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Metastasis and angiogenesis in cancer are dependent on a variety of biologically active compounds including serine proteases. 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 diamine or a lysine analog diamino acid side chain was undertaker. The diamine and diamino acid inhibitors have inhibition constants of 500 µM and 40 µM respectively. Synthesis of a piperidone derivative inhibitor was completed.

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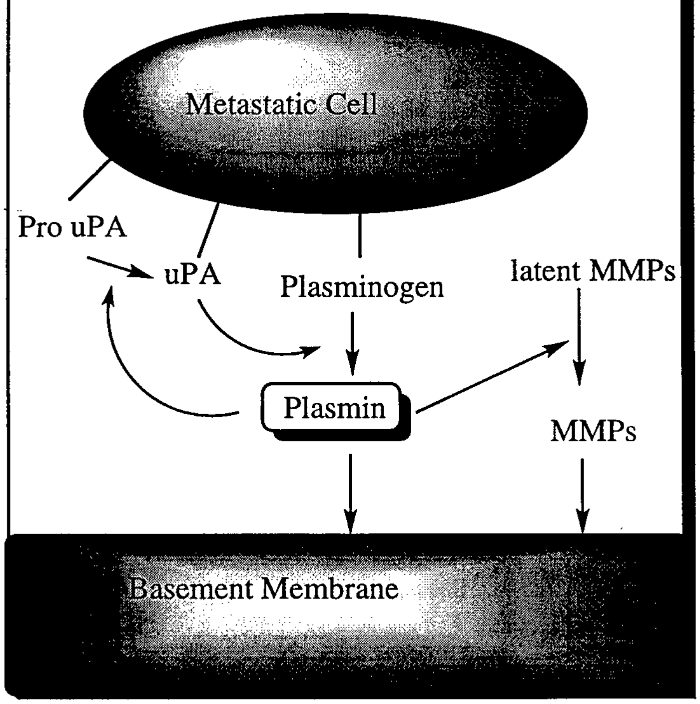
Introduction

Angiogenesis and metastasis are attracting attention as 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 extention of the epithelial cells into the surrounding tissue and formation of new blood vessels.[3] During metastasis the cancerous cell itself becomes inplanted 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 proliferation or 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 both activation of MMPs and degradation of fibrin or collagen in the basement membrane (Figure 1).[3] Current pharmaceuticals aimed at deactivating plasmin are dependant on inhibiting its fibrinolytic activity by blocking the lysine binding site.[5] In this case plasminogen, the inactive precursor of plasmin, is restricted from binding with fibrin and being 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.[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 reversable inhibitor of the cysteine protease papain which we determined does 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. Primary recognition is between enzyme and inhibitor provided by the addition of a Iysine side chain analog at the P_1 site. Secondary recognition involve phenyalanine in the P_2 site and D-isoleucine in the P_3 site. 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. 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.

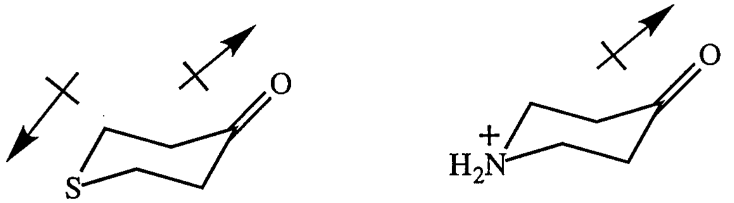


Figure 2. dipole-dipole and dipole-charge repulsive interactions.

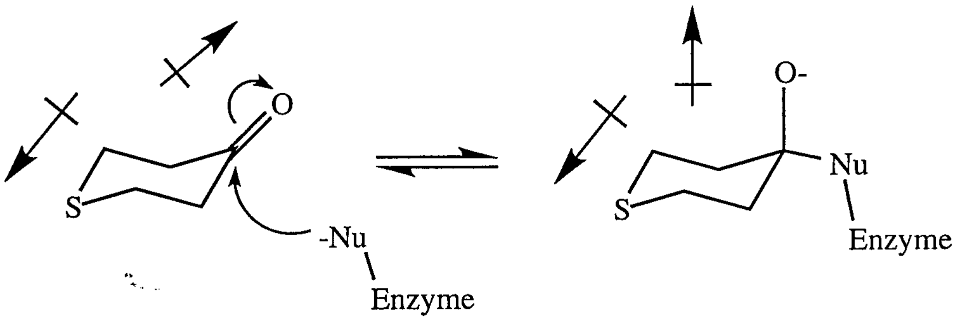


Figure 3. Nucleophilic attack on carbonyl forms tetrahedral intermediate.

The main function of the parent ring structure is the positioning of the heteroatom gamma to the carbonyl. Preliminary molecular modeling shows no apparent unfavorable steric interactions with the ring which extends from the catalytic triad out into the active site cleft. 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 create steric interference to addition of the actove 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. We chose phenylalanine and D-isoleucine as the secondary recognition residues based upon a study by Okada et al.[5] Altogether the general structure of the inhibitor consists of a

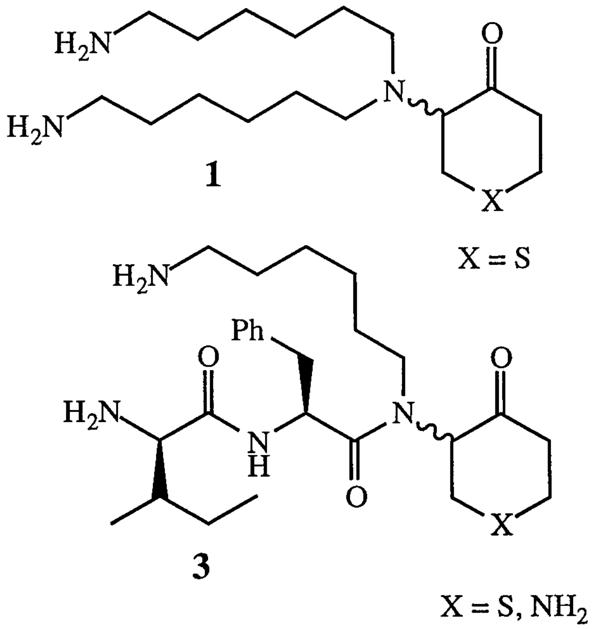


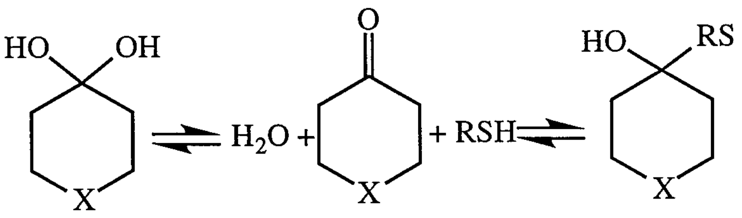
Figure 4. General structure of plasmin inhibitors

tetrahydrothiopyranone ring appended with either two aminoalkyl chains (1) or one aminoalkyland one peptide chain containing phenylalanine and D-Isoleucine (2, Figure 4).

Preliminary Results

To investigate the influence of the heteroatom in the parent ring structure, equilibrium constant experiments were undertaken. The addition of both water (K_{H20}) and 3-mercaptopropionic acid (K_{RSH}) were measured as mimics of the nucleophiles present in serine and cysteine proteases respectively (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

Table 1 Hydration Constants

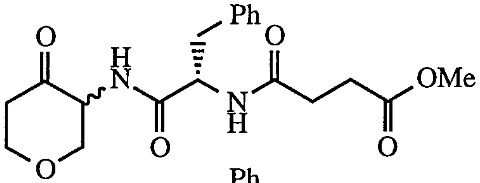
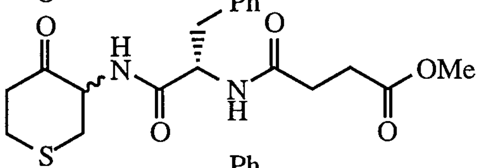
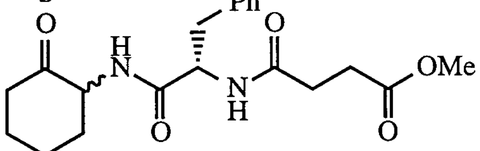
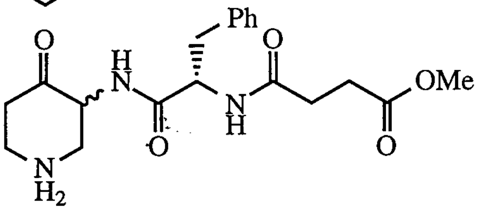
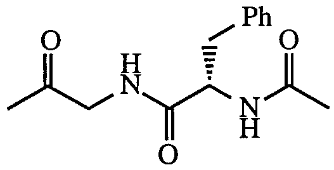


X	$K_{H2O}(M^{-1})$	$K_{RSH}(M^{-1})$
CH_2	8.1×10^{-4}	0.22
S	9.0×10^{-3}	1.5
O	8.0×10^{-3}	1.8
NH_2^+	0.18	27.6
SO	0.068	11.7
SO_2	0.30	60.2

formed during enzymatic hydrolysis of an amide bond.

A series of papain inhibitors consisting of the cyclohexanone, tetrahydropyranone, tetrahydrothiopyranone and piperidone were synthesized to test this hypothesis. The compounds were good inhibitors of papain (K_i =10-120 μM). 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

Table 2

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

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

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. With the 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

Month 1-3

Continuing with the final synthesis of the initial dialkyl chain compounds, the deprotection and purification of the six carbon analog (compound 1) were worked out. The best deprotection conditions were determined to be 6M HCl in MeOH. Purification was provided by reverse phase HPLC using a C-18 column with a gradient eluent ranging from 0%-100% MeCN in water. Compound I was assayed against plasmin, thrombin, kallikrein and trypsin (Table 3).[11] Oxidation of the sulfide inhibitor with PCC led to

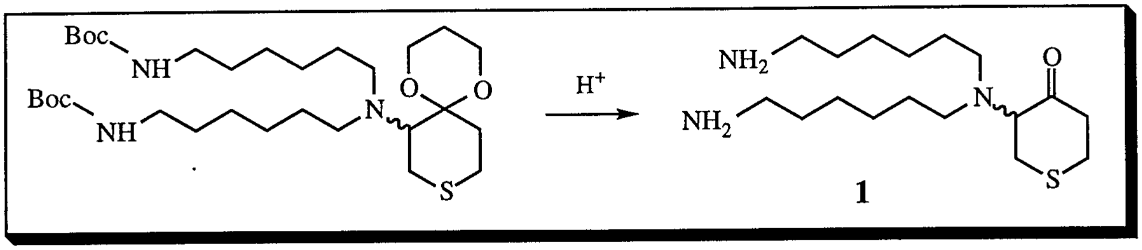
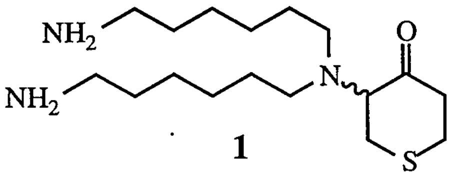
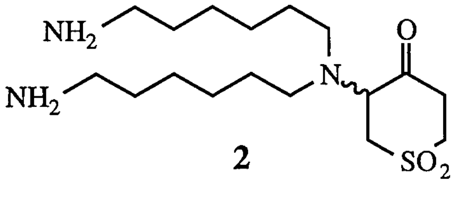


Table 3	Ki (µM)			
	Plasmin	Thrombin	Kallikrein	Trypsin
 **1**	500	25,000	25,000	1700
 **2**	510			

the sulfone (compound **2**). An assay of sulfone **2** against plasmin resulted in a K_i of 510 μM. Based on the hydration, constants the inhibition constant of the sulfone should be considerably lower than its sulfide equivalent. The similarity between the two lead us to believe there may not be a correct alignment of the inhibitor in the enzyme active site, preventing formation of the covalent intermediate. In a paper by Okada, a plasmin substrate did not undergo cleavage when only a lysine residue was used for recognition.[5] The attachment of a phenylalanine in the secondary recognition site allowed natural cleavage to procede implying the molecule was now correctly positioned within the active site.[5] Attaching the two amino acids, phenylalanine and D-isoluceine, to the secondary recognition chain of our inhibitor was a potential solution to the problem.

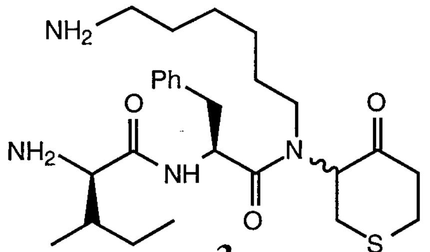
Month 4-6

To produce the monoalkylated amine, a necessary precursor of the diamino acid inhibitor **3**, we had to turn back to the reductive amination (Scheme 1). We used sodium triacetoxyborohydride, a reducing agent specific to imines, which lead to the conversion of the primary amine **10** to the secondary amine **11**. Acid fluoride coupling lead to the Fmoc ketal **12**. Deprotection with piperidine gave the amino ketal **13.** A tradition coupling using HOBT, EDC and 4-methylmorpholine afforded the Boc ketal **14.**

Month 7-9

Deprotection of the Boc ketal **14** was accomplished using transketalization chemistry. Dry *p*-TsOH in acetone provided the sulfide inhibitor **3**. Final purification and separation of the two diastereomers was accomplished with reverse phase HPLC (C-18 column, 0%-100% MeCN/H_2O over 45 min). The inhibitor **3** was assayed against

Table 4



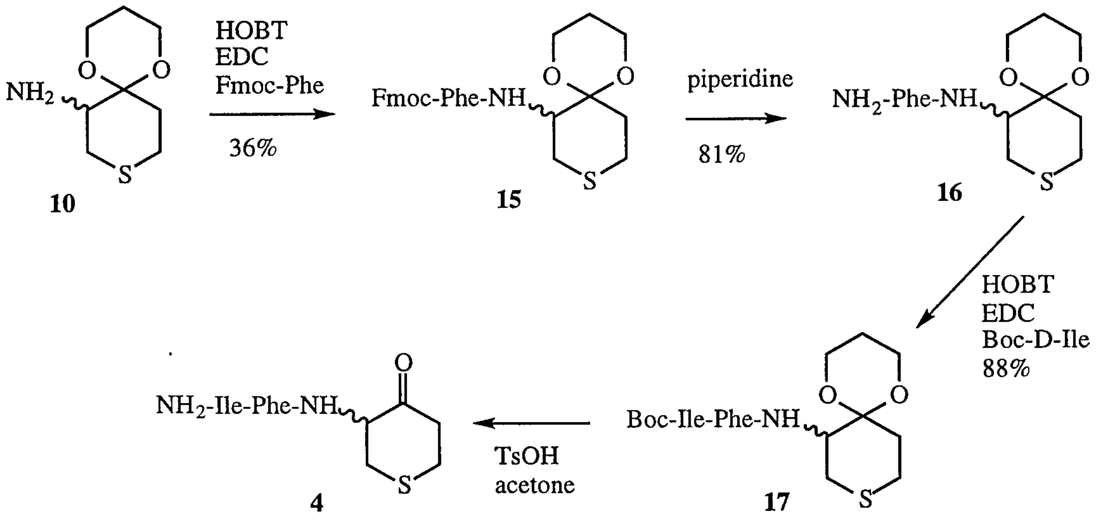
3

* more potent diastereomer

K_i (μM)			
Plasmin	Thrombin	Kallikrein	Trypsin
40*	1100*	1300*	2500*
170			

plasmin, kallikrein, thrombin and trypsin (Table 4). A decrease in the K_i from the dialkyl chain inhibitor **1** (500 μM) to the diamino acid inhibitor **3** (40 μM) illustrates the importance of the amino acids in the binding of the inhibitor. The sulfide compound **3** may now be lining up correctly in the active site leading to the covalent binding on which these inhibitors are dependent. Inhibitor **3** exhibits selectivity for plasmin over thrombin by 28 times and kallikrein by 33 times. Synthesis of a control compound without the lysine

Scheme 2



side chain on the tertiary amide was undertaken (Scheme 2). Traditional coupling using HOBT, EDC chemistry led to the Fmoc ketal **15**. After removal of the Fmoc with piperidine to give the amino ketal **16**, the diastereomers were separated by flash chromatography. A second traditional coupling under similar conditions led to the Boc ketal **17**.

Month 10-12

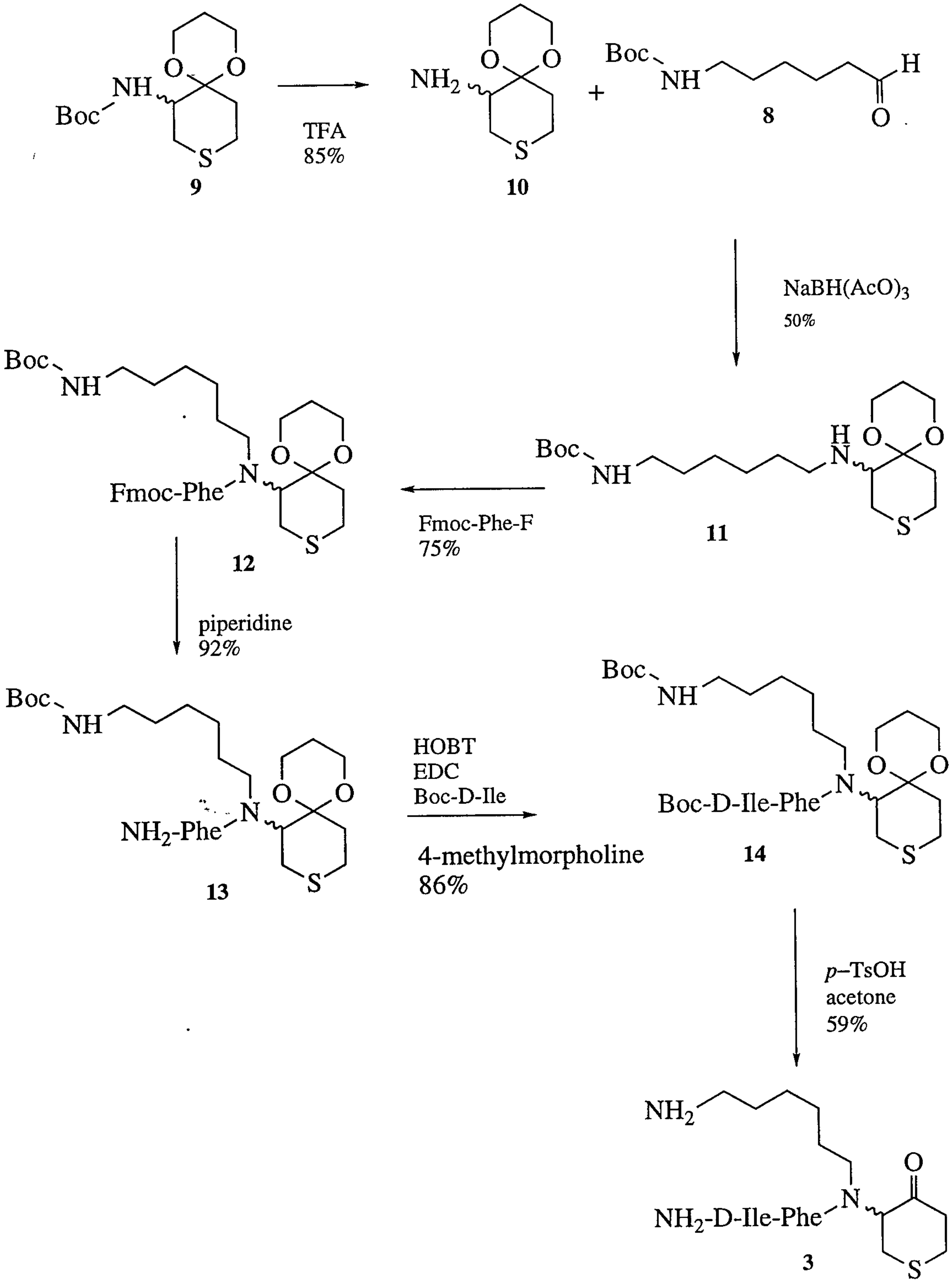
The final deprotection of the control compound was attempted using *p*-TsoH in acetone. It was discovered at this point that the transketalization using ten equivalents of acid was causing racemization of the amino acids present. We are currently experimenting to determine if lowering the concentration of *p*-TsOH to 0.5 equivalents will solve the racemization problem with the synthesis of inhibitor **4**.

Synthesis of the piperidone inhibitor was also undertaken during this period (Scheme 3). The hydrochloride salt **18** was treated with benzyl chloroformate to give the Cbz piperidone **19**. Compound **19** was protected to form the ketal **20** before being hydrolyzed with NaOH to form the acid **21**. A Curtius rearrangement of the acid **21** provided the carbamate **22**. Deprotection of the carbamate **22** led to the amino ketal **23** which when combined with aldehyde **8**, underwent reductive amination in the presence of triacetoxyborohydride to provide the secondary amine **24**. Acid fluoride coupling provided the Fmoc phenylalanine ketal **25,** followed by a piperidine deprotection to produce the amino ketal **26**. The final coupling was achieved with an HOBT, EDC peptide coupling to produce the Boc ketal **27**. Removal of the ketal and Boc groups was acheived with *p*-TsOH and acetone to produce compound **28**. After the final deprotection using palladium and hydrogen, inhibitor **5** was purified by RPHPLC. The diastereomers were not separated due to a racemization potential with the piperidone derivatives under assay conditions. The assay performed with the piperidone inhibitor against plasmin showed unexpected results and will have to be studies more thoroughly at a future date.

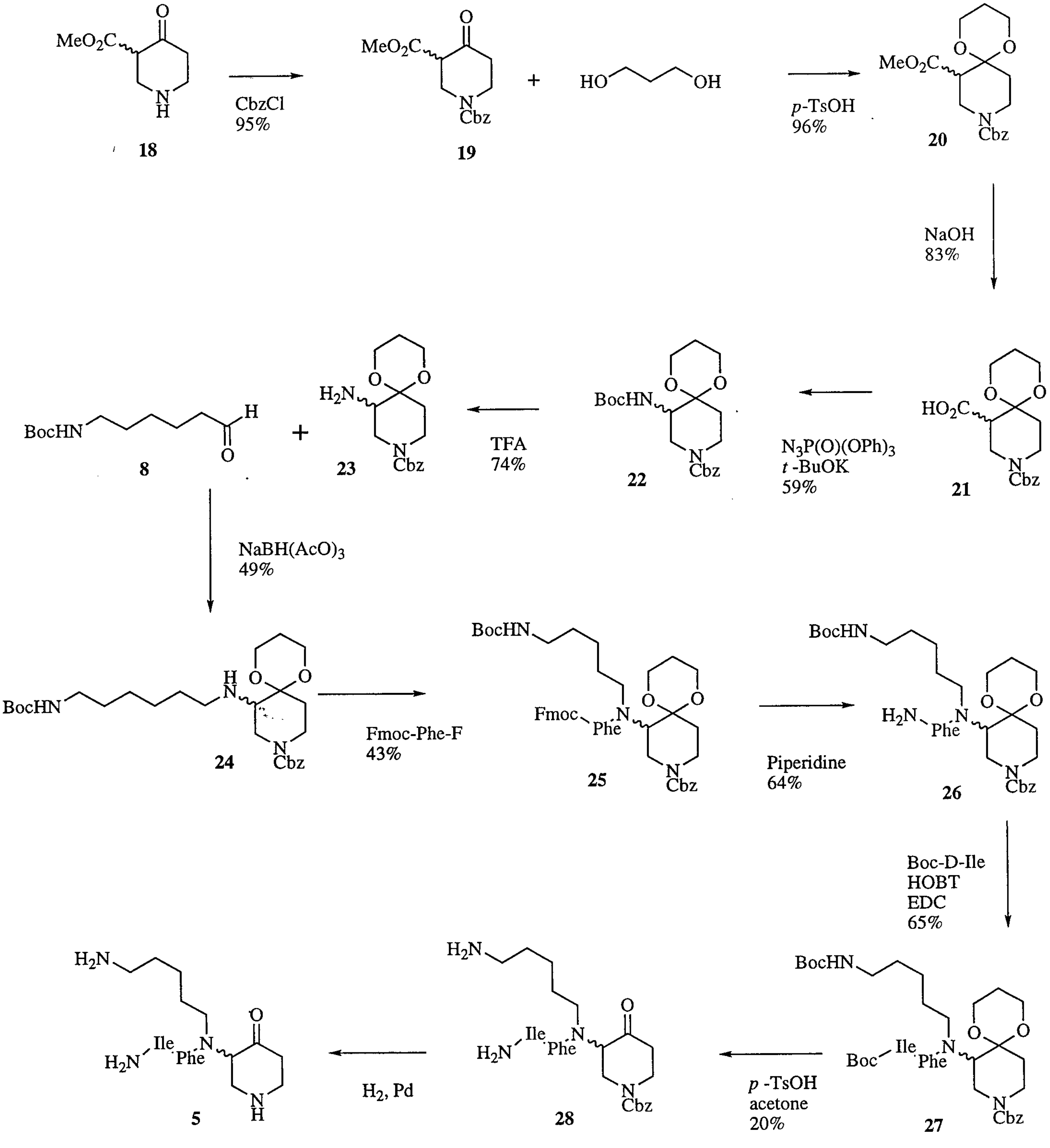
Summary

We have completed the synthesis of the sulfide plasmin inhibitor **3**. However, we will have to determine whether racemization of the amino acid side chain has taken place. The control compound to test the necessity of the lysine recognition chain is essentially done with just the assays to be completed. The piperidone inhibitor was assayed once with unexpected results, and will have to be more thoroughly investigated.

Scheme 1


9
TFA
85%
10
+
8
NaBH(AcO)$_3$
50%
11
Fmoc-Phe-F
75%
12
piperidine
92%
13
HOBT
EDC
Boc-D-Ile
4-methylmorpholine
86%
14
p–TsOH
acetone
59%
3
Boc
NH
NH$_2$
Fmoc-Phe
NH$_2$-Phe
Boc-D-Ile-Phe
NH$_2$-D-Ile-Phe

Scheme 3


MeO2C
O
N
H
18
CbzCl
95%
19
Cbz
+
HO
OH
p-TsOH
96%
20
NaOH
83%
HO2C
21
N3P(O)(OPh)3
t-BuOK
59%
BocHN
22
TFA
74%
H2N
23
BocHN
H
O
8
+
NaBH(AcO)3
49%
24
Fmoc-Phe-F
43%
Fmoc
Phe
25
Piperidine
64%
H2N
Phe
26
Boc-D-Ile
HOBT
EDC
65%
Boc
Ile
27
p-TsOH
acetone
20%
H2N
Ile
28
H2, Pd
5

Procedures

General Methods. NMR spectra were recorded on Brucker 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) 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 angstrom molecular sieves. All other reagents were used as received. Organic solutions were dried with $MgSO_4$ unless otherwise noted. Solvent removal was performed by rotary evaporation at water aspirator pressure.

Alcohol 7

6-Amino-1-hexanol **6** (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 then added and the reaction mixture was allowed to warm to room temperature and then 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 **7** (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 8

The alcohol **7** (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 **8**: 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.

Primary amine 10

Trifluoroacetic acid (TFA, 25 mL) was added to a solution of the carbamate **9** (4.6 g, 16 mmol) dissolved in CH_2Cl_2. After stirring at room temperature for 2 h the solvent was evaporated under reduced pressure. The remaining material was partitioned between 10 mL of EtOAc and 20 mL of 1N HCl. After separation, the aqueous layer was made basic with 1N NaOH and extracted 6 times with 50 mL of CH_2Cl_2. The combined CH_2Cl_2 layers were dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was recrystallized from CH_2Cl_2 affording 2.6 g (14 mmol, 85%) of the primary amine **10**. ^{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.

Secondary amine 11

Aldehyde **8** (0.52 g, 2.4 mmol) was dissolved in 2 mL of 1,2-dichloroethane (DCE) and added to a solution of primary amine **10** (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 allowed to stir for an additional 3 h at 25°C. 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-EI Calcd for $C_{19}H_{36}N_2NaO_4S$ ($M+Na^+$) 411.2294 found 411.2306.

Fmoc ketal 12

Fmoc-Phe-F (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 1N NaOH (10 mL), 1N HCl (15 mL) and saturated $NaHCO_3$ solution (15 mL). 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-EI ($M+Na^+$) calcd for $C_{43}H_{55}N_3NaO_7S$ 780.3659, found 780.3663.

Amino ketal 13

A DMF solution (10 mL) of Fmoc ketal **12** (0.38 g, 0.50 mmol) and piperidine (0.30 mL, 3.0 mmol) was stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure and t crude material was purified by flash chromatography (98:2 CH_2Cl_2) to give a mixture of two diastereomers of amino ketal **13** (0.25 g, 0.46 mmol, 92%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.05-2.04 (m, 23H), 2.35 (m, 1H), 2.62-4.11 (m, 14H), 4.59 (bs, 1H), 7.16-7.33 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.3, 25.5, 26.8, 26.85, 26.90, 27.5, 28.0, 28.8, 29.4, 29.5, 30.4, 31.8, 40.9, 42.6, 43.1, 45.0, 45.2, 53.2, 53.8, 59.1, 59.3, 59.4, 59.7, 62.9, 63.4, 79.0, 97.8, 97.9, 126.6, 126.8, 128.7, 128.8, 128.9, 129.7, 129.75, 129.82, 138.3, 139.4, 156.4, 175.7, 176.5, 177.6; HRMS-EI ($M+Na^+$) calcd for $C_{28}H_{45}N_3NaO_5S$ 558.2978, found 558.2973.

Boc ketal 14

A DMF solution (10 mL) containing HOBT (0.062 g, 0.46 mmol), EDC (0.088 g, 0.46 mmol) and Boc-D-Ile (0.092 g, 0.46 mmol) was stirred at room temperature for 30 min. A solution of the amino ketal **13** (0.25 g, 0.46 mmol) and 4-methylmorpholine (0.10 mL, 0.92 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 (1:1 EtOAc/ hexanes) afforded a mixture of two diastereomers of Boc ketal **14** (0.28 g, 138 mmol, 81%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.63-1.86 (m, 39H), 2.26-2.32 (m, 1H), 2.60-4.13 (m, 13H), 4.57 (m, 1H), 4.92-5.28 (m, 2H), 6.40-6.87 (m, 1H), 7.16-7.28 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.6, 15.2, 15.5, 15.6, 15.7, 24.7, 24.8, 25.0, 26.5, 27.2, 27.3, 28.1, 28.3, 28.5, 29.2, 30.0, 30.1, 31.0, 31.2, 38.0, 38.2, 40.2, 40.6, 41.0, 41.1, 45.0, 50.3, 50.8, 51.0, 58.8, 58.9, 59.0, 59.2, 63.0, 79.0, 79.7, 97.1, 97.6, 126.8, 127.0, 128.3, 128.4, 128.5, 128.7, 129.4, 129.7, 129.8, 136.4, 155.6, 169.9, 172.6, 172.7; HRMS-EI ($M+Na^+$) calcd for $C_{37}H_{64}N_4NaO_8S$ 771.4343, found 771.4334.

Fmoc ketal 15

A DMF solution (75 mL) containing HOBT (0.37 g, 2.8 mmol), EDC (0.69 g, 3.6 mmol) and Fmoc-phe (1.1 g, 2.8 mmol) was stirred at room temperature for 1 h. A solution of the primary amine **10** (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 $MgSO_4$, concentrated under reduced pressure. Flash chromatography (1:1 EtOAc/ hexanes) afforded a mixture of two diastereomers of Fmoc ketal **15** (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 16

A solution of piperidine (0.6 mL, 6.0 mmol) and Fmoc ketal **15** (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 chromotography (2:98 MeOH / CH_2Cl_2) to afford the two separate diastereomers of amino ketal **16a** (0.17 g, 0.50 mmol) and **16b** (0.11 g, 0.32 mmol) in a combined yield of 81%. For **16a**: ^{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); **16b**: ^{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); **16a+16b**: ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.6, 25.1, 30.8, 32.7, 41.1, 46.9, 47.2, 56.5, 56.7, 59.2, 59.27, 59.31, 96.2, 126.7, 126.8, 128.7, 129.4, 137.9, 138.0, 173.8, 173.9; HRMS-FAB (M+Na$^+$) calcd for $C_{17}H_{24}N_2NaO_3S$ 359.1406, found 359.1412.

Boc ketal 17a & 17b

Compound **17a** (156 mg, 0.28 mmol, 88%) was prepared from Compound **16a** (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 discribed in **14**. The crude material was purified by flash chromatography (4:1 EtOAc/ hexanes). **17a**: ^{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).

Compound **17b** (148 mg, 0.27 mmol, 86%) was prepared from Compound **16b** (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 discribed in **14**. The crude material was purified by flash chromatography (4:1 EtOAc/ hexanes). **17b**: ^{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).

Inhibitor 3

To a solution of Boc ketal **14** (82 mg, 0.11 mmol) in 10 mL of acetone-d_6 was added dry *p*-toluenesulfonic acid (TsOH, 190 mg, 1.10 mmol). The reaction was stirred at room temperature for 12 h and the solvent was removed under reduced pressure. The crude material was partially purified by flash chromatography (1:10:89 30% aqueous NH_4OH/ MeOH/ CH_2Cl_2). Final purification and separation of diastereomers was accomplished by

RPHPLC (0%-50% gradient of MeCN in H_2O with 0.1% TFA over 45 min) to afford 15 mg (0.030 mmol) of the earlier eluting diastereomer **3a** and 17 mg (0.034 mmol) of the later eluting diastereomer **3b** for a combined yield of 59%. **3a**: ^1H NMR (300 MHz, MeOH-d_4) δ 0.6–1.1 (m, 7H), 1.2-1.8 (m, 11H), 2.6-3.3 (m, 10H), 3.3-3.5 (m, 1H), 4.1 (dd, *J* = ??, 1H), 5.1 (dd, *J* = ??, 1H), 7.1-7.3 (m, 5H); ^{13}C NMR (75 MHz, MeOH-d_4) δ 11.7, 14.8, 24.9, 27.2, 28.5, 30.6, 32.2, 37.8, 38.7, 40.6, 44.8, 50.3, 52.5, 59.0, 67.7, 128.2, 129.8, 130.3, 138.0, 169.2, 172.7, 203.6. 2nd diastereomer ^1H NMR (300 MHz, MeOH-d_4) δ 4.08 (dd, *J* = 11.1, 6.3 Hz, 1H), 5.08 (dd, *J* = 9.0, 5.7 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.6, 14.9, 25.0, 27.1, 27.2, 28.4, 28.5, 30.3, 31.9, 37.7, 39.4, 40.6, 44.8, 50.4, 52.4, 59.0, 67.4, 128.3, 128.8, 130.4, 137.8, 169.1, 172.3, 204.1.

Cbz piperidone 19

The hydrochloride salt **18** (10 g, 52 mmol)was dissolved in 400 mL of CH_2Cl_2 containingtTriethylamine (TEA, 14 mL, 100 mmol) . After cooling the mixture to 0°C, benzyl chloroformate (8.8 mL, 62 mmol) was added and the reaction allowed to stir for 2 h. The reaction was then quenched with 3.1 N AcOH and washed with a saturated solution of $NaHCO_3$. After drying over $MgSO_4$ and concentrating the crude mixture was purified by flash chomotography (1:4 EtOAc/ hexanes) affording the Cbz piperidone **19** (14 g, 49 mmol, 95%). Predominantly enol form: ^1H NMR (400 MHz, $CDCl_3$) δ 2.39 (bs, 2H), 3.64 (t, *J* = 5.8 Hz, 2H), 3.77 (s, 3H), 4.11 (s, 2H), 5.17 (s, 2H), 7.33 (m, 5H), 11.98 (s, 1H); keto and enol forms both visible: ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.7, 39.9, 40.4, 43.5, 45.6, 51.5, 52.3, 56.1, 67.3, 67.8, 95.5, 127.9, 128.0, 128.2, 128.4, 136.1, 136.5, 155.2, 168.1, 169.5, 170.1, 170.8; HRMS-CI (M+H$^+$) calcd for $C_{15}H_{18}NO_5$ 292.1185, found 292.1189.

Ketal 20

The Cbz piperidone **19** (14 g, 47 mmol), propylene glycol (7 mL, 94 mmol) and *p*-toluenesulfonic acid (*p*-TsOH, 1.7 g, 9.0 mmol) were dissolved in 300 mL of benzene. The reaction was refluxed in an apparatus fitted with a Dean-Stark trap for azeotropic removal of water. After 24 h the reaction was quenched with saturated $NaHCO_3$ solution. The organic layer was dried over $MgSO_4$ and concentrated before purification of the crude product by flash chromotography (2:3 EtOAc/ hexanes) afforded the ketal **20** (16 g, 45 mmol, 96%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.63 (s, 1H), 1.80-1.89 (bm, 2H), 2.41 (ddd, J = 14.1, 9.9, 4.4 Hz, 1H), 2.95-3.06 (m, 1H), 3.31 (ddd, J = 13.3, 10.1, 3.4 Hz, 1H), 3.47 (bt, J = 13.1 Hz, 1H), 3.59-3.66 (m, 3H), 3.80-4.06 (m, 6H), 5.09 (m, 2H), 7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.1, 28.4, 29.2, 40.5, 42.6, 46.8, 47.7, 51.7, 59.4, 59.5, 67.1, 96.3, 127.8, 127.9, 128.4, 129.8, 136.8, 155.2, 170.4; HRMS-FAB (M+Na$^+$) calcd for $C_{18}H_{23}NNaO_6$ 372.1423, found 372.1417.

Carboxylic Acid 21

The ketal **20** (15 g, 44 mmol) was dissolved in 400 mL of MeOH and 60 mL of 1N NaOH. After heating at reflux for 42 h the reaction was diluted with 200 mL of H_2O before the MeOH was evaporated under reduced pressure. The resultant aqueous solution was extracted with EtOAc to remove the neutral impurities before being acidified with 1N HCl. A second extraction with EtOAc was performed to recover the carboxylic acid. The second organic layer was dried over $MgSO_4$ and concentrated. The carboxylic acid **21** (12 g, 36 mmol, 83%) was used without further purification: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.57 (m, 2H), 2.03 (bm, 1H), 2.62 (bm, 1H), 2.77 (bm, 1H), 3.21 (bm, 1H), 3.49 (bm, 1H), 3.74-4.12 (m, 6H), 5.15 (s, 2H), 7.32 (m, 5H) 8.97 (bs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.7, 27.7, 40.3, 41.9, 49.1, 59.6, 60.0, 67.3, 97.2, 127.8, 127.9, 128.4, 136.4, 155.1, 171.2; HRMS-FAB ($M+Na^+$) calcd for $C_{17}H_{21}NNaO_6$ 358.1267, found 358.1268.

Carbamate 22

A benzene solution (600 mL) containing diphenylphosphorylazide (9.0 mL, 42 mmol), TEA (7.0 mL, 50 mmol) and the carboxylic acid **21** (13 g, 38 mmol) was heated at reflux for 3 h. To follow the progress of the reaction aliquots of the solution were monitored by FTIR for the disappearance of an azide peak at 2171 cm^{-1} and the appearance of an isocyanide peak at 2244 cm^{-1}. The reaction was then added slowly to a 0°C solution of THF (200 mL) containing potassium-*t*-butoxide (21 g, 190 mmol). After 30 min the mixture was quenched with 2.2 N AcOH. The organic layer was washed with saturated $NaHCO_3$ solution, dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography (1:1 EtOAc/ hexanes) providing the carbamate **22** (9.1 g, 22 mmol, 59%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.32-1.81 (m, 11H), 2.24 (bm, 1H), 3.35-3.93 (m, 9H), 4.86 (d, $J = 8.1$ Hz, 1H), 5.12 (m, 2H), 7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.1, 28.0, 28.3, 29.2, 40.3, 45.1, 50.4, 59.4, 59.5, 67.1, 79.4, 96.0, 127.7, 127.9, 128.4, 136.6, 155.4, 155.6; HRMS-FAB ($M+Na^+$) calcd for $C_{21}H_{30}N_2NaO_6$ 429.2002, found 429.2005.

Amino Ketal 23

Carbamate **22** (8.6 g, 21 mmol) was dissolved in 400 mL CH_2Cl_2 before TFA (30 mL, 400 mmol) was added. After 1h the solvent was evapoated under reduced pressure. The resultant material was partitioned between 100 mL of CH_2Cl_2 and 10 mL of 1N NaOH. The crude product was purified by flash chromotography (1:10:89 NH_4OH/

MeOH/ CH_2Cl_2) provided the amino ketal **23** (4.6 g, 15.6 mmol, 74%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40-2.00 (bm, 3H), 2.40-4.34 (bm, 10H), 5.02-5.16 (m, 2H), 7.28-8.00 (bm, 7H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.6, 26.7, 40.3, 42.4, 52.7, 52.9, 53.4, 59.8, 59.9, 66.4, 67.7, 94.4, 119.0, 128.0, 128.5, 136.3, 155.3, 162.3, 162.7, 187.3; HRMS-FAB (M+Na$^+$) calcd for $C_{16}H_{22}N_2NaO_4$ 329.1447, found 329.1475.

Secondary Amine 24

The amino ketal **23** (0.54 g, 1.8 mmol) and the aldehyde **8** (0.34 g, 1.6 mmol) were dissolved in 4 mL of dichloroethane (DCE) before sodium triacetoxyborohydride (0.5 g, 2.5 mmol) was added. After 4h the reaction was 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 chromotography (2:1:7 EtOAc/ MeOH/ Et_2O) to afford the secondary amine **24** (0.44 g, 0.86 mmol, 49%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25-1.64 (m, 20H), 1.95 (bm, 1H), 2.35 (bm, 1H), 2.73-2.83 (bm, 3H), 3.07 (bq, *J* = 5.8 Hz, 2H), 3.38-3.69 (bm, 5H), 3.90 (bm, 4H), 4.60 (bs, 1H), 5.12 (s, 2H), 7.32 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 18.9, 25.5, 26.5, 26.8, 27.6, 28.4, 29.2, 29.9, 40.5, 43.3, 48.0, 59.4, 59.5, 67.2, 77.3, 79.0, 97.0, 127.8, 128.0, 128.5, 136.8, 155.6, 156.1.

Fmoc Ketal 25

Compound **25** (0.21 g, 0.24 mmol, 43%) was prepared from compound **24** (0.28 g, 0.55 mmol), Fmoc-phe-F (0.86 g, 2.2 mmol) and diisopropylethylamine (0.38 mL, 2.2 mmol) by the method discribed in **12**. The crude product was purified by flash chromatography (4:1 hexanes/ EtOAc). **25**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23-2.05 (m, 20H), 2.93-3.25 (m, 8H), 3.80-4.43 (m, 10H), 5.17 (m, 4H), 5.61-5.90 (m, 1H), 7.19-

7.78 (m, 18H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 14.2, 21.0, 24.7, 25.0, 25.2, 26.0, 26.4, 26.6, 26.7, 27.7, 28.1, 28.41, 28.44, 29.0, 29.7, 30.0, 31.6, 38.9, 40.3, 40.5, 40.6, 40.9, 43.6, 44.8, 45.1, 46.6, 47.1, 47.2, 52.3, 52.5, 52.7, 52.9, 55.2, 59.3, 59.4, 60.3, 66.6, 67.0, 67.3, 67.5, 77.6, 78.9, 97.1, 97.5, 98.1, 119.5, 120.0, 125.1, 125.2, 126.8, 126.97, 127.02, 127.6, 127.9, 128.0, 128.2, 128.3, 128.4, 128.47, 128.53, 129.5, 135.9, 136.3, 136.6, 136.7, 141.2, 141.3, 143.8, 143.9, 143.96, 144.01, 155.09, 155.14, 155.5, 155.6, 156.0, 172.6, 173.3; HRMS-FAB (M+Na$^+$) calcd for $C_{51}H_{62}N_4NaO_9$ 897.4415, found 897.4410.

Amino Ketal 26

Compound **26** (0.1 g, 0.15 mmol, 64%) was prepared from compound **25** (0.21 g, 0.24 mmol) and piperidine (0.14 mL, 1.4 mmol) by the method discribed in **13**. The crude product was purified by flash chromatography (5% MeOH/ CH_2Cl_2). **26**: ^{1}H NMR (300 MHz, CDCl$_3$) δ 1.32-2.17 (m, 23H), 2.69-3.13 (m, 8H), 3.52-4.00 (m, 8H), 4.58-4.81 (m, 1H) 5.21 (m, 2H), 7.13-7.35 (m, 10H); HRMS-FAB (M+Na$^+$) calcd for $C_{36}H_{52}N_4NaO_7$ 675.3734, found 675.3732.

Boc Ketal 27

Compound **21**(76 mg, 0.088mmol, 65%) was prepared from compound **26** (100 mg, 0.15 mmol), HOBT (21 mg, 0.15 mmol), EDC (38 mg, 0.20 mmol), Boc-D-Ile (35 mg, 0.15 mmol) and 4-methylmorpholine (0.03 mL, 0.30 mmol) by the method discribed in **14**. The crude product was purified by flash chromatography (1:1 EtOAc/ hexanes)

Cbz piperidone 28

Compound **28** (14 mg, 0.016 mmol, 20%) was prepared from compound **27** (74 mg, 0.085 mmol), *p*-TsOH (150 mg, 0.85 mmol) by the method discribed in **3**. Purification and was accomplished by RPHPLC (0%-50% gradient of MeCN in H_2O with 0.1% TFA present over 45 min). **28**: ^{13}C NMR (75 MHz, MeOH-d_4) δ 12.1, 15.5, 25.3, 26.6, 27.3, 27.5, 28.8, 33.3, 38.5, 38.6, 40.9, 42.4, 43.0, 46.9, 47.7, 55.8, 59.4, 60.6, 61.7, 69.4, 69.7, 95.5, 95.9, 128.3, 129.6, 129.8, 129.95, 130.04, 130.6, 138.1, 138.7, 162.1, 170.0, 174.4.

Piperidone Inhibitor 5

Palladium on carbon (5%, twice rinsed with EtOH) was diluted in 5 mL of EtOH before the Cbz protected amine **28** (14 mg, 0.023 mmol) was added. The system was flushed with H_2 and then stirred at room temperature for 1h. The Pd was removed by filtration through a 0.45 μm filter and the solvent removed under reduced pressure affording 8.5 mg of the piperidone inhibitor **5** (0.018 mmol, 78%).

Assay Conditions

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 without further purification. The increase of absorbance at 404 nm was minitored 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 **3** & **5** were assayed in a solution with a final concentration of 10% DMSO. The reaction was initiated by addition of the chromogenic substrate. K_i values were determined by non linear fit to the Michaelis-Menten equation for competitive inhibition.

K_m	with 10% DMSO	without DMSO
Plasmin	395 µM	217 µM
Thrombin	22 µM	9.5 µM
Kallikrein	63 µM	117 µM
Trypsin	50 µM	42 µM

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