed in this project will incorporate the reactive β-lactam functionality into the peptide backbone of a serine protease substrate. Molecules with the general structure depicted in figure 2, will be synthesized and tested in an inhibition assay with chymotrypsin,[8] the prototypical serine protease.

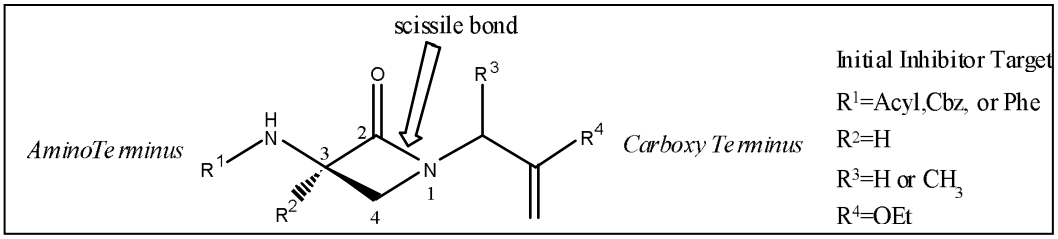


Figure 2: Design of Monocyclic β-lactam Peptidomimetics

These monocyclic β-lactam inhibitors will theoretically be recognized by the serine protease as a substrate, and bind in a manner which places the β-lactam at the active site (figure 3, page 7). In the active site, the β-lactam will occupy the S_1 subsite[9], and the substituent attached to the N-1 nitrogen will occupy the S_1' subsite, and extend in the direction of the carboxy terminus of a natural chymotrypsin substrate.

The β-lactam ring is inherently a very reactive structure. The atoms in the ring are constrained to bond angles of 90° by the four-membered ring. The sp^2 hybridized trigonal planar carbon (C-2) desires a bond angle of 120°, but is constrained to 90° by the ring. The other three atoms in the β- lactam ring are sp^3 hybridized (tetrahedral geometry) and have preferred bond angles of 109.5°. These atoms also experience a great deal of bond angle strain due to their inclusion in the β-lactam ring.[10] This bond angle strain makes the β-lactam ring reactive, and susceptible to nucleophilic attack at the electrophilic C-2.

As indicated by figure 3, once the inhibitor is in the active site, the protease's serine hydroxyl group will attack at position C-2 of the β-lactam and open the reactive ring. However, the C-4 methylene of the β-lactam will prevent the release of N-1 from the molecule. This will trap the enzyme in the acyl-enzyme intermediate form, which will be hydrolyzed slowly. In addition to the methylene bridge at C-4 holding the structure together, another reason for slow hydrolysis is the limited access of water into the crowded active site. As seen in figure 1, (page 5) the decomposition of the acyl-enzyme intermediate is driven by the attack of a water molecule. With the residues attached to N-1 still in place due to the methylene bridge, the attack

by water will be sterically hindered, thus further slowing down the hydrolysis of the acyl-enzyme intermediate.

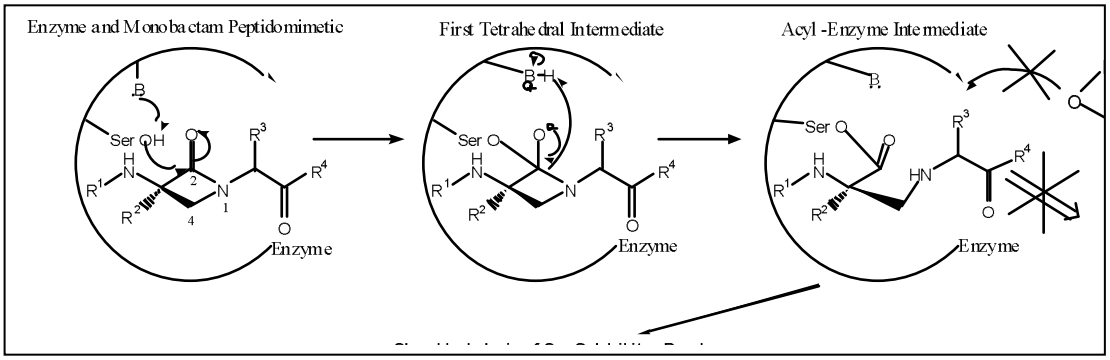


Figure 3: Proposed Mode of Serine Protease Inhibition By Monocyclic β-Lactams

These inhibitors have been rationally designed to inhibit serine proteases by this mechanism, however, previous research involving β-lactam type inhibitors of serine proteases,[11,12] has shown that inhibitors sharing the same functionality often behave differently in enzyme assays. Because of this, the exact mode of inhibition of these compounds will need to be determined experimentally.

As mentioned previously, serine protease inhibitors have been designed by creating peptidomimetics which include reactive moieties such as aldehydes,[13] boronic acids,[14] chloromethyl ketones,[15] fluoroketones,[16] α-keto esters,[17] or aza-peptides.[18] Most of these reported inhibitors (shown in figure 4) have incorporated a reactive functionality at the carboxy terminus of the peptide. Because of this, these peptidomimetics can only become involved in attractive interactions on one side of the scissile bond. The inhibitors synthesized in this project will be the first to incorporate the monocyclic β-lactam functionality into a peptide backbone. Similar to the fluoroketones,[16(c)] and aza-peptides,[18(b)] the monocyclic β-lactam peptidomimetics have the unique ability to exploit interactions with enzyme specificity pockets on both sides of the scissile bond.

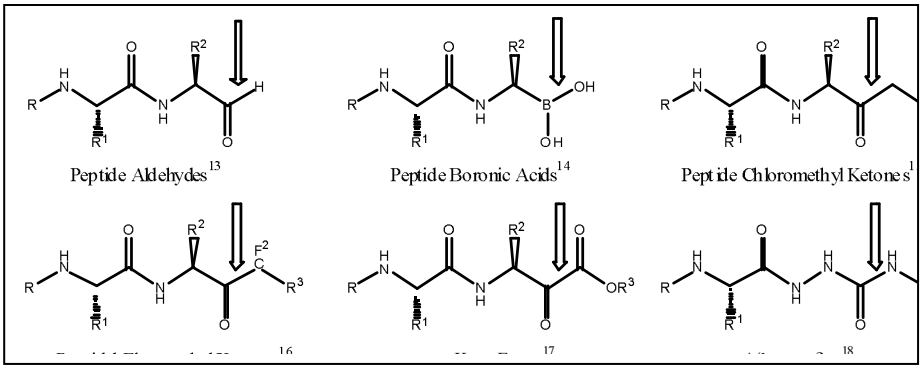


Figure 4: Rationally Designed Inhibitors of Serine Proteases

The groups which extend from both sides of the β-lactam will interact with the active site like a natural peptide substrate of chymotrypsin. If the interactions with the enzyme's active site are strongly attractive, the inhibitor should be effective against chymotrypsin. The scheme used to synthesize these inhibitors allows for the importance of these individual interactions to be explored in an incremental fashion. Once the initial inhibitor has been synthesized, accommodations in the synthesis can be made to alter all of the "R" groups (figure 2, page 6). By comparing inhibitory constants from the various inhibitors, it will be possible to see which groups are the most important for inhibition, and a pharmacophore for these types of inhibitors could be developed.

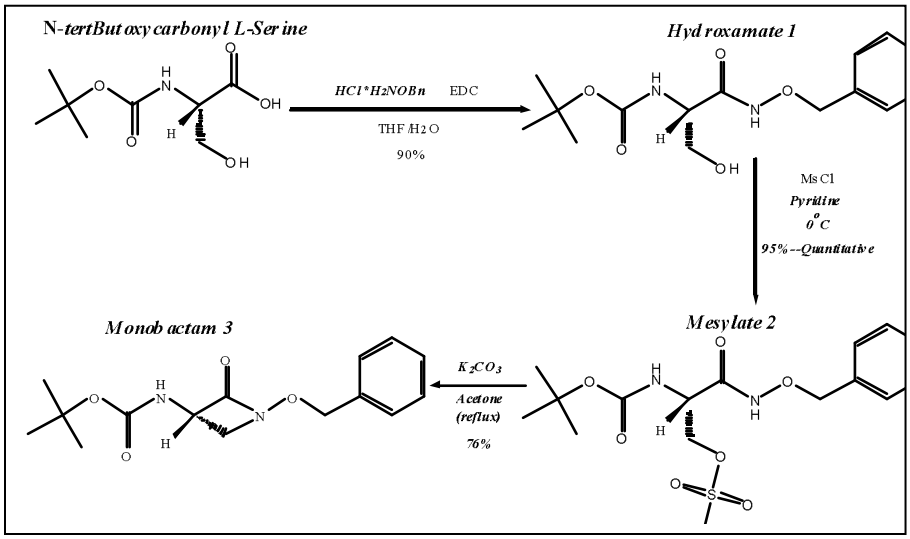
Preliminary molecular 3-D modeling studies have indicated that, in most cases, a peptidomimetic containing the β-lactam functionality is about 45 kilocalories per mol less stable than it's straight chain peptide counterpart. These calculations are based on steric energy minimization calculations, and the energy difference seems reasonable. The introduction of the β-lactam into the structure will limit some of the free rotation about the σ-bonds, and cause the steric energy to increase.

In addition, recent conformational studies,[19] with peptidomimetic monocyclic β-lactams have indicated that the most likely conformation of this type of structure is a β-turn. The degree of β-turn in the structure depends on the exact groups attached to the monocyclic β-lactam core. The effect that the β-turn conformation will have on the inhibitory properties of the proposed inhibitors will have to be ascertained directly through enzyme inhibitory studies.

If these rationally designed compounds are found to successfully inhibit chymotrypsin, the library of reactive functionality that can be used to combat serine protease activity will be expanded. Unlike many of their predecessors, these inhibitors will have the distinction of being able to exploit interactions with the enzyme on both sides of the scissile bond.

Results and Discussion

The first synthetic step was to assemble a fully protected monocyclic β-lactam (**3**) in a three step process (Scheme 1). This fully protected monocyclic β-lactam is the core from which all possible inhibitor targets are synthesized. Because this β-lactam core is such an important intermediate in the synthetic pathway, the development of Scheme 1 was a major focus of this project.



Scheme 1: Synthetic Pathway to the Monocyclic β-lactam Core

In the first step of Scheme 1, a hydroxamate serine derivative (**1**) was synthesized by the method of Miller et al,[20] coupling N-*tert*butoxycarbonyl L-serine (*t*-Boc-serine) to the hydroxylamine hydrochloride salt, HCl*H_2NOBn. This nucleophilic substitution on an acyl group involves the attack of a nucleophile (HCl*H_2NOBn) on the electrophilic carbon of the carbonyl from the carboxylic portion of *t*-Boc-serine. In order for this reaction to occur by this mechanism, the nucleophilic attack must be accompanied by the simultaneous departure of a leaving group, in this case, the hydroxyl group from the carboxylic acid would have to leave as a hydroxide anion. In general, strong bases are poor leaving groups, and weak bases are very good leaving groups.[10] Because the hydroxide anion (OH^-) is the prototypical strong base, it is therefore also a poor leaving group.

This obstacle is overcome by adding a coupling agent such as dicyclohexylcarbodiimide (DCC) to the reaction pot. Coupling reagents such as DCC will react with the free hydroxyl group of *t*-Boc-serine's carboxy terminus. This reaction modifies the OH group, and creates a good leaving group in its place.[10] The nucleophilic amine (HCl*H_2NOBn) can then be added, and the reaction will proceed to yield the serine hydroxamate derivative (**1**). Early attempts of this reaction involved using the aforementioned DCC as the coupling reagent. Although DCC is

a relatively inexpensive and readily available reagent, it presents a logistical problem in the synthesis. When DCC is used as a coupling reagent, dicyclohexylurea (DCU) is generated as a side product. DCU precipitates as a white solid, which must be removed via filtration. Removing all of the accumulated DCU via filtration was not always an easy task, and DCU had a tendency to appear as a contaminant in ensuing reactions. This observation, accompanied by the fact that the yields were not as high as they should ideally be in the first step of a synthesis (60-70%) led to the exploration of other coupling reagents as an alternative. The next coupling reagent attempted was 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC). EDC eliminated the precipitate problems that were inherent to DCC, however, the yields were still lower than expected (60%). It was concluded that the low yields were a result of factors other than the choice of carbodiimide coupling reagent.

Another aspect of this reaction that received considerable attention was the control of pH. Miller's protocol[10] performs this reaction in a pH range of 4-5. In order to maintain this pH range, careful consideration was taken. When the slightly acidic hydrochloride ($HCl*H_2NOBn$) was added to the stirring solution of *t*-Boc serine, the pH value quickly decreased to 1.5. To bring the solution back to the desired pH range, 1M NaOH was added dropwise. Then, when EDC was added, and the reaction had commenced, the pH began to gradually increase. This is due to the anionic nitrogen formed on the carbodiimide during its reaction with *t*-Boc-serine. In addition to this, when the nucleophillic substitution takes place, the carbodiimide leaves as an anion. To maintain the desired pH range of 4-5, 1M HCl was added dropwise over the course of the reaction (monitored by pH meter). Since the anions are formed throughout the entire course of the reaction, it was necessary to closely monitor the reaction progress via pH meter and adjust the pH as necessary.

Despite choosing the correct carbodiimide coupling reagent and monitoring the pH of the reaction carefully, yields were still less than satisfactory (60-70%). One possible explanation for this was problems with the work up and isolation of the hydroxamate (**1**). The Miller protocol calls for a total of nine aqueous washes during the work up. It was suspected that some of the product may have been brought into the aqueous layer by THF (THF solvates the hydroxamate product, and is miscible with H_2O) and lost during the washes. The solvent system, according to Miller's protocol, should be a 1:2 mixture of $THF:H_2O$. Based on the fact that the product (**1**) is soluble in THF, but insoluble in H_2O, it was proposed that using less THF might cause **1** to precipitate from the solution as it is formed. As a modification to the Miller procedure, the reaction was run with a minimum amount of THF in the solvent mixture. As predicted, when the solvent mixture contained just enough THF to dissolve all of the reagents, hydroxamate **1** precipitated as it formed. Upon completion, the white crystalline hydroxamate **1** was isolated via vacuum filtration in 90% yield. This compound needed no further purification as indicated by

TLC and melting point data. This highly reproducible protocol eliminated the need for the time consuming and difficult aqueous washes, and increased the average yield up to 90%, a very respectable yield for the first step in the synthetic pathway.

The next step in Scheme 1 is to cyclize hydroxamate **1** into the fully protected β-lactam **3**. This transformation occurs via an intramolecular S_N2 reaction with the O-Benzyl protected nitrogen atom as the reactive nucleophile. For this reaction to occur, the leaving group would be the hydroxyl group from serine's side chain. For reasons previously mentioned, a hydroxide anion is a very poor leaving group, which would need to be modified in order for the reaction to proceed. Since this is not a nucleophilic substitution on an acyl group, using a carbodiimide would not work to improve the hydroxide anion as a leaving group. Instead, methanesulfonyl chloride (MsCl) is used. MsCl reacts with the alcohol (from **1**) in a nucleophilic substitution reaction to form mesylate **2**.[10] A basic solvent, pyridine is used to neutralize the HCl that forms during the process. In this reaction, a poor leaving group, the hydroxide anion , is converted to the mesylate anion, a good leaving group. The reaction was run in pyridine, at 0^oC, under anhydrous conditions. It is very important to note that anhydrous conditions must be strictly adhered to. The mesylate anion is a good enough leaving group to possibly facilitate a side reaction in the presence of H_2O. If trace amounts of H_2O enter the reaction vessel from the atmosphere, undried solvent, or undried glassware, the H_2O would act as a nucleophile, and replace the mesylate anion via nucleophillic substitution. By this process, the reaction reverts back to the starting material, hydroxamate **1** as indicated below in figure 5.

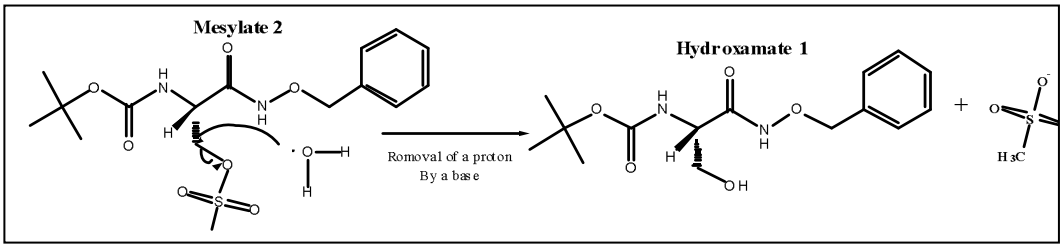


Figure 5: Decomposition of the Mesylate in the Presence of H_2O

However, when the anhydrous protocol is followed, this reaction has proven to be the easiest in this synthetic pathway. Typically, this reaction produces the mesylate **(2)** in yields above 90%.

Now that a good leaving group has been created, an intramolecular S_N2 cyclization can be run to yield the fully protected β-lactam (**3**). A closer look into the mechanism of this reaction indicates why the O-benzyl group attached to the nucleophilic nitrogen atom is so vital to this reaction. In order for this reaction to be spontaneous, the nucleophilicity of the amine must be enhanced. The O-benzyl group contributes to this enhancement in two ways.

The first way is through a phenomenon known as the α-effect.[22] Whenever the atom adjacent to the attacking nucleophile contains one or more pairs of unshared electrons, the strength of the nucleophile is enhanced. There is currently no concrete evidence to explain why this occurs, but two ideas have been hypothesized. One is that the ground state of the nucleophile is destabilized by repulsions from the unshared electron pairs on the adjacent atom. The other theory is that the transition state of the reaction could be stabilized by the adjacent unshared electron pairs.[22] Although neither of these explanations can currently be proven, the α-effect is a phenomenon that has repeatedly been experimentally observed. In the case of this reaction, the two pairs of unshared electrons on the adjacent oxygen atom contribute to the enhanced nucleophilicity of the nitrogen atom.

The second, and most important contribution to the amine's enhanced nucleophilicity is the fact that the O-benzyl group is an electron withdrawing group (EWG).[10] In order for the amine to participate in the intramolecular S_N2 reaction, it must be deprotonated (or the equilibrium must lie in favor of the deprotonated form). K_2CO_3 is the base used to achieve this deprotonation. However, K_2CO_3 is a fairly weak base, and would not be strong enough to deprotonate an amide (not benzyloxy protected) (pK_a~17). By adding the benzyloxy substituent to the amide, the pKa is reduced. The benzyloxy substituent withdraws electron density from the nitrogen atom. The reduced electron density of the nitrogen atom, due to the ability of the benzyloxy substituent to stabilize a negative charge, causes the pKa of the hydroxamate to decrease (pKa~6-10). In essence, the amine can now lose a proton more easily, because the anion will be stabilized. In the presence of K_2CO_3, the equilibrium of the benzyloxy substituted amine lies in favor of its deprotonated form.

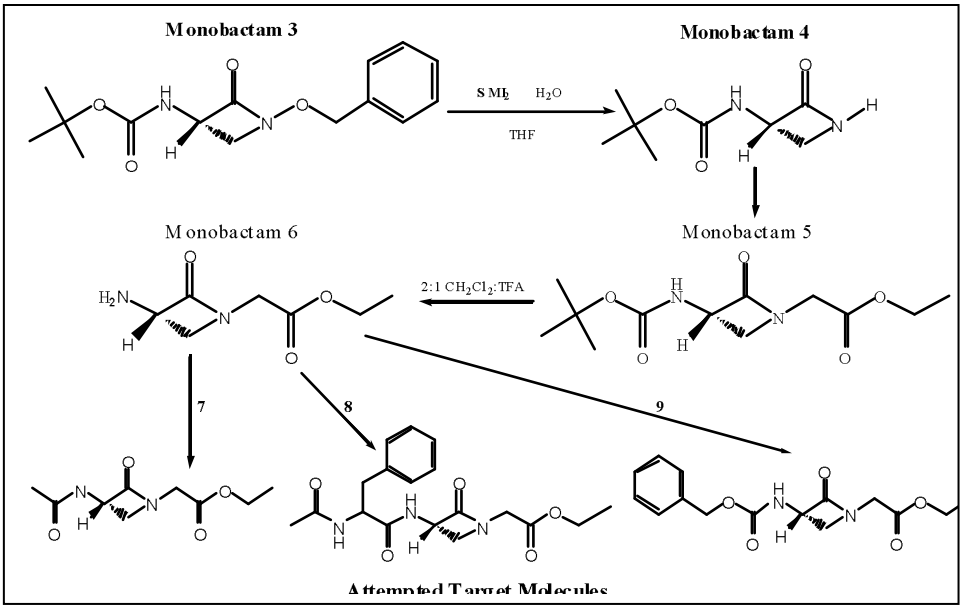
With the O-benzyl substitution the amine is sufficiently nucleophilic to take part in the intramolecular S_N2 reaction. In addition, the incorporation of the methyl sulfonate ester (OMs) into the molecule has enhanced the efficiency of the leaving group. This reaction was facilitated by refluxing the mesylate (**2**) with K_2CO_3 in dried acetone under anhydrous conditions as indicated by the protocol of Floyd et al.[21] Once again, the strict anhydrous environment was essential to the success of this reaction. As indicated in figure 5, mesylate **2** has a tendency to revert back to hydroxamate **1** in the presence of H_2O. After aqueous work up and column chromatography, yields of β-lactam **3** (the fully protected monobactam core) have been as good as 70-80%.

The only attempted modification to the reaction sequence was an attempt to transform hydroxamate **1** directly into **3** in a one step process.[23] The hydroxamate was reacted with 1-1'-sulfonyldiimidazole and sodium hydride (NaH) in a solution of dimethylformamide at low temperature. TLC showed that the crude product contained a large number of impurities, but the presence of the product was not identified. Due to the large number of impurities present and the

unconfirmed presence of product, this reaction was not pursued any further in the pathway to the β- lactam core.

Once **3** had been obtained in high purity and yield via Scheme 1, the next step in the synthesis was to deprotect and extend **3** on both sides of the scissile bond. Previous attempts in the lab focused on removing the *t*-Butoxycarbonyl group from **3** and coupling other residues to the resulting free amine. In all cases these reactions were unsuccessful due to a suspected opening of the strained β- lactam ring. The theoretical explanation for this draws on concepts that have previously been developed. The N-1 nitrogen atom of **3** is benzyloxy protected. As mentioned previously, the benzyloxy substituent reduces the pka of N-1, thereby making it a weaker base. Weaker bases have been known to be good leaving groups in reactions. The strong ability of N-1 to leave, causes the bond between N-1 and C-2 in the β-lactam to become weakened. In attempts to remove the *t*-Boc group from **3**, this labile bond is broken, destroying the β-lactam ring.

This evidence caused the focus of the synthesis to shift toward the removal of the benzyloxy protecting group from **3**. This reaction is the first step in Scheme 2, the elaboration of the monocyclic β-lactam core, and it has proven to be the most difficult reaction in the synthesis. Difficulties with this reaction have caused it to become the major focus of most recent research.



Scheme 2: The Elaboration of the Monocyclic β-lactam Core

Based on the work of Chiara et al,[24] β-lactam **3** was reacted with a 0.1 M solution of the Lewis acid samarium (II) iodide (SmI_2) and deoxygenated H_2O in THF. Although the exact reaction mechanism has not been experimentally determined, it is proposed to be a free radical

process. Early attempts of this reaction were successful, but with low yields (40-50%). Yields appeared to be higher when a freshly opened bottle of SmI_2 was used as opposed to when SmI_2 was used from a previously opened bottle. This is because SmI_2 has been known to react vigorously with the diradical O_2. Although extreme care was taken to always open bottles and transfer SmI_2 under an atmosphere of nitrogen gas, it was evident that even trace amounts of O_2 would react with SmI_2 in the bottle. This process could be detected by color. A fresh 0.1M solution of SmI_2 has a very distinct blue color to it, however immediately upon contact with O_2, the color changes to a pale yellow. To combat the decomposition of the SmI_2 reagent under storage, Molander's protocol[25] was attempted to generate SmI_2 in situ. In a glovebag, Sm metal and I_2 were combined with THF and allowed to stir under argon. The literature called for a deep blue green color in the solution, but even after three days of stirring, the solution was brown with a yellow-green precipitate. Although the solution was not the expected color, it was used in a benzyloxy cleavage reaction. However, it became apparent that the attempt to synthesize SmI_2 had not been a success. When this reagent was used to affect the benzyloxy removal, TLC indicated that no product was formed after three days of stirring at room temperature and the starting material remained unreacted.

Because the procedure to synthesize SmI_2 was not a success, the focus was turned to extending the shelf life of the Aldrich SmI_2 reagent. Instead of nitrogen, argon was used during the opening of the bottles and the dispensing of the reagent. Although the shelf life of the SmI_2 reagent was extended by doing this, there were still problems with the reaction. Chiara's protocol[24] indicates that the reaction should be stopped when TLC indicates the disappearance of all starting material. These reactions were run for as many as three days with TLC still indicating the presence of starting material. The low yields could be explained by the fact that the reaction was not going to completion. In an attempt to drive the reaction to completion, a reflux in THF was run. Although refluxing often drives reactions to completion, it did not work on this molecule. These harsh reaction conditions yielded a brown oil that contained no product based on TLC and IR.

The next attempt to drive the reaction to completion was based on SmI_2 research by Shabangi.[26] The cosolvent used with SmI_2, in this case, deoxygenated H_2O, can have a bearing on the reducing power of SmI_2. Shabangi determined that the reducing power of SmI_2 was the greatest when hexamethylphosphoramide (HMPA) was used as the cosolvent. As a modification to the Chiara protocol, deoxygenated H_2O was replaced by HMPA. Initially, the results appeared to be very encouraging as indicated by the total consumption of all starting material (**3**) based on TLC. However, although all of the starting material had been consumed, there were many more side products detected, and the overall amount of pure **4** isolated was about the same as in previous reactions. It appears that the HMPA made SmI_2 too strong a reducing agent to be used

in this reaction. Because this reaction and separation were logistically more difficult to perform, the Chiara protocol remains the most effective way to affect the transformation from **3** to **4**.

Once the benzyloxy protecting group was removed, the focus of the synthesis became extending the molecule from the N-1 position. The original reaction extends the molecule in the carboxy terminal direction by adding a group which mimics a glycine residue. This transformation was initially attempted via a rhodium-catalyzed carbenoid insertion reaction.[27] Carbenes are short lived reactive intermediates which have rarely been isolated, however certain reactions involving carbenes have been experimentally shown to react preferentially with specific bond types. Two of the most common types of bonds that carbenes react with are the carbon-carbon double bond, and the nitrogen-hydrogen bond.[22] This reaction attempted to alkylate **4** at N-1 via a carbenoid insertion reaction. One of the most common synthetic methods of generating carbenes is the decomposition of diazo compounds. When treated with a catalyst, the diazo compound decomposes to form nitrogen gas and a carbene.[22] In this reaction, ethyl diazoacetate was reacted with a rhodium (II) acetate catalyst to generate the carbene, which should insert into the N-1 bond of **4** to from the alkylated product (**5**). This reaction was run in a tolulene reflux for two days. The reaction formed four products based on TLC. Separation by column chromatography and analysis of each compound brought about the conclusion that none of these compounds were **5**. Because this reaction is logistically difficult to run, dangerous (diazo compounds have been known to explode in the presence of a rhodium catalyst at reflux), and inefficient, it was not explored further as a method of transforming **4** to **5**.

After the carbenoid insertion reaction had failed, the potential reason was found in the literature. Research by Davies[28] explored the relationship between the choice of diazo compound and the success of a carbenoid insertion reaction. A discovery was made that many diazo compounds have been known to dimerize in the presence of a rhodium catalyst. In one reaction involving ethyl diazoacetate, Davies reported that only 10% of the desired insertion product was formed, but 67% of the ethyl diazoacetate reagent formed a dimer. Although this reaction was not performed on a β- lactam, the results still bear significance. Because such a large proportion of the reagent formed the undesirable dimers, the yield would be extremely low no matter what compound it was reacted with, because there would not be enough monomeric ethyl diazoacetate to cause the insertion reaction to occur. Ethyl diazoacetate was reported to be the worst reagent to use in this type of insertion reaction for these reasons.[28] The research also reported that the best diazo compound for this type of insertion reaction was a phenyldiazoacetate methyl ester. Using this diazoacetate, Davies reported no dimerization products. This research explains why the rhodium catalyzed carbenoid insertion reaction did not work when ethyl diazoacetate was used. The evidence that phenyldiazoacetate is such an effective reagent for carbenoid insertions has potential future implications on this project.

Because **5** could not be synthesized by the rhodium catalyzed insertion, the focus was shifted to the removal of the proton at N-1 and reaction with the electrophile, ethyl bromoacetate. The base initially chosen for this task was lithium-hexamethyldisilizane (LiHMDS). LiHMDS was generated in situ by the reaction of 1,1,1,3,3,3-hexamethyldisilisane with butyllithium. LiHMDS was used to deprotonate **4** at -75^{o} and ethyl bromoacetate was added. The electronegative bromine atom withdraws electron density from the carbon that it is attached to. This enhances the electrophilicity of that carbon and consequently makes it more susceptible to nucleophilic attack. As the literature[29] had predicted, this reaction was a difficult transformation to accomplish. The success of this reaction was inconsistent, and yields were extremely low (10-20%). In addition, the successful attempts of this reaction never went to completion.

Initially, it was proposed that the deprotonation of N-1 was not being completed in these reactions. In order for this reaction to occur, N-1 must be in its deprotonated (most nucleophilic) form. If it is not fully deprotonated, N–1 will probably not be nucleophilic enough to react with ethyl bromoacetate, and product will not be formed. One way this could happen is if the generation of LiHMDS in situ did not go to completion. As mentioned above, one of the reagents used to generate LiHMDS is butyllithium. Butyllithium has been known to react vigorously with H_2O molecules present in atmospheric moisture. Because of this, solutions of butyllithium have been known to decrease in molarity under storage. If this was the case, the unpredictable nature of the reaction may be attributed to it. If the molarity of butyllithium is lower than expected, the full amount LiHMDS would not be generated. Consequently, N-1 from **4** would not be fully deprotonated and the reaction would not go to completion. For these reasons, the molarity of butyllithium was investigated further.

The butyllithium used in these reactions was a 1.6 M solution in hexanes from Aldrich. The molarity of butyllithium can be determined by titrating it with 2,5-dimethoxybenzyl alcohol.[30] When treated with organolithium reagents, 2,5-dimethoxybenzyl alcohol has been observed change from a colorless solution to a red solution when as little as 0.01 mmol equivalents of excess organolithium reagent is added.[30] This titration makes it possible to accurately determine the molarity of compounds such as butyllithium. This procedure was done previously in the lab by a colleague, and the molarity of the butyllithium solution was determined to be 1.07 M. Because this value was a considerable amount lower than the Aldrich value of 1.6 M, this titration was performed again on the butyllithium to see if it had undergone any further decrease in molarity.

A total of three titrations were done with butyllithium reacting with 2,5-dimethoxybenzyl alcohol in THF. Based on these titrations, the molarity of the butyllithium reagent was 1.39 M. Although this value was lower than the 1.6 M value indicated by Aldrich, it was higher than the 1.07 M which was previously calculated. Since all calculations in these reactions had been based

on a molarity of 1.07, the theory that the molarity had dropped below 1.07 M and detrimentally affected the reaction was proven wrong.

The butyllithium titration discounted the theory that LiHMDS had not been obtained in quantitative yield during its generation in situ. However, since the alkylation reaction had yielded product with a very modest rate of success, a new reaction was attempted based on the same principles. In an organic synthesis such as this one, an easy way to circumvent this type of problem is to use a different base. Based on the literature[31], sodium hydride (NaH) is a base that has been used to deprotonate similar molecules. An alkylation reaction involving **4** and ethyl bromoacetate with sodium hydride gave **5** in a 64% yield on its first attempt. Subsequent attempts of this reaction were successful, but in lower yields than the original 64% yield. Typical reactions using NaH as the base produced **5** in approximately 35-45% yield.

The results from the NaH reactions were substantially better than the results when LiHMDS was used as the base. In an attempt to further improve yields, the same reaction was performed with potassium hydride (KH) as the base. K^+ is larger than Na^+, therefore it holds the hydride ion more loosely. Because of this, reactions using KH often yield better results. However, when KH was used, **5** was isolated, but the yield was slightly lower than the typical 40% yield of the NaH reaction. To date, the alkylation using NaH as the base continues to be the most efficient way to transform **4** into **5**.

In addition to the alkylation reactions involving ethyl bromoacetate to yield **5**, the use of ethyl- 2-bromopropionate was originally proposed. As mentioned previously, reaction of **4** with ethyl bromoacetate adds a glycine ethyl ester to N-1 to create **5**. If this same reaction was done with ethyl- 2-bromopropionate, the group added to N-1 would be a methylated derivative of **5** which mimics an alanine ethyl ester. Both of these compounds would be useful intermediates in this synthetic pathway. With either of these compounds, removal of the *t*-Boc group, and coupling of another group in its place would yield a proposed chymotrypsin inhibitor. Enzyme testing two compounds which are different only in one functionality such as these two, is an important step in this project. If the addition of the methyl group improves the inhibition of chymotrypsin, it can be proposed that the methyl group is interacting in the S_1' pocket of the enzyme.

However, to date, this reaction has not been attempted in the lab. The fact that the yields of the alkylation reaction using ethyl bromoacetate have been so low indicates the reaction using ethyl-2-bromopropionate would not work well. In nucleophilic substitutions, primary alkyl halides (ethyl bromoacetate) are much more reactive than secondary alkyl halides (ethyl-2-bromopropionate).[10] The reason for this is explained by the mechanism of these reactions. When the nucleophile (N-1) attacks the electrophilic carbon on the alkyl halide, the bond between N-1 and the electrophilic carbon is being formed as the bond between the bromine atom and the

electrophillic carbon is being simultaneously broken. The transition state of this reaction involves five groups being attached to the electrophilic carbon.[10] In the primary alkyl halide, two of these five groups are hydrogen atoms. Since the hydrogen atoms don't occupy much space, there is not a great deal of steric hindrance in the transition state, and the reaction occurs easily. In the case of a secondary alkyl halide such as ethyl-2-bromopropionate, one of the hydrogen atoms has been replaced with a methyl group. This methyl group is much larger than the hydrogen atom which it replaced, and it adds steric hindrance to the transition state, making it a higher energy intermediate. This increases the activation energy needed to reach the transition state, and cause the reaction to proceed. In many cases, the energy level of the transition state is too high to be overcome, and the reaction does not occur.[10] If the ethyl bromoacetate reaction had worked in high yield, this reaction would be attempted to produce another group of compounds for enzyme inhibition.

Once **5** had been synthesized, the next step in Scheme 2 was to remove the *tert*-butoxycarbonyl protecting group, and couple various residues to the nitrogen atom to yield potential chymotrypsin inhibitors. As was mentioned previously, the *t*-Boc group removal from **3** was not possible because the benzyloxy substituent at N-1 made the β-lactam susceptible to ring opening. This factor was not a problem with **5**, as the *t*-Boc removal proved to be a rapid and facile reaction. The reagent used for this transformation was trifluoroacetic acid (TFA) in dry dichloromethane (1:2 ratio). TFA is perhaps the most universal reagent in *t*-Boc removal reactions.[32] TFA protonates the oxygen atom from the carbamate, which subsequently decomposes to form the free amine (**6**) along with 2- methylpropene and CO_2 as side products.[10] Occasionally, an alcohol such as p-cresol is used to scavenge for liberated *t*-butyl cations produced by the reaction,[32] but it was found to be unnecessary in this case. The reaction of **5** with TFA in CH_2Cl_2 produced **6** in quantitative yield after one hour of stirring at room temperature. The success of this reaction is easily determined by ^{1}H NMR. The ^{1}H NMR spectrum of **5** has a very large singlet peak at 1.41 ppm which represents the nine hydrogen atoms associated with the *t*-Boc group. If the reaction successfully yields **6**, the ^{1}H NMR spectrum will be missing this signature peak, a telltale sign that the reaction was a success.

The extension of **6** from the free amine, would yield a potential peptidomimetic chymotrypsin inhibitor. To date, three different reactions of this type have been attempted, but have not met with satisfactory results. The first attempt was to acetylate the free amine to yield **7**. Due to the small amount of **6** available at that time, this appeared to be easier than running a coupling reaction with another amino acid residue. In addition, the acyl group would accomplish two more things: it would protect the free amine, which is known to be a very reactive species and it would add another carbonyl group which would cause the molecule to more closely resemble a peptide; this would increase the probability of **7** being recognized by chymotrypsin in

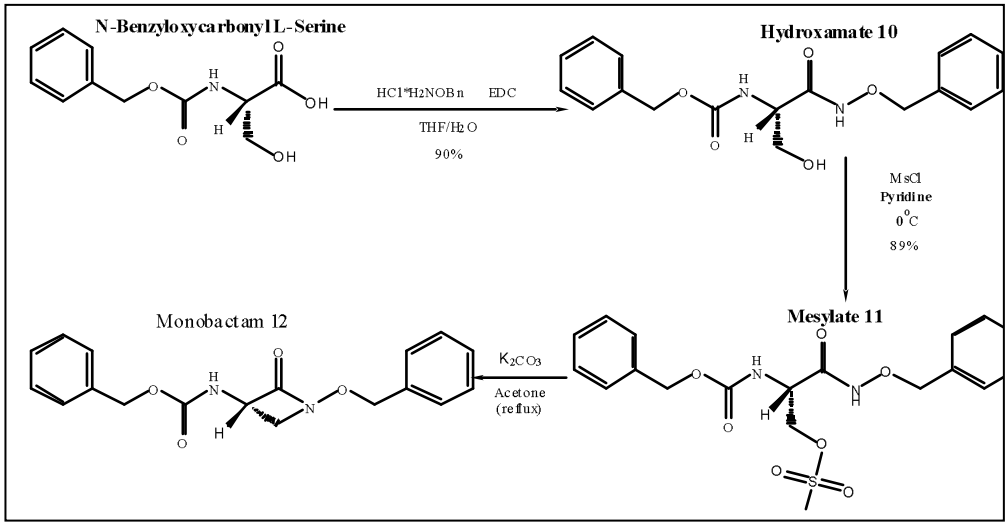
an enzyme assay. This transformation was attempted by reacting **6** with acetic anhydride. After aqueous work-up, TLC indicated that all **6** had been consumed, and many side products had formed. After separation by flash chromatography, none of these compounds isolated appeared to be product.

The next attempt to elaborate on **6** was to couple it to N-acetyl-phenylalanine. The addition of phenylalanine would increase the peptidomimetic character of the molecule which would enhance its chance of being recognized by chymotrypsin in an enzyme assay. More importantly, the addition of phenylalanine would introduce an aromatic phenyl group into the molecule. As mentioned previously, the phenyl group is a common recognition element for chymotrypsin. This fact, along with the background information previously developed, indicates that **8** would potentially be a good competitive inhibitor of chymotrypsin. As was the case with the attempted synthesis of **7**, this reaction was logistically difficult to perform on such a small scale. The intricate coupling reaction involving N-N-disopropylethylamine, hydroxybenzotriazole (HOBT) and EDC, did not conclusively yield any measurable amount of **8**. Due to the small scale on which this reaction was performed, the result was inconclusive. If a slightly larger quantity of **6** was available to run this reaction on, a conclusive determination of its success could be obtained.

The other attempted reaction was to transform **6** into its N-Cbz (benzyloxycarbonyl) derivative (**9**). The addition of the benzyloxycarbonyl group would add the phenyl group to the molecule as a potential chymotrypsin recognition element. This reaction was chosen because there was only a small amount of **6** available, and this reaction had proven to be easier based on work of a colleague in the lab. TLC upon completion of this reaction indicated that a product had formed that was less polar than the starting material. Infrared spectroscopy indicated that the β-lactam is most likely still intact, and that an aromatic group is present within the molecule. However, before this molecule can be tested in a chymotrypsin assay, it will have to be sent to the University of New Hampshire for 300 MHz ^{1}H NMR testing. Once the 300 MHz ^{1}H NMR data from UNH becomes available, the purity of β-lactam **9** can be determined. If this compound is found to be pure **9**, then it will be immediately tested in a chymotrypsin assay.

Recently, scheme 3 has also been explored as a method for generating a different inhibitor. Scheme 3 is identical to scheme 1, with the exception that the N-protecting group used is a benzyloxycarbonyl (Cbz) instead of a *tert*-Butoxycarbonyl group. All reactions are run under the same conditions as in scheme 1, but the final product is the Cbz-protected β-lactam **12**. It is proposed that this compound may inhibit chymotrypsin based on its structure. It has an extended structure similar to a peptide, the β-lactam core has been established, and two phenyl groups are present as potential recognition elements for chymotrypsin. This compound can be made in a facile three step synthesis, and could be beneficial in the development of the enzyme assay

techniques. The reason that this scheme is not a major focus of this project is because β-lactam **12** is a synthetic dead end in this pathway. β-lactam **12** is protected at its N-terminus by a



Scheme 3: Benzyloxycarbonyl Protected β-lactam

benzyloxycarbonly (Cbz) group, and at its C-terminus by an O-benzyloxy (OBn) protecting group. The next step in the synthesis would be to remove one of these protecting groups and extend the molecule in that direction. Unfortunately, these two protecting groups are both removed under the same conditions, and there are currently no synthetic methods available to distinguish between them when they are present in the same molecule. Hydroxamate **10** and mesylate **11** have been synthesized by scheme 3. Currently, the transformation of mesylate **11** to β-lactam **12** is being performed in the lab. Once this compound is isolated and tested for purity, it will be tested in a chymotrypsin assay.

Conclusions and Future Directions

Enzyme assays of the preliminary inhibitors (**9** and **12**) will provide useful information about the utility of β-lactam peptidomimetics as serine protease inhibitors. In order to better understand how these inhibitors work and obtain a pharmacophore, the library of potential inhibitors must be expanded. This will be accomplished by continuing to follow schemes 1 and 2. In order to improve the efficiency of scheme 2, some new reaction conditions have been proposed.

First, as an alternative to the SmI_2 reaction, the N-O reduction could be achieved by reacting β-lactam **3** with a solution of sodium in ammonia at low temperature. Previous work by Floyd et al[33] has shown these reaction conditions to be effective for the reduction of N-O bonds attached to β- lactams. Floyd's group used these conditions to yield the reduced product in

essentially quantitative yield. However, the bond that Floyd's group was reducing was an N-OCH_3 bond, not an N-OBn bond like the one in β-lactam **3**. The effect that the benzyl group will have on this transformation will have to be determined experimentally. However, if this reaction occurs in high yield, it will replace the SmI_2 reduction in scheme 2.

Although the alkylation of β-lactam **4** to yield β-lactam **5** has been achieved with some degree of success with NaH as the base, recent findings in the literature[34,35] indicate that Cs_2CO_3 may be a more effective base. Both Gala[34] and Palomo[35] have reported satisfactory results in N-alkylations of β-lactams when Cs_2CO_3 is used as the base. In the future, Cs_2CO_3 will be used in the transformation of **4** to **5**. If the yields are higher and more consistent than in previous attempts with NaH, then, Cs_2CO_3 will be permanently incorporated into scheme 2.

The N-alkylation reactions in this pathway lead to a glycine mimic attached to N-1 of the β- lactam. As previously mentioned, other alkylating agents (such as ethyl-2-bromopropionate) will not react at N-1 due to steric hindrance in the transition state. Because of this, the utility of the N-alkylation step is limited in that it can only add a glycine residue to the β-lactam core. Two other methods (figure 6) will be explored to alkylate N–1 under different conditions. Once these compounds are synthesized, their *t*-Boc groups can be removed, and other residues can be coupled to the amine by using the reactions from scheme 2. Inhibition assays of these different compounds will hopefully provide insight on the binding affinity of chymotrypsin, and lead to the development of a pharmacophore.

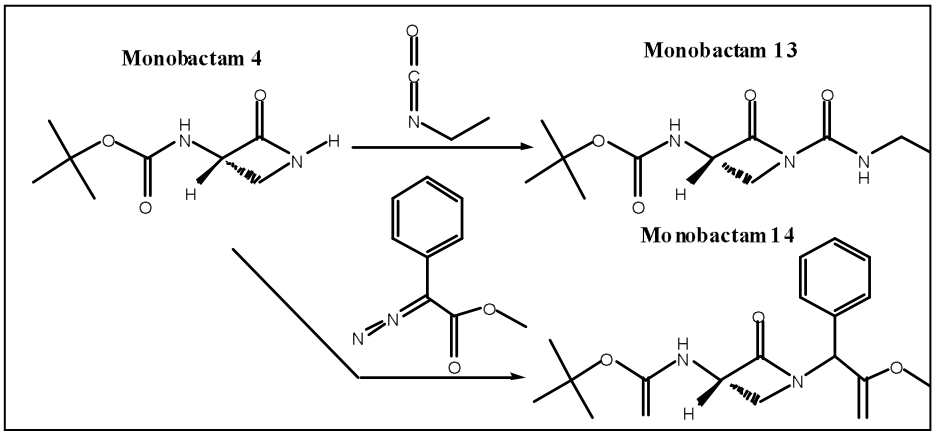


Figure 6: Alternatives to the N-Alkylation Using Ethyl Bromoacetate

Based on the work of Shah et al[36], isocyanates can be used to N-alkylate β-lactams. The reaction of β-lactam **4** with the isocyanate shown in figure 6 and triethylamine, would yield β-lactam **13**. This molecule would not directly mimic a peptide, but it would incorporate an additional carbonyl group, and an ethyl group which could possibly interact in a hydrophobic

pocket of chymotrypsin. This reaction should be an easier alternative to the difficult N-alkylation using ethyl bromoacetate based on the results of Shah et al.[36]

As mentioned previously, research by Davies[28] has indicated that phenyldiazoacetate methyl ester is an excellent reagent for a rhodium catalyzed carbenoid insertion reaction. Reaction of β- lactam **4** with phenyldiazoacetate methyl ester in the presence rhodium (II) acetate would yield β- lactam **14** (figure 6). The product of this reaction does not directly mimic a peptide, but it does have a carbonyl group, a phenyl group, and a methyl group, all which could be recognized by chymotrypsin in an assay. In order to perform this reaction, a method to make phenyl diazoacetate methyl ester would need to be ascertained.

In addition to this, another goal is to produce 100 mg of each compound involved in schemes 1,2, and 3. Obtaining 100 mg of each product in purified form will allow these molecules to be subjected to further analysis of their purity. In order to prove that each compound has been obtained, these molecules have to be sent out for elemental analysis and ^{13}C NMR. This information, combined with ^{1}H NMR, IR, melting points, and TLC data will determine the exact structure of each intermediate in this synthesis.

Experimental Section

General: Infrared spectra were recorded on a Bio-Rad FTS-7 Infrared Spectrometer with CH_2Cl_2 as the solvent. All ^{1}H NMR experiments were recorded on a Varian T-60 NMR Spectrometer in $CDCl_3$ unless otherwise noted. (Some of the more important molecules were tested on a 300MHz NMR spectrometer from the University of New Hampshire). TLC experiments were performed on Aldrich General Purpose TLC Plates-Silica on Glass with a UVP Short Wave Ultraviolet Lamp (254nm), ninhydrin (solution in butyl-alcohol) and I_2 as detection methods. All column chromatography separations were performed with Aldrich Silica Gel (200-400 mesh). All melting points were recorded on a Thomas Hoover Uni-Melt Capillary Melting Point Apparatus.

Hydroxamate (1): 2000 mg (9.74mmol) of N-*tert*Butoxycarbonyl-L-serine was dissolved in 10 mL THF: 20mL H_2O. Then 2333mg of O-Benzylhydroxylamine-hydrochloride (14.61mmol) was added. An additional 4mL (minimum amount) of THF was added to dissolve the remaining O-Benzylhydroxylamine-hydrochloride. The pH of the solution was 1.75. 3M NaOH was added until the pH was 4.46. 2333 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride was dissolved in H_2O, and added to the stirring solution. To combat the climb in pH, 1M HCl was periodically added to maintain a pH range of 4-5. After 2 hours, the product was collected by vacuum filtration, and dried in a 110^{o}C oven. The product was recrystallized in ethyl acetate and hexanes to give 2654 mg of the white crystalline hydroxamate **1**: mp 129.5-131^{o}C (literature value[20] 130-131^{o}C). TLC: Rf 0.51 in ethyl acetate.

Mesylate (2): Under argon, 1.07mL (13.88mmol) of methanesulfonyl chloride was added to a stirred solution of **2** (3550mg, 11.57mmol) in 20mL dry pyridine at 0°C. After 2 hours at 0°C, the reaction pot was warmed to room temperature. Ethyl acetate was added, and the organic layer was washed with 1M HCl until the aqueous wash was acidic to pH paper. The organic layer was then washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to obtain mesylate **3** as an oil (4195mg 94% yield). TLC: Rf .73 in ethyl acetate.

β-lactam (3): Under argon, 565 mg (4.063mmol) of K_2CO_3 was dissolved in 20mL dry acetone and brought to a reflux. 526mg of **2** was dissolved in 10mL dry acetone and added to the refluxing solution. After 2.5 hours, the reaction was cooled to room temperature, and filtered through celite. The reaction mixture was then concentrated to obtain a yellow oil which was dissolved in ethyl acetate and washed one time each with 1M HCl, 1M $NaHCO_3$, and brine. The ethyl acetate layer was dried over $MgSO_4$ and concentrated to yield a yellow oil. The oil was purified on a silica column with a 1/3 ethyl acetate/hexanes mobile phase. Fractions from the column were collected, concentrated, and recrystallized in ethyl acetate and hexanes to yield 294mg (75% yield) of **3** as a white crystalline solid: mp 89.5-91°C (literature value[21] 91-92°C). TLC: Rf 0.56 in 2:1 ethyl acetate: hexanes, Rf 0.26 in 1:3 ethyl acetate: hexanes. ^{1}H NMR (60MHz, $CDCl_3$): 7.40(s,5H); 5.23(s,1H; 4.95(s,2H); 4.36(s,1H); 3.45(t, J=2Hz, 1H); 3.23-3.08(m,1H); 1.41(s,9H). IR (CH_2Cl_2): 3433, 2900, 1779, 1723, 1518 cm^{-1}

β-lactam (4): Under argon, 30mL (2.97mmol) of SmI_2 was combined with 0.40mL of deoxygenated water. Then 290mg of **3** was dissolved in THF and added to the reaction pot. After 42 hours, the reaction was partitioned between saturated $NaHCO_3$ and ethyl acetate. The aqueous layer was extracted three times with ethyl acetate. The combined organic extracts were washed once each with 10% $Na_2S_2O_3$ and brine, dried over $MgSO_4$, and concentrated to yield a white solid which was purified on a silica column with 2/1 ethyl acetate/hexanes as the mobile phase. β-lactam **4** was isolated as a white solid (72mg, 42% yield). mp 173-174°C (literature value[24] 173-174°C). TLC Rf 0.13 in 2/1 ethyl acetate/hexanes. ^{1}HNMR (300MHz, $CDCl_3$): 5.85(s,1H); 5.17(s,1H); 4.84(s,1H); 3.63(t,1H); 3.35-3.30(m,1H); 1.43(s,9H). IR(CH_2Cl_2): 3400, 1779, 1716, 1513 cm^{-1}.

β-lactam (5): Method A: Under nitrogen, 0.106mL (0.503mmol) of 1,1,1,3,3,3-Hexamethyldisilizane was dissolved in THF and cooled to -75°C in a dry ice/acetone bath. 0.32mL of butyllithium (1.07M solution, 0.503mmol) was added, and the solution was stirred at -75°C for 15 minutes to yield 0.443mmols of lithium-hexamethyldisilizane (LiHMDS) in situ. β-lactam **4**, (35mg 0.201mmol) was added to the stirring solution of LiHMDS at -75°C. 0.247mL

(0.233mmol) of ethyl bromoacetate was added via syringe. The reaction was run at -75^{o}C for 1 hour, then it was warmed to room temperature and stirred until TLC indicated consumption of all starting material (2 hours at room temperature. The reaction pot was partitioned between dichloromethane and deionized water. The aqueous layer was extracted twice with dichloromethane. The combined dichloromethane extracts were washed once with brine, dried over $MgSO_4$, and concentrated to yield 59 mg of a yellow oil. The oil was purified by flash chromatography with a 2/1 ethyl acetate/hexanes mobile phase to yield 10mg of **5** as a white solid (64% yield). TLC: Rf 0.43 in 2/1 ethyl acetate/hexanes. ^{1}HNMR (300MHz, $CDCl_3$): 5.15(s,1H); 4.93(s, 1H); 4.20(q, J=7.2Hz, 2H); 4.05(s, 2H); 3.75(t, J=6.6Hz, 1H); 3.43-3.40(m, 1H); 1.45(s, 9H). IR(CH_2Cl_2): 3437, 2597, 1775, 1721, 1508 cm^{-1}.

Method B: Under argon, 31mg of **4** (0.176mmol) was dissolved in 5mL of THF. Then 18mg (0.445mmol) of sodium hydride (60% dispersion in mineral oil) was washed with hexanes and added to the reaction pot at 0^{o}C. After 15 minutes, 0.0217ml (0.196mmol) of ethyl bromoacetate was added via syringe. The reaction was allowed to warm to room temperature. After 2 hours, TLC still indicated presence of starting material. An additional 0.01ml of ethyl bromoacetate was added. After 1 additional hour at room temperature, the THF volume was concentrated to 3 mL, and the reaction pot was partitioned between ethyl acetate and saturated NH_4Cl. The aqueous layer was extracted twice with ethyl acetate. The combined organic extracts were then washed once with brine, dried over $MgSO_4$, and concentrated to obtain 29mg of a yellow oil (64% crude yield). TLC: Rf 0.44 in 2:1 ethyl acetate/hexanes, Rf 0.20 in 1:1 ethyl acetate/hexanes, Rf 0.38 in 5% methanol: dichloromethane. ^{1}HNMR (60 MHz, $CDCl_3$) 5.15(s,1H); 4.93(s, 1H); 4.20(q,2H); 4.05(s, 2H); 3.75(t, 1H); 3.43- 3.40(m, 1H); 1.45(s, 9H). IR(CH_2Cl_2): 3437, 2597, 1775, 1721, 1508 cm^{-1}.

Method C: The protocol was identical to that of method B with the exception that potassium hydride (KH) was used as the base as opposed to sodium hydride. Column chromatography yielded **5** as a clear oil. TLC: Rf 0.43 in 2/1 ethyl acetate/hexanes. ^{1}HNMR (60 MHz, $CDCl_3$) 5.15(s,1H); 4.93(s, 1H); 4.20(q,2H); 4.05(s, 2H); 3.75(t, 1H); 3.43-3.40(m, 1H); 1.45(s, 9H). IR(CH_2Cl_2): 3437, 2597, 1775, 1721, 1508 cm^{-1}. Inconclusive % yield.

β-lactam (6): Under argon, 16mg (0.062mmol) of **5** was dissolved in 2 mL of dry dichloromethane and cooled to 0^{o}C. Trifluoroacetic acid, 0.25 mL was added and the solution was warmed to room temperature and stirred for 1 hour. After 1 hour, TLC indicated that the starting material had been consumed, and a much more polar product had been formed. The reaction pot was cooled to 0^{o}C and quenched with saturated $NaHCO_3$. Then, the solution was

partitioned between dichloromethane and saturated $NaHCO_3$. The aqueous layer was removed, and the organic layer was washed once each with saturated $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated to yield10 mg (100% yield) **6** as a clear oil. TLC: Rf 0.1 in 2/1 ethyl acetate/hexanes (with a very distinct orange color in its ninhydrin spot). IR(CH_2Cl_2): 3437, 2597, 1760, 1508 cm^{-1}.

Hydroxamate (10): 515mg (2.10mmol) of N-benzyloxycarbonyl-L-serine was dissolved in 10 mL THF: 20mL H_2O. Then 500mg of O-Benzylhydroxylamine-hydrochloride (3.14mmol) was added. The pH of the solution was 1.3. Then 3M NaOH was added until the pH was 4.5. Then 508mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride was dissolved in H_2O, and added to the stirring solution. To combat the climb in pH, 1M HCl was periodically added to maintain a pH range of 4-5. After 2 hours, the product was collected by vacuum filtration, and dried in a 105^oC oven. The product was recrystallized in ethyl acetate and hexanes to give 515mg of the white crystalline hydroxamate **10**: mp 125-127^oC. TLC: Rf 0.19 in 2:1 ethyl acetate/hexanes.

Mesylate (11): Under argon, 0.07mL (0.87mmol) of methanesulfonyl chloride was added to a stirred solution of **10** (250mg, 0.725mmol) in 5mL dry pyridine at 0^oC. After 2 hours at 0^oC, the reaction pot was warmed to room temperature. Ethyl acetate was added, and the organic layer was washed with 1M HCl until the aqueous wash was acidic to pH paper. The organic layer was then washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to obtain mesylate **11** as a white solid (274mg 90% yield): mp 106-108^oC. TLC: Rf 0.46 in 2:1 ethyl acetate/hexanes.

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9. This refers to the terminology originally proposed by L. Schechter and A. Berger (*Biochem. Biophys. Res. Commun.* **1967**, *27*, 157). The S_1, S_2,etc refer to the enzyme subsites that correspond to P_1, P_2, etc of the amino acid residues of the substrate, or inhibitor in this case. The subscript numbers increase from the scissile bond in the direction of the amino terminus. S_1', S_2', etc and P_1'. P_2', etc are the subsites and residues respectively, that proceed in the direction of the carboxy terminus beginning from the scissile bond.

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