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Metastasis and angiogenesis in cancer are dependent on a variety of biologically active compounds including serine protease. Inhibition of proteases such as plasmin is a potential route for suppression of both metastasis and angiogenesis. Synthesis of tetrahydrothiopyran-4-one based inhibitors containing either a dialkylamino chain, a lysine analog side chain and a dipeptide (D-ile-phe or D-ile-Trp), or just the D-ile-phe dipeptide was accomplished. A compound with both the lysine analog side chain and the dipeptide but lacking a carbonyl was also produced. The best inhibitor had a Ki of 50 μM.

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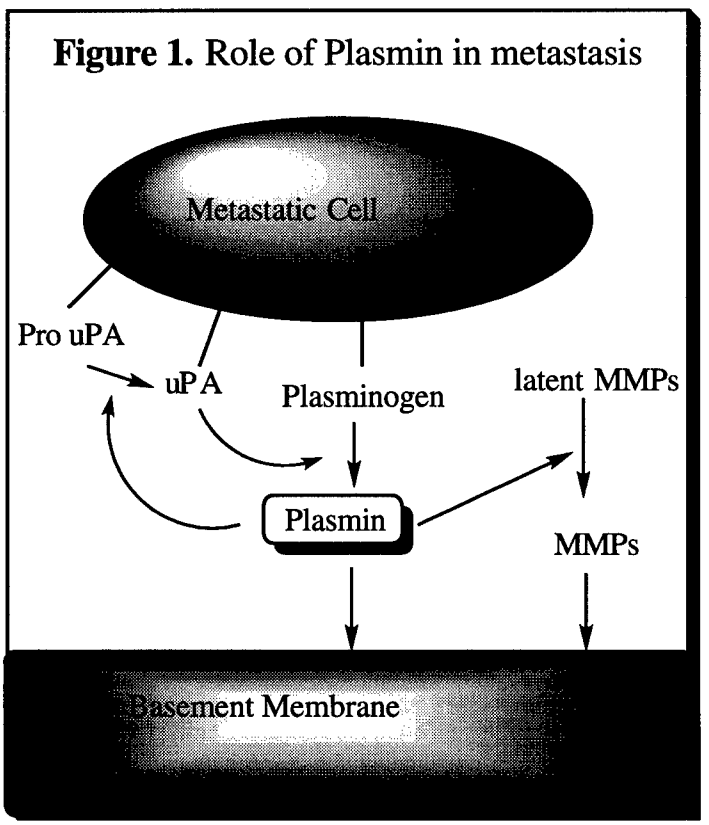
Table of Contents

Introduction

Angiogenesis and metastasis are attracting attention among the latest targets in the continuing battle against the proliferation of cancer. Recent publications prove that suppression of angiogenesis limits primary tumor growth and produces dormancy in secondary metastases.[1,2] The basic mechanism of both systems includes a proteolytic cascade which causes degradation of the basement membrane surrounding the blood vessels. In angiogenesis the resulting lesion allows for extension of the epithelial cells into the surrounding tissue and formation of new blood vessels.[3] During metastasis the cancerous cell itself becomes implanted in the extracellular matrix by way of the newly degraded basement membrane.[4] Serine and cysteine proteases and matrix metalloproteases (MMPs) are all involved in the proteolytic cascade. An agent which blocks the action of any of these enzymes could slow the growth or prevent distant relocation of a tumor.

Figure 1. Role of Plasmin in metastasis



Plasmin is one of the best characterized of the serine proteases present in the degradative pathway. Its central role in the proteolytic cascade includes activation of MMPs which go on to digest the basement membrane themselves or degradation of either fibrin or collagen both of which are present in the basement membrane (Figure 1).[3] Initially plasminogen must bind to its substrate by the lysine binding site before it can be activated to plasmin and begin amidolytic action to disrupt the proteins of the surrounding tissue. Current pharmaceuticals aimed at deactivating plasmin are dependent on inhibiting its fibrinolytic activity by blocking the lysine binding site.[5] In this case plasminogen is unable to bind with fibrin and does not become activated. The amidolytic activity of plasmin however is not affected by this treatment. The bodies chief defense against free plasmin is α2-antiplasmin. This natural inhibitor is also dependent on the lysine binding site for bonding with the enzyme and blocking its activity.[6] In neither case can plasmin which is already activated be inhibited. Therefore we set out to produce an active site directed plasmin inhibitor which can provide inhibition against both fibrinolysis and amidolysis.

We have previously reported a reversible inhibitor of the cysteine protease papain which was proven to form a covalent adduct with the enzyme.[7,8] The question arose whether the same central 4-heterocyclohexanone ring structure could also be used to produce a serine protease inhibitor. Similar to the cysteine inhibitors, a dipeptide side chain is attached to the ring which is designed to extend down the active site cleft and provide hydrophobic interactions with the enzymes three recognition subsites. In the plasmin inhibitor the primary recognition is dependent on a lysine analog, while the P_2 and P_3 sites are occupied by phenylalanine and D-isoleucine respectively. The resulting compound proved to be a good reversible selective inhibitor for plasmin (K_i = 40 μM).

Inhibitor Design

The main factor in the design of the central core of the inhibitor is the inclusion of through-space interactions to activate the carbonyl.[9] A heteroatom gamma to the carbonyl of a 4-heterocyclohexanone produces repulsive electrostatic strain within the molecule. In a compound such as tetrahydrothiopyran-4-one the dipole moment of the carbonyl is geometrically opposed to the dipole created by the electronegative sulfur atom (Figure 2). The greater the dipole created by the polarity of the group at the gamma position, the greater the strain. This repulsive interaction increases the electrophilicity of the carbonyl carbon, which in turn provides a stronger attraction for a nucleophile.

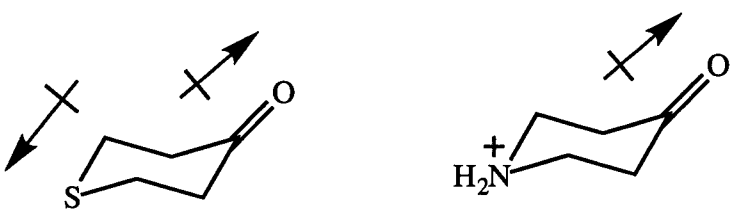


Figure 2. dipole-dipole and dipole-charge repulsive interactions.

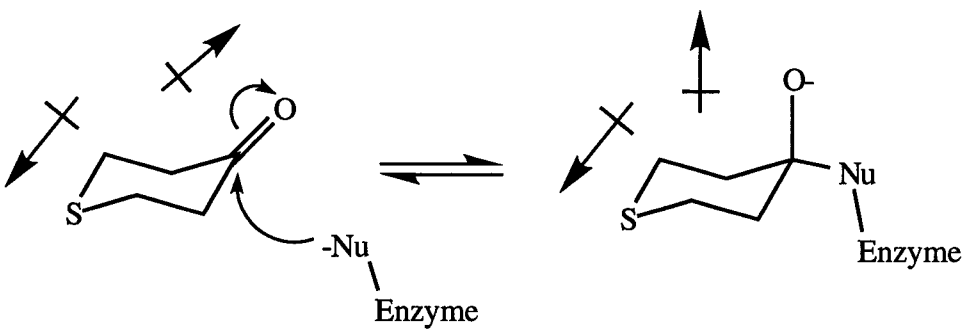


Figure 3. Nucleophilic attack on carbonyl forms tetrahedral intermediate.

Addition of such a nucleophile to the ketone creates a tetrahedral center which relieves the

strain (Figure 3). Serine and cysteine proteases use a mechanism to cleave peptide bonds which involves addition of a catalytic site nucleophile to the substrate's amide carbonyl to form a tetrahedral intermediate. The inhibitor, with its activated carbonyl, provides a more attractive binding site for the enzyme active site nucleophile when compared to the natural substrate. Due to relief of the repulsive electrostatic interactions the compound prefers the covalently bound tetrahedral form over the planar carbonyl of the free inhibitor resulting in increased binding affinity with the enzyme and therefore a more potent inhibitor.

Molecular modeling (Quanta, Molecular Simulations Inc.) was used to illustrate the fit of the inhibitor in the active site. The main function of the parent ring structure is the positioning of the heteroatom gamma to the carbonyl. The preliminary modeling shows no apparent unfavorable steric interactions between the enzyme and the ring which extends from the catalytic triad out into the active site cleft. The central ring also serves as an attachment point for the peptide recognition chains. For the plasmin inhibitor we provided a side chain which was designed to bind to the S_1 sites on the enzyme. For the cathepsin B inhibitor two side chains contacted both the S_1 and S_1' sites. The S_1 recognition site of plasmin is specific for lysine, and somewhat less for arginine.[9] Attachment of a lysine side chain directly to the position alpha to the carbonyl on the ring would closely approximate the geometry of the natural substrate. However formation of a quaternary center at that location could produce steric interference with addition of the active site nucleophile to the ketone. The steric strain is greatly reduced when the side chain attachment is shifted to the amide nitrogen. Molecular modeling studies suggest that a five or six methylene chain

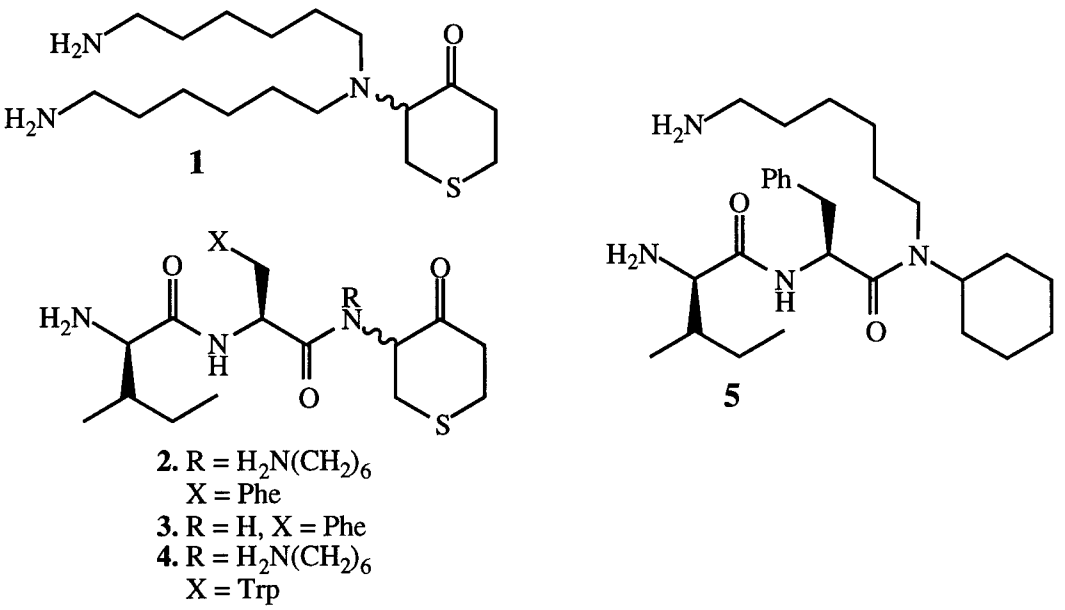


Figure 4. Structure of plasmin inhibitors.

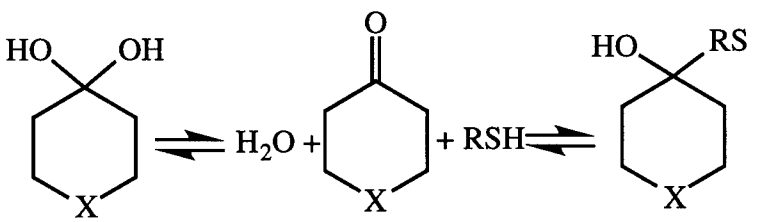
ending with an amino group approximates the length of a naturally positioned lysine residue.

A study by Okada et al showed that an alkyl chain ending with an amine group could be substituted for the peptide chain.[5] We decided to synthesize both the inhibitors, one with two alkyl chains for recognition and one with one alkyl chain designed to interact with the primary recognition site and a dipeptide chain to interact with the secondary recognition sites. We chose phenylalanine and D-isoleucine as the secondary recognition residues based upon the same study.[5] Altogether the general structure of the inhibitor consists of a tetrahydrothiopyranone ring appended with either two aminoalkyl chains (**1**) or one aminoalkyl and one peptide chain containing phenylalanine and D-isoleucine (**2**, Figure 3).

Preliminary Results

To investigate the influence of the heteroatom in the parent ring structure, equilibrium constant experiments were undertaken. Water (K_{H20}) and 3-mercaptopropionic acid (K_{RSH}) were used as mimics of the nucleophiles present in serine and cysteine proteases respectively. Their addition to the carbonyl of the parent ring structure indicates the potential electrophilic nature of the planar carbon (Table 1). As the electronegativity of the heteroatom increases so does the equilibrium constant, implying that the electrostatic repulsive forces between heteroatom and ketone also

Table 1 Hydration Constants



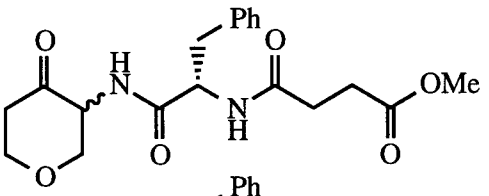
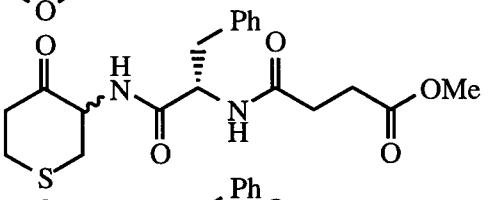
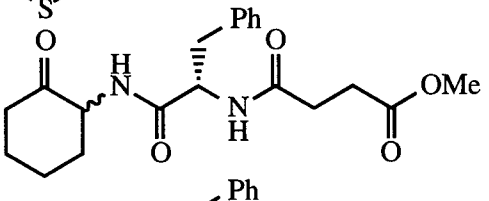
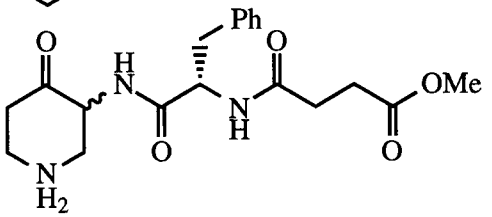
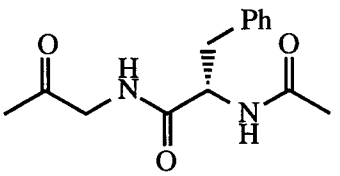
X	$K_{H2O}(M^{-1})$	$K_{RSH}(M^{-1})$
CH_2	8.1×10^{-4}	0.22
S	9.0×10^{-3}	1.5
O	8.0×10^{-3}	1.8
NH_2^+	0.18	27.6
SO	0.068	11.7
SO_2	0.30	60.2

increases. Ring strain also influences the concentration of hydrate. Cyclohexanone exhibits a strained ring system which destabilizes the ketone. Production of the tetrahedral intermediate by addition of a nucleophile to the carbonyl relieves this ring strain. The electronegativity and ring strain combine to stabilize the hydrate form which mimics the tetrahedral intermediate which is formed during enzymatic hydrolysis of an amide bond.

A series of papain inhibitors consisting of the cyclohexanone, tetrahydropyranone,

tetrahydrothiopyranone and piperidone was synthesized to test this hypothesis. The compounds were good inhibitors of papain (K_i =10-120 μM). Their potency generally

Table 2

	K_i (μM) More Potent Diastereomer	K_i (μM) 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.

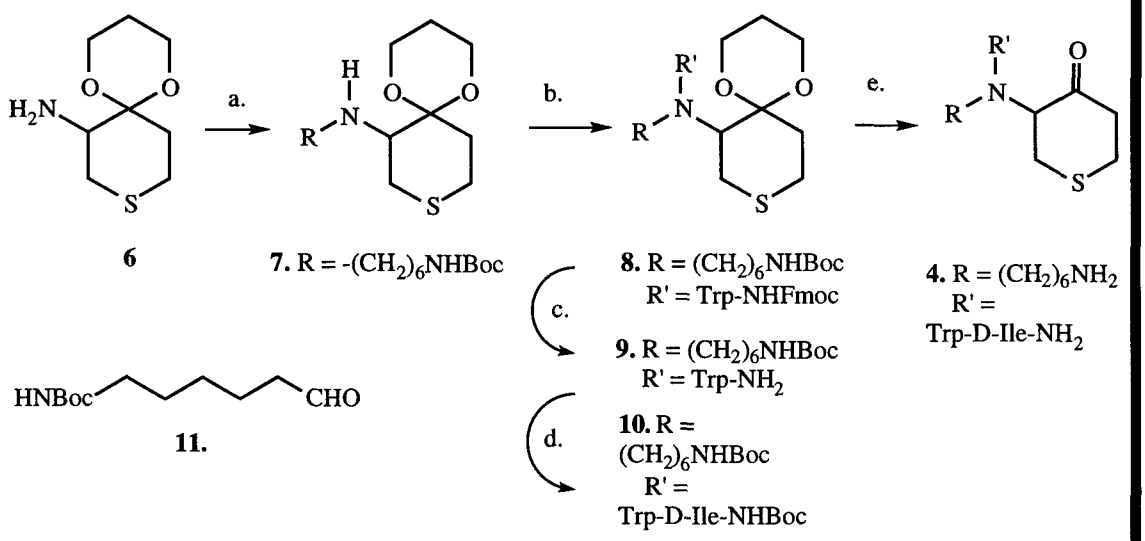
followed the expected trend as dictated by the hydration constants (Table 2). That is, the compounds that contained the most electronegative heteroatom were the most potent inhibitors. The potential of the nitrogen compound which we expected to be the best inhibitor due to the increased repulsion of the charge-dipole interaction, was not realized. There is the possibility of a unfavorable electrostatic interaction between the

positive charge on the nitrogen of the piperidone inhibitor and an enzymatic side chain in the active site cleft. With these preliminary results in hand we decided to use tetrahydrothiopyranone as the central ring structure for the preliminary plasmin inhibitors. 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 provide both sulfoxide-based and sulfone-based inhibitors.

Current Results

Months 1-6: During this period our research group ran a concurrent project consisting of the production of an inhibitor library which was then assayed against a set of enzymes. Initial results indicated two things which had a bearing on this project. The cyclohexanone ring structure is not a very effective core for cathepsin B inhibitors. An inhibitor with two side chains designed to bind to both the S2 and S2' recognition sites showed poor inhibition (1.1 mM). The study also implied that a tryptophan residue in place of the phenylalanine could lead to a more potent final inhibitor. The production of a plasmin inhibitor with a tryptophan (trp) residue was the first order of business in this year (Scheme 1). The synthesis of the new inhibitor closely followed the previously reported schemes. However, a comparison of the inhibition constants (K_i =100 μm) from

Scheme 1



6

7. R = -$(CH_2)_6$NHBoc

8. R = $(CH_2)_6$NHBoc
R' = Trp-NHFmoc

9. R = $(CH_2)_6$NHBoc
R' = Trp-NH_2

10. R = $(CH_2)_6$NHBoc
R' = Trp-D-Ile-NHBoc

4. R = $(CH_2)_6NH_2$
R' = Trp-D-Ile-NH_2

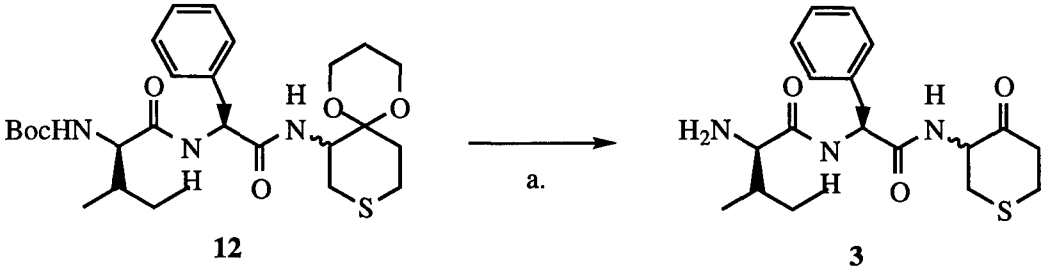
11.

a. $NaBH(OAc)_3$, DCE, **11,** 29%; b. Fmoc-Trp-F, DIEA, CH_2Cl_2, 41%; c. piperidine, DMF, 57%; d. HOBT, EDC, Boc-D-Ile, DMF,methylmorpholine, 89%; e. TFA, 35%.

the new tryptophan inhibitor showed it to be less potent than the phenylalanine inhibitor. Therefore the tryptophan compounds were not further investigated.

Another complication which had to be addressed at this time is the impurity of the control compound **3** without the lysine analog side chain. The product of the initial synthesis of the control proved to be a mixture of compounds when purification by reverse phase HPLC was attempted. The original deprotection scheme was altered to provide a scavenger for the carbocation formed during degradation of the protecting group. Purity of the final compounds was improved with this alteration (Scheme 2). The newly purified control compounds provided proof that the lysine side chain is extremely important to the binding of the inhibitor. The K_i for the two diastereomers of the control compound were 16 mM and 9 mM which is 320 times worse than the inhibitor containing the lysine analog side chain.

Scheme 2



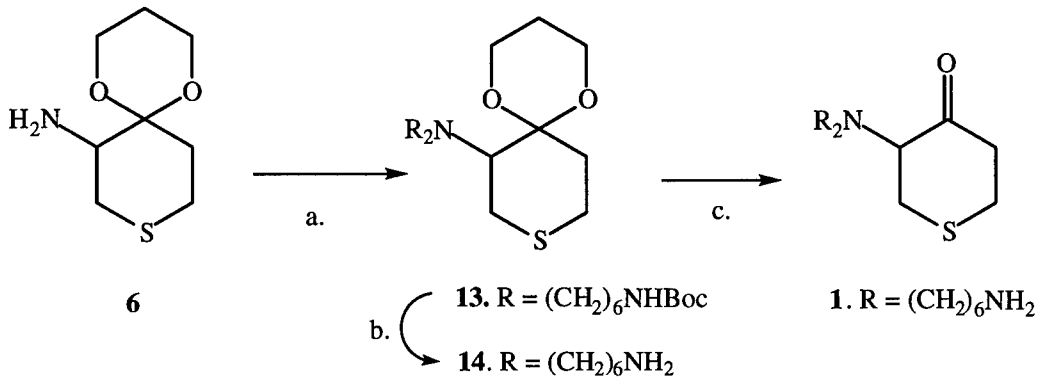
a. 92.5% TFA, 2.5% H_2O, 2.5% triisopropylamine, 2.5% thioanisole, 63%.

The new deprotection scheme was also used successfully to provide pure samples of the two targeted plasmin inhibitors. Final Ki values for the two diastereomer of that compound are 50 μM and 120 μM (See table 1).

Months 6-12: With the optimization of the reductive amination, it became possible to improve the synthesis of the dialkyl inhibitor (Scheme 3). The new synthetic route provided a cleaner final compound. The final inhibition constant for the dialkylated

compound was 410 μM. Again the number is almost 10 times worse than compound **1** showing that the addition of the dipeptide chain greatly improves the inhibition.

Scheme 3



6

b. **13.** R = $(CH_2)_6NHBoc$

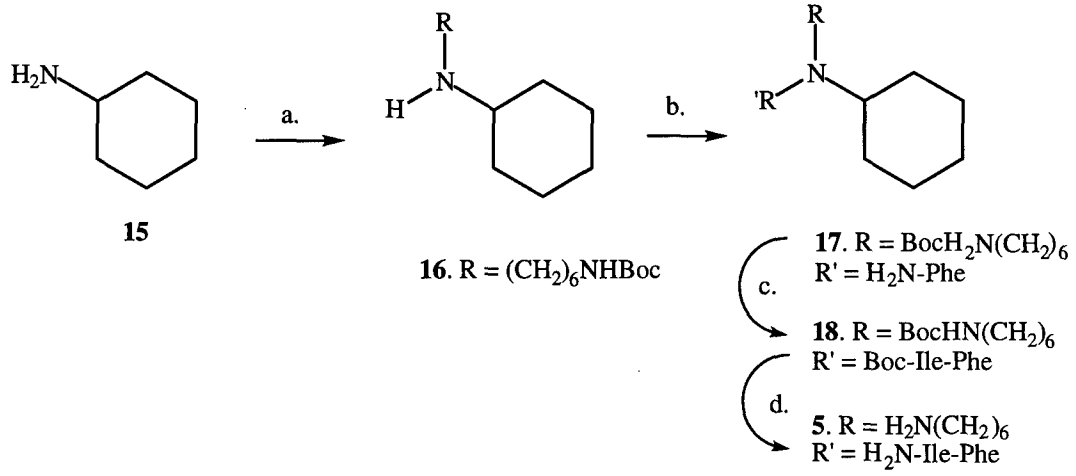
14. R = $(CH_2)_6NH_2$

1. R = $(CH_2)_6NH_2$

a. $NaBH(OAc)_3$, DCE, $_{22}$%; b. 92.5% TFA, 2.5% TIS, 2.5% H_2O, 2.5% thioanisole, 50%; c. HCl, MeOH, 100%.

Finally another control compound was designed to show the importance of the activated carbonyl. Earlier work proved that the cysteine inhibitor was indeed forming the covalent adduct with the enzyme, however we have no such proof with this inhibitor. The second control compound is missing the carbonyl on the central ring thereby denying the active site nucleophile an electrophilic binding site. Synthesis of the cyclohexane control compound consisted of a reductive amination to attach the lysine side chain and peptide couplings to provide the dipeptide recognition chain (Scheme 4). The inhibition constant for the second control compound (515 μM) implies that the carbonyl is an extremely important moiety on the inhibitor and that covalent attachment at that site is likely.

Scheme 4



15

16. R = $(CH_2)_6NHBoc$

c.

17. R = $BocH_2N(CH_2)_6$
R' = H_2N-Phe

18. R = $BocHN(CH_2)_6$
R' = Boc-Ile-Phe

d.

5. R = $H_2N(CH_2)_6$
R' = H_2N-Ile-Phe

a. $NaBH(OAc)_3$, DCE, 25%; b. Fmoc-Phe-F, DIEA, 66%; c. HOBT, EDC, methyl morpholine, DMF, 91%; d. 95% TFA, 2.5% TIS, 2.5% ethanedithiol, 59%.

Conclusions

At this point the project is essentially finished. The central cyclohexanone core has proven to be an acceptable core for a plasmin inhibitor. Compound **2** is a good inhibitor of plasmin (**2A:** K_i = 50 μM; **2B:** K_i = 120 μM). The lysine side chain and the ketone are both necessary for the potency of the inhibitor as shown by the lower potency of the inhibitors lacking those essential elements (**3A:** K_i = 9 mM; **3B:** K_i = 16 mM; **5:** K_i = 0.5 mM). Likewise, the dipeptide recognition chain is equally important for the binding of the compounds as shown by the results of the dialkyl chain inhibitor (K_i = 0.5 mM). The cathepsin B inhibitors are not as potent with an inhibition constant of 1.1 mM.

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Procedures

General Methods. NMR spectra were recorded on Bruker WM-250, Avance-300 or Avance-400 instruments. Spectra were calibrated using TMS (δ = 0.00 ppm) for ^{1}H NMR and $CDCl_3$ (δ = 77.0 ppm) or CD_3OD (δ = 49.0 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 glasswear. Anhydrous procedures were conducted using standard syringe and cannula transfer techniques. THF and toluene were distilled from sodium and benzophenone. Other solvents were of reagent grade and were stored over 4 Å molecular sieves. All other reagents were used as received. Organic solutions were dried over $MgSO_4$ unless otherwise noted. Solvent removal was performed by rotary evaporation at water aspirator pressure.

Secondary amine 7. Aldehyde **11** (0.52 g, 2.4 mmol) was dissolved in 2 mL of 1,2-dichloroethane (DCE) and added to a solution of primary amine **6** (0.51 g, 2.7 mmol) dissolved in 3 mL of DCE. After 10 min sodium triacetoxyborohydride (0.80 g, 3.8 mmol) was added and the reaction was allowed to stir for an additional 3 h at room temperature. The reaction was then quenched with saturated $NaHCO_3$ solution and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (2:1:7 EtOAc/ MeOH/ Et_2O) providing the secondary amine **7** (0.53 g, 1.40 mmol, 50%). ^{1}H NMR (300

MHz, MeOH-d_4) δ 1.36-1.72 (m, 21H), 2.02 (m, 1H), 2.50 (dm, J = 13.7 Hz, 1H), 2.69 (ddd, J = 9.7, 9.7, 2.6 Hz, 1H), 2.76-2.89 (m, 4H), 3.00-3.07 (m, 4H), 3.87 (m, 2H), 4.02 (ddd, J = 11.9, 9.3, 2.5 Hz, 1H), 4.12 (ddd, J = 12.0, 12.0, 2.7 Hz, 4H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 24.7, 25.2, 25.5, 25.9, 26.7, 28.2, 30.2, 40.4, 46.0, 60.0, 60.1, 62.8, 79.3, 96.1, 158.0; HRMS-EI (M+Na$^+$) Calcd for $C_{19}H_{36}N_2NaO_4S$ 411.2294, found 411.2306.

Fmoc-Trp amine 8. Secondary amine **7** (1.2 g, 3.1 mmol) was dissolved in 100 mL CH_2Cl_2 before DIEA (1.1 mL, 6.3 mmol) was added. After 10 min. Fmoc-Trp-F (5.0 g, 9.5 mmol) was added and the reaction mixture was heated at reflux for 4 h. The mixture was then washed with 50 mL of each 1N NaOH, 2.2 N AcOH and saturated $NaHCO_3$ solution before being dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (2:3 EtOAc/ hexanes) to afford a mixture of diastereomers of the Fmoc-Trp amine **8** (1.2 g, 1.2 mmol, 41%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.16-1.32 (m, 7H), 1.40-1.45 (m, 10H), 1.61-1.65 (m, 10H), 2.15 d, J = 12 Hz, 0.5H), 2.30-2.56 (m, 1H), 2.65-3.26 (m, 7.5H), 3.35-3.86 (m, 5.5H), 3.94-4.77 (m, 5H), 4.96 (m, 0.5H), 5.05-5.18 (m, 1H), 5.74 (m, 0.5H), 6.01 (d, J = 7 Hz, 0.5H), 7.18-7.77 (m, 12H), 8.08-8.17 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.2, 25.3, 26.8, 27.4, 27.7, 28.6, 28.9, 29.1, 29.4, 30.4, 30.8, 31.3, 31.5, 40.9, 45.2, 47.5, 47.7, 52.2, 59.0, 59.3, 63.2, 67.1, 67.4, 79.3, 83.9, 97.4, 97.9, 115.7, 115.9, 116.0, 119.4, 119.7, 120.3, 120.4, 123.25, 123.33, 124.6, 125.1, 125.5, 125.6, 127.4, 127.5, 128.1, 130.7, 135.8, 141.6, 141.7, 144.1, 144.2, 144.3, 144.4, 149.9, 155.7, 156.4, 173.4, 173.6.

Primary amine 9. Compound **8** was dissolved in 15 mL of DMF before piperidine (0.7 mL, 7.0 mmol) was added. After stirring at room temperature for 1.5 h the reaction mixture was partitioned between EtOAc and H_2O. The organic layer was dried over MgSO4 and concentrated before being purified by flash chromatography (2% MeOH/ CH_2Cl_2) to afford a total yield of 0.40 g, (0.59 mmol, 51%). The diastereomers were separated at this stage providing the primary amines **9A** and **9B**. **9A:** ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.87-0.92 (m, 1H), 1.26 (m, 6H), 1.36 (m, 13H), 1.59 (m, 10H), 1.79-1.97 (m, 1H), 2.29-2.35 (m, 1H), 2.46 (d, J = 12.6 Hz, 1H), 2.55-2.70 (m, 1H), 2.74-2.98 (m, 6H), 3.07 (dd, J = 14.1, 5.4 Hz, 1H), 3.28-3.49 (m, 2H), 3.57-3.78 (m, 1H), 3.82-4.06 (m, 3H), 7.13-7.60 (m, 4H), 7.90-8.07 (m, 1H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 12.9, 24.1, 24.3, 24.5, 25.6, 26.1, 26.7, 26.89, 26.92, 27.3, 27.5, 28.0, 28.2, 29.3, 29.4, 30.2, 30.4, 30.9, 31.2, 31.5, 39.6, 39.8, 43.9, 44.4, 50.2, 51.0, 53.3, 58.0, 58.1, 58.4, 58.6, 59.3, 60.0, 63.5, 83.1, 83.5, 97.2, 97.4, 99.9, 114.6, 114.8, 116.2, 116.4, 118.4, 118.7, 122.0, 122.4, 123.6, 123.8, 124.18, 124.24, 130.2, 130.6, 149.5, 175.6, 176.6; **9B**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.76-0.88 (m, 2H), 1.06-1.23 (m, 7H), 1.36 (m, 13H), 1.59 (m, 9H), 1.70-1.83 (m, 1H), 2.11-2.20 (m, 1H), 2.47 (t, J = 13.1 Hz, 1H), 2.71-3.24 (m, 8H), 3.47 (d, J = 9.1 Hz, 1H), 3.62-3.80 (m, 3H), 3.91-4.05 (m, 2H), 4.12-4.17 (m, 1H), 7.13-7.60 (m, 4H), 7.99 (d, J = 7.9 Hz, 1H).

Boc-Ile amine 10A. A solution containing 3 mL of DMF, HOBT (23 mg, 0.17 mmol), EDC (42 mg, 0.22 mmol) and Boc-D-ile (39 mg, 0.17 mmol) was stirred for 15 min. before primary amine **9A** (0.12 g, 0.17 mmol) and 4-methylmorpholine (0.09, 0.34 mmol) was added. The reaction mixture was stirred at room temperature for 18 h before being partitioned between EtOAc and H_2O. The organic layer was washed with 10 mL of each H_2O, saturated $KHSO_4$ and saturated Na_2CO_3 solutions, dried over $MgSO_4$ and concentrated. The crude product was purified fy flash chromatography (4:1 EtOAc/

hexanes) to afford the Boc-D-ile amine**10A** (0.13 g, 0.15 mmol, 89%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.63-0.76 (m, 2H), 0.87-1.00 (m, 7H), 1.26-1.46 (m, 30H), 1.65 (m, 12H), 1.86-2.01 (m, 3H), 2.41 (m, 1H), 2.65-2.83 (m, 1H), 2.92-3.22 (m, 6H), 3.30-3.53 (m, 2H), 3.71-4.07 (m, 4H), 4.52 (m, 1H), 4.88-5.41 (m, 2H), 7.18-7.43 (m, 3H), 7.61-8.16 (m, 2H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 11.8, 12.0, 15.5, 15.9, 16.0, 24.8, 24.9, 25.1, 25.3, 26.1, 26.3, 26.7, 26.9, 27.4, 28.4, 28.6, 28.66, 28.69, 28.8, 28.9, 29.1, 29.4, 29.7, 29.9, 30.3, 31.3, 31.4, 37.6, 38.1, 40.8, 44.6, 44.9, 45.2, 48.9, 49.8, 59.0, 59.2, 59.4, 59.5, 63.1, 77.9, 79.4, 80.1, 80.2, 83.7, 84.1, 97.9, 98.1, 115.5, 115.6, 116.0, 116.8, 119.45, 119.53, 122.3, 122.8, 123.3, 124.4, 124.5, 124.6, 125.1, 129.6, 130.7, 131.1, 135.9, 149.8, 150.0, 155.9, 156.4, 171.1, 173.0, 173.3.

Boc-Ile amine 10B. Compound **10B** was synthesized from **9B** (0.15 g, 0.22 mmol), HOBT (30 mg, 0.22 mmol), EDC (54 mg, 0.28 mmol), Boc-D-ile (51 mg, 0.22 mmol) and 4-methylmorpholine (0.05 mL, 0.44 mmol) with the procedure for the synthesis of **10A**. The product was purified by flash column (4:1 EtOAc/ hexanes) to afford compound **10B** (0.17 g, 0.19 mmol, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.81-0.98 (m, 6H), 1.19-1.71 (m, 52H), 2.12 (m, 2H), 2.26-2.53 (m, 1H), 2.70-3.43 (m, 9H), 3.51-4.09 (m, 4H), 4.58-5.41 (m, 2H), 7.00-8.10 (m, 5H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 11.5, 11.6, 14.2, 15.5, 24.7, 24.8, 24.9, 25.1, 25.9, 26.3, 26.5, 27.1, 27.2, 28.2, 28.3, 28.5, 29.0, 29.4, 29.7, 30.0, 31.0, 31.9, 37.4, 37.7, 38.0, 40.5, 44.9, 50.5, 58.4, 58.9, 62.7, 79.0, 79.6, 83.5, 97.0, 97.8, 115.3, 115.6, 119.2, 119.4, 122.5, 123.0, 124.1, 124.6, 124.8, 130.2, 135.4, 149.5, 149.6, 155.6, 156.0, 170.1, 172.9, 173.1.

Tertiary amine 13. Amine **6** (0.15 g, 0.79 mmol) was dissolved in 5 mL of 1,2-dichloroethane (DCE) before the aldehyde **11** (0.38 g, 1.7 mmol) and sodium triacetoxyborohydride (0.23 g, 1.1 mmol) were added. After 6.5 h at room temperature the reaction was partitioned between saturated $NaHCO_3$ solution and EtOAc. The organic layer was dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography (EtOAc) affording 0.10 g (0.18 mmol, 22%) of the dialkyl amine **13**. ^1H NMR (400 MHz, MeOH-d_4) δ 1.24–1.35 (m, 37H), 1.96 (m, 1H), 2.28 (m, 1H), 2.51-2.63 (m, 3H), 2.78 (m, 3H), 2.93-3.00 (m, 5H), 3.23-3.27 (m, 2H), 3.75-4.02 (m, 4H); ^{13}C NMR (100 MHz, MeOH -d_4) δ 25.9, 26.5, 27.1, 27.9, 28.2, 28.8, 30.2, 31.1, 33.0, 41.4, 53.4, 59.6, 59.8, 68.2, 79.8, 100.8, 158.6; HRMS-EI ($M+H^+$) calcd for $C_{28}H_{54}N_3O_6S$ 560.3733, found 560.3721.

Alkyl Ketal 14. Tertiary amine **13** (100 mg, 0.17 mmol) was dissolved in 1 mL of a solution containing 92.5% TFA, 2.5% TIS, 2.5% thioanisole and 2.5% H_2O. The reaction was stirred at room temperature for 1 h before the TFA was removed under reduced pressure. The resultant liquid was dissolved in MeOH to which Et_2O was added until the solution turned cloudy. The ketal **14** (53 mg, 0.86 mmol, 50%) which settled out of the solution as an oily liquid was used without further purification. ^1H NMR (250 MHz, MeOH-d_4) δ 1.46 (m, 9H), 1.66-1.85 (m, 10H), 2.07 (m, 1H), 2.49-2.54 (m, 1H), 2.79-3.01 (m, 7H), 3.25 (m, 2H), 3.45 (m, 1H), 3.59-3.64 (dd, J = 12.0, 2.9 Hz, 1H), 3.93-4.28 (m, 5H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 23.8, 25.9, 26.0, 26.5, 26.6, 27.4, 27.5, 27.8, 28.7, 28.8, 32.6, 40.9, 54.6, 55.6, 61.2, 61.4, 69.9, 98.3, 118 (q), 163.2 (q).

Secondary amine 16. Compound **16** was prepared from **15** (3.8 mL, 33.5 mmol), compound **11** (6.6 g, 30.5 mmol) and sodium triacetoxyborohydride (3.7 g, 17.5 mmol) in 20 mL DCE using the method described for the synthesis of **7**. The crude material was purified by flash chromatography (2:1:7 EtOAc/ MeOH/ Et_2O) affording the secondary amine **16** (1.3 g, 4.4 mmol, 25%). ^1H NMR (300 MHz, $CDCl_3$) δ 0.96-1.47 (m, 23H), 1.57-1.73 (m, 3H), 1.84 (m, 2H), 2.32-2.42 (m, 1H), 2.58 (t, J = 7.1 Hz, 2H), 3.07 (q, J = 6.5 Hz, 2H), 4.60 (bs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.5, 26.6, 27.1, 27.5, 28.5, 30.4, 30.8, 34.0, 40.7, 47.3, 57.3, 79.3, 156.4; HRMS-FAB ($M+Na^+$) cald for $C_{17}H_{34}N_2NaO_2$ 321.2518, found 321.2522.

Primary amine 17. A solution of Fmoc-Phe-F (5 g, 13.2 mmol), DIEA (2.3 mL, 13.2 mmol) and the secondary amine **16** (1.3 g, 4.4 mmol) in 100 mL of CH_2Cl_2 was refluxed for 18 hrs. The mixture was then diluted with 100 mL of EtOAc, and washed with 100 mL each of 1N NaOH, 1N HCl and saturated $NaHCO_3$ solution. The resultant organic layer was dried over $MgSO_4$ and concentrated before being purified by flash chromatography (1:1 EtOAc/ hexanes) which provided a mixture of Fmoc-Phe and the alkylated amine. The mixture was dissolved in 85 mL of DMF to which piperidine (2.2 mL, 22 mmol) was added. After 15 min the solution was partitioned between 150 mL of EtOAc and 150 mL of H_2O. The organic layer was dried over $MgSO_4$ and concentrated before being purified by flash chromatography (2% MeOH/ CH_2Cl_2) to afford a mixture of diastereomers of the primary amine **17** (1.3 g, 2.9 mmol, 66%). ^1H NMR (300 MHz, $CDCl_3$) δ 1.02-1.74 (m, 27H), 2.65-3.34 (m, 6H), 3.67 (t, J = 7.0 Hz, 0.5H), 3.89 (t, J = 7.0 Hz, 0.5H), 4.22 (m, 0.5H), 4.67 (m, 0.5H), 7.16-7.33 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.6, 25.9, 26.3, 26.4, 26.7, 26.8, 27.4, 28.8, 29.7, 30.4, 30.9, 31.2, 31.7, 32.1, 32.6, 42.7, 43.9, 53.3, 54.2, 54.4, 57.1, 127.0, 128.8, 128.9, 129.1,

129.7, 138.3, 156.4, 174.5; HRMS-FAB (M+Na_+) calcd for $C_{26}H_{43}N_3NaO_3$ 468.3202, found 468.3216.

Boc dipeptide 18. A DMF solution (40 mL) containing HOBT (0.39 g, 2.9 mmol), EDC (0.73 g, 3.8 mmol) and Boc-D-Isoleucine (0.68 g, 2.9 mmol) was stirred at room temperature for 20 h. A solution of the amino ketal **17** (1.3 g, 2.9 mmol) and 4-methyl morpholine (0.66 g, 6 mmol) was dissolved in 35 mL DMF was then added to the reaction mixture. After 4 h the reaction mixture was pwrtitioned between EtOAc and H_2O. The organic layer was washed with H_2O, dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (4:1 EtOAc/ hexanes) to afford a mixture of diastereomers of Boc dipeptide **17** (1.7 g, 2.6 mmol, 91%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.76-0.82 (m, 7H), 0.94-1.77 (m, 36H), 2.60-3.29 (m, 6H), 4.00-4.12 (m, 2H), 4.69-5.53 (m, 3H), 7.07-7.28 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.9, 15.8, 24.87, 24.94, 25.5, 26.0, 26.1, 26.8, 27.3, 28.7, 28.79, 28.81, 30.3, 31.0, 31.4, 40.4, 40.5, 42.7, 50.5, 54.6, 57.4, 59.5, 79.8, 127.2, 128.8, 129.8, 129.9, 136.8, 137.0, 155.9, 170.7, 171.0, 171.3; HRMS-FAB (M+Na^+) calcd for $C_{37}H_{62}N_4NaO_6$ 681.4567, found 681.4550.

Inhibitor 1. Ketal **7** (53 mg, 0.09 mmol) was dissolved in a solution of 5 mL MeOH and 10 mL 6 N HCl. The reaction was heated at reflux for 1 h before the solvent was removed under reduced pressure. The crude mixture was dissolved in a small amount of MeOH to which Et_2O was added until the solution turned cloudy. The Et_2O was pipetted off and the oily residue further purified by RPHPLC (H_2O with 0.1 % TFA) to afford 53 mg (0.09 mmol, 99%) of inhibitor **1**. ^{1}H NMR (300 MHz, MeOH -d_4) δ 1.38 (m, 10H), 1.50-1.90 (m, 10H), 2.83-3.25 (m, 10H), 4.51 (dd, J = 11.5, 5.3 Hz, 1H); ^{13}C NMR (75

MHz, MeOH-d_4) δ 24.9, 25.9, 26.06, 26.12, 26.3, 27.3, 28.1, 29.0, 39.5, 44.5, 53.0, 53.2, 69.9, 201.9.

Inhibitor 3A & 3B. Compound **12A** (53 mg, 0.10 mmol) was dissolved in 1 mL of a solution containing 92.5% TFA, 2.5% TIS, 2.5% H_2O, and 2.5% thioanisole. After 1h the TFA was removed under reduced pressure. The crude mixture was purified by flash chromatography (10:89:1 MeOH, CH_2Cl_2, concentrated NH_4OH) before the final purification was performed using RPHPLC (0%-100% MeCN/ H_2O over 45 min) affording the inhibitor **3A** (17 mg, 0.03 mmol, 34%). In MeOH-d_4 the inhibitors are visible as an approximately 1:1 mixture of hemiketal and ketone. **3A**: ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.70–0.78 (m, 7H), 1.15 (m, 1H), 1.65 (m, 1H), 1.83 (m, 0.5H), 1.99 (m, 0.5H), 2.17 (m, 0.5H), 2.38 (m, 0.5H), 2.59-3.00 (m, 5H), 3.13 (ddd, J = 13.2, 2.8, 5.6 Hz, 0.5H), 3.28 (m, 0.5H), 3.67 (m, 1H), 4.13 (m, 0.5H), 4.72 (dd, J = 11.6, 4.8 Hz, 0.5H), 4.82 (dd, J = 11.2, 7.2 Hz, 0.5H), 7.23-7.35 (m, 5H); ^{13}C NMR (100 MHz, MeOH-d_4) δ 10.7, 13.6, 24.0, 24.7, 25.0, 25.1, 30.45, 30.49, 34.7, 35.2, 36.7, 37.8, 38.0, 44.4, 64.99, 55.02, 58.00, 58.03, 59.8, 95.5, 96.2, 115.5 (q, J = 42 Hz), 160.3 (q, J = 34 Hz), 168.3, 168.4, 172.4, 172.68, 172.71, 204.4; HRMS-EI (M+Na$^+$) calcd for $C_{20}H_{29}N_3NaO_3S$ 414.1827, found 414.1823.

Inhibitor **3B** was prepared from compound **12B** (60 mg, 0.11 mmol) and 1 mL of the TFA solution specified in the synthesis of **3A**. The crude product was purified by RPHPLC (0%-100% MeCN/H_2O over 45 min) to afford inhibitor **3B** (45 mg, 0.090 mmol, 82%). ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.58-1.05 (m, 7H), 1.16-1.40 (m, 1H), 1.65-1.81 (m, 1H), 1.94-2.14 (m, 1H), 2.39-3.05 (m, 6H), 3.15-3.23 (m, 1H), 3.69 (d, J = 5.2 Hz, 1H), 4.05-4.11 (m, 0.5H), 4.67 (dd, J = 11.5, 5.3 Hz, 0.5H), 4.73 (dd, J = 10.1, 5.8 Hz, 0.5H), 4.84 (dd, J = 10.2, 5.2 Hz, 0.5H), 7.22-7.32 (m, 5H); ^{13}C NMR

(75 MHz, MeOH-d_4) δ 14.2, 14.3, 27.7, 27.8, 28.5, 33.8, 34.0, 38.1, 38.8, 40.30, 40.33, 41.4, 41.7, 48.0, 58.5, 59.0, 61.5, 61.7, 63.6, 99.2, 130.6, 130.7, 132.2, 132.8, 140.6, 140.8, 172.0, 172.4, 175.6, 176.1, 207.9; HRMS-EI ($M+Na^+$) calcd for $C_{20}H_{29}N_3NaO_3S$ 414.1827, found 414.1834.

Trp Inhibitor 4A. The Boc-ile amine **11A** (29 mg, 0.053 mmol) was dissolved in 1 mL of CH_2Cl_2 before 1 mL of a solution containing 92.5% TFA, 2.5 % TIS, 2.5% H_2O and 2.5% ethanedithiol was added. After 1.5 h the solvent was removed via vacuum line. The crude product was purified by RPHPLC (0%-35% MeCN/ H_2O) to afford the Trp inhibitor **4A**.

Trp Inhibitor 4B. The Boc-ile amine **11B** (44 mg, 0.053 mmol) was dissolved in 1 mL of CH_2Cl_2 before 1 mL of a solution containing 92.5% TFA, 2.5 % TIS, 2.5% H_2O and 2.5% ethanedithiol was added. After 1.5 h the solvent was removed via vacuum line. The crude product was purified by RPHPLC (0%-35% MeCN/ H_2O) to afford the Trp inhibitor **4B** (14 g, 0.019 mmol, 35%).

Amine 5. The Boc dipeptide **18** (180 mg, 0.27 mmol) was dissolved in 1 mL of CH_2Cl_2 before 1 mL of a solution containing 95% TFA, 2.5 % TIS, 2.5% ethanedithiol was added. After 30 min the solvent was removed via vacuum line. The crude product was purified by flash chromatography (10:89:1 MeOH/ CH_2Cl_2/ NH_4OH) to afford a mixture of diastereomers of amine **5** (0.11 g, 0.16 mmol, 59%). ^1H NMR (300 MHz, $CDCl_3$) δ 0.83-0.92 (m, 6H), 1.00-1.20 (m, 3H), 1.33-1.87 (m, 19H), 2.89-3.12 (m, 5H), 3.24-3.34 (m, 3H), 3.38 (m, 0.5H), 3.68 (dd, J = 8.3, 5.7 Hz, 1H), 3.94-4.18 (m, 1H), 5.15 (t, J = 7.7 Hz, 1H), 7.23-7.35 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.67, 10.69, 13.9, 14.0, 24.1, 25.2, 25.5, 25.8, 25.9, 26.0, 26.1, 26.2, 26.4, 26.8, 27.4,

27.5, 29.1, 30.6, 31.0, 31.2, 31.5, 37.0, 38.4, 38.9, 39.58, 39.63, 42.5, 43.9, 51.3, 52.2, 55.5, 58.0, 116.0 (q, J = 292 Hz), 127.1, 127.22, 127.24, 128.6, 128.7, 128.8, 129.4, 129.5, 129.6, 136.8, 137.0, 162.1 (q, J = 34 Hz), 168.6, 168.7, 171.6, 171.9; HRMS-FAB ($M+Na^+$) calcd for $C_{27}H_{46}N_4NaO_2$ 481.3519, found 481.3523.

Appendix 1

Conclusions

- The results indicate that the 4-heterocyclohexanone ring is a viable scaffold on which to build both cysteine and serine protease inhibitors.
- The potency of these inhibitors can be improved by harnessing the through space influence of the electronegative heteroatom.
- Both the lysine side chain and the carbonyl moieties are essential to the potency of the inhibitor.
- The dipeptide side chain is more advantageous for binding in the active site cleft and results in a more potent inhibitor.

Publications

Sanders, T.; Seto, C. 4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin. *J. Med. Chem.* **1999** in press.

Degrees

This research contributed toward the degree of PhD for the PI.

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4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

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Table of Contents Graphic for:

4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

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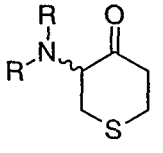

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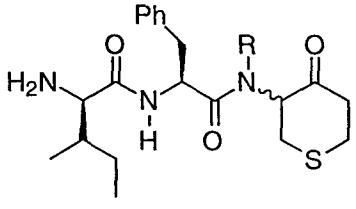

2 R = $(CH_2)_6NH_2$
3 R = H

Abstract for:

4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

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Abstract: Three inhibitors that are based upon a 4-heterocyclohexanone nucleus were synthesized and evaluated for activity against the serine protease plasmin. Inhibitors of plasmin have potential as cancer chemotherapeutic agents that act by blocking both angiogenesis and metastasis. Inhibitor **1** has moderate activity against plasmin, but shows good selectivity for this enzyme compared to other serine proteases including trypsin, thrombin, and kallikrein. Inhibitor **2** shows both good activity and selectivity for plasmin. Inhibitor **3**, which does not incorporate an aminohexyl group that can interact with the S1 subsite, has poor activity. These results, along with previous work, demonstrate that the 4-heterocyclohexanone nucleus can effectively serve as the basis for designing inhibitors of both serine and cysteine proteases.

4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

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Introduction

Angiogenesis and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of chemotherapeutic agents. Several recent reports in the literature have demonstrated that suppressing angiogenesis is an effective method for limiting the growth of primary tumors and producing dormancy in secondary metastases.[1,2] Both angiogenesis and metastasis require a proteolytic cascade that involves serine, cysteine, and metalloproteases. This proteolytic cascade degrades the basement membrane which surrounds blood vessels.[3] During angiogenesis the resulting lesion in the basement membrane allows epithelial cells to extend into the neighboring tissues and form new blood vessels. During metastasis cancer cells penetrate through the degraded basement membrane and extracellular matrix, become implanted in the underlying tissues, and subsequently form secondary tumors.[4] Compounds which inhibit enzymes in the proteolytic cascade may be useful for blocking these processes.

Plasmin is a serine protease that plays an important role in the proteolytic cascade. This protease acts directly by hydrolyzing components of the basement membrane such as fibrin, type IV collagen, fibronectin, and laminin, and also acts indirectly by activating other enzymes in the cascade such as matrix metalloproteases.[3] Degradation of the basement membrane by plasmin is a multi-step process. For example, during the first step in fibrin hydrolysis, plasminogen, which is the inactive precursor to plasmin, binds to fibrin via a lysine binding site. Next plasminogen is

converted to active plasmin in a reaction that is catalyzed by urokinase plasminogen activator. Finally catalytic residues in the active site of plasmin, which is separate from the lysine binding site, hydrolyze fibrin via the mechanism that is common to serine proteases.[5] Most current pharmaceutical agents that are designed to inhibit plasmin are targeted to the lysine binding site.[6] These agents inhibit fibrinolysis by blocking the binding of plasminogen to fibrin, and thus halting production of new plasmin. α2-Antiplasmin, a natural plasmin inhibitor, is also targeted to the lysine binding site.[7] However these fibrinolysis inhibitors have no effect on the active site of the enzyme, which retains its catalytic activity. Thus plasmin that is already activated retains its catalytic activity even after treatment with inhibitors that are directed toward the lysine binding site. To overcome this problem, we are interested in developing inhibitors that are targeted to the active site of plasmin and are designed to shut down catalytic activity. In this paper we report the synthesis and evaluation of compounds **1 - 3** which are active site directed inhibitors of plasmin. Compound **2** has both good activity and specificity against plasmin when compared to several other serine proteases.[8]

[Structures of Inhibitors **1 - 3**]

Design of Inhibitors

We have recently reported a new class of inhibitors for cysteine proteases that are based upon a 4-heterocyclohexanone pharmacophore.[9] ^{13}C NMR studies using a ^{13}C-labeled inhibitor confirm that these molecules react with the enzyme to give a reversibly-formed covalent hemithioketal adduct between the active site cysteine residue and the ketone of the inhibitor.[10] The key design feature in these molecules is the through-space electrostatic repulsion that occurs between the heteroatom and ketone functionalities in the 4-heterocyclohexanone pharmacophore. This repulsive interaction controls the electrophilicity of the ketone, which in turn controls the potency of the inhibitors.[9]

Because serine and cysteine proteases share a similar mechanism for hydrolyzing amide bonds, we expect that 4-heterocyclohexanones should be good inhibitors of both classes of enzymes. Reaction of the active site nucleophile of a serine protease with a 4-heterocyclohexanone-based inhibitor would give a reversibly formed hemiketal adduct. However, several reversible protease inhibitors show activity against one class of enzyme and not the other. For example, trifluoromethyl ketones and boronic acids are good inhibitors of serine protease[11] but not cysteine proteases.[12,13] Nitriles have the opposite specificity, while aldehydes and α-dicarbonyl compounds are good inhibitors of both classes of enzymes.[13] Thus one of our motivations for synthesizing compounds **1 - 3** was to determine if 4-heterocyclohexanones would prove to have activity against serine proteases, in addition to cysteine proteases as we have shown previously.[9]

Plasmin has a strong specificity for substrates with positively charged side chains in the P1 position. To accommodate this specificity we have included a lysine-like side chain in the structure of compounds **1** and **2**. However, attachment of this side chain in its "natural" peptide-like position would place it on the tetrahydrothiopyranone ring between the ketone and the exocyclic nitrogen (Figure 1). This placement would create a sterically demanding quaternary center alpha to the reactive ketone. Space filling molecular models suggest that this quaternary center would sterically inhibit addition of an active site nucleophile to the ketone, and thus decrease the potency of the inhibitor. To overcome this difficulty we have attached the P1 side chain to the amide nitrogen that is connected to the ring. This type of modification is well precedented in peptoids.[14] In order to ensure that the lysine-like side chain of the inhibitor makes good contact with the aspartic acid at the base of the S1 binding site, we have increased the length of the aminoalkyl chain to six carbons. This chain length is based upon molecular modeling studies of inhibitor **2** bound in the active site of trypsin. The X-ray crystal structure of the active site of plasmin has not been solved, however the active sites of plasmin and trypsin share significant homology.[15]

[Figure 1]

Compound **1** contains three functionalities that are designed to make specific contacts with the active site. The ketone will react with the active site nucleophile to give a hemiketal. In addition one of the aminoalkyl chains will bind in the S1 subsite, while the second aminoalkyl chain will extend along the main channel of the active site to make contacts with the S3 subsite. Okada and coworkers have shown that peptide-based substrates and inhibitors that contain a free N-terminus at the P3 position bind well to the enzyme.[16] In compound **2**, one of the aminoalkyl chains has been replaced by phenylalanine and D-isoleucine in order to include additional functionality that will interact with the S2 and S3 subsites.[16] The sulfur atom was incorporated into the cyclohexanone rings of the three inhibitors because the related tetrahydrothiopyranone-based inhibitor of the cysteine protease papain had good activity and its synthesis was relatively straightforward.[9] Compound **3**, which lacks an aminoalkyl functionality, was synthesized in order to determine how much the P1 side chain contributes to the affinity of the inhibitors for plasmin. We have also synthesized compound **22** (Scheme 4), which is similar in structure to **3**, but lacks the electrophilic ketone functionality. This molecule provides a useful control for probing the mechanism of inhibition by inhibitors **1** - **3**.

Chemistry

The synthesis of inhibitor **1**, which is outlined in Scheme 1, began with deprotection of the Boc-protected nitrogen in compound **4** with trifluoroacetic acid to give amine **5**. The synthesis of **4** has been reported previously.[9] Dialkylation of **5** by reductive amination with two equivalents of aldehyde **10** gave the tertiary amine **6**. The Boc protecting groups were removed with TFA to give **7** and the ketal was hydrolyzed using aqueous HCl to give inhibitor **1**. Aldehyde **10** was synthesized starting from 6-amino-1-hexanol **8** (Scheme 2). The amino group in **8** was first protected using $(Boc)_2O$ to give alcohol **9**, followed by oxidation of the alcohol using pyridinium chlorochromate.

[Schemes 1 and 2]

The synthesis of inhibitors **2** and **3** began with reductive amination of **5** with one equivalent of aldehyde **10** using sodium triacetoxyborohydride in dichloroethane to give secondary amine **11** (Scheme 3).[17] An alternate strategy for the preparation of **11** involving monoalkylation of **5** with the appropriate alkyl bromide gave a poor yield of the secondary amine. Amine **5** was coupled to Fmoc-Phe using a standard peptide coupling procedure that employed 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT). However, the more sterically hindered secondary amine **11** did not couple under these conditions and required reaction with the acid fluoride of Fmoc-Phe using methodology developed by Carpino.[18] These reactions gave compounds **12** and **13**, each as a mixture of two diastereomers. The Fmoc protecting groups in **12** and **13** were removed using piperidine in DMF to give amines **14** and **15**. The diastereomers of both compounds were separated at this stage using flash chromatography, and each of the diastereomers was carried separately through the remainder of the synthesis. The amines were next coupled to Boc-D-Ile using EDC and HOBT to give compounds **16** and **17**. Final removal of the Boc and ketal protecting groups in **16** and **17** was accomplished by treatment with trifluoroacetic acid and H_2O to give inhibitors **2** and **3**.

Control compound **22** was synthesized in a manner similar to that of inhibitor **2** (Scheme 4). Reductive alkylation of cyclohexylamine with aldehyde **10** gave secondary amine **19**. This material was coupled to Fmoc-Phe-F followed by removal of the Fmoc protecting group to give compound **20**. After a second coupling reaction with Boc-D-Ile, the Boc protecting groups were removed to give **22**.

[Schemes 3 and 4]

Results and Discussion

Compound **1**, which incorporates two simple aminohexyl side chains, was assayed against four different serine proteases; plasmin, trypsin, thrombin, and kallikrein (Table 1). All of these proteases have a strong specificity for positively charged side chains such as lysine or arginine at the P1 position, and thus provide a reasonable test of the specificity of the inhibitors for plasmin compared to other related enzymes. Compound **1** has modest activity against plasmin with an inhibition constant of 400 μM. It has greater than 25-fold selectivity for this protease when compared to thrombin and kallikrein, and a three-fold selectivity when compared to trypsin. The similar affinity of this inhibitor for plasmin and trypsin is reasonable based upon the sequence homology between the two enzymes.[15]

[Table 1]

In order to increase both the potency and specificity of the inhibitors, we have replaced one of the aminohexyl chains in compound **1** with a D-Ile-L-Phe dipeptide to give inhibitors **2A** and **2B**. The free N-terminus of the D-Ile residue in these compounds positions a positive charge in the S3 enzyme subsite, which has been shown to be beneficial for binding.[16] In addition, the D-Ile and Phe side chains provide hydrophobic contacts with the S2 and S3 subsites. Compound **2A** is a good inhibitor of plasmin with an inhibition constant of 50 μM. By comparison it has significantly lower affinity for trypsin, thrombin and kallikrein. The low activity of this inhibitor against trypsin is somewhat surprising given the reported similarity between the active sites of plasmin and trypsin.[16] Lineweaver-Burk analysis of **2A** against plasmin demonstrates that it is a reversible competitive inhibitor. This observation is consistent with a mechanism of inhibition that involves addition of the active site serine residue to the tetrahydrothiopyranone carbonyl group of the inhibitor to give a reversibly formed hemiketal. This mechanism has been demonstrated for related inhibitors of the cysteine protease papain.[10]

Compound **2B** has an affinity for plasmin that is 2 - 3 times lower than its diastereomer **2A**. In contrast, there is a 100-fold difference in the binding of two diastereomers of analogous inhibitors for papain.[9] Papain has a relatively deep and narrow binding cleft[19] that discriminates strongly between two diastereomers that differ in stereochemistry at the position alpha to the reactive ketone. Based upon the similarities between plasmin and trypsin,[16] it is likely that plasmin has a binding cleft that is much more open and shallow when compared to papain. This sterically unrestrictive active site can accommodate both diastereomers of inhibitor **2**, and it is reasonable to expect that both diastereomers can find a conformation in the active site that allows reaction with the active site serine residue. Thus **2A** and **2B** can bind to plasmin with similar affinities.

We have synthesized compounds **3 A** and **B** in order to determine how much the aminohexyl side chain contributes to the potency of the inhibitors. These compounds are similar in structure to **2 A** and **B**, but are missing the side chain which interacts with the S1 pocket in the enzyme active site. Since plasmin is specific for substrates that incorporate a lysine residue at P1, we expected that removing the aminohexyl group from the inhibitor should have a significant negative impact on its ability to bind. Compounds **3 A** and **B** have inhibition constants against plasmin of 9.0 and 16.0 mM, respectively. These values are 200 - 300 times higher than the inhibition constant for **2A**. This result confirms that the aminohexyl side chain, which mimics a lysine residue at the P1 position of the inhibitor, is critical for good recognition and affinity for the protease.

The design of these 4-heterocyclohexanone-based inhibitors depends upon the supposition that the ketone of the inhibitors reacts in a reversible covalent fashion with the active site nucleophile. This mechanism has been confirmed for cysteine proteases,[10] but remains unproven for serine proteases. Thus it is possible that compounds **1 - 3** are inhibiting plasmin through simple noncovalent interactions. In order to further explore the mechanism of inhibition, we have synthesized compound **22** (Scheme 4) which is missing the thioether and ketone functionalities that are present in inhibitor **2**. If the 4-heterocyclohexanones are interacting with the enzyme solely through noncovalent interactions such as salt bridges, hydrogen bonds, and hydrophobic

interactions, control compound **22** should be a good mimic of inhibitor **2**, and the two molecules would be expected to have similar affinities for plasmin. However, if the ketone of inhibitor **2** is also reacting with the active site serine to give a reversibly-formed covalent adduct, we would expect **22** to be a significantly weaker inhibitor. Table 1 shows that compound **22** has an inhibition constant against plasmin of 520 μM; a value that is 10-fold higher than that observed for inhibitor **2**. Although this results does not unambiguously prove the mechanism of inhibition, it is consistent with the reasonable mechanistic hypothesis that inhibitors **1** - **3** react with the active site serine to give a hemiketal adduct.

In conclusion, this work has shown that the 4-heterocyclohexanone nucleus can serve as the basis for designing good inhibitors of plasmin. In addition, our experiments highlight the versatility of the 4-heterocyclohexanone nucleus because we have now confirmed that it can be used to synthesize inhibitors of both serine and cysteine proteases. We have also demonstrated the feasibility of attaching P1 recognition elements to the inhibitors using the amide nitrogen in a strategy that is borrowed from peptoids.[14] Our future work will focus on extending the noncovalent interactions of these inhibitors into the leaving group subsites of plasmin in order to increase both their potency and specificity.

Experimental Section

General Methods. NMR spectra were recorded on Bruker WM-250, Avance-300 or Avance-400 instruments. Spectra were calibrated using TMS ($\delta = 0.00$ ppm) for ^{1}H NMR and $CDCl_3$ ($\delta = 77.0$ ppm) or CD_3OD ($\delta = 49.0$ 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 glasswear. Anhydrous procedures were conducted using standard syringe and cannula transfer techniques. THF was distilled from sodium and benzophenone. Other solvents were of reagent grade and were stored over 4 Å molecular sieves. All other reagents were used as received. Organic solutions were dried over $MgSO_4$ unless otherwise noted. Solvent removal was performed by rotary evaporation at water aspirator pressure.

Primary amine 5. A solution containing 9.5 mL of trifluoroacetic acid (TFA), 0.25 mL of triisopropylsilane (TIS), and 0.25 mL of thioanisole was added to the carbamate **4**[9] (4.8 g, 17 mmol) dissolved in 2 mL of CH_2Cl_2. After stirring at room temperature for 10 min the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (1:10:89 concentrated NH_4OH, CH_3OH, CH_2Cl_2) affording 2.8 g (15 mmol, 89%) of the primary amine **5**. ^{1}H NMR (300 MHz, MeOH-d_4) δ 1.44 (dm, J = 13.5 Hz, 1H), 1.65 (ddd, J = 15.0, 11.6, 3.5 Hz, 1H), 1.94-2.11 (m, 1H), 2.50 (dm, J = 13.8 Hz, 1H), 2.61 (ddd, J = 13.1, 5.6, 1.8 Hz, 1H), 2.73 (ddd, J = 13.9, 11.4, 2.6 Hz, 1H), 2.96 (dd, J = 13.0, 11.2 Hz, 1H), 3.21 (dm, J = 14.4 Hz, 1H), 3.30 (m, 1H), 3.89 (m, 2H), 4.10 (m, 2H); ^{13}C NMR (75 MHz, MeOH-

d_4) δ 25.9, 26.4, 28.5, 31.1, 57.9, 60.9, 61.1, 96.5; HRMS-EI (M^+) calcd for $C_8H_{15}NO_2S$ 189.0824, found 189.0827.

Tertiary amine 6. Amine **5** (0.15 g, 0.79 mmol) was dissolved in 5 mL of 1,2-dichloroethane (DCE) before the aldehyde **10** (0.38 g, 1.7 mmol) and sodium triacetoxyborohydride (0.23 g, 1.1 mmol) were added. After 6.5 h at room temperature the reaction was partitioned between saturated $NaHCO_3$ solution and EtOAc. The organic layer was dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography (EtOAc) affording 0.10 g (0.18 mmol, 22%) of the tertiary amine **6**. ^{1}H NMR (400 MHz, MeOH-d_4) δ 1.24-1.35 (m, 37H), 1.96 (m, 1H), 2.28 (m, 1H), 2.51-2.63 (m, 3H), 2.78 (m, 3H), 2.93-3.00 (m, 5H), 3.23-3.27 (m, 2H), 3.75-4.02 (m, 4H); ^{13}C NMR (100 MHz, MeOH-d_4) δ 25.9, 26.5, 27.1, 27.9, 28.2, 28.8, 30.2, 31.1, 33.0, 41.4, 53.4, 59.6, 59.8, 68.2, 79.8, 100.8, 158.6; HRMS-FAB ($M+Na^+$) calcd for $C_{30}H_{57}N_3NaO_6S$ 610.3866, found 610.3882.

Ketal 7. Tertiary amine **6** (100 mg, 0.17 mmol) was dissolved in 1 mL of a solution containing 92.5% TFA, 2.5% TIS, 2.5% thioanisole and 2.5% H_2O. The reaction was stirred at room temperature for 1 h before the TFA was removed under reduced pressure. The resultant material was dissolved in MeOH to which Et_2O was added until the solution turned cloudy. The ketal **7** (53 mg, 0.86 mmol, 50%) which settled out of the solution as an oily liquid was used without further purification. ^{1}H NMR (250 MHz, MeOH-d_4) δ 1.46 (m, 9H), 1.66-1.85 (m, 10H), 2.07 (m, 1H), 2.49-2.54 (m, 1H), 2.79-3.01 (m, 7H), 3.25 (m, 2H), 3.45 (m, 1H), 3.59-3.64 (dd, J = 12.0, 2.9 Hz, 1H), 3.93-4.28 (m, 5H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 23.8, 25.9, 26.0, 26.5, 26.6, 27.4, 27.5, 27.8, 28.7, 28.8, 32.6, 40.9, 54.6, 55.6, 61.2, 61.4, 69.9, 98.3, 118 (q), 163.2 (q).

Alcohol 9. 6-Amino-1-hexanol **8** (2.0 g, 17 mmol) was dissolved in a 5:1 mixture of 1,4-dioxane/H_2O and cooled to 0 °C. Di-*t*-butyl dicarbonate (7.5 g, 34 mmol) was added and the

reaction mixture was allowed to warm to room temperature and stirred for 12 h. The dioxane was evaporated under reduced pressure and the remaining material was partitioned between EtOAc and saturated $NaHCO_3$ solution. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (4:1 EtOAc/ hexanes) to afford alcohol **9** (3.2 g, 15 mmol, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35-1.76 (m, 18H), 3.11 (q, J = 6.1 Hz, 2H), 3.64 (t, J = 6.3 Hz, 2H), 4.60 (bs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.4, 26.4, 26.5, 28.4, 30.0, 32.6, 40.0, 62.4, 79.1, 156.2; HRMS-CI ($M+H^+$) calcd for $C_{11}H_{24}NO_3$ 218.1756, found 218.1760.

Aldehyde 10. The alcohol **9** (7.2 g, 33 mmol) was added to a CH_2Cl_2 solution (500 mL) containing 51 g of neutral alumina and pyridinium chlorochromate (11 g, 50 mmol). The reaction was allowed to stir at room temperature for 3 h and then was loaded directly onto a flash chromatography column. The product was eluted with 1:1 EtOAc/ hexanes to afford 6.5 g (30 mmol, 91%) of the aldehyde **10**. IR 1704 cm^{-1} (CO); ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.26-1.49 (m, 13H), 1.61 (pent, J = 7.2 Hz, 2H), 2.40 (t, J = 7.2 Hz, 2H), 3.08 (q, J = 6.6 Hz, 2H), 4.59 (bs, 1H), 9.76 (t, J = 1.7 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.5, 27.1, 29.2, 30.7, 41.4, 44.5, 79.8, 156.8, 203.0; HRMS-CI ($M+H^+$) calcd for $C_{10}H_{22}NO_2$ 216.1600, found 216.1600.

Secondary amine 11. Aldehyde **10** (0.52 g, 2.4 mmol) was dissolved in 2 mL of DCE and added to a solution of primary amine **5** (0.51 g, 2.7 mmol) dissolved in 3 mL of DCE. After 10 min sodium triacetoxyborohydride (0.80 g, 3.8 mmol) was added and the reaction was allowed to stir for an additional 3 h at room temperature. The reaction was then quenched with saturated $NaHCO_3$ solution and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (2:1:7 EtOAc/ MeOH/ Et_2O) providing the secondary amine **11** (0.53 g, 1.40 mmol, 50%). ^{1}H NMR (300 MHz, MeOH-d_4) δ 1.36-1.72 (m, 21H), 2.02 (m, 1H), 2.50 (dm, J = 13.7 Hz, 1H), 2.69 (ddd, J = 9.7, 9.7, 2.6 Hz, 1H), 2.76-2.89 (m, 4H), 3.00-3.07 (m, 4H),

3.87 (m, 2H), 4.02 (ddd, J = 11.9, 9.3, 2.5 Hz, 1H), 4.12 (ddd, J = 12.0, 12.0, 2.7 Hz, 4H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 24.7, 25.2, 25.5, 25.9, 26.7, 28.2, 30.2, 40.4, 46.0, 60.0, 60.1, 62.8, 79.3, 96.1, 158.0; HRMS-FAB (M+Na$^+$) calcd for $C_{19}H_{36}N_2NaO_4S$ 411.2294, found 411.2306.

Fmoc ketal 12. Fmoc-Phe-F[18] (0.34 g, 0.89 mmol) and diisopropylethylamine (DIEA, 0.10 mL, 0.60 mmol) were added to a solution of the secondary amine **11** (0.11 g, 0.30 mmol) dissolved in 15 mL of CH_2Cl_2. The reaction was heated at reflux for 5 h then cooled and washed with 10 mL of 1N NaOH, 15 mL of 1N HCl and 15 mL of saturated $NaHCO_3$ solution. The organic layer was then dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (2:3 EtOAc/ hexanes) of the resultant material afforded a mixture of two diastereomers of Fmoc ketal **12** (0.17 g, 0.22 mmol, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.02-2.07 (m, 21H), 2.28-2.45 (m, 1H), 2.56-5.09 (m, 18H), 5.39-5.90 (m, 1H), 7.20-7.83 (m, 13H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.0, 26.5, 27.1, 27.3, 28.4, 28.7, 29.1, 30.1, 31.3, 40.5, 41.4, 44.9, 47.1, 47.3, 52.1, 52.4, 58.8, 59.0, 63.0, 66.6, 66.9, 97.1, 120.0, 125.1, 125.2, 126.5, 126.8, 127.0, 127.6, 128.3, 128.4, 128.5, 128.6, 129.4, 129.7, 136.4, 136.8, 141.3, 143.9, 144.0, 155.2, 156.0, 172.8; HRMS-FAB (M+Na$^+$) calcd for $C_{43}H_{55}N_3NaO_7S$ 780.3659, found 780.3663.

Fmoc ketal 13. A DMF solution (75 mL) containing hydroxybenzotriazole (HOBT, 0.37 g, 2.8 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 0.69 g, 3.6 mmol) and Fmoc-phenylalanine (1.1 g, 2.8 mmol) was stirred at room temperature for 1 h. A solution of the primary amine **5** (0.52 g, 2.8 mmol) and 4-methylmorpholine (0.60 mL, 5.5 mmol) dissolved in 25 mL DMF was then added to the reaction mixture. After 2 h the reaction mixture was partitioned between 100 mL of EtOAc and 100 mL of H_2O. The organic layer was washed with 100 mL of H_2O, 50 mL of saturated $KHSO_4$ solution, 50 mL of saturated Na_2CO_3 solution, dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (1:1

EtOAc/ hexanes) afforded a mixture of two diastereomers of Fmoc ketal **13** (0.56 g, 1.0 mmol, 36%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.62-1.81 (m, 3H), 2.28-3.19 (m, 7H), 3.72-3.93 (m, 4H), 4.10-4.46 (m, 5H), 5.44 (m, 1H), 6.24-6.48 (m, 1H), 7.24-7.79 (m, 13H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.0, 25.3, 25.4, 30.6, 30.7, 32.0, 32.2, 39.2, 39.5, 47.6, 56.6, 56.9, 59.4, 59.5, 59.6, 67.5, 96.3, 96.4, 120.4, 125.47, 125.54, 127.3, 127.5, 128.1, 129.0, 129.1, 129.8, 129.9, 136.8, 137.0, 141.7, 144.2, 156.2, 170.4, 170.6; HRMS-FAB ($M+Na^+$) calcd for $C_{32}H_{34}N_2NaO_5S$ 581.2086, found 581.2099.

Amino ketal 14A & 14B. A DMF solution (35 mL) of Fmoc ketal **12** (1.75 g, 2.3 mmol) and piperidine (1.4 mL, 14 mmol) was stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure and the crude material was purified by flash chromatography (98:2 CH_2Cl_2/ MeOH) to give the two separate diastereomers of the amino ketal **14A** (0.43 g, 0.50 mmol) and **14B** (0.41 g, 0.76 mmol) with a combined yield of 67%. **14A**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.08 (m, 1H), 1.09-1.34 (m, 7H), 1.36-1.52 (m, 14H), 1.68-1.73 (m, 4H), 1.84-1.97 (m, 1H), 2.36 (dq, J = 13.5, 1.8 Hz, 1H), 2.65-2.82 (m, 2H), 2.91-3.02 (m, 2H), 3.09-3.19 (m, 4H), 3.44-3.59 (m, 1H), 3.63 (dd, J = 11.4, 3.3 Hz, 1H), 3.71-3.91 (m, 4H), 4.02 (dt, J = 11.9, 2.4 Hz, 1H), 4.10 (dd, J = 10.0, 5.7 Hz, 1H), 4.60 (bm, 1H), 7.19-7.39 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 27.6, 29.2, 29.8, 30.3, 31.2, 31.9, 32.8, 34.1, 45.4, 47.5, 55.5, 61.6, 62.0, 65.2, 100.1, 129.1, 131.2, 132.2, 140.6, 180.0; HRMS-ESI ($M+H^+$) calcd for $C_{28}H_{46}N_3O_5S$ 536.3158, found 536.3163. **14B**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25-1.67 (m, 27H), 1.96-2.02 (m, 2H), 2.43-2.51 (m, 2H), 2.67 (dd, J = 13.6, 9.3 Hz, 1H), 2.79-2.83 (m, 1H), 3.11-3.30 (m, 6H), 3.36-3.52 (m, 2H), 3.69-4.05 (m, 6H), 4.27 (dd, J = 11.2, 2.9 Hz, 1H), 4.61 (bs, 1H), 7.20-7.34 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.0, 24.1, 24.3, 25.6, 25.8, 26.1, 26.4, 27.5, 27.7, 28.0, 28.3, 29.3, 30.2, 30.7, 30.9, 39.8, 41.5, 42.0, 43.6, 43.8, 52.6, 52.9, 57.9, 58.0, 58.1, 58.3, 62.3, 78.2, 78.3, 96.7, 97.2, 125.5, 125.9, 127.6, 127.7, 128.6, 128.7, 137.6, 138.6, 155.2, 155.5, 175.0, 175.8; HRMS-ESI ($M+H^+$) calcd for $C_{28}H_{46}N_3O_5S$ 536.3158, found 536.3140.

Amino ketal 15A & 15B. A solution of piperidine (0.6 mL, 6.0 mmol) and Fmoc ketal **13** (0.56 g, 1.0 mmol) in 5 mL of DMF was allowed to stir at room temperature for 5 h. The reaction mixture was then partitioned between 50 mL of EtOAc and 50 mL of H_2O. The organic layer was washed with H_2O, dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography (2:98 MeOH/CH_2Cl_2) to afford the two separate diastereomers of the amino ketal **15A** (0.17 g, 0.50 mmol) and **15B** (0.11 g, 0.32 mmol) with a combined yield of 81%. **15A**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26 (s, 2H), 1.47 (m, 2H), 1.68 (m, 1H), 2.30 (bm, 1H), 2.50 (m, 2H), 2.73 (dd, *J* = 13.7, 9.2 Hz, 5H), 2.93 (m, 1H), 3.26 (dd, *J* = 13.7, 3.9 Hz, 1H), 3.67 (dd, *J* = 9.2, 4.0 Hz, 1H), 3.81 (m, 1H), 3.93 (m, 2H), 4.14 (m, 1H), 4.66 (bm, 1H), 7.23-7.34 (m, 5H), 8.01 (d, *J* = 8.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.9, 26.7, 31.3, 32.7, 42.6, 57.6, 60.5, 60.8, 97.9, 128.2, 129.9, 131.0, 134.3, 139.2, 176.8; HRMS-FAB ($M+H^+$) calcd for $C_{17}H_{25}N_2O_3S$ 337.1586, found 337.1579. **15B**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.53-1.72 (m, 4H), 2.29 (bs, 1H), 2.52 (m, 2H), 2.76 (dd, *J* = 13.7, 9.1 Hz, 2H), 2.96 (dd, *J* = 10.8, 2.1 Hz, 1H), 3.26 (dd, *J* = 13.7, 4.5 Hz, 1H), 3.64 (dd, *J* = 9.1, 4.6 Hz, 1H), 3.82 (m, 1H), 3.94 (m, 2H), 3.96 (m, 1H), 4.70 (bm, 1H), 7.22-7.35 (m, 5H), 7.90 (d, *J* = 9.2 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.9, 26.6, 31.2, 32.7, 42.8, 57.9, 60.5, 60.7, 97.8, 128.1, 130.0, 130.9, 139.4, 176.8; HRMS-FAB ($M+H^+$) calcd for $C_{17}H_{25}N_2O_3S$ 337.1586, found 337.1592.

Boc ketal 16A. A DMF solution (10 mL) containing HOBT (103 mg, 0.76 mmol), EDC (192 mg, 1.0 mmol) and Boc-D-Isoleucine (172 mg, 0.76 mmol) was stirred at room temperature for 20 h. A solution of the amino ketal **14A** (0.41 g, 0.76 mmol) and 4-methylmorpholine (0.17 mL, 1.5 mmol) dissolved in 10 mL DMF was then added to the reaction mixture. After 4 h the reaction mixture was partitioned between EtOAc and H_2O. The organic layer was washed with H_2O, dried over $MgSO_4$ and concentrated under reduced pressure. Flash chromatography (4:1 EtOAc/ hexanes) afforded the Boc ketal **16A** (0.44 g, 0.59 mmol, 78%).

This compound appears in the NMR spectra as a mixture of two conformational isomers. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.75-0.99 (m, 6H), 1.06-1.36 (m, 34H), 2.27-2.32 (m, 1H), 2.62 (t, J = 13.1 Hz, 1H), 2.75 (t, J = 12.3 Hz, 1H), 2.87 (t, J = 11.5 Hz, 1H), 2.97-3.17 (m, 5H), 3.46-4.03 (m, 6H), 4.56 (m, 0.5H), 4.97-5.30 (m, 1.5H), 6.90 (d, J = 7.1 Hz, 1H), 7.17-7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.9, 15.6, 15.77, 15.83, 25.0, 25.2, 25.3, 26.3, 26.8, 27.4, 27.5, 28.6, 28.7, 29.4, 30.4, 31.4, 37.8, 38.2, 40.4, 40.8, 41.2, 41.3, 45.3, 51.2, 51.3, 59.2, 63.3, 79.3, 79.9, 97.3, 98.3, 127.1, 128.7, 129.0, 129.7, 130.0, 130.1, 136.6, 137.0, 155.9, 170.3, 170.4, 173.0, 192.2, 201.5; HRMS-FAB ($M+Na^+$) calcd for $C_{39}H_{64}N_4NaO_8S$ 771.4166, found 771.4334 for a mixture of diastereomers **16A** and **16B**.

Boc ketal 16B. Compound **16B** was prepared from **14B** (270 mg, 0.51 mmol), HOBT (68 mg, 0.51 mmol), EDC (130 mg, 0.67 mmol), Boc-D-Ile (120 mg, 0.51 mmol) and 4-methylmorpholine (0.11 mL, 1.0 mmol) in 20 mL DMF using the method described for the synthesis of **16A.** The crude material was purified by HPLC (1.5% MeOH/CH_2Cl_2 over 45 min) to afford **16B** (240 mg, 0.32 mmol, 63%). This compound appears in the NMR spectra as a mixture of two conformational isomers. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.67-1.07 (m, 6H), 1.32-1.48 (m, 26H), 1.62-1.79 (m, 2H), 1.91-2.03 (m, 2H), 2.44 (m, 1.5H), 2.76-3.49 (m, 8H), 3.66 (m, 0.5H), 3.80-4.05 (m, 4H), 4.57 (m, 1H), 4.92-5.29 (m, 3H), 6.48 (m, 0.5H), 6.65 (m, 0.5H), 7.18-7.30 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.9, 12.1, 15.6, 15.8, 24.8, 24.9, 25.0, 25.36, 25.41, 26.5, 26.9, 27.4, 28.4, 28.7, 28.8, 28.9, 29.2, 30.1, 31.3, 31.6, 37.7, 38.2, 38.5, 40.6, 40.9, 44.8, 45.2, 50.7, 51.1, 53.8, 59.1, 59.2, 59.3, 59.4, 59.5, 63.0, 77. 7, 79.4, 80.0, 97.9, 98.2, 126.8, 127.4, 128.6, 128.9, 129.8, 129.9, 137.0, 138.0, 155.9, 171.1, 173.1; HRMS-FAB ($M+Na^+$) calcd for $C_{39}H_{64}N_4NaO_8S$ 771.4166, found 771.4334 for a mixture of diastereomers **16A** and **16B**.

Boc ketal 17A. Compound **17A** was prepared from Compound **15A** (110 mg, 0.32 mmol), HOBT (43 mg, 0.32 mmol), EDC (80 mg, 0.42 mmol), Boc-D-Ile (74 mg, 0.32 mmol)

and 4-methylmorpholine (0.070 mL, 0.64 mmol) in 15 mL of DMF by the method described for the synthesis of **16A**. The crude material was purified by flash chromatography (4:1 EtOAc/ hexanes) to afford **17A** (156 mg, 0.28 mmol, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.80-0.95 (m, 7H), 1.24-1.44 (m, 10H), 1.60-1.81 (m, 6H), 2.46-2.59 (m, 3H), 2.82-3.13 (m, 3H), 3.76-4.01 (m, 5H), 4.44 (m, 1H), 4.73 (q, J = 6.9 Hz, 1H), 5.01 (m, 1H), 6.58 (m, 2H), 7.19-7.32 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.9, 15.8, 24.9, 25.4, 28.7, 30.6, 31.9, 37.8, 38.6, 54.8, 59.6, 80.4, 96.3, 127.3, 129.0, 129.8, 136.8, 156.0, 170.4, 171.8; HRMS-FAB ($M+H^+$) calcd for $C_{28}H_{44}N_3O_6S$ 550.2951, found 550.2961.

Boc ketal 17B. Compound **17B** was prepared from Compound **15B** (106 mg, 0.32 mmol), HOBT (43 mg, 0.32 mmol), EDC (78 mg, 0.41 mmol), Boc-D-Ile (73 mg, 0.32 mmol) and 4-methylmorpholine (0.070 mL, 0.64 mmol) in 15 mL of DMF by the method described for the synthesis of **16A**. The crude material was purified by flash chromatography (4:1 EtOAc/ hexanes) to give compound **17B** (148 mg, 0.27 mmol, 86%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.81-1.02 (m, 7H), 1.23-1.80 (m, 16H), 2.27-2.74 (m, 4H), 2.95-3.18 (m, 2H), 3.70-3.99 (m, 5H), 4.38 (s, 1H), 4.67 (q, J = 8.2 Hz, 1H), 5.11 (m, 1H), 6.41 (m, 1H), 6.81 (d, J = 7.5 Hz, 1H) 7.20-7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.0, 15.8, 24.9, 25.3, 28.7, 30.5, 32.0, 38.1, 38.8, 55.2, 59.5, 59.6, 80.3, 96.3, 127.4, 129.1, 129.7, 137.0, 156.0, 170.1, 171.7; HRMS-FAB ($M+H^+$) calcd for $C_{28}H_{44}N_3O_6S$ 550.2951, found 550.2953.

Inhibitor 1. Ketal **7** (53 mg, 0.09 mmol) was dissolved in a solution of 5 mL MeOH and 10 mL 6 N HCl. The reaction was heated at reflux for 1 h before the solvent was removed under reduced pressure. The crude material was dissolved in a small amount of MeOH to which Et_2O was added until the solution turned cloudy. The Et_2O was pipetted off and the oily residue further purified by RPHPLC (H_2O with 0.1 % TFA) to afford 53 mg (0.09 mmol, 99%) of inhibitor **1**. ^{1}H NMR (300 MHz, MeOH -d_4) δ 1.31 (m, 10H), 1.67-1.83 (m, 10H), 2.92-3.04

(m, 9H), 3.14 (m, 1H), 4.61 (dd, J = 11.6, 5.3 Hz, 1H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 24.9, 25.9, 26.06, 26.12, 26.3, 27.3, 28.1, 29.0, 39.5, 44.5, 53.0, 53.2, 69.9, 201.9.

Inhibitor 2A. The Boc ketal **16A** (100 mg, 0.14 mmol) was dissolved in 1 mL of a solution containing 92.5% TFA, 2.5% TIS, 2.5% thioanisole and 2.5% H_2O. After 18 h the TFA was removed under reduced pressure. The crude mixture was purified by reverse-phase HPLC (0%-50% MeCN/H_2O over 45 min) affording 49 mg (0.068 mmol, 50%) of the inhibitor **2A**. ^{1}H NMR (300 MHz, MeOH-d_4) δ 0.83-0.97 (m, 7H), 1.31-1.41 (m, 7H), 1.71-1.79 (m, 4H), 2.79-2.99 (m, 7H), 3.05-3.21(m, 2H), 3.39-3.48 (m, 2H), 3.69 (d, J = 5.5 Hz, 1H), 4.10 (dd, J = 11.1, 5.9 Hz, 1H), 5.12 (dd, J = 9.3, 5.8 Hz, 1H), 7.26-7.38 (m, 5H); ^{13}C NMR (75 MHz, MeOH -d_4) δ 12.0, 15.3, 25.4, 27.5, 27.6, 27.9, 28.0, 28.8, 28.9, 30.7, 32.3, 34.6, 38.1, 39.8, 41.0, 45.2, 50.8, 52.1, 52.8, 53.7, 54.2, 59.4, 61.3, 67.8, 128.7, 130.2, 130.3, 130.8, 131.1, 138.0, 138.2, 163.4, 169.5, 172.7, 190.3, 204.6; HRMS-FAB (M+H$^+$) calcd for $C_{26}H_{43}N_4O_3S$ 491.3056, found 491.3067.

Inhibitor 2B. Inhibitor **2B** was prepared from compound **16B** (240 mg, 0.32 mmol) and 1 mL of the TFA solution specified in the synthesis of **2A**. The crude product was purified by RPHPLC (0%-50% MeCN/H_2O over 45 min) to afford inhibitor **2B** (21 mg, 0.030 mmol, 9%). ^{1}H NMR (300 MHz, MeOH-d_4) δ 0.52-0.82 (m, 7H), 1.06-1.12 (m, 1H), 1.19-1.36 (m, 5H), 1.49-1.64 (m, 5H), 2.64-2.85 (m, 6H), 2.91-2.98 (m, 1H), 3.08 (dd, J = 14.5, 4.6 Hz, 1H), 4.93 (dd, J = 10.2, 4.6 Hz, 1H), 7.00-7.24 (m, 5H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 12.1, 15.2, 25.4, 27.6, 28.9, 31.0, 32.6, 38.2, 39.1, 41.0, 45.3, 50.8, 52.9, 59.4, 68.1, 128.6, 130.1, 130.5, 130.7, 138.5, 169.6, 173.1, 204.0; HRMS-ESI (M+H$^+$) calcd for $C_{26}H_{43}N_4O_3S$ 491.3056, found 491.3065.

Inhibitor 3A. Compound **17A** (53 mg, 0.10 mmol) was dissolved in 1 mL of a solution containing 92.5% TFA, 2.5% TIS, 2.5% H_2O, and 2.5% thioanisole. After 1h the TFA was

removed under reduced pressure. The crude mixture was purified by flash chromatography (10:89:1 MeOH, CH_2Cl_2, concentrated NH_4OH) before the final purification was performed using RPHPLC (0%-100% MeCN/H_2O over 45 min) affording the inhibitor **3A** (17 mg, 0.03 mmol, 34%). In MeOH-d_4 solution, the inhibitor is visible as an approximate 1:1 mixture of hemiketal and ketone. ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.70–0.78 (m, 7H), 1.15 (m, 1H), 1.65 (m, 1H), 1.83 (m, 0.5H), 1.99 (m, 0.5H), 2.17 (m, 0.5H), 2.38 (m, 0.5H), 2.59-3.00 (m, 5H), 3.13 (ddd, J = 13.2, 5.6, 2.8 Hz, 0.5H), 3.28 (m, 0.5H), 3.67 (m, 1H), 4.13 (m, 0.5H), 4.72 (dd, J = 11.6, 4.8 Hz, 0.5H), 4.82 (dd, J = 11.2, 7.2 Hz, 0.5H), 7.23-7.35 (m, 5H); ^{13}C NMR (100 MHz, MeOH-d_4) δ 10.7, 13.6, 24.0, 24.7, 25.0, 25.1, 30.45, 30.49, 34.7, 35.2, 36.7, 37.8, 38.0, 44.4, 64.99, 55.02, 58.00, 58.03, 59.8, 95.5, 96.2, 115.5 (q, J = 284 Hz), 160.3 (q, J = 34 Hz), 168.3, 168.4, 172.4, 172.68, 172.71, 204.4; HRMS-FAB (M+Na$^+$) calcd for $C_{20}H_{29}N_3NaO_3S$ 414.1827, found 414.1823.

Inhibitor 3B. Inhibitor **3B** was prepared from compound **17B** (60 mg, 0.11 mmol) and 1 mL of the TFA solution specified in the synthesis of **3A**. The crude product was purified by RPHPLC (0%-100% MeCN/H_2O over 45 min) to afford inhibitor **3B** (45 mg, 0.090 mmol, 82%). In MeOH-d_4 solution, the inhibitor is visible as an approximate 1:1 mixture of hemiketal and ketone. ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.58-1.05 (m, 7H), 1.16-1.40 (m, 1H), 1.65-1.81 (m, 1H), 1.94-2.14 (m, 1H), 2.39-3.05 (m, 6H), 3.15-3.23 (m, 1H), 3.69 (d, J = 5.2 Hz, 1H), 4.05-4.11 (m, 0.5H), 4.67 (dd, J = 11.5, 5.3 Hz, 0.5H), 4.73 (dd, J = 10.1, 5.8 Hz, 0.5H), 4.84 (dd, J = 10.2, 5.2 Hz, 0.5H), 7.22-7.32 (m, 5H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 14.2, 14.3, 27.7, 27.8, 28.5, 33.8, 34.0, 38.1, 38.8, 40.30, 40.33, 41.4, 41.7, 48.0, 58.5, 59.0, 61.5, 61.7, 63.6, 99.2, 130.6, 130.7, 132.2, 132.8, 140.6, 140.8, 172.0, 172.4, 175.6, 176.1, 207.9; HRMS-FAB (M+Na$^+$) calcd for $C_{20}H_{29}N_3NaO_3S$ 414.1827, found 414.1834.

Secondary amine 19. Compound **19** was prepared from **18** (3.8 mL, 33.5 mmol), compound **10** (6.6 g, 30.5 mmol) and sodium triacetoxyborohydride (3.7 g, 17.5 mmol) in 20 mL

of DCE using the method described for the synthesis of **11**. The crude material was purified by flash chromatography (2:1:7 EtOAc/ MeOH/ Et_2O) to afford the secondary amine **19** (1.3 g, 4.4 mmol, 25%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96-1.47 (m, 23H), 1.57-1.73 (m, 3H), 1.84 (m, 2H), 2.32-2.42 (m, 1H), 2.58 (t, J = 7.1 Hz, 2H), 3.07 (q, J = 6.5 Hz, 2H), 4.60 (bs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.5, 26.6, 27.1, 27.5, 28.5, 30.4, 30.8, 34.0, 40.7, 47.3, 57.3, 79.3, 156.4; HRMS-FAB (M+Na^+) calcd for $C_{17}H_{34}N_2NaO_2$ 321.2518, found 321.2522.

Primary amine 20. A solution of Fmoc-Phe-F (5 g, 13.2 mmol), DIEA (2.3 mL, 13.2 mmol) and the secondary amine **19** (1.3 g, 4.4 mmol) in 100 mL of CH_2Cl_2 was heated at reflux for 18 h. The mixture was then diluted with 100 mL of EtOAc, and washed with 100 mL each of 1N NaOH, 1N HCl and saturated $NaHCO_3$ solution. The resultant organic layer was dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography (1:1 EtOAc/ hexanes) which provided a mixture of Fmoc-Phe and the expected coupling product. The mixture was dissolved in 85 mL of DMF, and piperidine (2.2 mL, 22 mmol) was added. After 15 min the solution was partitioned between 150 mL of EtOAc and 150 mL of H_2O. The organic layer was dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography (2% MeOH/ CH_2Cl_2) to afford the primary amine **20** as a mixture of two conformational isomers that interconvert slowly on the NMR time scale (1.3 g, 2.9 mmol, 66%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.02-1.74 (m, 27H), 2.65-3.34 (m, 6H), 3.67 (t, J = 7.0 Hz, 0.5H), 3.89 (t, J = 7.0 Hz, 0.5H), 4.22 (m, 0.5H), 4.67 (m, 0.5H), 7.16-7.33 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.6, 25.9, 26.3, 26.4, 26.7, 26.8, 27.4, 28.8, 29.7, 30.4, 30.9, 31.2, 31.7, 32.1, 32.6, 42.7, 43.9, 53.3, 54.2, 54.4, 57.1, 127.0, 128.8, 128.9, 129.1, 129.7, 138.3, 156.4, 174.5; HRMS-FAB (M+Na^+) calcd for $C_{26}H_{43}N_3NaO_3$ 468.3202, found 468.3216.

Boc dipeptide 21. Compound **21** was prepared from **20** (1.3 g, 2.9 mmol), HOBT (0.39 g, 2.9 mmol), EDC (0.73 g, 3.8 mmol), Boc-D-Ile (0.68 g, 2.9 mmol), and 4-methylmorpholine (0.66 g, 6 mmol) in 75 mL DMF using the method described for the synthesis

of **16A**. The crude material was purified by flash chromatography (4:1 EtOAc/ hexanes) to afford Boc dipeptide **21** (1.7 g, 2.6 mmol, 91%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.76-0.82 (m, 7H), 0.94-1.77 (m, 36H), 2.60-3.29 (m, 6H), 4.00-4.12 (m, 2H), 4.69-5.53 (m, 3H), 7.07-7.28 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.9, 15.8, 24.87, 24.94, 25.5, 26.0, 26.1, 26.8, 27.3, 28.7, 28.79, 28.81, 30.3, 31.0, 31.4, 40.4, 40.5, 42.7, 50.5, 54.6, 57.4, 59.5, 79.8, 127.2, 128.8, 129.8, 129.9, 136.8, 137.0, 155.9, 170.7, 171.0, 171.3; HRMS-FAB ($M+Na^+$) calcd for $C_{37}H_{62}N_4NaO_6$ 681.4567, found 681.4550.

Amine 22. The Boc dipeptide **21** (180 mg, 0.27 mmol) was dissolved in 1 mL of CH_2Cl_2 before 1 mL of a solution containing 95% TFA, 2.5 % TIS, 2.5% ethanedithiol was added. After 30 min the solvent was removed under vacuum. The crude product was purified by flash chromatography (10:89:1 MeOH/ CH_2Cl_2/ concentrated aqueous NH_4OH) to afford amine **22** (0.11 g, 0.16 mmol, 59%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.83-0.92 (m, 6H), 1.00-1.20 (m, 3H), 1.33-1.87 (m, 19H), 2.89-3.12 (m, 5H), 3.24-3.34 (m, 3H), 3.38 (m, 0.5H), 3.68 (dd, J = 8.3, 5.7 Hz, 1H), 3.94-4.18 (m, 1H), 5.15 (t, J = 7.7 Hz, 1H), 7.23-7.35 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 10.67, 10.69, 13.9, 14.0, 24.1, 25.2, 25.5, 25.8, 25.9, 26.0, 26.1, 26.2, 26.4, 26.8, 27.4, 27.5, 29.1, 30.6, 31.0, 31.2, 31.5, 37.0, 38.4, 38.9, 39.58, 39.63, 42.5, 43.9, 51.3, 52.2, 55.5, 58.0, 116.0 (q, J = 293 Hz), 127.1, 127.22, 127.24, 128.6, 128.7, 128.8, 129.4, 129.5, 129.6, 136.8, 137.0, 162.1 (q, J = 34 Hz), 168.6, 168.7, 171.6, 171.9; HRMS-FAB ($M+Na^+$) calcd for $C_{27}H_{46}N_4NaO_2$ 481.3519, found 481.3523.

Enzyme Assays. The amidolytic activity of plasmin, thrombin, kallikrein and trypsin were determined using chromogenic substrates D-Val-Leu-Lys-pNA, H-D-Phe-Pip-Arg-pNA, H-D-Pro-Phe-Arg-pNA, and H-D-Phe-Pip-Arg-pNA, respectively.[20] Enzymes and substrates were used as received from Sigma-Aldrich or Chromogenix (distributor: DiaPharma Group, Inc.) without further purification. Reaction progress was monitored on a Perkin-Elmer 8452A diode array UV-vis spectrometer. All enzymes were assayed at 25 °C in 50 mM sodium phosphate

buffer (pH 7.4) with or without inhibitor. Due to solubility, inhibitors **2** & **3** were assayed in a solution with a final concentration of 10% DMSO. Initial rates were determined by monitoring the change in absorbance at 404 nm from 60-120 sec after mixing. None of the inhibitors showed evidence of slow binding behavior. Inhibitor **2A** was subjected to full kinetic analysis against plasmin. For each inhibitor concentration examined (**2A**: 0, 8.6, 43, 86, 170, 260 μM) five substrate concentrations were used (75, 150, 300, 600, 1200 μM) with at least two independent determinations at each concentration. K_i values were determined by nonlinear fit to the Michaelis-Menten equation for competitive inhibition using simple weighing. Competitive inhibition was confirmed by Lineweaver - Burk analysis using simple statistical weighing to the linear fit of 1/v vs. 1/[S]. For the less potent compounds (**1, 2B, 3A, 3B**) a substrate concentration of 300 μM was monitored with six different inhibitor concentrations (**1**: 0, 110, 210, 430, 860, 1700 μM; **2B**: 0, 110, 230, 460, 690, 920 μM; **3A**: 0, 0.8, 1.6, 2.4, 3.1, 3.9 mM; **3B**: 0, 0.9, 1.8, 2.7, 3.6, 4.5 mM). For inhibitor **2A** assayed against thrombin (Thr), Kallikrein (Kal) and trypsin (Try), a single substrate concentration (Thr: 50 μM; Kal: 100 μM; Try: 50 μM) was monitored with six different inhibitor concentrations (Thr: 0, 26, 53, 79, 110, 160 μM; Kal: 0, 48, 96, 144, 190, 240 μM; Try: 0, 26, 53, 79, 110, 160 μM). Competitive inhibition was assumed and K_i values were calculated using a Dixon analysis. Data analysis was performed with the commercial graphing package Grafit (Erithacus Software Ltd.). K_m values for the substrates were determined both with and without 10% DMSO (without DMSO: plasmin 220 μM, Thr 10 μM, Kal 117 μM, Try 42 μM; with DMSO: plasmin 370 μM, Thr 22 μM, Kal 63 μM, Try 50 μM).

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Supporting Information Available: HPLC characterization for compounds **1**, **2 A**, **3 A** and **B**, and **22**. Lineweaver-Burk plots for the inhibition of plasmin by compound **2A** (7 pages). This material is available free of charge via the Internet at http://pubs.cas.org.

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Table 1. Inhibition of Serine Proteases by Inhibitors **1**- **3**, and **22**.

	K_i (μM)			
Compound[a]	Plasmin	Trypsin	Thrombin	Kallikrein
1	400 ± 35	1,400 ± 110	>10,000	>10,000
2A	50 ± 5	1,700 ± 1,500	720 ± 550	630 ± 125
2B	130 ± 10			
3A	9,000 ± 1,000			
3B	16,000 ± 1,300			
22	520 ± 30			

[a] A and B represent two different diastereomers.

Figure and Scheme Legends

Figure 1. Shifting of the P1 side chain from the position alpha to the ketone to the exocyclic nitrogen to avoid formation of the quaternary center. R = $(CH_2)_6NH_2$.

Scheme 1[a]

[a] Reagents and conditions: (a) TFA, 85%; (b) **10**, $NaBH(OAc)_3$, 22%; (c) TFA, H_2O, triisopropylsilane, thioanisole, 50%; (d) 6N HCl, 99%.

Scheme 2[a]

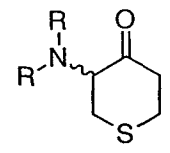
[a] Reagents and conditions: (a) $(Boc)_2O$, 88%; (b) pyridinium chlorochromate, 91%.

Scheme 3[a]

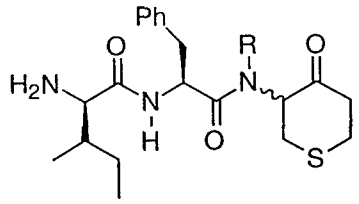
[a] Reagents and conditions: (a) **10**, $NaBH(OAc)_3$, dichloroethane, 50%; (b) Fmoc-Phe-F, DIEA, **12** 75%; (c) FmocPhe, EDC, HOBT, **13** 36%; (d) piperidine, DMF, **14 A** and **B** 67%, **15 A** and **B** 81%; (e) Boc-D-Ile, EDC, HOBT, **16A** 78%, **16B** 63%, **17A** 88%, **17B** 86%; (f) TFA, H_2O, TIS, thioanisole, **2A** 50%, **2B** 9%, **3A** 34%, **3B** 82%. A and B represent two different diastereomers.

Scheme 4[a]

[a] Reagents and conditions: (a) **10**, $NaBH(OAc)_3$, dichloroethane, 25%; (b) Fmoc-Phe-F, DIEA; (c) piperidine, DMF, 66% (2 steps); (d) Boc-D-Ile, EDC, HOBT, 91%; (e) TFA, triisopropylsilane, ethanedithiol, 59%.

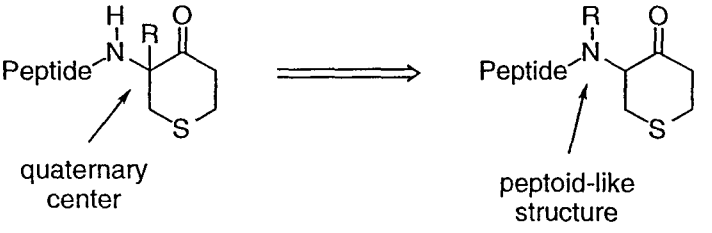


1 R = $(CH_2)_6NH_2$



2 R = $(CH_2)_6NH_2$
3 R = H

Structures of Inhibitors **1** - **3**.



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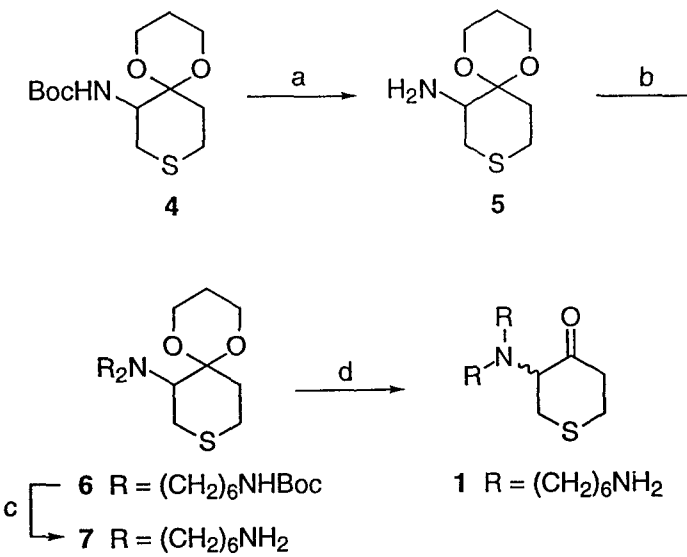

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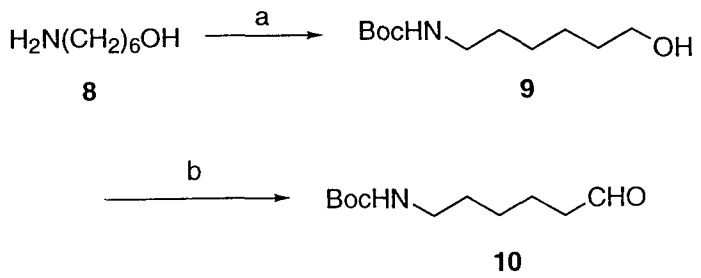

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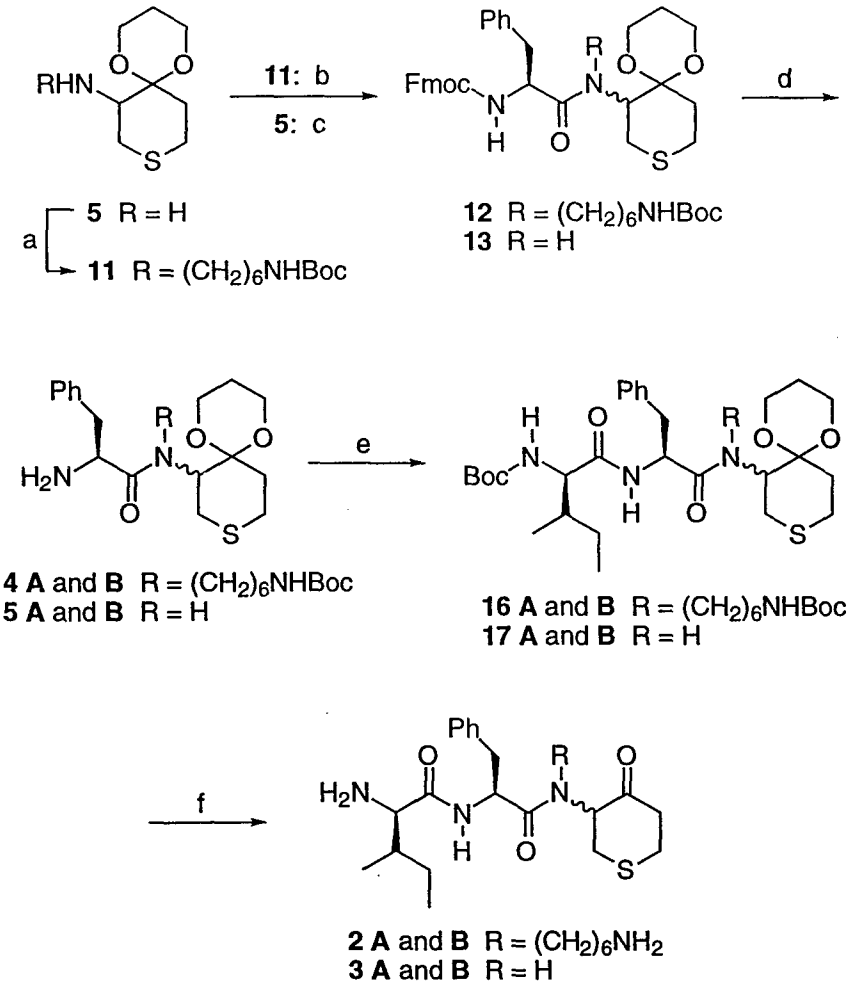

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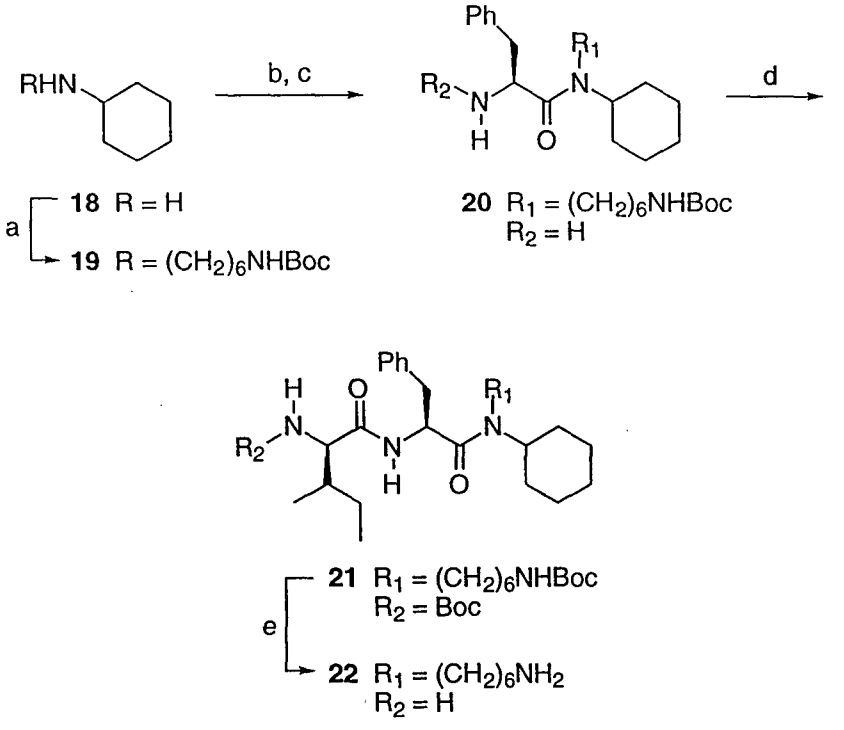

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AD_____

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PRINCIPAL INVESTIGATION: Tanya C. Sanders

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