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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**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**

![Diagram of Metastatic Cell, Pro uPA, uPA, Plasminogen, latent MMPs, Plasmin, MMPs, Basement Membrane]
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). 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. 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. 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. 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 P₂ and P₃ sites are occupied by phenylalanine and D-isoleucine respectively. The resulting compound proved to be a good reversible selective inhibitor for plasmin (Kᵢ = 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. 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 $S_1^\prime$ sites. The $S_1$ recognition site of plasmin is specific for lysine, and somewhat less for arginine. 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
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. 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. 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 ($K_{H_2O}$) 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 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 ($K_i = 10-120 \mu M$). Their potency generally 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

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1. Mixture of diastereomers
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 straightforward 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 ($K_i = 100 \mu m$) from

![Scheme 1](image)

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.

The new deprotection scheme was also used successfully to provide pure samples of the two targeted plasmin inhibitors. Final $K_i$ values for the two diastereomer of that compound are 50 $\mu$M and 120 $\mu$M (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.

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.
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: $K_i = 50 \mu M$; 2B: $K_i = 120 \mu M$). 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.
References


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 1H NMR and CDCl₃ (δ = 77.0 ppm) or CD₃OD (δ = 49.0 ppm) for 13C 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 MgSO₄ 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 NaHCO₃ solution and extracted with EtOAc. The organic layer was dried over MgSO₄ and concentrated under reduced pressure. The crude product was purified by flash chromatography (2:1:7 EtOAc/MeOH/ Et₂O) providing the secondary amine 7 (0.53 g, 1.40 mmol, 50%). 1H NMR (300
MHz, MeOH-d₄) 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); ¹³C NMR (75 MHz, MeOH-d₄) 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 C₁₉H₃₆N₂NaO₄S 411.2294, found 411.2306.

**Fmoc-Trp amine 8.** Secondary amine 7 (1.2 g, 3.1 mmol) was dissolved in 100 mL CH₂Cl₂ 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 NaHCO₃ solution before being dried over MgSO₄ 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%). ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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, 144.4, 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 H2O. The organic layer was dried over MgSO4 and concentrated before being purified by flash chromatography (2% MeOH/CH2Cl2) 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:** 1H NMR (300 MHz, CDCl3) \( \delta \) 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); 13C NMR (75 MHz, MeOH-\( d_4 \)) \( \delta \) 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:** 1H NMR (300 MHz, CDCl3) \( \delta \) 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 H2O. The organic layer was washed with 10 mL of each H2O, saturated KHSO4 and saturated Na2CO3 solutions, dried over MgSO4 and concentrated. The crude product was purified by flash chromatography (4:1 EtOAc/
hexanes) to afford the Boc-D-ile amine\textbf{10A} (0.13 g, 0.15 mmol, 89%). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 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-$d_4$) $\delta$ 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 \textbf{10B} was synthesized from \textbf{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 \textbf{10A}. The product was purified by flash column (4:1 EtOAc/ hexanes) to afford compound \textbf{10B} (0.17 g, 0.19 mmol, 88%). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 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-$d_4$) $\delta$ 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 NaHCO₃ solution and EtOAc. The organic layer was dried over MgSO₄ 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-d₄) 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); ¹³C NMR (100 MHz, MeOH-d₄) 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 C₂₈H₅₄N₃O₆S 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% H₂O. 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 Et₂O 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-d₄) 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); ¹³C NMR (75 MHz, MeOH-d₄) 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/Et₂O) affording the secondary amine 16 (1.3 g, 4.4 mmol, 25%). ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₁₁H₂₄N₂NaO₂ 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 CH₂Cl₂ 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 NaHCO₃ solution. The resultant organic layer was dried over MgSO₄ 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 H₂O. The organic layer was dried over MgSO₄ and concentrated before being purified by flash chromatography (2% MeOH/CH₂Cl₂) to afford a mixture of diastereomers of the primary amine 17 (1.3 g, 2.9 mmol, 66%). ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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,
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 partitioned between EtOAc and H₂O. The organic layer was washed with H₂O, dried over MgSO₄ 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%). ^1H NMR (300 MHz, CDCl₃) 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); ^13C NMR (75 MHz, CDCl₃) 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 C₃₇H₆₂N₄NaO₆ 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 Et₂O was added until the solution turned cloudy. The Et₂O was pipetted off and the oily residue further purified by RPHPLC (H₂O with 0.1 % TFA) to afford 53 mg (0.09 mmol, 99%) of inhibitor 1. ^1H NMR (300 MHz, MeOH-d₄) 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); ^13C NMR (75 MHz, CDCl₃) δ 11.9, 12.8, 24.9, 25.5, 26.0, 26.1, 26.8, 27.3, 28.7, 28.9, 30.3, 31.0, 31.4, 40.4, 40.5, 42.7, 50.5, 54.6, 57.3, 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 C₂₆H₄₃N₃NaO₃ 468.3202, found 468.3216.
MHz, MeOH-\textit{d}_4 \delta 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\textsubscript{2}O, 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\textsubscript{2}Cl\textsubscript{2}, concentrated NH\textsubscript{4}OH) before the final purification was performed using RPHPLC (0%-100% MeCN/ H\textsubscript{2}O over 45 min) affording the inhibitor 3A (17 mg, 0.03 mmol, 34%). In MeOH-\textit{d}_4 the inhibitors are visible as an approximately 1:1 mixture of hemiketal and ketone. 3A: \textsuperscript{1}H NMR (400 MHz, MeOH-\textit{d}_4) \delta 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, \textit{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, \textit{J} = 11.6, 4.8 Hz, 0.5H), 4.82 (dd, \textit{J} = 11.2, 7.2 Hz, 0.5H), 7.23-7.35 (m, 5H); \textsuperscript{13}C NMR (100 MHz, MeOH-\textit{d}_4) \delta 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, \textit{J} = 42 Hz), 160.3 (q, \textit{J} = 34 Hz), 168.3, 168.4, 172.4, 172.68, 172.71, 204.4; HRMS-EI (M+Na\textsuperscript{+}) calcd for C\textsubscript{20}H\textsubscript{29}N\textsubscript{3}NaO\textsubscript{3}S 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\textsubscript{2}O over 45 min) to afford inhibitor 3B (45 mg, 0.090 mmol, 82%). \textsuperscript{1}H NMR (400 MHz, MeOH-\textit{d}_4) \delta 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, \textit{J} = 5.2 Hz, 1H), 4.05-4.11 (m, 0.5H), 4.67 (dd, \textit{J} = 11.5, 5.3 Hz, 0.5H), 4.73 (dd, \textit{J} = 10.1, 5.8 Hz, 0.5H), 4.84 (dd, \textit{J} = 10.2, 5.2 Hz, 0.5H), 7.22-7.32 (m, 5H); \textsuperscript{13}C NMR
(75 MHz, MeOH-\textit{d}_4) \delta 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\textsubscript{2}Cl\textsubscript{2} before 1 mL of a solution containing 92.5% TFA, 2.5% TIS, 2.5% H\textsubscript{2}O 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\textsubscript{2}O) 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\textsubscript{2}Cl\textsubscript{2} before 1 mL of a solution containing 92.5% TFA, 2.5% TIS, 2.5% H\textsubscript{2}O 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\textsubscript{2}O) 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\textsubscript{2}Cl\textsubscript{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\textsubscript{2}Cl\textsubscript{2}/ NH\textsubscript{4}OH) to afford a mixture of diastereomers of amine 5 (0.11 g, 0.16 mmol, 59%). \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \delta 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); \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \delta 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 \( \text{C}_{27}\text{H}_{46}\text{N}_{4}\text{NaO}_{2} \) 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


Degrees

This research contributed toward the degree of PhD for the PI.
4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

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Department of Chemistry, Brown University
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Table of Contents Graphic for:

4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

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1 $R = (\text{CH}_2)_6\text{NH}_2$
2 $R = (\text{CH}_2)_6\text{NH}_2$
3 $R = \text{H}$
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. 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. 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. 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. 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. Most current pharmaceutical agents that are designed to inhibit plasmin are targeted to the lysine binding site. 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. 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.

[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. 13C NMR studies using a 13C-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. 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.
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 proteases but not cysteine proteases. Nitriles have the opposite specificity, while aldehydes and α-dicarbonyl compounds are good inhibitors of both classes of enzymes. 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.

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 precededent in peptoids. 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.
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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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)\textsubscript{2}O to give alcohol 9, followed by oxidation of the alcohol using pyridinium chlorochromate.
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). 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. 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 H₂O 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.
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 \( \mu M \). 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.\(^5\)

[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.\(^6\) 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 \( \mu M \). 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.\(^6\) 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. Papain has a relatively deep and narrow binding cleft 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, 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 3A 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 2A 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 3A 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, 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. 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 (δ = 0.00 ppm) for ¹H NMR and CDCl₃ (δ = 77.0 ppm) or CD₃OD (δ = 49.0 ppm) for ¹³C 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 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 MgSO₄ 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° (4.8 g, 17 mmol) dissolved in 2 mL of CH₂Cl₂. 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 NH₄OH, CH₃OH, CH₂Cl₂) affording 2.8 g (15 mmol, 89%) of the primary amine 5. ¹H NMR (300 MHz, MeOH-d₄) 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); ¹³C NMR (75 MHz, MeOH-
$d_4$ $\delta$ 25.9, 26.4, 28.5, 31.1, 57.9, 60.9, 61.1, 96.5; HRMS-EI (M$^+$) calcd for $C_{9}H_{15}NO_{2}S$ 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-$d_4$) $\delta$ 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-$d_4$) $\delta$ 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 $C_{30}H_{57}N_{3}NaO_{6}S$ 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$_2$O. 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$_2$O 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-$d_4$) $\delta$ 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-$d_4$) $\delta$ 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$_2$O 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 NaHCO₃ solution. The organic layer was washed with brine, dried over MgSO₄ 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%). ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) δ 25.4, 26.4, 26.5, 28.4, 30.0, 32.6, 40.0, 62.4, 79.1, 156.2; HRMS-Cl (M+H+) calcd for C₁₁H₂₄NO₃ 218.1756, found 218.1760.

**Aldehyde 10.** The alcohol 9 (7.2 g, 33 mmol) was added to a CH₂Cl₂ 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); ¹H NMR (250 MHz, CDCl₃) 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); ¹³C NMR (100 MHz, CDCl₃) δ 22.5, 27.1, 29.2, 30.7, 41.4, 44.5, 79.8, 156.8, 203.0; HRMS-Cl (M+H⁺) calcd for C₁₉H₂₂NO₂ 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 NaHCO₃ solution and extracted with EtOAc. The organic layer was dried over MgSO₄ and concentrated under reduced pressure. The crude product was purified by flash chromatography (2:1:7 EtOAc/ MeOH/ Et₂O) providing the secondary amine 11 (0.53 g, 1.40 mmol, 50%). ¹H NMR (300 MHz, MeOH-d₄) 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}\text{C} \text{NMR (75 MHz, MeOH-\text{d}_4)} \delta 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; \text{HRMS-FAB (M+Na}^+) \text{ calcd for } C_{19}H_{36}N_2NaO_4S 411.2294, \text{ 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\(_2\)Cl\(_2\). 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%). \(^1\text{H} \text{NMR (300 MHz, CDCl}_3\) \delta 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); \(^{13}\text{C} \text{NMR (75 MHz, CDCl}_3\) \delta 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; \text{HRMS-FAB (M+Na}^+) \text{ calcd for } C_{43}H_{55}N_3NaO_7S 780.3659, \text{ 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\(_2\)O. The organic layer was washed with 100 mL of H\(_2\)O, 50 mL of saturated KHSO\(_4\) solution, 50 mL of saturated Na\(_2\)CO\(_3\) 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%). ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₃₂H₃₄N₂NaOS 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 CH₂Cl₂/ 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: ¹H NMR (300 MHz, CDCl₃) 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), 2.91-3.19 (m, 2H), 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₂₈H₄₆N₃O₅S 536.3158, found 536.3163. 14B: ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₂₈H₄₆N₃O₅S 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 H₂O. The organic layer was washed with H₂O, dried over MgSO₄ and concentrated. The crude material was purified by flash chromatography (2.98 MeOH/CH₂Cl₂) 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: ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₁₇H₂₁N₂O₃S 337.1586, found 337.1579. 15B: ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₁₇H₂₁N₂O₃S 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-Issoleucine (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 H₂O. The organic layer was washed with H₂O, dried over MgSO₄ 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\(_3\)) \(\delta\) 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); \(^1^3\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 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\(_4\)NaO\(_8\)S 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\(_2\)Cl\(_2\) 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\(_3\)) \(\delta\) 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); \(^1^3\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 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, 137.0, 138.0, 155.9, 171.1, 173.1; HRMS-FAB (M+Na\(^+\)) calcd for C\(_{39}\)H\(_{64}\)N\(_4\)NaO\(_8\)S 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%). H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (100 MHz, CDCl₃) 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 C₂₈H₄₄N₃O₆S 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%). H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (100 MHz, CDCl₃) 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 C₂₈H₄₄N₃O₆S 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 Et₂O was added until the solution turned cloudy. The Et₂O was pipetted off and the oily residue further purified by RPHPLC (H₂O with 0.1 % TFA) to afford 53 mg (0.09 mmol, 99%) of inhibitor 1. H NMR (300 MHz, MeOH-d₄) 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$_2$O. After 18 h the TFA was removed under reduced pressure. The crude mixture was purified by reverse-phase HPLC (0%-50% MeCN/H$_2$O 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$_4$O$_3$S 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$_2$O 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$_4$O$_3$S 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$_2$O, 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$_2$Cl$_2$, concentrated NH$_4$OH) before the final purification was performed using RPHPLC (0%-100% MeCN/H$_2$O 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-$d_4$) $\delta$ 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-$d_4$) $\delta$ 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$_3$NaO$_3$S 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$_2$O 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-$d_4$) $\delta$ 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-$d_4$) $\delta$ 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$_3$NaO$_3$S 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/Et₂O) to afford the secondary amine 19 (1.3 g, 4.4 mmol, 25%). ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₁₇H₃₄N₂NaO₃ 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 CH₂Cl₂ 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 NaHCO₃ solution. The resultant organic layer was dried over MgSO₄ 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 H₂O. The organic layer was dried over MgSO₄ and concentrated. The crude material was purified by flash chromatography (2% MeOH/CH₂Cl₂) 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%). ¹H NMR (300 MHz, CDCl₃) 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); ¹³C NMR (75 MHz, CDCl₃) 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 C₂₆H₄₃N₃NaO₃ 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$_3$) $\delta$ 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$_3$) $\delta$ 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$_4$NaO$_6$ 681.4567, found 681.4550.

Amine 22. The Boc dipeptide 21 (180 mg, 0.27 mmol) was dissolved in 1 mL of CH$_2$C$_2$ 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$_2$C$_2$ / concentrated aqueous NH$_4$OH) to afford amine 22 (0.11 g, 0.16 mmol, 59%). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 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$_3$) $\delta$ 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$_4$NaO$_4$ 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. 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).
Acknowledgment. This research was supported by the NIH (Grant 1 R01 GM57327-01), the Petroleum Research Fund administered by the American Chemical Society (Grant 30544-G4), and the U.S. Army Medical Research and Materiel Command - Breast Cancer Research Initiative (Grant DAMD17-96-1-6161, Career Development Award to C.T.S.). T.C.S. was supported by a Predoctoral Fellowship from the U.S. Army Medical Research and Materiel Command - Breast Cancer Research Initiative (Grant DAMD17-96-1-6037).

Supporting Information Available: HPLC characterization for compounds 1, 2A, 3A 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.
References


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

<table>
<thead>
<tr>
<th>Compound $^a$</th>
<th>Plasmin</th>
<th>Trypsin</th>
<th>Thrombin</th>
<th>Kallikrein</th>
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<tr>
<td>1</td>
<td>400 ± 35</td>
<td>1,400 ± 110</td>
<td>&gt;10,000</td>
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<tr>
<td>2A</td>
<td>50 ± 5</td>
<td>1,700 ± 1,500</td>
<td>720 ± 550</td>
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<tr>
<td>2B</td>
<td>130 ± 10</td>
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<tr>
<td>3A</td>
<td>9,000 ± 1,000</td>
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<tr>
<td>3B</td>
<td>16,000 ± 1,300</td>
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<tr>
<td>22</td>
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</table>

$^a$ 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 = (CH₂)₆NH₂.

Scheme 1

" Reagents and conditions: (a) TFA, 85%; (b) 10, NaBH(OAc)₃, 22%; (c) TFA, H₂O, triisopropylsilane, thioanisole, 50%; (d) 6N HCl, 99%.

Scheme 2

" Reagents and conditions: (a) (Boc)₂O, 88%; (b) pyridinium chlorochromate, 91%.

Scheme 3

" 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, H₂O, TIS, thioanisole, 2A 50%, 2B 9%, 3A 34%, 3B 82%. A and B represent two different diastereomers.

Scheme 4

" 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.
Structures of Inhibitors 1 - 3.

1 \( R = (\text{CH}_2)_6\text{NH}_2 \)

2 \( R = (\text{CH}_2)_6\text{NH}_2 \)

3 \( R = \text{H} \)
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. $R = (\text{CH}_2)_6\text{NH}_2$. 
Scheme 1

\[ \text{Reagents and conditions: (a) TFA, 85%; (b) 10, NaBH(OAc)₃, 22%; (c) TFA, H₂O, triisopropylsilane, thioanisole, 50%; (d) 6N HCl, 99%}. \]
Scheme 2

\[ \text{H}_2\text{N(CH}_2\text{)}_6\text{OH} \xrightarrow{a} \text{BocHN} - \text{OH} \]

\[ \xrightarrow{b} \text{BocHN} - \text{CHO} \]

* Reagents and conditions: (a) (Boc)_2O, 88%; (b) pyridinium chlorochromate, 91%.
Scheme 3

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$_2$O, TIS, thioanisole, 2A 50%, 2B 9%, 3A 34%, 3B 82%. A and B represent two different diastereomers.
Scheme 4

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%.
GRANT NUMBER DAMD17-96-1-6037

TITLE: A New Class of Serine and Cysteine Protease Inhibitor with Chemotherapeutic Potential

PRINCIPAL INVESTIGATION: Tanya C. Sanders

CONTRACTING ORGANIZATION: Brown University
Providence, Rhode Island 02912

REPORT DATE: June 1999

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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