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Metastasis of cancer is dependent on a variety of biologically active compounds including serine proteases. Inhibition of proteases such as plasmin is a potential route for both suppression of metastatic lesions and control of primary tumor growth. Synthesis of tetrahydrothiopyran-4-one based inhibitors of plasmin containing either a diamine or amide/amine side chain was undertaken. Initial attempts to produce the inhibitors through amide alkylation and reductive amination proved futile. The diamine inhibitor was finally produced using amine alkylation chemistry. A monoamine side product of this reaction forms the starting material for the other inhibitor. A simple peptide coupling reaction converts the monoamine to the amide/amine inhibitor. Preliminary testing on the crude products shows both inhibitors to have some activity against plasmin with a Ki of 6 mM for the diamine inhibitor and 3 mM for the amide/amine inhibitor.

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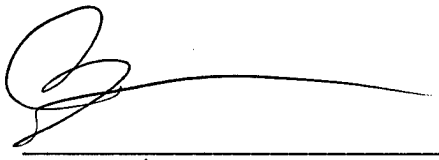

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Contents

Introduction Cancer is a complex disease of many stages. Many different compounds play intricate roles in the metabolic pathways associated with this deadly disease. Serine proteases are present in the metastatic stage and also in angiogenesis. The inhibition of serine proteases could be used to suppress both the formation of secondary tumors and the continued growth of tumors already present. Plasmin and urokinase plasminogen activator are two of the most prevalent enzymes in the degradative scheme which leads to the disruption of the extracellular matrix. Of these two proteases proven to be present in malignant carcinomas, plasmin is the best choice for an inhibitor study because inhibitors of this enzyme are straight forward to synthesize.

General cancer background The primary tumor of a malignant cancer with the exception of brain tumors does not usually cause fatality. The secondary tumors resulting from metastasis produce the dangerous lesions within the vital organs which more often result in death. The danger results when the uncontrolled replication of cells of any tumor creates a cell mass large enough to interfere with the function of the host tissue. During continued growth the formation of new blood vessels by angiogenesis is necessary to support the cells within the inner reaches of the tumor. Without the new blood vessels to remove waste and provide nutrients the center of the tumor will die. A cell mass can only attain a small bicellular layer without angiogenesis. These new blood vessels however are not as structurally sound as the natural arteries within the body. As the aggressive tumor grows the quickly formed blood vessels leak, allowing the transportation of cells across the endothelial layer and into the circulatory system. In this way cells can access the blood transportation network and travel throughout the body forming remote secondary tumors.

When cells leave the primary tumor they tend to lodge in the next capillary bed "downstream" from the cell mass.[1] The endothelial cells lining the blood vessels retract when a tumor cell binds to them, allowing access to the basement membrane. At this point the invading cell must conduct a complex series of events in order to penetrate the tissue. Initially, the cell degrades the basement membrane at the point of contact.

The cell then extends pseudopodia into the newly vacated site while using the still viable sides of the newly formed tunnel to push itself forward. After crossing the basement membrane into the extracellular matrix, the cell begins to replicate and form the new tumor. As with the primary tumor, growth past a bicellular layer is dependent on angiogenesis.

Introduction of proteases Throughout the process of metastasis , the invading cells manufacture proteases. For a viable secondary tumor to form, the cell must transverse the basement membrane, invade the extracellular matrix, and allow angiogenesis to occur. The matrices between cells are constructed from a dense network of carbohydrates and proteins. A complex cascade of proteases permit degradation of the proteins present in both the basement membrane and the extracellular matrix. In a narrow junction between the front edge of the invading cell and the host tissue lies an area of lysis where the proteases are active. Three kinds of proteases are found in this zone of lysis: Metalloproteases, Cysteine proteases and Serine proteases (Table **1**).

Table 1

Proteases	**Inhibitors**
Metalloproteases:	
Type IV Collagenases (gelatinases)	Tissue inhibitors of Metalloproteases (TIMP)
Interstitial Collagenases	
Styromelysins	
Cysteine proteases:	
Cathepsin B	Leupeptin
Cathepsin L	
Serine protease:	
* Plasmin	Antiplasmin
* Urokinase Plasminogen Activator	Anti-uPA IgG
Tumor-associated Trypsin	

* Commercially available

The main mechanistic action of the inhibitor with which we are working is not suited to the metalloproteases, and we have already studied a cysteine protease. Therefore, the initial focus of this research will be aimed toward the serine proteases, specifically the two best understood proteases, plasmin and urokinase plasminogen activator.

Mechanism of proteolytic degradation of basement membrane The invasion of the interstitial matrix by metastatic cells and angiogenesis both contain the same proteolytic cascade.[2] Initially plasminogen, the inactive form of plasmin, becomes bound to the exposed extracellular matrix. Prourokinase plasminogen activator is a regulatory enzyme which has a very short half life in the blood stream. The prourokinase is activated to urokinase by plasmin along with multiple other proteases not entirely characterized as of yet. The resultant urokinase plasminogen activator (uPA) reacts with the bound plasminogen to activate it to plasmin. The plasmin can then activate the prourokinase still present in the blood stream forming a feedback regulatory system which greatly increases the production of plasmin and uPA. Plasmin itself can degrade the proteins within the basement membrane and also activate other proteases to cooperate in the matrix lysis. Natural inhibitors for both of these proteases are present within the system at all times. It is only when the balance between protease and inhibitor is shifted toward the enzyme that degradation of the basement membrane can occur.[2] Small molecule inhibitors introduced at this point could change the ratio of active to inactive proteases and suppress both new metastatic growths and the angiogenesis necessary for the continued growth of tumors already present.

Protease inhibitors Mignatti's study in 1986 determined that multiple proteases are involved in the cascade and their inhibition can limit tumor invasion through the human amniotic membrane.[3] He showed that both serine protease inhibitors and metalloprotease inhibitors could both suppress the invasion of the tumor, but cysteine protease inhibitors

could not. He tested antiplasmin, leupeptin, tissue inhibitor of metalloproteases (TIMP), and antibodies for both urokinase plasminogen activator and tissue plasminogen activator inhibitor. While the addition of the leupeptin and tissue plasminogen activator inhibitor did not appreciatively affect the amount of tumor invasion, the antiplasmin, TIMP, and the antibody for the uPA significantly lowered the ability of the tumor cell line to cross the amniotic barrier. These results proved that the inhibition of plasmin, certain metalloproteases and uPA will suppress metastasis. While recent interest in the metalloproteases has been high, similar interest in the serine proteases has not been as much in evidence. Two of the serine proteases which are being investigated for their roles in metastasis are uPA and Plasmin (Plm). Multiple studies have shown the presence of uPA in the blood serum can be used as a marker and prognostic evaluator of breast cancer.[4-8] Only plasminogen, the inactive form of plasmin, is observed in the blood, and therefore is not useful as an indicator.

Scientists have studied the application of serine protease inhibitors on a cancerous system.[9-11] The protease inhibitors suppress the runaway growth of the cancerous cells. However, there is no way to prove which of the multiple serine proteases involved in the cascade is being inhibited. Therefore these studies are not especially useful for the purpose of inhibitor design. Therapy with serine proteases inhibitors has been proposed for both the prevention of metastasis and the inhibition of angiogenesis.[12,13] The suppression of angiogenesis is especially useful as it limits the ability of the cells which have already metastasized to grow into dangerous secondary tumors. The serine protease inhibitors which could potentially suppress angiogenesis would also inhibit the invasion of any additional metastatic cells in the blood stream.

It has been known for years that certain primary tumors suppress the growth of secondary tumors. A study by Folkman in 1994 showed that a peptide fragment with a 98% homology for an interior segment of plasminogen was present in the blood of mice infected with Lewis Lung Carcinoma. The fragment inhibited both angiogenesis and

metastasis development. Interestingly, intact plasminogen does not produce the same result. Even though the author does not put forth any explanation for the effect, I believe a competitive inhibition between the natural plasminogen and the fragment is taking place. The intact plasminogen would not inhibit the system since it would be activated to plasmin. The fragment contains the lysine binding site for the plasminogen so it could be attaching to the wall of the basement membrane in the place of the natural enzyme. However it is missing the active site so the enzymatic activity is completely absent. When a fragment which is also missing the lysine binding site is introduced to the system, the secondary tumors do not show any suppression. Therefore I believe this study shows that the down regulation of active plasmin either by blocking its binding which is currently being done with available pharmaceuticals or by inhibiting it will lead to the suppression of angiogenesis.

Direct comparison between plasmin and uPA At this point it is useful to look more closely at the serine proteases which are present in cancerous lesions. Both Plm and uPA are commercially available. The preferred residue for recognition at the S_1 site of uPA is an arginine residue while for Plm it is a lysine. The synthesis of a lysine side chain is considerably easier. Okamoto has determined not only the best P'_3 to P_3 residues for a plasmin inhibitor,[14,15] but he has also shown that the P_2 and P_3 residues can be replaced with an alkylamine.[16] The replacement of two peptides from the end of the inhibitor with an amine will decrease the number of steps and therefore improve the overall efficiency of the total synthesis. The structure of the two enzymes is fundamentally similar, consisting of five repeated sequences termed kringles and a serine active site on a separate chain. The X-ray structure for the Plm does not yet exist on public access. However, Umeyama's study implies that plasmin is an enzyme with a relatively wide binding pocket for most of the recognition sites when compared to thrombin, factor Xa, and trypsin while simultaneously retaining a narrow binding site for the P_1 residue.[17] This allows for a

certain flexibility in the design of a molecule which is capable of binding at the active site while retaining the preference for lysine at the P_1 site on the inhibitor.

I believe an inhibitor for the plasmin could be useful in cancer therapy, especially the aggressive phenotypes which lead to multiple deadly secondary tumors. There is a potential market for long term pharmaceuticals for the continued suppression of secondary tumors. Finally, the overall synthetic ease of the plasmin inhibitors compared to the uPA inhibitors makes it the preferred choice for the initial phase of this study.

Inhibitor Design Design of the inhibitor depends on a number of factors. One of these factors is the influence of *through-space* interactions.[18] A heteroatom gamma to the carbonyl on a 4-heterocyclohexanone produces a repulsive electrostatic strain within the molecule. This opposing *dipole-dipole* interaction in a compound such as tetrahydrothiopyran-4-one or its oxidized forms, consisting of the more polar sulfoxide and the sulfone, produce this strain (Figure **1**). The greater the dipole created by the polarity of the group at the gamma position, the greater the strain. This repulsive

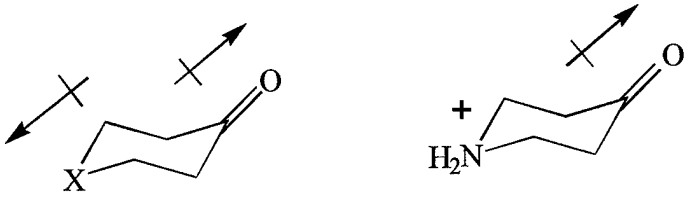


Figure 1 *Dipole-dipole* and *Dipole-charge* repulsive interactions.

interaction increases the electrophilicity of the carbonyl carbon, which in turn provides a stronger attraction for a nucleophile. Addition of nucleophilies to the ketone creates a tetrahedral center, and relieves the strain (Figure **2**). Serine and cysteine

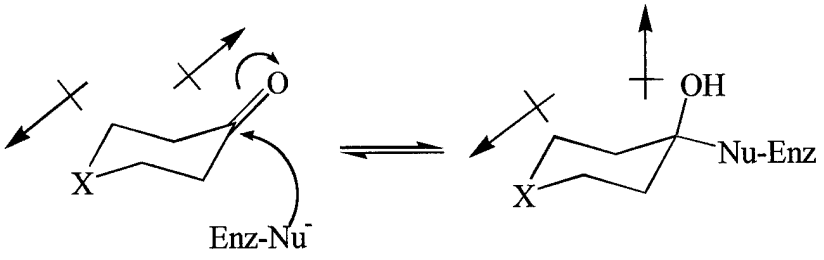


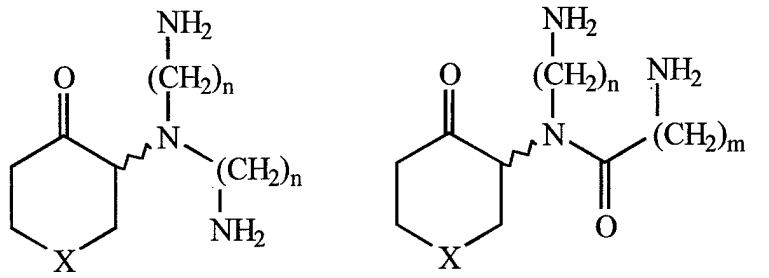
Figure 2 Relief of strain upon addition of enzymatic nucleophile

proteases have a nucleophilic residue in the active site. The mechanism by which these enzymes cleave peptide bonds involves addition of the catalytic site nucleophile to an amide carbonyl of a substrate to form a tetrahedral intermediate. The inhibitor, which has a carbonyl group with enhanced electrophilicity, provides a more attractive binding site for the enzyme active site nucleophile when compared to the natural substrate. The relief of repulsive electrostatic interactions produces a compound which prefers having a tetrahedral center at the carbonyl's site and therefore stays covalently bound to the enzyme active site nucleophile for relatively prolonged periods.

Ring structure While the main function of the parent ring structure is the positioning of the heteroatom gamma to the carbonyl, it also provides a scaffold for future modifications. Unlike the trifluoromethyl ketones or aldehydes, the cyclohexanone inhibitors are bi-directional. A cyclohexanone inhibitor can be designed to include both P_1 and P'_1 residues, thereby increasing both the binding affinity and the potency of the inhibitor. For preliminary studies only the P_1 residues are attached to the parent cyclohexanone ring.

Side chains The primary recognition site on plasmin is specific for lysine, and somewhat less for arginine. While the attachment of a four methylene chain directly to the position alpha to the carbonyl on the ring would closely approximate the geometry of the

natural substrate, formation of a quaternary center at that location could create steric problems. The steric strain is greatly reduced if the side chain attachment is shifted to the amide nitrogen. Molecular modeling studies suggest that a five or six carbon chain ending with an amino group is a good mimic for the natural lysine side chain attached to the ring. Okamoto proved that the P_2 and P_3 residue could be replaced with a pentylamine moiety without loss of affinity.[16] Therefore the preferred P_2 amino acids were replaced with a straight methylene chain in order to simplify the synthesis. Altogether, the general structure of the plasmin inhibitor consists of a tetrahydrothiopyranone ring with an alpha side chain containing either an alkylamino diamine (**19**) or amide-amine (**20**) combination (Figure **3**).



19 **20**

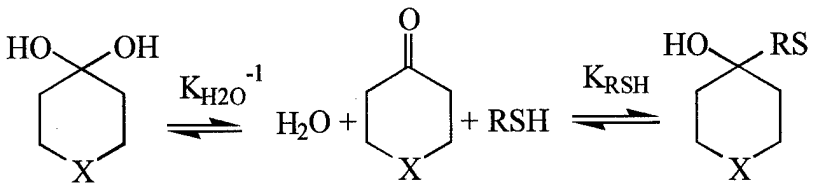
X = S, SO, SO_2
n = 4,5,6
m = 5

Figure 3 General inhibitor structures
19 = Diamine
20 = Amide

Preliminary Results

Equilibrium Constant Experiments To investigate the influence of the heteroatom in the parent ring structure, equilibrium constant experiments were undertaken. The addition of both water (K_{H2O}) and 3-mercaptopropionic acid (K_{RSH}) were measured to compare the nucleophiles present in serine and cysteine proteases respectively (Table **2**).

Table **2**

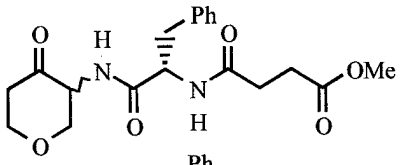
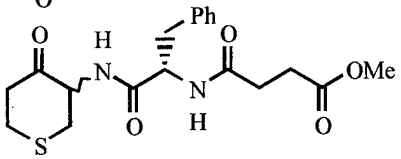
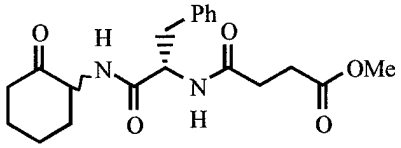
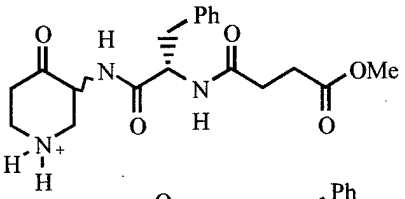
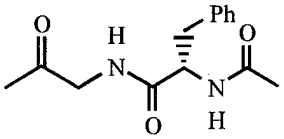


X	K_{H2O} (M^{-1})	K_{RSH} (M^{-1})
CH_2	8.1×10^{-4}	0.22
S	9.0×10^{-3}	1.5
O	8.0×10^{-3}	1.8
NH_2^+	0.18	27.6
SO	0.068	11.7
SO_2	0.30	60.2

As the electronegativity of the heteroatom increases so does the equilibrium constant, implying that the electrostatic repulsive forces between heteratom and ketone also increase with electronegativity. Ring strain also influences the concentration of hydrate. Cyclohexanone exhibits a strained ring system which destabilizes the ketone. Addition of a nucleophile to the carbonyl produces a tetrahedral center which relieves the ring strain. The electronegativity and ring strain combine to stabilize the hydrate form which mimics the tetrahedral intermediate that is formed during enzymatic hydrolysis of the amide bonds.

A series of papain inhibitors containing the 4-heterocyclohexanone ring were synthesized to test this hypothesis. The heteroatoms included sulfur, oxygen and nitrogen. The compounds were good inhibitors of papain. Their potency generally followed the expected trend as dictated by the hydration constants (Table **3**). That is, the compounds that contained the most electronegative hereoatom were the most potent inhibitors.

Table 3

	K_i (μM)	
	More Potent Diastereomer	Less Potent Diastereomer
	11	3300
	26	2400
	78	3200
	120[1]	
For Comparison 	1550[2]	

1. Mixture of Diastereomers
2. Bendall, M.R.; Cartwright, I.L.; Clart, P.I.; Lowe, G.; Nurse, D. *Eur. J. Biochem.* **1977**, *79*, 201.

The potential of the nitrogen compound was initially assumed to be the greatest due to the increased repulsion of the *charge-dipole* interaction. This potential was not realized possibly due to a electrostatic interference by nearby residues in the active site. With the preliminary results in hand we decided to use the tetrahydrothiopyranone as the basic ring structure for the first plasmin inhibitor synthesized. The sulfur based inhibitor, while having one of the better inhibition constants, also has a relatively straight forward synthesis and the potential to be further oxidized to produce both the sulfoxide and the sulfone inhibitors.

Current Results

Month 1- 4: Completion of Preliminary Results Conclusion of the preliminary results included the oxidation of the sulfide papain inhibitors and the synthesis and assay of the piperidone papain inhibitor. Results of the assays appear in Table **3**.

Month 5 - 6: Hydration Experiments Hydration studies of model compounds containing a quaternary center at the alpha position were performed to determine the suitability of the structure. Compounds **16,17** and **18** were synthesized (Figure **4**).

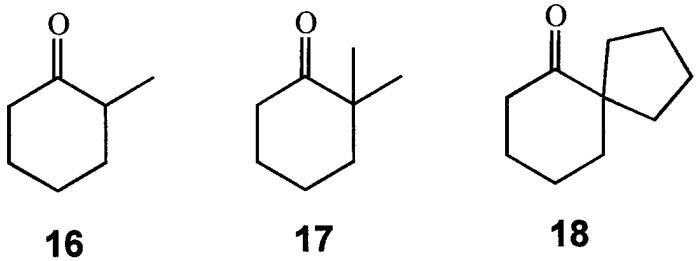


Figure **4** Hydration experiment compounds

The resulting compounds were then subjected to nuclear magnetic resonance (NMR) experiments to determine the hydration constants. Unluckily the NMR signal used to determine the relative concentration of hydrate was masked by other signals. Therefore the determination of the hydration constants was not possible for any of the three compounds.

Molecular modeling of the compounds was also performed. A public access x-ray crystallographic structure of trypsin, a serine protease closely related to plasmin, was imported into the Quanta Molecular Modeling program from the Brookhaven National Laboratory Protein Data Bank. Comparison of the molecular energy data suggested that there may be steric interference resulting from the quaternary center on the ring. The

separate inhibitors were constructed within the modeling program and then covalently linked to the enzyme active site nucleophile. We then minimized the energy of these inhibitors in the active site. The compounds which contained a quaternary center had higher energies and therefore were less stable. From this data we determined that shifting the attachment site for the primary recognition chain to avoid the quaternary structure would benefit the inhibitor. Models of the proposed inhibitors did not show any apparent steric interference (Figure **5**).

Month 7 - 8: Attempted synthesis of the amide linked inhibitor The modeling studies implied that the best site for the attachment of the primary recognition side chain was the nitrogen of the amide linkage (Figure **3**). The structural similarity between the amide and the amine linkages suggested inhibitors of both types should be synthesized.
With this in mind, we began synthesis of initial representatives of structures **19** and **20**.

Synthesis of these inhibitors proceeds in two converging pathways. First synthesis of the main ring structure of the inhibitor is accomplished with a proven synthetic route already performed in this lab.[19] Scheme **1** depicts the steps of this synthesis. Initially the

Scheme 1

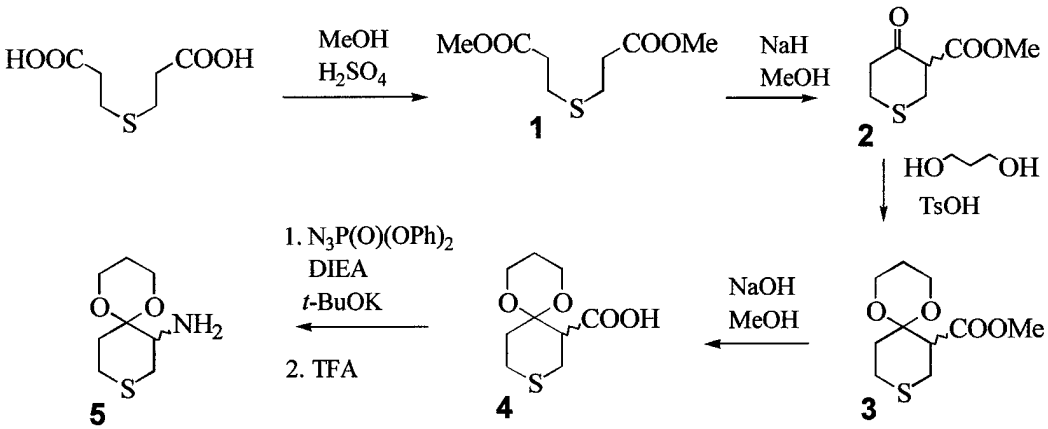
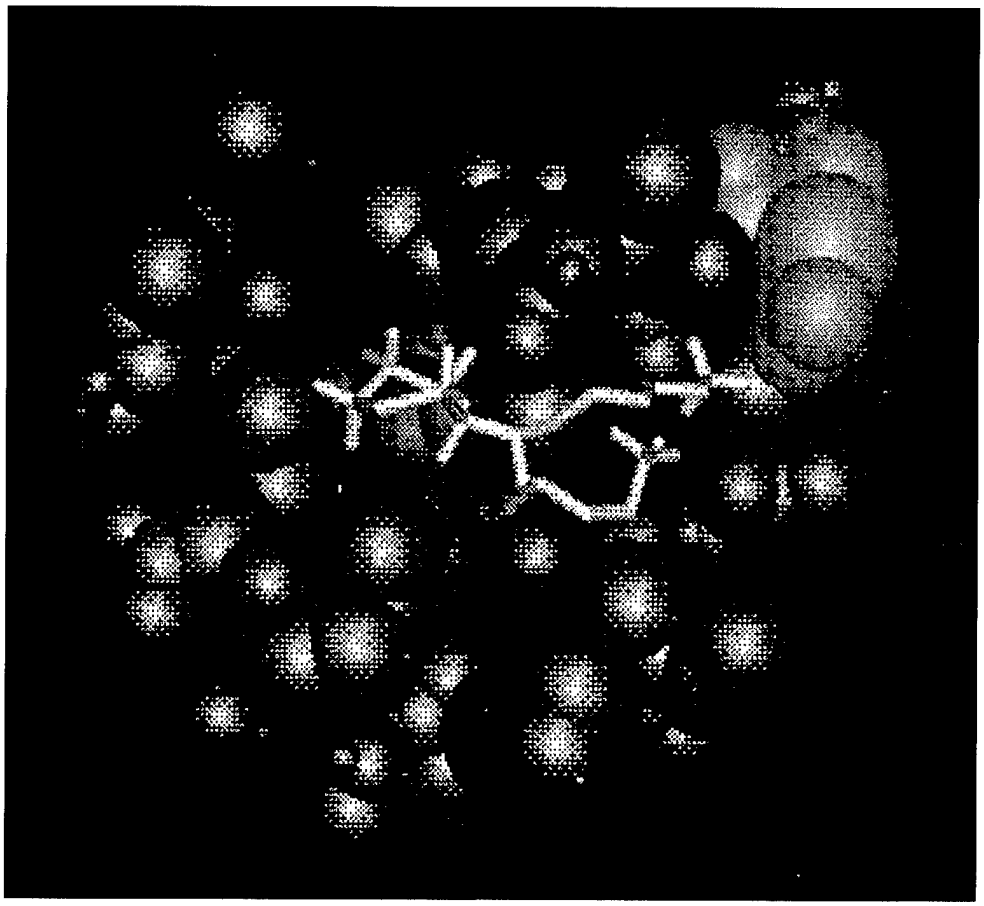


Figure 5:

Five carbon diamine inhibitor bound to the active site of trypsin

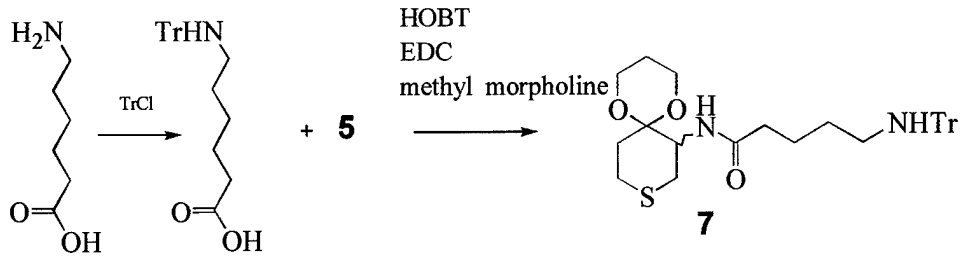


Dark purple = Active site serine
Pink = Aspartate binding site of P1 residue
White = Inhibitor
Blue = Enzyme

commercially available thiodipropionate is esterified by reaction with acidic methanol reaction. The resultant diester **1** is cyclized with sodium hydride and catalytic quantities of methanol in ethyl ether to afford the cyclic ester **2**. Protection of the active carbonyl is accomplished by formation of the ketal using an acid catalyzed addition of 1,3 propanediol. The ketal ester **3** is hydrolyzed in an alkaline methanol solution to produce the acid ketal **4**. A Curtius rearrangement to create an isocyanate is accomplished using diphenylphosphorylazide and diisopropylethylamine (DIEA) in benzene. The isocyanate is quenched in a tetrahydrofuran (THF) solution containing potassium *t*-butoxide to give a cyclic carbamate. Finally, the carbamate is converted to free amine **5** by the addition of trifluoroacetic acid. The free amine is then used in the production of the amide and diamine inhibitors.

Month 9: Synthesis of the amide linkage inhibitors With two different chains attached to the nitrogen it is possible to envision two distinct synthetic routes: addition of the amine chain first or addition of the amide chain first. The initial attempt at synthesis of the amide inhibitor **20** included addition of the amide side chain first. Scheme **2** details this synthetic

Scheme 2

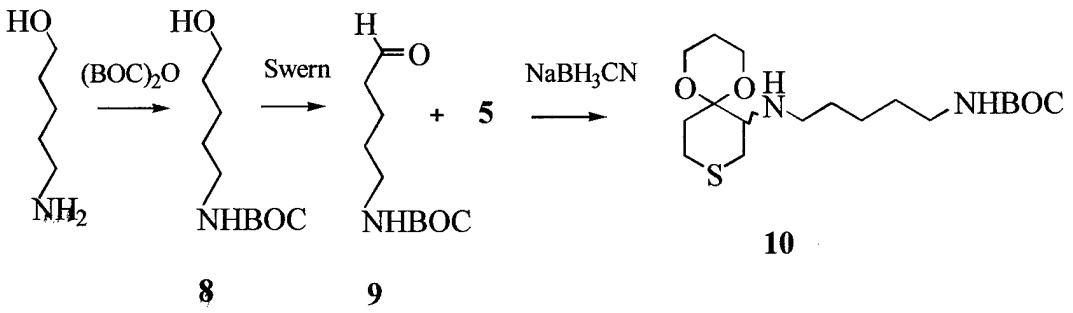


route. Commercially available 6-aminocaprioic acid is reacted with trityl chloride to protect the amine and produce compound **6**. For the coupling between compounds **6** and

5, a methylene chloride solution of hydroxybenzotriazole, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and methyl morpholine are used to produce the amide **7**. Subsequent attempts to deprotonate the amide nitrogen to bond an alkylamine proved to be unsuccessful. Neither the combination of sodium hydride and sodium iodide nor the phase transfer catalyst tetrabutylammonium sulfate in a slurry of potassium carbonate solution and methylene chloride succeeded in deprotonating the amide for subsequent reaction with an alkyl bromide. This scheme was dropped because of the failure of this step to provide the final compound.

Month 10: Monoalkylation and reductive amination The other main route for attachment of the two side chains consists of the addition of the amine chain first. In an attempt to produce a monoalkylated amine, scheme **3** was attempted. First, commercially

Scheme 3

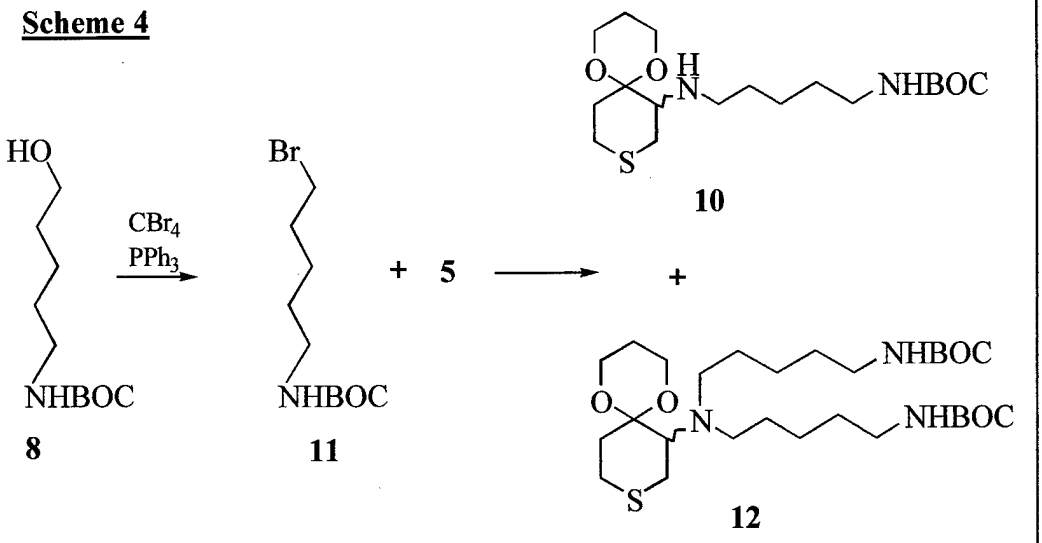


8 **9** **10**

available 5-amino-1-pentanol was reacted with di-*t*-butyl dicarbonate to produce the BOC protected alcohol **8**. This compound in turn was oxidized with oxalyl chloride and dimethyl sulfoxide to provide the aldehyde **9**. Compounds **9** and **5** where combined in a THF solution and the product was immediately reduced with sodium borohydride. This reaction proved to be of very poor quality and yielded a great number of side products. For this reason this scheme was abandoned.

Next, the monoalkylation of the ring structure was attempted via nucleophilic substitution. Scheme **4** shows the synthetic route used to provide the alkylated products.

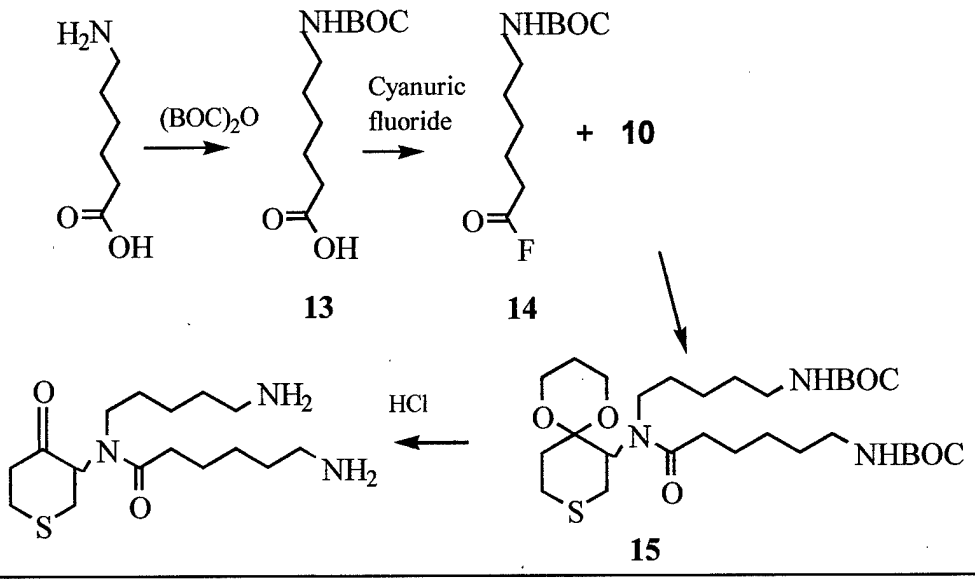
Scheme 4



The BOC protected alcohol **8** is converted to the bromide **11** with carbon tetrabromide and triphenylphosphine. The bromide is then combined with the amine ring structure in an acetonitrile solution containing DIEA. Heating of the reaction for up to five days results in the formation of both monoalkylated amine **10** and dialkylated amine **12**. Unfortunately it is difficult to influence the ratio of mono to dialkylated products produced with this method. Likewise the alkyl bromide used in this reaction has the tendency to undergo elimination, thereby reducing the efficiency of the reaction. At this point, however this reaction is our only method for producing the dialkylated product.

Month 11: Production of the amide linkage Following the formation of the monoalkylated amine using scheme **4**, the way was clear for producing the amide linked inhibitor. Scheme **5** illustrates the methodology used to access the final amide inhibitor **20**. Commercially available 6-aminocaprioic acid was reacted with

Scheme 5



di-*t*-butyl dicarbonate to produce acid **13**. Cyanuric fluoride was then used to produce acid fluoride **14**. When the acid fluoride is heated with the monoalkylated amine it results in formation of the BOC protected amide inhibitor. After production of both the protected inhibitors, the final step involves the deprotection of both compounds. While both a transketalization using *p*-toluenesulfonic acid in acetone and hydrolysis of the ketal using the pyridinium salt of toluenesulfonic acid resulted in the final product, a cleaner reaction consisting of a hydrochloric acid hydrolysis is preferred.

Month 12: Km studies and preliminary assays The final synthetic route for inhibitors **19** and **20** are essentially worked out. I have synthesized a number of the inhibitors which are to be studied, though the final purification is still troublesome. The removal of the 1,3-propanediol from the deprotection of the ketal has proven to be very difficult. Initial attempts to assay the crude inhibitors have proven successful showing the crude mixture to have some activity for porcine plasmin. Crude inhibitor **19** exhibits a K_i of 6 mM. The K_i of the crude inhibitor **20** is 3 mM.

In conclusion, I spent the first six months of the project finishing the preliminary results by oxidizing the sulfide papain inhibitors, and conducting the hydration and modeling experiments. The final six months I devoted to the synthesis of the initial inhibitors including examples of both the diamine and amide-amine structures.

Procedures

General Methods. NMR spectra were recorded on Brucker WM-250 or AM-400 instruments. Spectra were calibrated using TMS ($\delta = 0.00$ ppm) for ^1H NMR and $CDCl_3$ ($\delta = 77.0$ ppm) for ^{13}C NMR. IR spectra were recorded on a Perkin-Elmer 1700 series FT-IR spectrometer. Mass spectra were recorded on a Kratos MS 80 under electron impact (EI), chemical ionization (CI) or fast-atom bombardment (FAB) conditions. HPLC analyses were performed on a Rainin HPLC system with Rainin Microsorb silica or C18 columns, and UV detection. Semi-preparative HPLC was performed on the same system using a semi-preparative column (21.4 x 250 mm).

Reactions were conducted under an atmosphere of dry nitrogen in oven dried glassware. Anhydrous procedures were conducted using standard syringe and cannula transfer techniques. THF and toluene were distilled from sodium and benzophenone. Methylene chloride was distilled from CaH_2. Other solvents were of reagent grade and were stored over 4 angstrom molecular sieves. All other reagents were used as received. Organic solutions were dried with $MgSO_4$ unless otherwise noted. Solvent removal was performed by rotary evaporation at water aspirator pressure.

Dimethyl thiodipropionate (1) To a stirred solution of 3,3'-thiodipropionic acid (5.99 g, 33.6 mmol) in 200 mL methanol (MeOH) was added 1 mL concentrated sulfuric acid. The flask was fitted with a condenser and heated at reflux for 3.5 h. The resultant solution was concentrated to 20% of the original volume, and after diluting with 200 mL ethyl acetate (EtOAc), was washed twice with 100 mL 1:1 H_2O/sat. $NaHCO_3$ solution, and once with brine. After drying over $MgSO_4$ and evaporation of the solvent, the clear oily liquid was purified by column chromatography (silica, 1:4 EtOAc / Hexanes) giving 6.89 g (33.39 mmol, 99%). ^1H NMR ($CDCl_3$, 250 MHz) δ 2.6 (td, 4H, $J = 1.1, 7.1$ Hz), 2.8 (td, 4H, $J = 1.1, 7.8$Hz), 3.7 (s, 6H); IR (NaCl) ν 1728.4 (carbonyl); HRMS-EI (M^+) Calcd for $C_8H_{14}O_4S$ 206.0613, found 206.0617.

Tetrahydrothiopyran methyl ester (2) To a solution of NaH (1.46 g, 60% dispersion in mineral oil, 36.5 mmol) in 40 mL dry ethyl ether was added 8 drops of MeOH. The diester (**1**) was added after being diluted with 5 mL of ether. After heating at reflux for 2 h, the solution was allowed to cool to room temperature and NaH (1.28 g, 60% in oil, 32.0 mmol) was added. After heating at reflux for an additional hour, the solution was quenched with 40 mL of 3.1 N AcOH. The aqueous layer was washed three times with ether. Combined organic layers were washed with 1:1 H_2O/sat. $NaHCO_3$ sol., and dried. The oily liquid resulting from the concentration of the organic layer was purified by column chromatography (silica, 1:4 EtOAc / Hexanes) giving 3.60 g (20.7 mmol, 69%). Cmpd (**2**) was present in approximately a 3:1 mixture of the keto and enol forms. ^1H NMR ($CDCl_3$, 250 MHz) enol: 2.6 (m, 2H), 2.8 (m,2H), 3.3 (s, 2H), 3.7 (s, 3H) keto: 2.8-2.9 (m), 3.2 (m), 3.6 (m); HRMS-EI (M^+) Calcd for $C_7H_{10}O_3S$ 174.0351, found 174.0348.

Tetrahydrothiopyran methyl ester ketal (3) A flask containing benzene (100 mL) solution of the cyclic ester (**2**, 1.47 g, 8.42 mmol), ethylene glycol (1.0 mL, 16.8 mmol) and TsOH (0.33 g, 1.68 mmol) was fitted with a Dean-Stark trap and condenser. After heating at reflux for 3.5 h, the solution was washed with 80 mL 1:1 H_2O/sat. $NaHCO_3$ solution, dried, and concentrated. The resultant oily liquid was purified by column chromatography (silica, 2:3 EtOAc/Hexanes) giving 1.78 g (8.16 mmol, 97%). ^1H NMR ($CDCl_3$, 250 MHz) δ 1.8 (ddd, 1H, J = 3.6, 9.3, 13.3 Hz) 2.2 (ddd, 1H, J = 3.5, 7.1, 13.5 Hz), 2.6 - 3.0 (m, 5H), 3.7 (s, 3H), 3.9 (m, 4H); IR (NaCl) ν 1734.6 (carbonyl); HRMS-EI (M^+) Calcd for $C_9H_{14}O_4S$ 218.0613, found 218.0612.

Tetrahydrothiopyran acid (4) A solution containing 100 mL MeOH, 1N NaOH (1.2 mL, 1.2 mmol) and the cyclic ester (**3**, 0.26 g, 1.18 mmol) was heated at reflux for 24 h. After extracting the unreacted ester with methylene chloride (CH_2Cl_2), the solution was acidified with 1N HCl and the carboxylic acid was extracted with CH_2Cl_2. The white solid resulting from concentration of the solution was recrystallized from 1:2 EtOAc/Hexanes affording 0.14 g of the carboxylic acid **4** (0.68 mmol, 57%). ^1H NMR ($CDCl_3$, 250 MHz) δ 1.8 (ddd, 1H, J = 4.0, 8.4, 13.0 Hz), 2.2 (ddd,1H, J = 3.7, 7.2,13.5 Hz), 2.6-3.1 (m, 5H), 4.0 (m, 4H), 10.2 (bs, 1H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 26.6, 29.2, 35.6, 50.7, 64.6, 65.1, 107.2, 175.2; HRMS-EI (M^+) Calcd for $C_8H_{12}O_4S$ 204.0456, found 204.0458.

Thio carbamate To a solution of 100 mL benzene and thioacid (**6**, 1.29 g, 6.30 mmol) was added Et_3N (1.05 mL, 7.56 mmol) and diphenyl phosphoryl azide (1.29 mL, 6.0 mmol). The solution was heated at reflux until the IR spectra showed no further conversion from azide (ν = 2171 nm) to isocyanate (ν = 2250 nm). After 3 h the solution was transferred to a flask containing lithium *t*-butoxide formed from *t*- butyl alcohol (2.41 mL, 25.2 mmol) and 1.7 M *n*-butyllithium (7.88 mL, 12.6 mmol) which was cooled to 0 °C. After 1.2 h the flask was removed from the ice bath and the solution was washed with brine, dried and concentrated. The resultant oily yellow liquid was purified by column chromatography (silica, 2:3 EtOAc/Hexanes) giving 0.74 g (2.69 mmol, 42%). ^1H NMR ($CDCl_3$, 250 MHz) δ 1.4 (s, 9H), 1.8 (m, 1H), 2.0 (m, 1H), 2.6 (m, 3H), 2.8 (d, 2H, J =19.7 Hz), 3.9 (m, 4H), 4.0 (m, 1H), 5.1 (bd, 1H, J = 8.9 Hz); IR (NaCl) ν 3353.4 (amide) 1713.8 (carbonyl); HRMS-EI ($M+Na^+$) Calcd for $C_{13}H_{23}NNaO_4S$ 312.1246, found 312.1257.

Tetrahydrothiopyran amine (5) The carbamate (4.6 g, 16 mmol) was diluted in 350 mL of CH_2Cl_2 before trifluoroacetic acid (TFA, 24 mL, 320 mmol) was added at room temp. After 1.75 h the solvent and TFA were removed under reduced pressure. The resultant oily liquid was diluted with 10 mL EtOAc and extracted into 20 mL of 1N HCl. The aqueous layer was rendered basic with 1N NaOH and then extracted repeatedly with CH_2Cl_2 (6 x 50 mL). The combined organic layers were dried and concentrated providing 2.6 g (13 mmol, 85%) of the amine (**5**). ^1H NMR ($CDCl_3$, 250 MHz) δ 1.5 (m, 1H), 1.8-2.0 (m, 4H), 2.5-2.7 (m, 4H), 2.9 (dd, 1H, J = 3.0, 13.3 Hz), 3.1 (dd, 1H, J = 3.0, 7.5 Hz) 3.8-4.0 (m, 4H).

Tritylamino hexanoic acid (6) A CH_2Cl_2 solution (150 mL) of 6-aminocaproic acid (5.0 g, 38 mmol) was stirred with triphenylmethyl chloride (21 g, 76 mmol) and diisopropylethylamine (DIEA, 13 g, 76 mmol) for 2 days. The solution was washed with 1N HCl (50 mL) and sat. $NaHCO_3$ solution (30 mL) to remove the DIEA. Then the remaining solution was stirred overnight with 50 mL 1N NaOH and 50 mL MeOH forming a homogeneous solution. After removing the volatile solvents under reduced pressure the resultant liquid was partitioned between EtOAc and brine. The aqueous layer was acidified with 1N HCl and extracted with CH_2Cl_2. The resultant organic layer was dried and concentrated. A chromatography column (silica, 10% MeOH/ CH_2Cl_2) was run on the oily liquid resulting in the hexanoic acid. ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.3-1.6 (m, 6H), 2.1 (t, 2H), 2.3 (t, 2H), 4.6 (bs, 1H), 7.2-7.5 (m, 24H).

Tetrahydrothiopyran amide hexanoic acid (7) To a CH_2Cl_2 solution (10 mL) was added hydroxybenzotriazole (0.07 g, 0.53 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 0.13 g, 0.69 mmol) and the acid **6** (0.20 g, 0.53 mmol). The solution was stirred at room temp for 25 min before the amine **5** (0.1 g, 0.53 mmol) diluted in 5 mL CH_2Cl_2 and methyl morpholine (0.11 g, 1.0 mmol) were added. After an additional hour the solution was washed with water, sat $KHSO_4$ solution, and sat. Na_2CO_3 solution before being dried over $MgSO_4$ and concentrated. The resultant oily liquid was purified by column chromatography (silica, 1:19 EtOAc/ Hexanes) affording **7** (0.06 g, 0.11 mmol, 20%). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.3-1.5 (m, 15H), 1.5-1.7 (m, 4H), 1.7-1.8 (m, 1H), 2.2 (t, 2H, J = 7.4 Hz), 2.4-2.8 (m, 3H), 2.9 (dd, 1H, J = 2.3, 13.2 Hz), 3.0 (q, 2H, J = 6.4 Hz), 3.8 (pent, 1H, J = 5.8 Hz), 3.9 (t, 2H, J = 5.6 Hz), 4.1 (pent, 1H, J = 5.8 Hz), 4.6 (bm, 1H), 6.2 (d, 1H, J = 8.9 Hz).

5-BOC amino pentanol (8) A 1,4-dioxane solution of 5-amino-1-pentanol (1 mL, 10.2 mmol) was cooled to 0°C before di-*t*-butyl dicarbonate (5.3 g, 24 mmol) was added. The solution was allowed to warm up to room temp. and stirred overnight. The solution was then concentrated under reduced pressure. The resultant liquid was dissolved in EtOAc and washed with sat. $NaHCO_3$ solution and brine before being dried and concentrated. The final compound was purified by column chromatography (silica, 10% MeOH/CH_2Cl_2) affording 2.1 g (10 mmol, 99%) of the alcohol (**8**). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.4-1.8 (m, 18H), 3.1 (q, 2H, J = 6.4 Hz), 3.6 (t, 2H, J = 6.3 Hz), 4.6 (bs, 1H).

5-BOC aminopentyl aldehyde (9) Oxalyl chloride (1.9 mL, 3.7 mmol) was diluted with 15 mL CH_2Cl_2 before being cooled to -78°C. Dimethylsulfoxide (DMSO, 0.52 mL, 7.3 mmol) and the alcohol **8** (0.53 g, 2.6 mmol) diluted in 10 mL of CH_2Cl_2 were added. After the reaction vessel was warmed to -40°C, triethylamine (Et_3N, 1.6 mL, 11.4 mmol) was added. After 15 min. the reaction mixture was washed with 10 mL sat. $NaHCO_3$ solution and 10 mL H_2O, then dried and concentrated. The resultant liquid was purified by column chromatography (silica, 3:2 EtOAc/Hexanes) affording 0.31 g (1.6 mmol) of the aldehyde **9**. The compound was classified by reaction with 2,4-dinitrophenylhydrazine.

5-BOC aminopentyl bromide (11) The BOC protected alcohol (1.1 g, 5.3 mmol) was dissolved in 50 mL of CH_2Cl_2 before carbon tetrabromide (2.5 g, 7.4 mmol) was added, and after 10 min. the solution was cooled to 0°C. Triphenylphosphine (2.8 g, 10.6 mmol) recrystallized out of hexanes as added turning the reaction mixture a bright yellow. After 2 h the solution was loaded directly onto a silica column charged with 2.5% MeOH/CH_2Cl_2 producing the bromide **11** (1.4 g, 5.1 mmol, 97%). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.4-1.5 (m, 9H), 1.8 (m, 2H), 3.1 (q, 2H, J = 6.1 Hz) 3.4 (t, 2H, J = 6.7 Hz), 4.6 (bs, 1H); HRMS-FAB ($M+Na^+$) Calcd for $C_{10}H_{20}NNaO_2Br$ 288.0575, found 288.0574.

Mono & Dialkylamino tetrahydrothiopyran (10, 12) To an acetonitrile solution (70 mL) of the cyclic amine (**5**, 0.49 g, 2.6 mmol) was added the alkyl bromide (**11**, 1.4 g, 5.1 mmol) and DIEA (0.67 g, 5.2 mmol). After 4 days at reflux the acetonilrile was removed under reduced pressure before being diluted with EtOAc. The solution was washed with 50 mL of 2.2 N AcOH, sat. $NaHCO_3$ solution, dried and concentrated. The resultant oily liquid was purified by column chromatography (silica, EtOAc) affording 0.15 g (0.41 mmol) of the monoalkyl amine **10** and 0.25 g (0.46 mmol, combined yield of 34%) of the dialkylamine **12**. Monoalkylamine: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.2-1.6 (m, 16H), 1.8-1.9 (m, 2H), 2.5-2.8 (m, 7H), 2.9 (dd, 1H, J = 3.1, 7.8 Hz), 3.0 (q, 2H, J = 6.4 Hz), 3.8-4.0 (m, 4H), 4.5 (bs, 1H); HRMS-EI ($M+H^+$) Calcd for $C_{18}H_{35}N_2O_4S$ (CI) 375.2317, found 375.2328. Dialkylamine: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.2-1.6 (m, 30H), 2.3 (m, 2H), 2.5 (m, 2H), 2.7-2.8 (m, 4H), 3.0-3.2 (m, 6H), 3.8-4.0 (m, 4H); HRMS-EI ($M+H^+$) Calcd for $C_{28}H_{54}N_3O_6S$ 560.3733, found 560.3721.

BOC amino caproic acid (13) To a solution of 1,4-dioxane (150 mL) was added 6-aminocaproic acid (5.0 g, 49 mmol) and di-*t*-butyl dicarbonate (21 g, 97 mmol). The reaction was stirred at room temp. overnight before the dioxane was removed under reduced pressure. The resultant gelatinous solid was partitioned between EtOAc and 1N NaOH. The aqueous layer was acidified with 1N HCl and extracted again with EtOAc. The final organic layer was dried and concentrated affording an oily liquid (7.7g, 33 mmol, 69%) which was used without further purification. ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.1-1.7 (m, 15H), 2.4 (dt, 2H, J = 1.6, 7.2 Hz), 3.1 (q, 2H, J = 6.5 Hz), 4.5 (bs, 1H), 9.7 (s, 1H).

BOC amino caprioic acid fluoride (14) The acid (**13**, 4.3 g, 18.5 mmol) was diluted with CH_2Cl_2 (90 mL) before being cooled in a brine ice bath and pyridine was added (1.8 g, 18.5 mmol). After 15 min cyanuric fluoride (5.0 g, 37 mmol) was added and the reaction was allowed to stir for 3 h. The solution was diluted with 50 mL CH_2Cl_2 and washed with 3 x 100 mL ice cold water, dried and concentrated. This compound was used without further purification.

Amide linked tetrahydrothiopyran (15) To a CH_2Cl_2 solution of the monoalkylamine (58 mg, 0.16 mmol) was added the acid fluoride (**14**, 72 mg, 0.31 mmol) and DIEA (0.022 mL, 0.17 mmol). After refluxing for 2 days the reaction was washed with 2.2 N AcOH and sat. $NaHCO_3$ solution before being dried and concentrated affording 29 mg of the amide (**15**, 0.050 mmol, 32%). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.1-

1.7 (m, 34H), 2.2-2.4 (m, 4H), 2.8 (m, 1H), 3.0-3.5 (m, 8H), 3.7-4.0 (m, 4H), 4.1 (q, J = 7.1 Hz), 4.6 (bs, 1H); HRMS Calcd for $C_{29}H_{53}N_3NaO_7S$ 610.3602 found 610.3497.

Enzyme Assays. Plasmin (porcine) and D-Val-Leu-Lys p-nitroanilide were used as received from Sigma Chemical Co. Reaction progress was monitored at 404 nm with a Perkin-Elmer 8452A diode array UV-Vis spectrometer. Plasmin was assayed at 25°C in 50 mM sodium phosphate buffer (pH 7.4). Concentration of the substrate was 225 μM. Determination of the K_i was performed by nonlinear fit to the Michaelis-Menton equation. K_m of the substrate was measured at 220 μM (reported value 270 μM).

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