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In this report we describe our efforts to develop a new class of competitive inhibitors for serine and cysteine proteases. These compounds are potential anticancer agents that would act by inhibiting metastasis and angiogenesis. The first part of this report describes solution and solid phase methods for the synthesis of inhibitors of the cysteine protease cathepsin B. These inhibitors are based on a cyclohexanone pharmacophore and are designed to interact with both the S and S' subsites of the enzyme active site.

In the second part we demonstrate that the 4-heterocyclohexanone pharmacophore can be used to synthesize effective inhibitors of serine proteases. We have constructed an inhibitor of the serine protease plasmin, and shown that it has good activity and significant specificity for plasmin over other proteases.

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Introduction. This report describes our efforts, over the last year, to systematically investigate a new class of serine and cysteine protease inhibitors as potential anticancer agents. Cancer cells release a number of serine and cysteine proteases that have been shown to stimulate angiogenesis (vascularization of tumor tissue) and to promote the proliferation and migration of tumor cells. These enzymes either act directly by degrading components of the extracellular matrix and basement membrane such as collagen, elastin, fibronectin, laminin, and entactin, or indirectly by activating other proteolytic enzymes. Inhibition of these proteases has been shown to be an effective method for blocking tumor invasion of the extracellular matrix and basement membrane by cancer cells, *including human mammary epithelial cells.* Thus development of a new class of potent and specific inhibitors for these enzymes should have a direct impact on the treatment of breast cancer by providing chemotherapeutic agents which are designed to inhibit tumor growth and metastasis.

During the first year (1997) of this grant, we demonstrated that 4-heterocyclohexanones could be used to synthesized inhibitors of cysteine proteases, and investigated the mechanism of inhibition using ^{13}C NMR spectroscopy. This work has been published in two papers (*Journal of the American Chemical Society* **1997**, *119*, 4285 - 4291 and *Journal of Organic Chemistry* **1998**, *63*, 2367 - 2370), reprints of which are provided at the end of this report. Most of this work was described in our first annual report, and corresponds to completion of Tasks 1, 8, and 9 from our Statement of Work.

This, our second annual report, is organized into two sections. Part 1 describes our work to extend the structure of our inhibitors so that they contact both the S and S' enzyme subsites. This strategy should lead to more potent and specific inhibitors. We have applied this strategy to the synthesis of inhibitors of cathepsin B, an enzyme that is implicated in both angiogenesis and metastasis. This corresponds to the completion of Tasks 4 - 6 from the Statement of Work.

Part 2 describes our efforts to demonstrate that the 4-heterocyclohexanone pharmacophore can be used to synthesize effective inhibitors of serine proteases. We have constructed an inhibitor of the serine protease plasmin, and shown that it has good activity and significant specificity for

plasmin over other proteases. This work comprises a combination of tasks 2 and 10 from the Statement of Work. We have decided to use plasmin instead of chymotrypsin (as was originally proposed in task 2) to test the effectiveness of our inhibitors against serine proteases. We have made this decision because the biochemical relationship between chymotrypsin and cancer development is not well understood, while the role played by plasmin in angiogenesis and metastasis is better established.

Part 1. Synthesis of Cyclohexanone-Based Cathepsin B Inhibitors that Interact with Both the S and S' Binding Sites

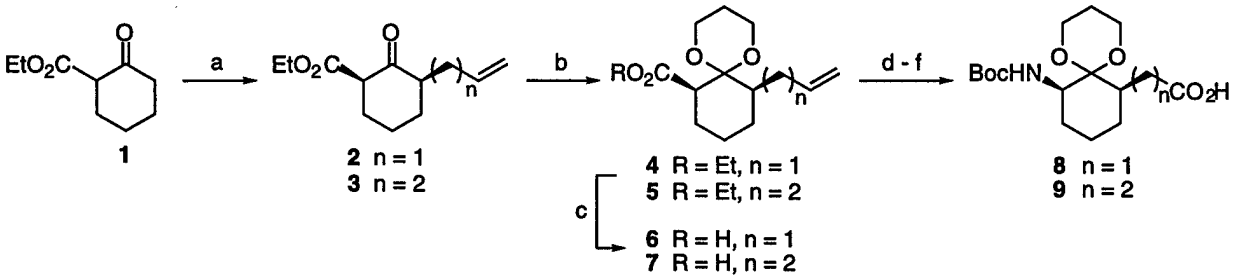
Abstract: Solution and solid phase methods are described for the synthesis of inhibitors of the cysteine protease cathepsin B. These inhibitors are based on a cyclohexanone pharmacophore and are designed to interact with both the S and S' subsites of the enzyme active site.

Introduction

The cysteine proteases cathepsin B, cathepsin K, and the ICE-like proteases are involved in disease processes that include metastasis of cancer,[2] bone resorption in osteoporosis,[3] and the control of programmed cell death.[4] These proteases are important targets for the development of inhibitors, both as therapeutic agents and as tools that can help to clarify the biological function of the enzymes.[5] We recently reported a new class transition-state analog inhibitors for cysteine proteases that are based upon a 4-heterocyclohexanone pharmacophore.[6] These inhibitors react with the enzyme active site nucleophile to give a reversibly formed hemithioketal adduct.[7] The 4-heterocyclohexanone nucleus was derivatized on one side of the reactive ketone so that the inhibitors made contacts with only the S subsites of the enzyme active site. However, inhibitors that extend interactions into both the S and S' subsites may have increased potency and specificity when compared to their singly-sided counterparts.[8] In this paper we describe solution and solid phase methods for synthesizing inhibitors of cathepsin B that are designed to interact with both the S and S' subsites. Development of a solid phase protocol for synthesis makes possible the construction of a combinatorial library of protease inhibitors based upon the cyclohexanone pharmacophore.

Compound **16** (Scheme 2) was designed as an inhibitor for cathepsin B using a combination of molecular modeling studies[9] and data from an X-ray crystal structure of the enzyme

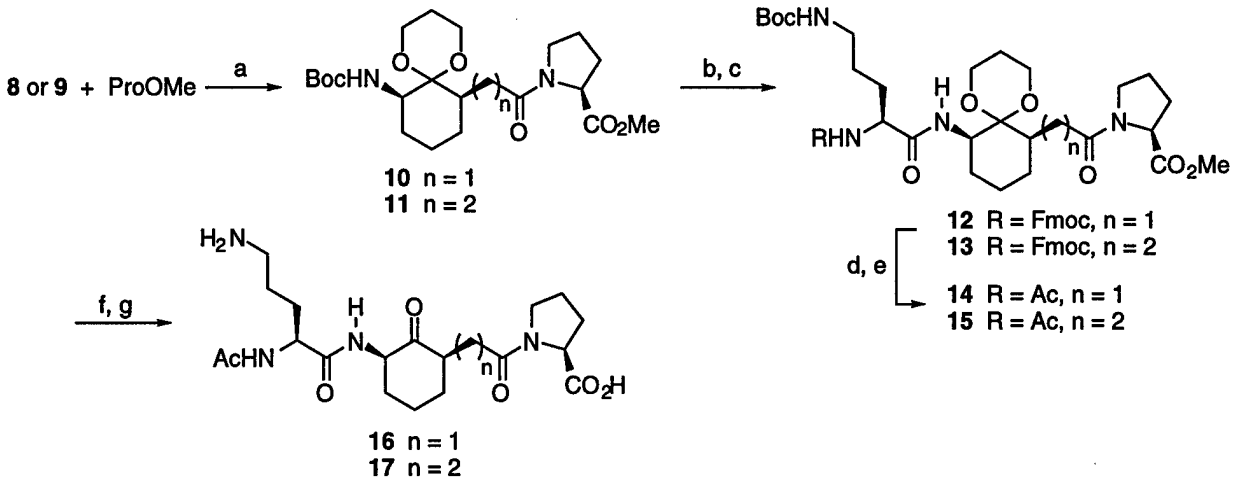
with an epoxysuccinyl inhibitor irreversibly bound to the active site nucleophile.[10] The ornithine side chain at the P2 position of **16** is designed to form a salt bridge with Glu 245 at the base of the S2 binding pocket of the enzyme. Proline is meant to fit into the shallow S2' binding site, with the free C-terminal carboxylate of the inhibitor forming hydrogen bonds with His 110 and His 111 of the protease. The structure of inhibitor **16** is intended to mimic the backbone of a natural peptide substrate. However, modeling studies suggested that this compound may be slightly too short to interact optimally with the two His residues. Therefore we have also synthesized compound **17**, which is one methylene unit longer than **16**, in order to account for this possibility.



Scheme 1. Reagents and Conditions: a) LDA (2 equiv.), 3-bromo-1-propene or 4-bromo-1-butene (1 equiv.), **2**: 64%, **3**: 60%; b) 1,3-propanediol, TMSCl, **4**: 70%, **5**: 62%; c) NaOH, MeOH, **6**: 58%, **7**: 80%; d) $(C_6H_5O)_2PON_3$, benzene, reflux; e) *t*-BuOK, THF; f) $KMnO_4$, $NaIO_4$, **8**: 70%, **9**: 59% (3 steps). One of two enantiomers is shown.

Synthesis of the cyclohexanone nucleus (Scheme 1) began with double deprotonation of ketoester **1**, followed by alkylation of the more reactive enolate with the appropriate bromoalkene to give compounds **2** and **3**.[11,12] Protection of the ketone with 1,3-propanediol and TMSCl,[13] followed by saponification of the ester gave carboxylic acids **6** and **7**. Reaction of the acids with diphenylphosphoryl azide in refluxing benzene induced the Curtius rearrangement.[14] The isocyanate product of these reactions was trapped with potassium *tert*-butoxide to yield the corresponding Boc protected amines. Finally, oxidative cleavage of the alkenes gave protected

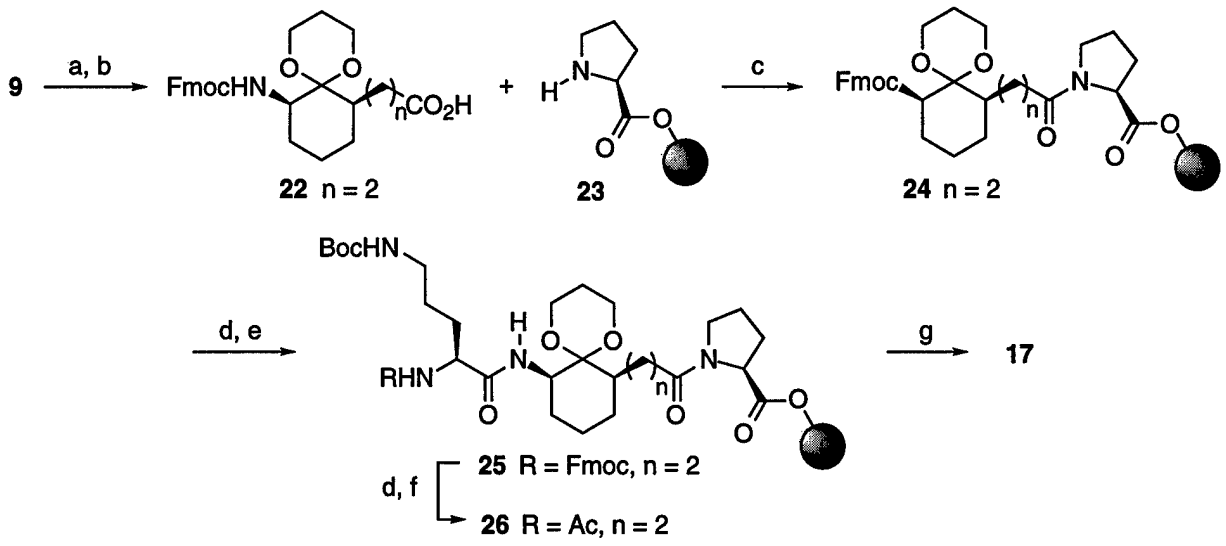
amino acids **8** and **9**. Analysis of the conformation of compound **7** by NMR studies using COSY and 1D-NOE experiments indicated that the carboxylic acid and butene substituents on the cyclohexanone ring were present in the thermodynamically favored *cis*-1,3 diequitorial orientation.



Scheme 2. Reagents and Conditions: a) EDC, HOBt, **10**: 84%, **11**: 92%; b) TFA; c) N-α-Fmoc-N-δ-Boc-Orn, EDC, HOBt, **12**: 56%, **13**: 62% (2 steps); d) tris(2-aminoethyl)amine; e) Ac_2O, **14**: 51%, **15**: 71% (2 steps); f) LiOH; g) TFA, H_2O, **16**: 97%, **17**: 82% (2 steps). One of two diastereomers is shown.

The cyclohexanone nucleus was next coupled to proline methyl ester to give compounds **10** and **11** as mixtures of two diastereomers (Scheme 2). Removal of the Boc group followed by coupling to N-α-Fmoc-N-δ-Boc-Orn gave compounds **12** and **13**. The N-terminus was subsequently deprotected and capped with acetic anhydride to yield **14** and **15**. Finally the methyl ester was saponified, and the ketal and Boc protecting groups were removed by treatment with TFA in the presence of a small amount of water to yield inhibitors **16** and **17**.

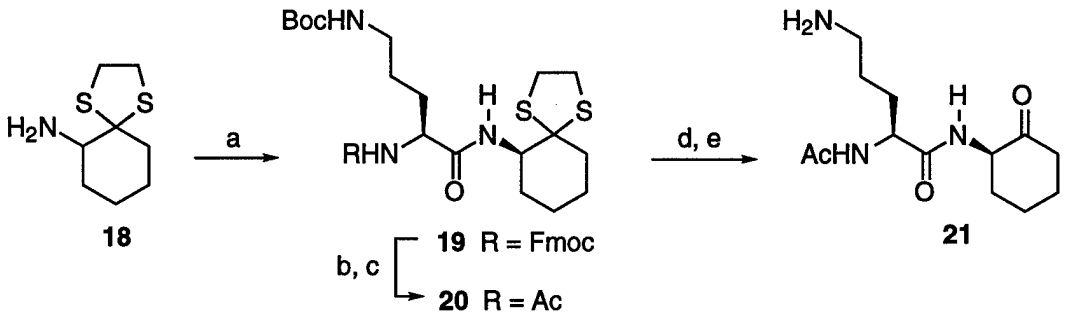
We have also developed a solid phase protocol for synthesizing these cyclohexanone-based protease inhibitors. The protocol, which is outlined in Scheme 3, is analogous to the Fmoc strategy for synthesizing peptides on a solid support. This synthesis required a derivative of the



Scheme 3. Reagents and Conditions: a) TFA; b) N-(9-fluorenylmethoxycarbonyloxy)-succinimide; c) HBTU, DIEA; d) piperidine; e) N-α-Fmoc-N-δ-Boc-Orn, HBTU, DIEA; f) Ac_2O; g) TFA, H_2O. One of two diastereomers is shown.

cyclohexanone pharmacophore that had a free C-terminal carboxylate, an Fmoc group on the N-terminus, and a protecting group on the ketone that could be removed under mild conditions. Compound **22** fulfilled these requirements. Solid phase synthesis of inhibitor **17** was performed on Wang resin that was preloaded with Fmoc-Pro. Standard coupling and Fmoc deprotection procedures were employed.[15] The N-terminus was capped with acetic anhydride, and TFA was used to cleave compound **26** from the solid support and to remove the Boc group. The ketal protecting group was removed by adding H_2O (30% v/v) to the cleavage cocktail and stirring the solution overnight at room temperature. The crude material was isolated by lyophilization and purified by reverse phase HPLC to yield inhibitor **17** that was identical to material obtained from the solution phase synthesis.

In order to determine how much the Pro residue in **16** and **17** contributes to the potency of the inhibitors, we have synthesized control compound **21** which lacks any binding interactions with the S' subsites of the enzyme. The synthesis of **21** (Scheme 4) began with amine **18**,[16] and



Scheme 4. Reagents and Conditions: a) N-α-Fmoc-N-δ-Boc-Orn, EDC, HOBt, 80%; b) tris(2-aminoethyl)amine; c) Ac_2O, 99% (2 steps); d) N-bromosuccinimide, H_2O; e) TFA, 80% (2 steps). One of two diastereomers is shown.

was similar to the synthesis of the N-terminal portion of inhibitors **16** and **17**. The only difference was that the ketone was carried through the synthesis as a thioketal, which was deprotected at the end of the sequence using N-bromosuccinimide and H_2O.[17]

Biological Assay and Conclusions

The inhibitors were assayed against cathepsin B using the methylcoumarylamide substrate Z-Arg-Arg-NMec.[18] The hydrolysis reactions were monitored by fluorescence spectroscopy using excitation and emission wavelengths of 350 and 440 nm respectively. Control compound **21** is a poor inhibitor of cathepsin B with an inhibition constant of 24 mM. Compounds **16** and **17** have K_i values of 6.6 and 6.1 mM, respectively.[19] These results demonstrate that the potency of cyclohexanone-based inhibitors can be improved significantly by building in functionality that interact with the S' binding sites. Although our design efforts have not yet yielded inhibitors with high potency against cathepsin B, this work has set the stage for the solid phase synthesis of a combinatorial library of inhibitors that are constructed around the 4-heterocyclohexanone pharmacophore.

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19. The error in the values of the inhibition constants is approximately ±20%.

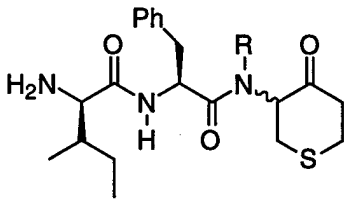
Part 2. 4-Heterocyclohexanone-Based Inhibitors of the Serine Protease Plasmin

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

Plasmin is a serine protease that plays an important role in the proteolytic cascade. This protease acts directly by hydrolyzing components of the basement membrane such as fibrin, type IV collagen, fibronectin, and laminin, and also acts indirectly by activating other enzymes in the cascade such as matrix metalloproteases.[3] Degradation of the basement membrane by plasmin is a multi-step process. For example, during the first step in fibrin hydrolysis, plasminogen which is the inactive precursor to plasmin, binds to fibrin via a lysine binding site. Next plasminogen is converted to active plasmin in a reaction that is catalyzed by urokinase plasminogen activator. Finally, catalytic residues in the active site of plasmin, which is separate from the lysine binding site, hydrolyze fibrin via the mechanism that is common to serine proteases.[5] Most current pharmaceutical agents that are designed to inhibit plasmin are targeted to the lysine binding site.[6] These agents inhibit fibrinolysis by blocking binding of plasminogen to fibrin, and thus halting

production of new plasmin. α2-Antiplasmin, a natural plasmin inhibitor, is also targeted to the lysine binding site.[7] However these fibrinolysis inhibitors have no effect on the active site of the enzyme, which retains its catalytic activity. Thus plasmin that is present in the body 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** and **2**, which are active site directed inhibitors of plasmin that show good activity and specificity.



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

Design of Inhibitors

We have recently reported a new class of inhibitors for cysteine proteases that are based upon a 4-heterocyclohexanone pharmacophore.[8] ^{13}C NMR studies using a ^{13}C-labeled inhibitor confirm that these molecules react with the enzyme to give a reversibly-formed covalent adduct between the active site cysteine residue and the ketone of the inhibitor.[9] 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.[8]

Because serine and cysteine proteases share a similar mechanism for hydrolyzing amide bonds, we expect that 4-heterocyclohexanones should be good inhibitors of both classes of enzymes. Reaction of the active site nucleophile of a serine protease with a 4-

heterocyclohexanone-based inhibitor would give a reversibly formed hemiketal adduct. However, several reversible protease inhibitors show activity against one class of enzyme and not the other. For example, trifluoromethyl ketones and boronic acids are good inhibitors of serine protease[10] but not cysteine proteases.[11, 12] Nitriles have the opposite specificity, while aldehydes and α-dicarbonyl compounds are good inhibitors of both classes of enzymes.[12] Thus one of our motivations for synthesizing compounds **1** and **2** was to determine if 4-heterocyclohexanones would prove to have activity against serine proteases, in addition to cysteine proteases as we have shown previously.[8]

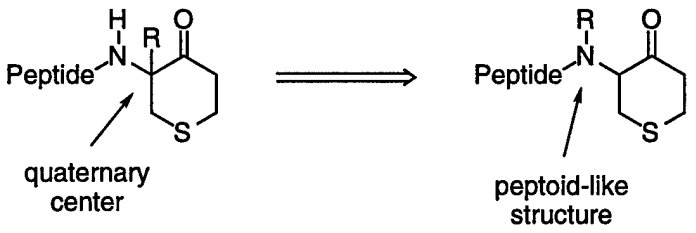


Figure 1. Shifting of the P1 side chain from the position alpha to the ketone to the exocyclic nitrogen to avoid formation of the quaternary center. R = $(CH_2)_6NH_2$.

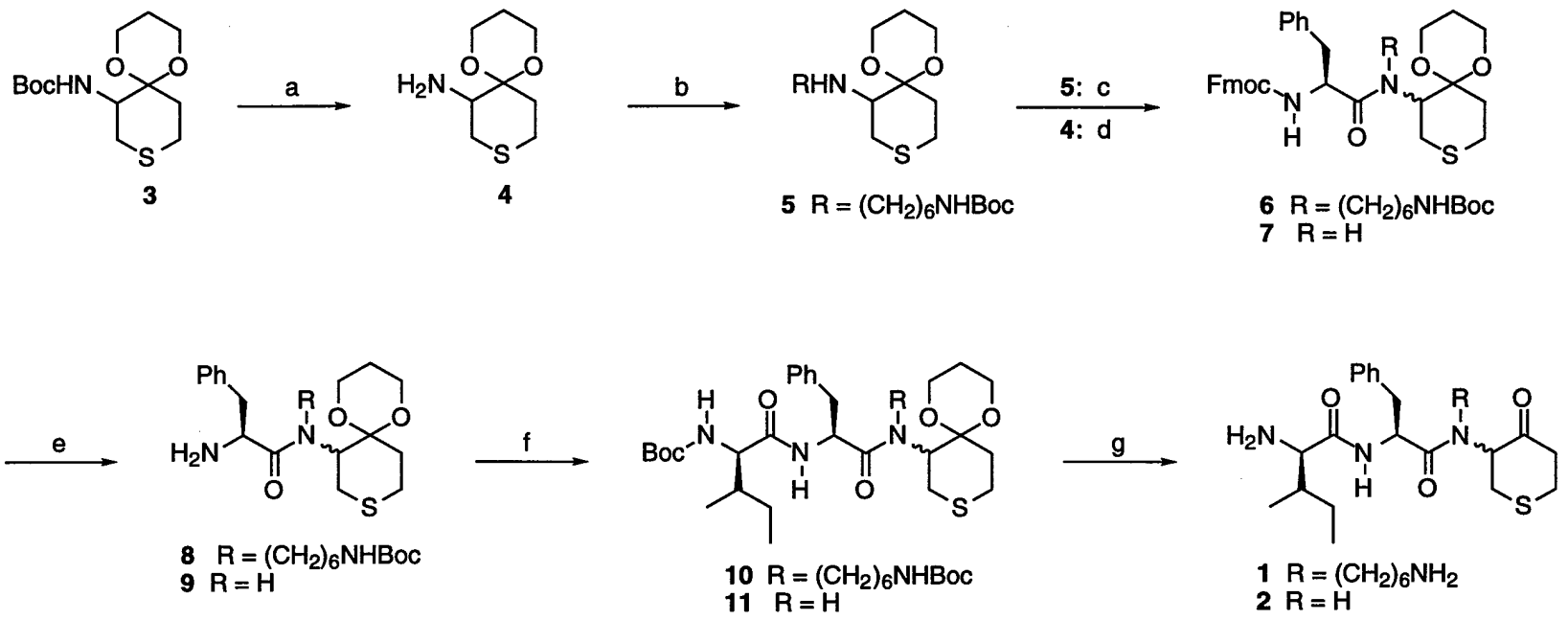
Plasmin has a strong specificity for substrates with positively charged side chains in the P1 position. To accommodate this specificity we have included a lysine-like side chain in the structure of compound **1**. However, attachment of this side chain in its "natural" peptide-like position would place it on the tetrahydrothiopyranone ring between the ketone and the exocyclic nitrogen (Figure 1). This placement would create a sterically demanding quaternary center alpha to the reactive ketone. Space filling molecular models suggest that this quaternary center would sterically inhibit addition of an active site nucleophile to the ketone, and thus decrease the potency of the inhibitor. To overcome this difficulty we have attached the P1 side chain to the amide nitrogen that is connected to the ring. This type of modification is well precedented in peptoids.[13] 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 **1** 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.[14] Phenylalanine and D-isoleucine were included in the structure of **1** because work by Okada has shown that substrates and inhibitors with these residues at the P2 and P3 positions bind tightly to the enzyme.[15] The sulfur atom was incorporated into the cyclohexanone ring because the related tetrahydrothiopyranone-based inhibitor of the cysteine protease papain had good activity and was significantly easier to synthesize than the corresponding tetrahydropyranone analog. Compound **2** was synthesized in order to determine how much the P1 side chain contributes to the affinity of the inhibitor for plasmin.

Chemistry

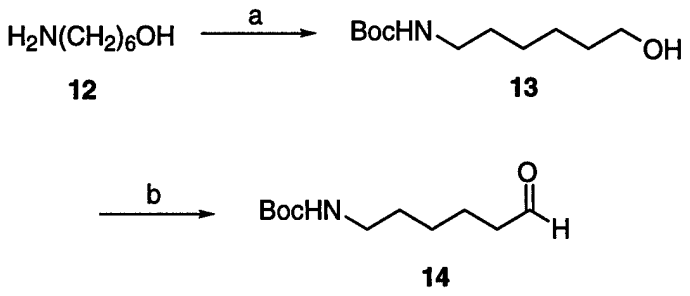
The synthesis of inhibitors **1** and **2**, which is outlined in Scheme 1, began with deprotection of the Boc group in compound **3** with trifluoroacetic acid to give amine **4**. The synthesis of **3** has been reported previously.[8] Reductive amination of **4** with aldehyde **14** (Scheme 2) using sodium triacetoxyborohydride in dichloroethane gave secondary amine **5**. An alternate strategy for the preparation of **5** involving monoalkylation of **4** with the appropriate alkyl halide gave a poor yield of the secondary amine. Aldehyde **14** was synthesized starting from 6-amino-1-hexanol (**12**, Scheme 2). The amine was first protected using $(Boc)_2O$ to give alcohol **13**, followed by oxidation of the alcohol with pyridinium chlorochromate to give the corresponding aldehyde.

Scheme 1[a]



[a] Reagents and conditions: (a) TFA, 85%; (b) **14**, $NaBH(OAc)_3$, dichloroethane, 50%; (c) FmocPhe-F, DIEA, **6** 75%; (d) FmocPhe, EDC, HOBT, **7** 36%; (e) piperidine, DMF, **8** 92%, **9** 81%; (f) Boc-D-Ile, EDC, HOBT, **10** 81%, **11** 87%; (g) *p*-TsOH, acetone, **1** 59%, **2** X%.

Scheme 2[a]



[a] Reagents and conditions: (a) $(Boc)_2O$, 97%; (b) pyridinium chlorochromate, 91%.

Amines **4** and **5** were coupled to Fmoc-Phe using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT) or the acid fluoride methodology of Carpino.[16] These reactions gave compounds **6** and **7**, each as a mixture of diastereomers. The Fmoc protecting groups in **6** and **7** were removed using piperidine in DMF to give amines **8** and **9**. Compound **8** was carried through the synthesis as a mixture of diastereomers, while the two diastereomers of compound **9** could be separated easily at this stage using flash chromatography. Each of the diastereomers of **9** were carried through the remainder of the synthesis separately. These amines were next coupled to Boc-D-Ile using EDC and HOBT to give compounds **10** and **11**. Final removal of the Boc and ketal protecting groups in **10** and **11** was accomplished by treatment with *p*-toluenesulfonic acid (*p*-TsOH) in acetone to give inhibitors **1** and **2**. If this double deprotection reaction is performed with 10 equivalents of *p*-TsOH, the reaction is rapid but gives a significant amount of racemization of the amino acids as measured by HPLC and ^1H NMR analysis. Performing the reaction with 1 equivalent gives a much slower reaction, but avoids the problem of racemization. The diastereomers of inhibitor **1** were separated by RP-HPLC.

Results and Discussion

We have evaluated the activity of inhibitor **1** against four different serine proteases; plasmin, thrombin, kallikrein, and trypsin. Because all of these enzymes share a specificity for positively charged residues at the P1 position, it is challenging to design inhibitors which are highly active against one specific enzyme while not inhibiting the others. Plasmin and trypsin prefer lysine at the P1 position, while thrombin and kallikrein prefer arginine. At the outset of these experiments, we expected compound **1** to show similar activity against plasmin and trypsin for several reasons. First, these enzymes have a similar specificity at the P1 position. Second, the active sites of these enzymes share significant homology. Finally, the X-ray crystal structure of trypsin was used in the design of inhibitor **1**, because the structure of the active site of plasmin has not yet been solved. However, as shown below, we were surprised and gratified to find that inhibitor **1** shows significant specificity against the intended target plasmin, and is a much poorer inhibitor of the other three serine proteases.

Table 1. Michaelis Constants in the Absence and Presence of DMSO.

		K_M (μM)	
Enzyme	Substrate	Without DMSO	With 10% DMSO
plasmin	D-Val-Leu-Lys-pNA	217	395
thrombin	D-Phe-Pip-Arg-pNA	10	22
kallikrein	D-Pro-Phe-Arg-pNa	117	63
trypsin	D-Phe-Pip-Arg-pNA	42	50

Pip = L-pipecolinic acid, pNA = *para*-nitroanilide

The enzymes were assayed using the nitroanilide substrates shown in Table 1. Hydrolysis of the substrates was monitored using UV spectroscopy by following the increase in absorbance at

404 nm. These experiments were carried out on a Perkin-Elmer 8452A diode array spectrophotometer at room temperature.

In order to increase the solubility of the inhibitor, the assays were performed in a solution of 50 mM phosphate buffer (pH 7.4) that contained 10% DMSO. Using a small percentage of an organic cosolvent in these types of assays with proteolytic enzymes is a fairly common practice. However before assaying the inhibitors, we needed to verify that this cosolvent did not perturb significantly the kinetic characteristics of the enzymes. Therefore we measured the Michaelis constants for the various enzyme-substrate combinations both in the presence and absence of DMSO. The results of these studies, shown in Table 1, demonstrate that the cosolvent does not change the binding characteristics of these enzymes by more than approximately a factor of two. This small change makes us confident that the organic cosolvent is not significantly altering the structure and activity of the proteases.

Table 2. Inhibition of Serine Proteases by Inhibitor **1**.

	K_i (μM)			
compound[a]	plasmin	thrombin	trypsin	kalikrein
1 A	40	1100	2500	1300
1 B	170	X	X	X

[a] A and B represent two different diastereomers.

The results of the inhibition assays that we have performed to date with the two diastereomers of compound **1** are shown in Table 2. Diastereomer **1A** is an excellent competitive reversible inhibitor of the intended target protease plasmin with an inhibition constant of 40 μM. By comparison it is a much poorer inhibitor of the other three serine proteases that were tested. This compound has a specificity for plasmin that is between 28 and 63 times higher than for thrombin, trypsin, or kallikrein. Diastereomer **1B** is a somewhat poorer inhibitor of plasmin with

an inhibition constant of 170 μM. We have not yet assayed **1B** against the other three proteases, nor have we completed assays of compound **2** against any of the enzymes.

In a series of analogous inhibitors of the cysteine protease papain, we observed that the enzyme bound one diastereomer of the inhibitors approximately 100-fold tighter than the other diastereomer.[8] However for the two diastereomers **1A** and **1B**, plasmin shows only a 4-fold difference in affinity. This result is somewhat unusual because we generally think of enzymes as having a high degree of selectivity for the absolute stereochemistry of substrates. From another viewpoint, these inhibition data may be providing us with important information concerning the active site of plasmin. Any insight that we can gain about the structure of the enzyme is important since, as noted earlier, the X-ray crystal structure of the active site of plasmin has never been solved.

The crystal structure of papain reveals that it has a very narrow and deep binding cleft which is reflected in its high specificity for one diastereomer of an inhibitor. By contrast, the low specificity of plasmin for the two diasteromers of inhibitor **1** suggest that it has a much wider and shallower active site. Such a structure would make it likely that both diastereomers could fit reasonably in the active site and react with the active site nucleophile to give a hemiketal adduct.

Conclusions

In this report we have demonstrated that the 4-heterocyclohexanone pharamcophore can be used effectively to construct potent inhibitors of serine proteases. We have used this strategy to synthesize two inhibitors for plasmin, which is an enzyme that is involved with both angiogenesis and metastasis. We have shown that one of these inhibitors is a potent inhibitor for plasmin and shows a high degree of selectivity for this enzyme compared to other serine proteases that have a similar preference for positively charged P1 residues. Our studies of the binding preference of the two diastereomers of inhibitor **1** suggest that the active site of plasmin is relatively broad and shallow and can accommodate significant stereochemical variation within the inhibitor. These

studies will be completed when we finish assaying inhibitors **1B**, **2A**, and **2B** against the four serine proteases.

Experimental Section

General Methods. NMR spectra were recorded on Bruker WM-250, Avance-300 or Avance-400 instruments. Spectra were calibrated using TMS ($\delta = 0.00$ ppm) for ^{1}H NMR and $CDCl_3$ ($\delta = 77.0$ ppm) for ^{13}C NMR. IR spectra were recorded on a Perkin-Elmer 1700 series FT-IR spectrometer. Mass spectra were recorded on a Kratos MS 80 under electron impact (EI), chemical ionization (CI) or fast-atom bombardment (FAB) conditions. HPLC analyses were performed on a Rainin HPLC system with Rainin Microsorb silica or C18 columns, and UV detection. Semi-preparative HPLC was performed on the same system using a semi-preparative column (21.4 x 250 mm).

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

Alcohol 13. 6-Amino-1-hexanol **12** (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 **13** (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 14. The alcohol **13** (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 aldehyde **14**: IR 1704 cm^{-1} (CO); ^1H 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 4. Trifluoroacetic acid (TFA, 25 mL) was added to a solution of the carbamate **3** (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 portions 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 primary amine **4**: ^1H 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 5. Aldehyde **14** (0.52 g, 2.4 mmol) was dissolved in 2 mL of 1,2-dichloroethane (DCE) and added to a solution of primary amine **4** (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 **5** (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 6. Fmoc-Phe-F[16] (0.34 g, 0.89 mmol) and diisopropylethylamine (DIEA, 0.10 mL, 0.60 mmol) were added to a solution of the secondary amine **5** (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 **6** (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 8. A DMF solution (10 mL) of Fmoc ketal **6** (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 the crude material was purified by flash chromatography (98:2 CH_2Cl_2/MeOH) to give a mixture of two diastereomers of amino ketal **8** (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 10. 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 **8** (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 **10** (0.28 g, 138 mmol, 81%): ^1H 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.

Inhibitor 1. To a solution of Boc ketal **10** (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 the diastereomers was accomplished by RPHPLC (gradient of 0-50% MeCN in H_2O with 0.1% TFA over 45 min) to afford 15 mg

(0.030 mmol) of the earlier eluting diastereomer **1a** and 17 mg (0.034 mmol) of the later eluting diastereomer **1b** for a combined yield of 59%. **1a**: ^{1}H 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. **1b**: ^{1}H 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.

Fmoc ketal 7. 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 **4** (0.52 g, 2.8 mmol) and 4-methylmorpholine (0.60 mL, 5.5 mmol) dissolved in 25 mL of DMF was then added to the reaction mixture. After 2 h the reaction mixture was partitioned between 100 mL of EtOAc and 100 mL of H_2O. The organic layer was washed with 100 mL of H_2O, 50 mL of saturated $KHSO_4$ solution, 50 mL of saturated Na_2CO_3 solution, dried over $MgSO_4$, and concentrated under reduced pressure. Flash chromatography (1:1 EtOAc/hexanes) afforded a mixture of two diastereomers of Fmoc ketal **7** (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 9. A solution of piperidine (0.6 mL, 6.0 mmol) and Fmoc ketal **7** (0.56 g, 1.0 mmol) in 5 mL of DMF was allowed to stir at room temperature for 5 h. The reaction mixture

was then partitioned between 50 mL of EtOAc and 50 mL of H_2O. The organic layer was washed with H_2O, dried over $MgSO_4$ and concentrated. The crude material was purified by flash chromatography (2:98 MeOH/CH_2Cl_2) to afford the two separate diastereomers of amino ketal **9a** (0.17 g, 0.50 mmol) and **9b** (0.11 g, 0.32 mmol) in a combined yield of 81%. **9a**: ^{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). **9b**: ^{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). Mixture of **9a** and **9b**: ^{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 11a. Compound **11a** was prepared from compound **9a** (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 compound **10**. The crude material was purified by flash chromatography (4:1 EtOAc/hexanes) to give 156 mg (0.28 mmol, 88%) of compound **11a**: ^{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).

Boc ketal 11b. Compound **11b** was prepared from compound **9b** (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 compound **10**. The crude material was purified by flash chromatography (4:1 EtOAc/ hexanes) to give 148 mg (0.27 mmol, 86%) of compound **11b**: ^{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).

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.[17] Enzymes and substrates were used as received without further purification. The increase of absorbance at 404 nm 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 **1** & **2** 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.

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Using the Electrostatic Field Effect to Design a New Class of Inhibitors for Cysteine Proteases

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Contribution from the Department of Chemistry, Brown University, Providence, Rhode Island 02912

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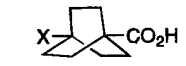
Contribution from the Department of Chemistry, Brown University, Providence, Rhode Island 02912

Received December 4, 1996[⊗]

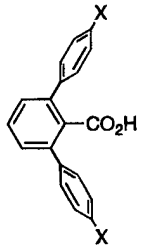
Abstract: A new class of competitive inhibitors for the cysteine protease papain is described. These inhibitors are based upon a 4-heterocyclohexanone ring and are designed to react with the enzyme active site nucleophile to give a reversibly formed hemithioketal. The electrophilicity of the ketone in these inhibitors is enhanced by ring strain and by through-space electrostatic repulsion with the heteroatom at the 1-position of the ring. Equilibrium constants for addition of water and 3-mercaptopropionic acid to several 4-heterocyclohexanones were measured by ^{1}H NMR spectroscopy. These reactions model addition of the active site nucleophile to the corresponding inhibitors. The equilibrium constants give a linear correlation with the field substituent constant F for the functional group at the 1-position of the heterocyclohexanone. These equilibrium constants also correlate well with the inhibition constants for the 4-heterocyclohexanone-based inhibitors, which range from 11 to 120 μM. Thus, the model system can be used to predict the potency of structurally related enzyme inhibitors.

Introduction

The Field Effect. The physical-organic literature contains many examples of chemical systems that use through-space electronic interactions to control equilibria or regio- and stereospecificity of organic reactions.[1,2] Molecules such as 4-substituted bicyclo[2.2.2]octane-1-carboxylic acid have been developed to investigate the Coulombic interaction between a polar substituent and a carboxylic acid.[3] The through-space electrostatic interaction between these groups perturbs the pK_a of the acid. More recently, Siegel and co-workers examined through-space polar π interactions in *para*-substituted 2,6-diphenylbenzoic acids.[4] In this system, the substituents alter the polarity of the phenyl rings, which in turn influence the acidity and hydrogen-bonding characteristics of the carboxylic acid. These examples demonstrate that through-space electrostatic interactions can exert a powerful influence on chemical reactions. Despite the importance of these studies, we and others[4] have noted that through-space interactions are seldom used as a rational design element in bioorganic and medicinal chemistry.[5] In this paper, we present a physical-organic strategy for designing a new class of inhibitors for cysteine proteases. These inhibitors are based on a 4-heterocyclohexanone nucleus and take advantage of through-space electrostatic repulsion to control the potency of enzyme inhibition.



bicyclo[2.2.2]octane-1-carboxylic acids



2,6-diphenylbenzoic acids

Other Cysteine Protease Inhibitors. Cysteine proteases are important targets in medicinal chemistry. They have been implicated in diseases such as rheumatoid arthritis,[6] muscular dystrophy,[7] and cancer metastasis.[8] Many types of chemical functionality have served as the central pharmacophore for reversible and irreversible inhibitors of cysteine proteases. Among the reversible inhibitors are aldehydes,[9] nitriles,[10] α-keto carbonyl compounds,[11] and cyclopropenones.[12] Aldehydes and nitriles inhibit proteases by forming a reversible covalent bond between the electrophilic functionality of the inhibitor and the nucleophilic sulfur atom of the active site cysteine residue.[13]

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.

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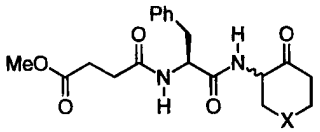
Chart 1. Structures of Cysteine Protease Inhibitors



X = CH_2, S, O, NH_2^+

This mechanism is also likely to be operative in the α-keto carbonyl[11] and cyclopropenone inhibitors.

Design of Inhibitors. Chart 1 shows the structures of 4-heterocyclohexanone-based inhibitors for the cysteine protease papain. These inhibitors consist of a 4-heterocyclohexanone core that is appended with an *N*-(methoxysuccinyl)phenylalanine side chain. We have chosen papain for our initial studies because its structure and mechanism have been thoroughly characterized. In addition, it provides a good model for evaluating the design of new inhibitors and for comparing them to previously reported compounds. The inhibitors include a phenylalanine residue because papain has a high specificity for this amino acid at the P2 position.[14] The methoxysuccinyl group was attached in order to increase solubility of the compounds in aqueous solution.

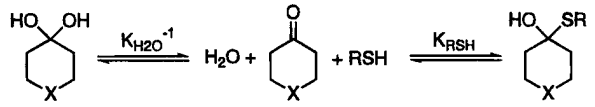
The inhibitors incorporate an electrophilic ketone moiety that is designed to give a reversibly formed hemithioketal with the enzyme active site nucleophile, in analogy with previously reported inhibitors. Compounds based upon unactivated ketones are not electrophilic enough to react with the active site cysteine nucleophile.[15] However, the carbonyl groups in 4-heterocyclohexanones are more electrophilic than standard ketones. Two factors increase their reactivity. First, there is an unfavorable dipole−dipole repulsion between the carbonyl and the heteroatom at the 1-position of the ring.[16−18] This interaction destabilizes the ketone, but is dissipated by addition of nucleophiles. Second, ring strain enhances the reactivity of 4-heterocyclohexanones. The cyclic compounds are more strained than their acyclic counterparts, and this strain is relieved by nucleophilic addition to the carbonyl to give a tetrahedral center.[18,19] Variations in the bond angles and bond lengths associated with the heteroatom will modulate this effect.[20]

An alternate method for increasing the electrophilicity of ketones is to add electron-withdrawing substituents to them. This strategy, which relies on through-bond inductive effects, has been implemented in the synthesis of potent trifluoromethyl ketone inhibitors of serine proteases.[21] However, these compounds have been found to be poor reversible inhibitors of cysteine proteases.[22]

We have synthesized a series of inhibitors that incorporate increasingly electronegative functional groups at the 1-position of the heterocyclohexanone ring. These compounds have allowed us to examine the relationship between the electronic characteristics of the X group (Chart 1) and the potency of the inhibitor. Electronegative X groups are expected to destabilize the ketone via through-space electrostatic repulsion, thereby shifting the ketone−hemithioketal equilibrium in favor of the hemithioketal and resulting in more potent inhibition.

The compounds reported in this paper are first-generation inhibitors that interact only with the S subsites of the enzyme active site. However, the 4-heterocyclohexanone nucleus can be derivatized on both sides of the electrophilic carbonyl to yield inhibitors that make contacts with both the S and S′ subsites. This is in contrast to aldehyde- and nitrile-based inhibitors that are limited to interactions with only half of the active site.

Table 1. Equilibrium Constants for Addition of Water and Thiol to Selected Ketones[a]



X	K_{H_2O} (M^{-1})	K_{RSH} (M^{-1})	$K_{RSH,app}$ (M^{-1})
CH_2	8.1×10^{-4}	0.22	0.21
S	9.0×10^{-3}	1.5	0.99
O	8.0×10^{-3}	1.8	1.3
NH_2^+	0.18	27.6	2.7
SO	0.068	11.7	2.5
SO_2	0.30	60.2	3.5
	Other Ketones		
CH_3COCH_3[b]	2.3×10^{-5}	0.0052	0.0052
CH_3COCO_2H[b]	0.031	58	22
$CH_3COCO_2CH_3$[b]	0.045	71	20

[a] RSH = $HO_2CCH_2CH_2SH$. [b] Data taken from reference 23.

Results

Model System. Before we undertook the multistep synthesis of our cysteine protease inhibitors, we first wanted to investigate the degree to which the heteroatom influences the reactivity of the ketone in these compounds. We have thus measured the equilibrium constants for addition of water and thiol nucleophiles to simple 4-heterocyclohexanones. These nucleophilic additions serve as a model for reaction of the enzyme active site nucleophile with the inhibitors.

Table 1 shows equilibrium constants for addition of water and 3-mercaptopropionic acid to a variety of ketones. The equilibrium constants were determined using ^1H NMR spectroscopy according to the procedure of Burkey and Fahey.[18,23] Figure 1 shows NMR spectra of tetrahydropyran-4-one as an example of how these measurements were made. The bottom spectrum, taken in acetone-d_6, shows resonances that correspond to tetrahydropyranone. The middle spectrum, taken in D_2O, shows resonances for the both the ketone (a and b) and the hydrate (c and d). These two species are in slow exchange on the NMR time scale. Integration of the resonances can be used to determine the hydration equilibrium constant. The top spectrum shows a mixture of tetrahydropyranone and 3-mercaptopropionic acid in D_2O. We observe resonances for ketone, hydrate, hemithioketal (e−h), and free thiol (i and j). Equilibrium constants for several of the ketones listed in Table 1 have been measured previously under different reaction conditions.[18,24] Our equilibrium constants are in reasonable agreement with the

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(20) Transannular anomeric interactions have been used previously to explain reaction rates and axial selectivities for addition of nucleophiles to 4-heterocyclohexanones. It is possible that these types of interactions also stabilize the hemithioketal that results from nucleophilic addition of a thiol to these ketones. (a) Cieplak, A. S. *J. Am. Chem. Soc.* **1981**, *103*, 4540. (b) Cieplak, A. S. *J. Am. Chem. Soc.* **1989**, *111*, 8447 and references therein.

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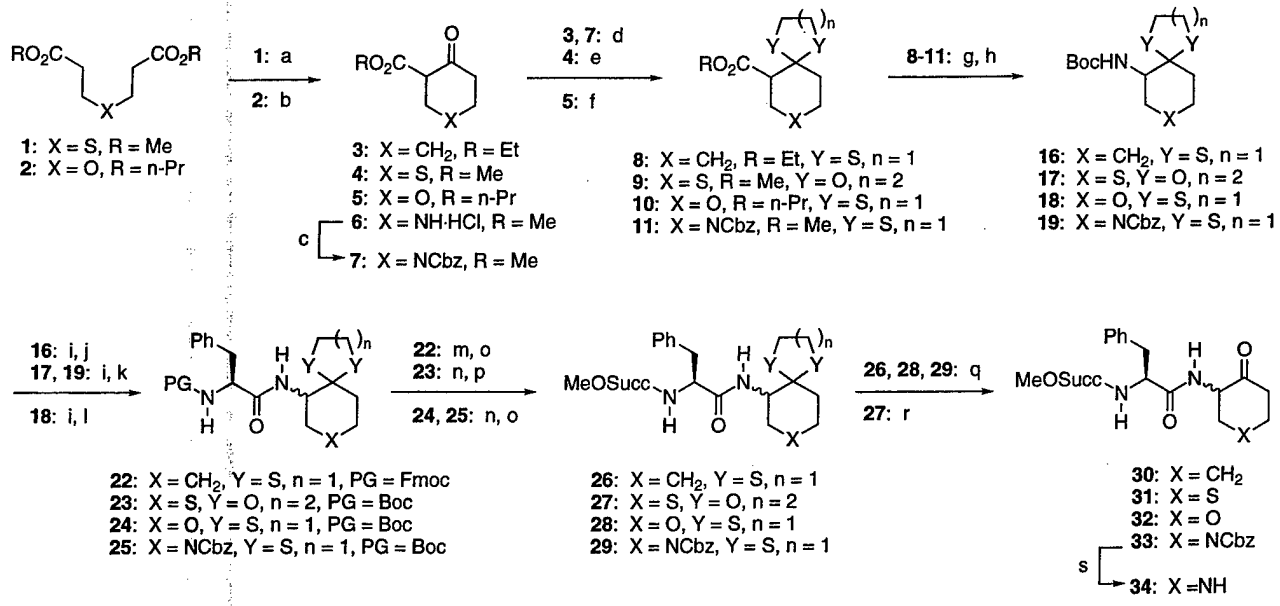
Scheme 1[a]



[a] (a) NaH, catalytic MeOH, 81%; (b) LDA, THF, −78 °C, 31%; (c) CbzCl, TEA, 95%; (d) ethanedithiol, TsOH, 94% from **3**, 74% from **7**; (e) 1,3-propanediol, TsOH, 77%; (f) ethanedithiol, BF_3·Et_2O, 43%; (g) NaOH, MeOH; (h) diphenylphosphoryl azide, benzene, followed by *t*-BuOK, THF, 60% from **8**, 37% from **9**, 44% from **10**, 71% from **11** (two step yields); (i) TFA, CH_2Cl_2; (j) FmocPhe-F, DIEA, 92% (two steps); (k) BocPhe-OH, EDC, HOBT, 84% from **17**, 81% from **19** (two step yields); (l) BocPhe-F, DIEA, 61% (two steps); (m) $N(CH_2CH_2NH_2)_3$, CH_2Cl_2; (n) TFA, CH_2Cl_2; (o) monomethyl succinate, EDC, HOBT, 70% from **22**, 70% from **24**, 89% from **25** (two step yields); (p) methyl(*N*-hydroxysuccinimidyl) succinate, DIEA, 77% (two steps); (q) NBS, H_2O, 80% from **26**, 66% from **28**, 68% from **29**; (r) acetone, TsOH, 79%; (s) H_2, 5% Pd/C, 79%.

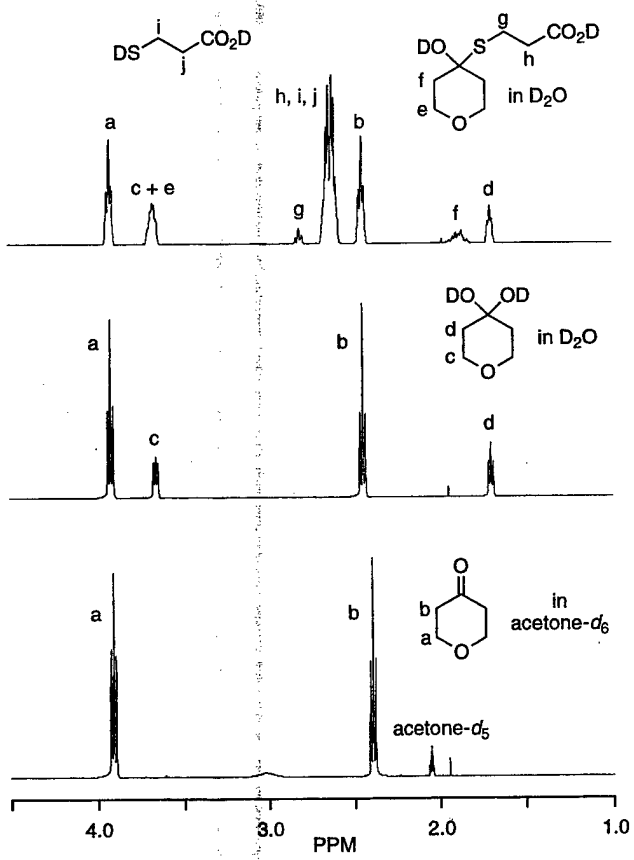


Figure 1. ^1H NMR spectra of the ketone, hydrate, and hemithioketal of tetrahydropyranone. The bottom spectrum shows the ketone in acetone-d_6 solution. The middle spectrum shows a mixture of ketone and hydrate in D_2O solution. The top spectrum shows a mixture of ketone, hydrate, hemithioketal, and free thiol in D_2O solution.

previously reported values. Equilibrium constants for acetone, pyruvic acid, and methyl pyruvate are taken from the literature.[23]

The hydration equilibrium constant for cyclohexanone is 35 times greater than that of acetone. In cyclohexanone, ring strain destabilizes the ketone and shifts the equilibrium by 2.1 kcal/mol in favor of hydrate when compared to acetone. Substituting electronegative functionality at the 4-position of the cyclohexanone ring leads to further destabilization of the ketone as a result of through-space electrostatic repulsion. For example, in the sulfone-containing molecule, the equilibrium is shifted by an additional 3.5 kcal/mol in favor of the hydrate. These results demonstrate that the electrostatic field effect, in combination with ring strain, can have a significant influence on the stability of hydrates. Similar trends are observed for the formation of hemithioketals.

The reaction between an enzyme and an inhibitor occurs in an aqueous environment. We must therefore consider that reaction between papain and the 4-heterocyclohexanone-based inhibitors will occur in competition with reaction between the inhibitor and water. This competition will lower the effective concentration of the inhibitor. We have calculated an *apparent* equilibrium constant for addition of thiol to ketone ($K_{RSH,app}$), first described by Jencks,[25] that accounts for the fact that the inhibitor will be present as a mixture of both ketone and hydrate in aqueous solution.

$$K_{RSH,app} = [\text{hemithioketal}]/[\text{ketone} + \text{hydrate}][\text{thiol}] = K_{RSH}/(1 + K_{H_2O}[H_2O]) \quad (1)$$

For molecules such as acetone that form a minimal amount of hydrate, the $K_{RSH,app}$ value is approximately equal to K_{RSH}. However, if a ketone forms a significant amount of hydrate, then $K_{RSH,app}$ is less than K_{RSH}. If the ketone, but not the hydrate form of these compounds, is the active inhibitory species, we would expect a correlation between the $K_{RSH,app}$ value of the parent ketone and the potency of the corresponding inhibitor.

Synthesis of Inhibitors. We have developed a generalized strategy for the synthesis of our papain inhibitors (Scheme 1). This strategy allows us to perform similar reactions in the

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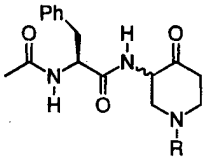
(25) Sanders, E. G.; Jencks, W. P. *J. Am. Chem. Soc.* **1968**, *90*, 6154.

preparation of each of the four target compounds. Dieckmann condensation of diesters **1** and **2** gives keto esters **4** and **5**. Compounds **3** and **6** are commercially available. The yield for cyclization of **2** is only 31%, presumably because of competing β-elimination. However, this represents a significant improvement over the previously reported synthesis of methyl tetrahydropyran-4-one-3-carboxylate, which proceeded in 8% yield.[26] The ketones in compounds **3**, **5**, and **7** are protected as thioketals. Since the oxidative conditions that are used for removal of this protecting group are not compatible with thioethers, compound **4** is protected as an oxygen ketal. The esters are hydrolyzed and the resulting carboxylic acids are treated with diphenylphosphoryl azide.[27] Curtius rearrangement followed by trapping of the isocyanates with *t*-BuOK gives carbamates **16**−**19**. The Boc protecting groups are removed with trifluoroacetic acid, and the resulting amines are coupled with an N-protected phenylalanine derivative.[28] After removing the phenylalanine protecting groups, the free amines are coupled to monomethyl succinate to give compounds **26**−**29**. The thioketal protecting groups in compounds **26**, **28**, and **29** are removed by treatment with *N*-bromosuccinimide,[29] and the diastereomers of inhibitors **30** and **32** are separated by HPLC. The Cbz protecting group in compound **33** is removed by catalytic hydrogenation to give inhibitor **34**, which is evaluated as a mixture of diastereomers. The diastereomers of **27** can be separated by flash chromatography, and each are then treated with acetone and *p*-toluenesulfonic acid to give the separate diastereomers of inhibitor **31**.

Racemization of Inhibitors. Papain is assayed in 100 mM phosphate buffer at pH 6.5. These conditions may catalyze the enolization of the ketone in our inhibitors and thus lead to their racemization. We have monitored this reaction using HPLC or ^{1}H NMR spectroscopy. The cyclohexanone-based inhibitor **30** was very stable under the assay conditions, showing less that 5% racemization after 24 h. Tetrahydropyranone **32** was somewhat less stable, with a half-time for racemization of 5.25 h. However, this reaction is slow enough so that over the time period of a typical enzyme assay, the compound racemizes less than 1%. We were unable to separate the diastereomers of piperidone inhibitor **34** or its precursor **33** by standard chromatographic techniques. However, the diastereomers of compound **35**, which has an acetyl group on its N-terminus rather than a methoxysuccinyl group, were readily separated by HPLC. We therefore chose to study racemization of compound **36** by ^{1}H NMR spectroscopy. Over the course of the 10 min required

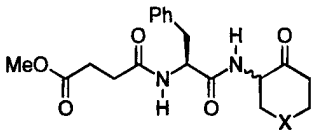


35 a and **b**: R = Cbz
36 a and **b**: R = H

to prepare the sample and acquire the spectrum, this compound was completely racemized. Therefore, we measured the inhibition constant for compound **34** as a mixture of diastereomers. We have not examined racemization of the tetrahydrothiopyranone-based inhibitor **31**, but observed reactivity trends and chemical intuition both suggest that it should have a racemization rate that falls between that of compounds **30** and **32**.

Inhibition Studies. The 4-heterocyclohexanone-based inhibitors **30**−**32** and **34** are all reversible competitive inhibitors of papain (Table 2).[30] The enzyme shows a clear preference for one diastereomer of each inhibitor, although we have not determined the absolute configuration of the tighter binding diastereomer. Data for the acetone-, pyruvic acid-, and methyl pyruvate-based inhibitors are included in Table 2 for comparison. Although these three reference compounds do not have a methoxysuccinyl group on their N-terminus, our previous work has demonstrated that inhibitors with *N*-acetyl or *N*-Cbz blocking groups have inhibition constants that are within a factor of two of the *N*-methoxysuccinyl compounds.

Table 2. Inhibition of Papain by 4-Heterocyclohexanone-Based Inhibitors



X	K_i (μM) more-potent diastereomer	K_i (μM) less-potent diastereomer
CH_2	78	3200
S	26	2400
O	11	3300
NH_2^+	121[a]	
Other Ketone-Based Inhibitors		
AcPhe-$NHCH_2COMe$		1550[b]
ZPhe-$NHCH_2COCO_2H$		7[c]
ZPhe-$NHCH_2COCO_2Me$		1[c]

[a] Assayed as a mixture of diastereomers. This compound racemizes under the assay conditions. [b] Data from ref 15. [c] Data from ref 11.

The cyclohexanone-based inhibitor (X = CH_2) is 20 times more potent than the noncyclic acetone-based inhibitor. This is a reflection of the ring strain in cyclohexanone that destabilizes the ketone relative to the hemithioketal that is formed by reaction of the inhibitor with the active site nucleophile. Substituting electronegative functionality into the ring (X = S, O) leads to even better inhibitors. This trend in inhibition constants mirrors the differences that we observe for reaction of the parent ketones with water and thiol nucleophiles. The only compound that does not fit the trend is the piperidone-based inhibitor **34**. This compound is protonated under the assay conditions (pH 6.5), and its low potency is likely caused by the unfavorability of placing this positive charge into the enzyme active site.[9]

Discussion

Linear Free-Energy Relationship. We observe a correlation between the reactivity of 4-heterocyclohexanones and the electronic properties of the heteroatom in these molecules. This correlation requires an appropriate description of the magnitude of the through-space electrostatic repulsion between the heteroatom and the ketone. Swain and Lupton[31] have constructed a modified Hammett equation (eq 2) in which they describe the electronic characteristics of substituents in terms of two parameters; a field substituent constant F, and a resonance substituent constant R.

$$\log(K_X/K_H) = \rho(fF + rR) \qquad (2)$$

The terms f and r are empirical weighing factors that are specific for the particular reaction and set of reaction conditions,

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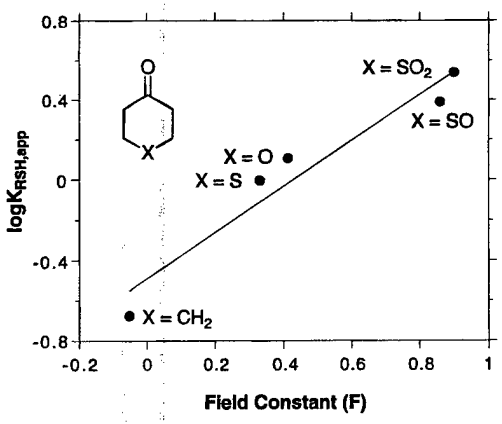

Figure 2. Correlation between the logarithm of the apparent equilibrium constant for addition of thiol to 4-heterocyclohexanones and the field substituent constant F (log $K_{RSH,app}$ = 1.1F − 0.5; correlation coefficient = 0.97).

while the F and R parameters are independent of the reaction. If the major interaction between the heteroatom and ketone is electrostatic, then the field substituent constant F should provide a good measure of this interaction.

The chemical systems that are used to define field substituent constants are designed so that the substituents are attached to the parent molecules through a single bond.[31] However, in 4-heterocyclohexanones the heteroatom is attached by two bonds. We have thus approximated the functionality at the 1-position of heterocyclohexan-4-ones by using the field constant for the substituents -CH_3, -SCH_3, -OCH_3, -$SOCH_3$, and -SO_2CH_3. Protonated piperidone has been omitted from our analysis because the F value for the corresponding substituent, -$NH_2CH_3^+$, has not been reported.

Figure 2 shows that there is a good correlation between the logarithm of the apparent equilibrium constants for addition of thiol to 4-heterocyclohexanones (log $K_{RSH,app}$) and the field substituent constants.[32] This correlation confirms that the interaction between the heteroatom and the ketone in 4-heterocyclohexanones is best described as a through-space electrostatic repulsion. Resonance effects, differences in ring strain, and transannular anomeric effects[20] have a relatively minor influence on the equilibria of the reversible addition of water and thiol nucleophiles to these ketones. The slope of the line in Figure 2 is 1.1. A similar plot for dissociation of 4-substituted benzoic acids has a slope of 0.49.[31] Comparison of these values suggests that addition of thiols to 4-heterocyclohexanones responds two times more strongly to the *field component* of the electronic effects exerted by substituents. The larger slope for the addition reaction is reasonable because the substituent and reaction center are closer together than they are in 4-substituted benzoic acids.

Correlation between Ketone Reactivity and Enzyme Inhibition. We have designed our cysteine protease inhibitors on the basis of the supposition that inhibitor potency is controlled by the stability of the hemithioketal that results from addition of the active site nucleophile to the inhibitor, although we have not proved the existence of this hemithioketal through structural studies. If this supposition is correct, we should observe a correlation between inhibition constants and the equilibrium constants for addition of thiol to the parent ketones. Because enzyme inhibition takes place in aqueous solvent, the most appropriate comparison is between inhibition constants and $K_{RSH,app}$ values.[33]

(32) A good correlation also exists between log K_{RSH} and F and between log K_{H_2O} and F. However, there is a poor correlation between log $K_{RSH,app}$ and the resonance substituent constant R (correlation coefficient = 0.41)

(33) For a similar analysis involving inhibitors of cathepsin B, see: ref 22a.

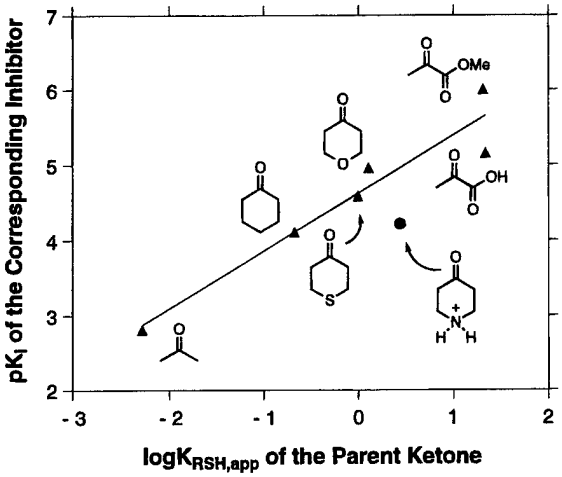

Figure 3. Correlation between inhibition constant (pK_i) of the ketone-based inhibitors and the logarithm of the apparent equilibrium constant for addition of thiol to the parent ketones (pK_i = 0.8 log $K_{RSH,app}$ + 4.6; correlation coefficient = 0.96).

The correlation shown in Figure 3 demonstrates that addition of 3-mercaptopropionic acid to simple ketones in aqueous solution is an appropriate model for addition of the enzyme active site cysteine residue to the corresponding ketone-based inhibitors. The apparent equilibrium constant for the model reaction provides a good prediction of inhibitor potency for this structurally homologous series of compounds. The plot of pK_i vs log $K_{RSH,app}$ has a slope of 0.8. This slope, which is less than unity, indicates that the enzymatic addition reaction responds less efficiently to the electrophilicity of the ketone than does the model system. The difference in reactivity is likely caused by the differences in steric, electronic, solvation, and orientational requirements of the enzymatic reaction compared to the reaction in solution.

We have omitted the piperidone-based inhibitor **34** from the linear regression in Figure 3 because the positive charge on this molecule perturbs its reactivity with the enzyme. As expected, this inhibitor does not fit well into a correlation that is based simply upon electrophilicity of the ketone in these molecules.

Conclusions. The results presented in this paper demonstrate that through-space electrostatic interactions can be useful and predictable design elements for construction of bioactive molecules. The physical-organic correlations point the way toward synthesis of more potent inhibitors. This goal can be achieved by choosing functionality that further increase the electrostatic repulsion between the heteroatom and the ketone in 4-heterocyclohexanones, such as a sulfoxide or sulfone. In addition, potency and specificity can be increased by functionalizing both the 3- and 5-positions of the heterocyclohexanone ring so that we extend noncovalent interactions of the inhibitor into the leaving group subsites. Future studies will be aimed toward proving formation of the hemithioketal intermediate using ^{13}C NMR spectroscopy in conjuction with an inhibitor that is labeled with ^{13}C at the ketone carbon.

Experimental Section

General Methods. NMR spectra were recorded on Brucker WM-250 or AM-400 instruments. Spectra were calibrated using TMS (δ = 0.00 ppm) for ^1H NMR and $CDCl_3$ (δ = 77.0) 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. Semipreparative HPLC was performed on the same system using a semipreparative column (21.4 × 250 mm).

Reactions were conducted under an atmosphere of dry nitrogen in oven-dried glassware. Anhydrous procedures were conducted using standard syringe and cannula transfer techniques. THF and toluene were distilled from sodium and benzophenone. Methylene chloride was distilled from CaH_2. Other solvents were of reagent grade and were stored over 4 Å 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.

Experimental details of the synthesis of inhibitors **30**, **31**, and **34** are available in the Supporting Information.

Di-*n*-propyl 4-Oxa-1,7-heptanedioate 2. A solution of 3,3′-oxydipropionitrile (18.9 g, 152 mmol) and *p*-toluenesulfonic acid (*p*-TsOH) monohydrate (115.8 g, 608 mmol) in *n*-propanol (200 mL) was refluxed for 24 h. The solution was cooled and concentrated to approximately 150 mL. The resulting solution was partitioned between 350 mL of water and 350 mL of hexanes. The organic layer was separated and washed with saturated $NaHCO_3$ (200 mL), water (300 mL), and brine (150 mL). The solution was dried, filtered, and concentrated, and the crude material was purified by flash chromatography (1:3 EtOAc/hexanes) to yield **2** (21.2 g, 57%) as a colorless liquid. The product can also be purified by vacuum distillation (bp 158 °C, 6 mm) in somewhat lower yields (45%): R_f 0.66 (1:1 EtOAc/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.94 (t, J = 7.4 Hz, 3H), 1.66 (dt, J = 7.0, 7.1 Hz, 2H), 2.57 (t, J = 6.1 Hz, 2H), 3.73 (t, J = 6.4 Hz, 2H), 4.04 (t, J = 6.7 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.3, 21.9, 35.0, 66.1, 66.4, 171.5; HRMS-FAB calcd for $C_{12}H_{22}O_5$ 246.1467, found 246.1467.

***n*-Propyl Tetrahydropyran-4-one-3-carboxylate 5.** To a solution of diispropylamine (4.65 g, 45.9 mmol) in THF (50 mL) at −78 °C was added *n*-butyllithium (4.38 mL of 10.0 M in hexanes). This solution was added via cannula to a solution of **2** (5.14 g, 20.9 mmol) in THF (300 mL) at −78 °C. The solution was stirred at −78 °C for 15 min, and then the reaction was quenched by the addition of 25 mL of H_2O. The solution was partitioned between 200 mL of 1 N HCl and 200 mL of hexanes. The resulting aqueous layer was extracted with EtOAc (150 mL), and the combined organic layers were washed with brine (300 mL). The solution was dried, filtered, and concentrated, and the crude material purified by flash chromatography (1:4 Et_2O/hexanes) to yield **5** (1.19 g, 31%) as a mixture of keto and enol tautomers: R_f = 0.54 (1:1 Et_2O/hexanes); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (t, J = 6.3 Hz, 3H), 1.27−1.31 (m), 1.61−1.75 (m, 2H), 2.37−2.41 (m, 3H), 2.52−2.59 (m), 2.66−2.73 (m), 3.46−3.50 (m), 3.71−3.75 (m), 3.85 (t, J = 5.7 Hz, 2H), 3.98−4.10 (m), 4.12 (t, J = 6.6 Hz, 2H), 4.16−4.25 (m), 4.28 (t, J = 1.7 Hz, 2H), 11.85 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.2, 21.9, 28.6, 41.8, 57.8, 63.9, 65.8, 66.3, 67.0, 68.1, 69.6, 97.4, 127.8, 129.7, 168.7, 170.1, 201.4; HRMS-EI (M^+) calcd for $C_9H_{14}O_4$ 186.0892, found 186.0894.

Tetrahydropyranone Thioketal 10. To a solution of **5** (1.26 g, 6.8 mmol) and 1,2-ethanedithiol (1.28 g, 13.6 mmol) in CH_2Cl_2 (20 mL) cooled in an ice bath was added BF_3·Et_2O (1.04 mL, 8.5 mmol). The solution was stirred at 0 °C for 4 h, and then it was washed with 10% aqueous NaOH solution, water, and brine (20 mL). The organic layer was dried, and concentrated, and the crude material was purified by flash chromatography (2:3 EtOAc/hexanes) to yield **10** (0.77 g, 43%) as a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.96 (t, J = 7.4 Hz, 3H), 1.63−1.73 (m, 2H), 1.93 (dm, J = 13.7 Hz, 1H), 2.84−2.88 (m, 1H), 2.91−2.92 (m, 1H), 3.24−3.32 (m, 4H), 3.64−3.69 (m, 1H), 3.90−3.93 (m, 2H), 4.08−4.14 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.4, 21.9, 38.3, 39.1, 40.0, 54.8, 65.7, 66.5, 67.8, 69.5, 171.0; HRMS-EI (M^+) calcd for $C_{11}H_{18}O_3S_2$ 262.0697, found 262.0707.

Tetrahydropyranone Carboxylic Acid 14. To a solution of **10** (0.41 g, 1.58 mmol) in MeOH (10 mL) was added 1 N NaOH (10 mL). The solution was stirred at 30 °C for 50 h. The solution was then cooled and diluted with 0.2 N NaOH (10 mL). The solution was washed with 1:1 EtOAc/hexanes (10 mL), and the aqueous layer was separated and acidified with 1 N HCl. The acidic aqueous solution was extracted with EtOAc (2 × 40 mL). These organic extracts were washed with brine (50 mL), dried, and concentrated. The resulting solid was recrystallized from EtOAc/hexanes to yield **14** (0.22 g, 68%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.98 (d, J = 13.8 Hz, 1H), 2.76 (m, 1H), 2.99 (t, J = 3.3 Hz, 1H), 3.29−3.35 (m, 4H), 3.68−3.74 (m, 1H), 3.88 (t, J = 4.3 Hz, 1H), 3.93 (t, J = 4.2 Hz, 1H), 3.99 (dd, J = 3.4, 12.2 Hz, 1H), 4.12 (dd, J = 3.4, 11.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 38.5, 39.2, 40.3, 54.2, 65.5, 67.8, 69.2; HRMS-EI (M^+) calcd for $C_8H_{12}O_3S_2$ 220.0228, found 220.0224.

Tetrahydropyranone Carbamate 18. A solution of **14** (0.22 g, 1.0 mmol), *N*,*N*-diisopropylethylamine (DIEA, 1.9 g, 1.50 mmol), and diphenylphosphoryl azide (DPPA, 0.28 g, 1.0 mmol) in benzene (10 mL) was refluxed overnight. Aliquots of the reaction mixture were monitored for disappearance of the acyl azide peak at 2168 cm^{-1} and appearance of the isocyanate peak at 2245 cm^{-1} by FT-IR. After the Curtius rearrangement was judged complete by IR, the solution was cooled in an ice bath and slowly added to an ice-cold solution of potassium *tert*-butoxide (0.34 g, 3.0 mmol) in THF (10 mL). The reaction was stirred for 15 min and then partitioned between 15 mL of 1 N HCl and 15 mL of EtOAc. The organic layer was separated and washed with 1 N NaOH and brine (15 mL). The solution was dried, filtered, and concentrated, and the crude material was purified by flash chromatography (1:4 EtOAc/hexanes) to yield **18** (0.19 g, 65%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.45 (s, 9H), 2.21 (t, J = 4.5 Hz, 2H), 3.26−3.35 (m, 4H), 3.61−3.64 (m, 1H), 3.79−3.82 (m, 1H), 3.89−3.99 (m, 2H), 5.04 (brd, J = 7.7 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 27.5, 37.7, 37.9, 40.9, 54.2, 66.1, 70.0, 78.1, 154.4; HRMS-EI (M^+) calcd for $C_{12}H_{21}NO_3S_2$ 291.0963, found 291.0959.

Aminotetrahydropyranone·Trifluoroacetic Acid Salt 21. Trifluoroacetic acid (TFA, 3.0 mL) was added to a solution of **18** (0.18 g, 0.62 mmol) in CH_2Cl_2 (10 mL) that was cooled in an ice bath. The reaction was stirred at 0 °C for 1 h, concentrated, redissolved in CH_2Cl_2, and then concentrated again to remove excess TFA. The crude oil was then triturated with ether to yield **21** (0.18 g, 95%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.14 (dt, J = 14.3, 5.6 Hz, 1H), 2.44 (dt, J = 14.1, 5.0 Hz, 1H), 3.32−3.47 (m, 5H), 3.70−3.76 (m, 3H), 4.04 (dd, J = 12.2, 3.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 39.9, 40.1, 41.1, 56.7, 68.1, 68.5, 68.6, 118.2 (q), 162.9 (q); HRMS-EI (M^+) calcd for $C_7H_{13}NOS_2$ 191.0439, found 191.0437.

Phenylalanyltetrahydropyranone 24. To a solution of **21** (250 mg, 0.82 mmol) and DIEA (529 mg, 4.1 mmol) in CH_2Cl_2 (10 mL) was added solid N-Boc-phenylalanyl fluoride[28] (240 mg, 0.90 mmol). The solution was stirred for 1 h and then washed with 1 N HCl, saturated $NaHCO_3$, and brine (10 mL). The solution was dried over Na_2CO_3 and concentrated, and the crude material was purified by flash chromatography (2:3 EtOAc/hexanes) to yield a mixture of diastereomers of **24** (218 mg, 61%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31 (s, 9H), 1.34 (s, 9H), 2.03−2.15 (m, 4H), 2.82 (brs, 1H), 2.94−3.16 (m, 13H), 3.46−3.51 (m, 2H), 3.57 (brm, 1H), 3.67−3.77 (m, 3H), 4.14 (brm, 1H), 4.19−4.27 (m, 2H), 4.37 (brm, 1H), 5.14 (brm, 1H), 5.31 (brm, 1H), 6.18 (brm, 1H), 6.58 (d, J = 9.0 Hz, 1H), 7.11−7.24 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 27.8, 28.0, 37.7, 38.1, 38.77, 38.79, 38.84, 41.8, 52.8, 52.9, 53.0, 53.1, 55.4, 55.9, 66.79, 66.84, 69.4, 69.6, 69.7, 69.9, 79.8, 126.5, 126.6, 128.3, 128.4, 129.0, 129.2, 155.1, 170.6, 170.9; HRMS-FAB (M + Na^+) calcd for $C_{21}H_{30}N_2NaO_4S_2$ 461.1545, found 461.1544.

(Methoxysuccinyl)tetrahydropyranone 28. A solution of **24** (200 mg, 0.46 mmol) and TFA (3 mL) in CH_2Cl_2 (7 mL) was stirred at 25 °C for 1 h. This solution was concentrated, and the resulting material was triturated with ether to precipitate the TFA salt as a white solid. This solid was washed with ether, dried under vacuum, and then added to a solution of methyl succinate (61 mg, 0.46 mmol), 1-hydroxybenzotriazole (HOBT, 72 mg, 0.46 mmol), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC, 114 mg, 0.60 mmol), and *N*-methylmorpholine (0.10 mL) in CH_2Cl_2 (5 mL). The reaction was stirred overnight at room temperature, and then it was washed with water, 1 M $KHSO_4$, saturated Na_2CO_3, and dried over Na_2CO_3. The dried solution was concentrated, and the crude material was purified by flash chromatography (7:3 EtOAc/hexanes) to yield a mixture of diastereomers of **28** (144 mg, 70%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.11−2.14 (m, 4H), 2.45−2.50 (m, 2H), 2.52−2.54 (m, 2H), 2.57−2.64 (m, 4H), 2.97−3.13 (m, 5H), 3.19−3.26 (m, 9H), 3.53−3.62 (m, 3H), 3.65 (s, 3H), 3.67 (s, 3H), 3.76−3.86 (m, 3H), 4.17−4.22 (m, 1H), 4.26−4.31 (m, 1H), 4.66−4.71 (m, 1H), 4.77−4.82 (m, 2H), 6.25 (d, J = 6.3 Hz, 1H), 6.68 (d, J = 9.1 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.96 (d, J = 7.7 Hz, 1H), 7.21−7.30 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.0, 29.1, 30.59, 30.61, 37.9, 38.2, 38.81, 38.88, 38.93, 41.9, 51.6, 53.0, 53.3, 54.3, 54.7, 66.9, 67.0, 69.48, 69.50, 69.7, 69.9, 126.6, 126.8, 128.4, 128.5, 129.1, 129.3, 136.5, 170.3,

170.7, 171.2, 171.4, 172.9, 173.0; HRMS-FAB (M + Na^+) calcd for $C_{21}H_{28}N_2NaO_5S_2$ 475.1338, found 475.1349.

Tetrahydropyranone Inhibitors 32a and 32b. A solution of *N*-bromosuccinimide (NBS, 440 mg, 2.47 mmol) in 80% aqueous MeCN (10 mL) was cooled in an ice bath. To this solution was added **28** (160 mg, 0.35 mmol) in MeCN (5 mL). The ice bath was removed, and the reaction mixture was stirred for 10 min. It was then partitioned between 1:1 CH_2Cl_2/EtOAc (25 mL) and saturated Na_2SO_3 (10 mL). The organic layer was separated, washed with saturated $NaHCO_3$ and brine, and dried over Na_2CO_3. The dried solution was concentrated, and the residue was redissolved in 1:1 MeCN/H_2O. This solution was filtered and extracted with 1:1 CH_2Cl_2/EtOAc. The resulting organic layer was dried and concentrated to yield a mixture of diastereomers of **32** (88 mg, 66%) as a white solid. The diastereomers were separated by HPLC (silica) with 3.5% 2-propanol in CH_2Cl_2 as the mobile phase. The retention times for diastereomers **32a** and **32b** were 13.1 and 14.1 min, respectively. For **32a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.45−248 (m, 3H), 2.59−2.74 (m, 3H), 2.93 (t, J = 9.9 Hz, 1H), 3.03 (m, 1H), 3.12−3.14 (m, 1H), 3.55 (t, J = 11.4 Hz, 1H), 3.67 (s, 3H), 4.29 (brm, 1H), 4.42 (brm, 1H), 4.61 (brm, 1H), 4.72 (brm, 1H), 6.28 (brm, 1H), 6.59 (brm, 1H), 7.17−7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.1, 30.9, 38.3, 42.2, 51.9, 54.4, 57.3, 68.8, 71.6, 127.1, 128.7, 129.2, 136.3, 170.7, 171.3, 173.4, 202.5; HRMS-FAB (M + Na^+) calcd for $C_{19}H_{24}N_2NaO_6$ 399.1532, found 399.1537. For **32b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.45−2.48 (m, 3H), 2.59−2.78 (m, 3H), 3.02−3.06 (m, 1H), 3.10−3.13 (m, 2H), 3.59 (t, J = 11.5 Hz, 1H), 3.67 (s, 3H), 4.27−4.32 (m, 1H), 4.54 (m, 2H), 4.73 (brm, 1H), 6.32 (brm, 1H), 6.66 (brm, 1H), 7.17−7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.1, 30.9, 38.2, 42.1, 51.8, 54.3, 57.5, 68.8, 71.8, 127.1, 128.7, 129.2, 136.1, 170.9, 171.3, 173.3, 202.2; HRMS-FAB (M + Na^+) calcd for $C_{19}H_{24}N_2NaO_6$ 399.1532, found 399.1521.

Measurement of K_{H_2O} and K_{RSH} by ^{1}H NMR Spectroscopy. These equilibrium constants were measured at 25 °C on a Brucker AM-400 NMR spectrometer according to the procedures of Burkey and Fahey.[18,23] Cyclohexanone, tetrahydropyran-4-one, tetrahydrothiopyran-4-one, and 4-piperidone hydrochloride were purchased from Aldrich Chemical Co. and used without further purification. NMR samples were prepared by dissolving the ketone (100 mM) in D_2O. For measurements of K_{RSH}, the concentration of 3-mercaptopropionic acid was 200 mM.

Racemization of Inhibitors. The racemization of the cyclohexanone inhibitors **30a** and **30b** was followed by RPHPLC using the conditions reported above for the separation of the two diastereomers. Each diastereomer was dissolved in 100 mM phosphate buffer at pH 6.5. Less than 5% racemization was detected after 24 h.

The racemization of the tetrahydropyranone inhibitors **32a** and **32b** was monitored using ^{1}H NMR spectroscopy by integration of the methyl ester signal at 3.47 ppm for **32a** and 3.45 ppm for **32b**. Each diastereomer was dissolved in 100 mM phosphate buffer at pH 6.5 that was prepared using D_2O. The observed first-order rate constant for racemization was measured to be $k_{obsd} = (2.2 \pm 0.5) \times 10^{-3}$ min^{-1}. This rate constant corresponds to a half-time for racemization of 5.25 h. Thus, over the time period of a typical enzyme assay, less than 1% of each diastereomer of the inhibitor will have racemized to the undesired diastereomer.

Racemization experiments for the piperidone-based inhibitor were performed using compounds **35a** and **35b**. These diastereomers were separated by HPLC with an eluent of 2% MeOH in CH_2Cl_2 (**35a** retention time 15.5 min; **35b** retention time 20.5 min). The Cbz protecting group in each diastereomer was removed using the procedure reported for the preparation of compound **34**, which yielded compounds **36a** and **36b**. ^{1}H NMR spectra demonstrated that these deprotections occurred with retention of stereochemistry. Diastereomer **36a** was split into two samples and each placed in an NMR tube. One sample was dissolved in 100 mM phosphate buffer (pH 6.5) that was prepared using D_2O. The ^{1}H NMR spectrum of this sample demonstrated that the compound was completely racemized within 10 min under these conditions. The second sample was dissolved in 1:1 acetone-d_6/D_2O. ^{1}H NMR of this sample showed relatively slow reaction, with complete racemization after approximately 22 h. Diastereomer **36b** gave similar results. For **36a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.98 (brs, 3H), 2.45−2.54 (brm, 3H), 3.06 (brs, 1H), 4.38−4.45 (brm, 2H), 4.75−4.82 (brm, 2H), 5.17 (brm, 2H), 6.34 (brs, 1H), 6.79 (brs, 1H), 7.18−7.38 (brm, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.2, 38.5, 40.4, 44.1, 48.7, 54.4, 56.6, 67.9, 127.1, 128.0, 128.2, 128.6, 128.7, 129.2, 136.2, 154.8, 170.0, 171.1, 202.88, 202.94; HRMS-FAB (M + Na^+) calcd for $C_{24}H_{27}N_3NaO_5$ 460.1849, found 460.1860. For **36b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.98 (brs, 3H), 2.44 (brm, 3H), 3.01 (brm, 3H), 4.46 (brs, 2H), 4.67−4.76 (brm, 2H), 5.18 (brm, 2H), 6.38 (brs, 1H), 6.68 (brs, 1H), 7.19−7.40 (brm, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.2, 38.7, 40.4, 44.2, 48.5, 54.5, 56.3, 67.9, 127.0, 128.0, 128.3, 128.6, 128.7, 129.3, 136.2, 136.4, 154.8, 170.2, 170.9, 203.2; HRMS-FAB (M + Na^+) calcd for $C_{24}H_{27}N_3NaO_5$ 460.1849, found 460.1849.

Papain Assays. Papain (recrystallized two times) and L-BAPNA (*N*α-benzoyl-L-arginine *p*-nitroanilide hydrochloride) were used as received from Sigma Chemical Co. Reaction progress was monitored with a Perkin-Elmer 8452A diode array UV-vis spectrometer. Papain was assayed at 25 °C in 100 mM phosphate buffer (pH 6.5) containing 5 mM EDTA and 5 mM cysteine. BAPNA and inhibitor stock solutions contained DMSO (10−100%), and all assay mixtures contained a final DMSO concentration of 10%. Papain stock solutions (0.5−1 mg/mL) were prepared in buffer (5×), and the enzyme was activated for 1 h before the assays were run. Initial rates were determined by monitoring the change in absorbance at 412 nm from 60 to 120 s after mixing. None of the inhibitors showed evidence of slow binding. The more potent diastereomer of each inhibitor was subjected to full kinetic analysis. For each inhibitor concentration examined (**30a** 0, 21, 53, 107, 160, 217 μM; **31a** 0, 2.7, 5.5, 27.4, 55, 110 μM; **32a** 0, 2, 25, 50, 75, 100 μM; **34a** 0, 13.9, 69.5, 139, 209, 417 μM) at least five substrate concentrations were used (**30a** 0.37, 0.53, 0.75, 1.5, 7.5 mM; **31a** 0.5, 0.66, 0.99, 2.0, 6.6 mM; **32a** 0.5, 0.65, 0.94, 1.7, 4.5, 8.0 mM; **34a** 0.5, 0.66, 0.99, 2.0, 6.6 mM) with at least two independent determinations at each concentration. K_m was measured to be 4.89 mM. The background hydrolysis rate was less than 1% of the slowest rate measure and thus ignored. 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 robust statistical weighing to the linear fit of 1/[*V*] vs 1/[*S*]. For the less-potent diastereomer of each inhibitor, a single substrate concentration (**30a** 5.28 mM; **31a** 3.30 mM; **32a** 4.22 mM) was monitored at with least 4 different inhibitor concentration (**30a** 0, 130, 410, 830 μM; **31a** 0, 0.14, 0.29, 0.57, 1.14, 1.72 mM; **32a** 0, 0.1, 0.56, 1.1, 1.5, 1.9 mM). Competitive inhibition was assumed, and K_i was calculated using a Dixon analysis. Data analysis was performed with the commercial graphing package Grafit (Erithacus Software Ltd).

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Supporting Information Available: Lineweaver−Burk plots for the inhibition of papain by compounds **30**−**32** and **34**; ^{1}H and ^{13}C NMR characterization for compounds reported in the Experimental Section; experimental details of the synthesis of inhibitors **30**, **31**, and **34** (55 pages). See any current masthead page for ordering and Internet access instructions.

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Demonstration by ^{13}C NMR Studies That Tetrahydropyranone-Based Inhibitors Bind to Cysteine Proteases by Reversible Formation of a Hemithioketal Adduct

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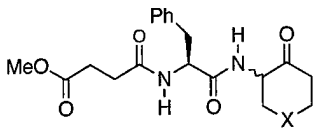
Introduction

Cysteine proteases are important targets in medicinal chemistry.[1] Members of this class of proteolytic enzymes, such as the calpains[2] and cathepsins B and L,[1] are implicated in a variety of diseases including rheumatoid arthritis, muscular dystrophy, and cancer. In addition, a new family of cysteine proteases have recently been discovered that are related to interleukin-1β converting enzyme (ICE) and CED-3.[3] These new proteases share a specificity for substrates with aspartic acid at the P1 position and have been shown to play key roles in both the regulation and initiation of programmed cell death or apoptosis. Excessive apoptosis causes neural damage in both Alzheimer's and Huntington's diseases, while insufficient apoptosis occurs in many cancers and in autoimmune disorders such as AIDS. The implication of cysteine proteases in such a large number of disease states provides a strong motivation for developing potent and specific inhibitors of these enzymes. Such compounds may serve as both new therapeutic agents and as tools for investigating the role of cysteine proteases in disease processes.

We have recently described a new class of cysteine protease inhibitors that are based upon a 4-heterocyclohexanone nucleus (compounds **1**–**4**).[4] The electrophilic ketone group in these compounds is designed to react with the enzyme-active-site nucleophile to give a reversibly formed hemithioketal adduct. This adduct mimics the tetrahedral intermediate that is formed during enzyme-catalyzed peptide hydrolysis. The reactivity of this carbonyl is enhanced by ring strain and by through-space electrostatic repulsion from the heteroatom at the 4-position of the ring. There is a good correlation between the electrophilicity of this ketone moiety and the potency of the inhibitors against the enzyme papain.[4]

Our interpretation of inhibition studies with compounds **1**–**4** was based upon the assumption that a hemithioketal does indeed form between the inhibitors and the active-site cysteine residue. This assumption is reasonable on the basis of the well-established mechanism by which papain catalyzes cleavage of amide bonds[1] and comparison of 4-heterocyclohexanones with other inhibitors, such as peptide aldehydes, that are known to give this type of covalent adduct.[5,6] However, there are at least two other plausible explanations for the reactivity trends that we observed. First the hydrate of the ketone, and not the ketone itself, could be the active inhibitory species. Hydrates of active carbonyl compounds are good inhibitors of both aspartic proteases such as pepsin and renin and metalloproteases such as angiotensin-converting enzyme and carboxypeptidase A.[7] Second, the differences in inhibition could have been caused by formation of a specific hydrogen bond or electrostatic interaction between the enzyme and the polar heteroatom at the 4-position of the ring. The goal of our current work is to determine if the mechanism by which 4-heterocyclohexanones inhibit papain is through formation of a hemithioketal adduct. Our approach is to synthesize an inhibitor, tetrahydropyranone **10** (Scheme 1), that incorporates a ^{13}C label at the ketone carbon. Reaction of this labeled inhibitor with a stoichiometric amount of papain is monitored by ^{13}C NMR spectroscopy. These experiments allow us to observe directly formation of the hemithioketal adduct between enzyme and inhibitor. The results demonstrate that, like peptide aldehydes, 4-heterocyclohexanones are transition-state analogue inhibitors of cysteine proteases.[5,8]



1: X = CH_2
2: X = S
3: X = O
4: X = NH_2^+

Results and Discussion

Synthesis of the Labeled Inhibitor. We have developed a synthesis of inhibitor **10** that places a single ^{13}C label specifically at the ketone carbon (Scheme 1). Reaction of bromoethyl ether **5** with $Et_4N^{13}CN$ gave dinitrile **6**.[9] The labeled reagent can be conveniently prepared from $K^{13}CN$ and Et_4NBF_4.[10] Alcoholysis of **6** followed by base-promoted cyclization of the resulting diester gave keto ester **7**. After protection of the ketone

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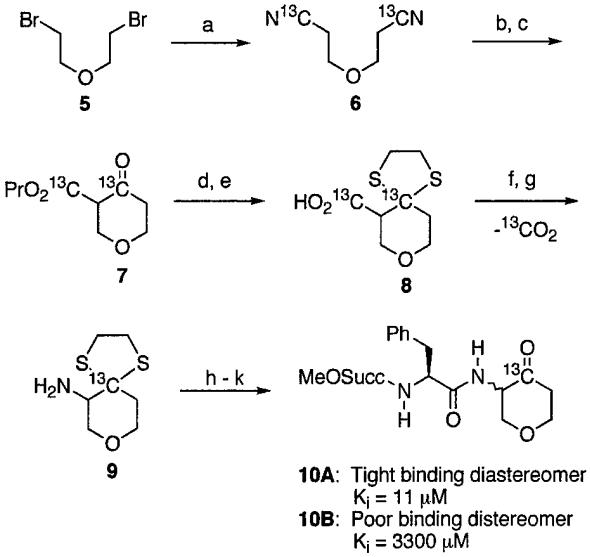
Scheme 1[a]



[a] Reagents: (a) $Et_4N^{13}CN$, 75%; (b) *n*-PrOH, *p*-TsOH; (c) LDA, THF, −78 °C; (d) ethanedithiol, $BF_3{\cdot}Et_2O$; (e) NaOH, MeOH; (f) $(C_6H_5O)_2P(O)N_3$, C_6H_6, followed by *t*-BuOK, THF; (g) TFA, CH_2Cl_2; (h) BocPheOH, EDC, HOBT; (i) TFA, CH_2Cl_2; (j) monomethyl succinate, EDC, HOBT; (k) NBS, H_2O.

and saponification of the ester, compound **8** was treated with diphenyl phosphorazidate to induce a Curtius rearrangement. Trapping of the resulting isocyanate with *t*-BuOK yielded the corresponding Boc-protected amine. Removal of the Boc group with TFA resulted in loss of 1 equiv of $^{13}CO_2$ from the molecule to give amine **9**. This compound contained a single ^{13}C label at the desired position. The phenylalanine residue and methoxysuccinyl group were attached using standard peptide coupling procedures, and the diastereomers of **10** were separated using preparative HPLC.

Racemization of Inhibitors. Inhibitors that are based upon 4-heterocyclohexanones racemize at a significant rate in 100 mM phosphate buffer at pH 6.5, conditions used for kinetic assays of papain. For example, the tetrahydropyranone-based inhibitor racemizes with a half-life of 5.3 h under these conditions.[4] In our current studies, we have found that the rate of racemization is inversely correlated with buffer concentration. In the experiments described below, which use 10 mM phosphate at pH 6.5, inhibitor **10A** has a half-life for racemization of 192 h. The stability of the inhibitor under conditions that employ low buffer concentration have allowed us to acquire ^{13}C NMR spectra of the separated diastereomers of **10** in the presence of papain, without significant interference from racemization.

Enzyme Purification. Commercial preparations of papain are contaminated with a large amount of inactive enzyme. Papain used in this study was purified by affinity chromatography on a mercurial agarose column.[11] Enzyme purified in this manner is greater than 95% active as judged by titration of the active-site cysteine-25 thiolate with the reagent 2,2′-dipyridyl disulfide (DDS).[12]

^{13}C NMR Experiments. The two diastereomers of inhibitor **10** have very different inhibition constants

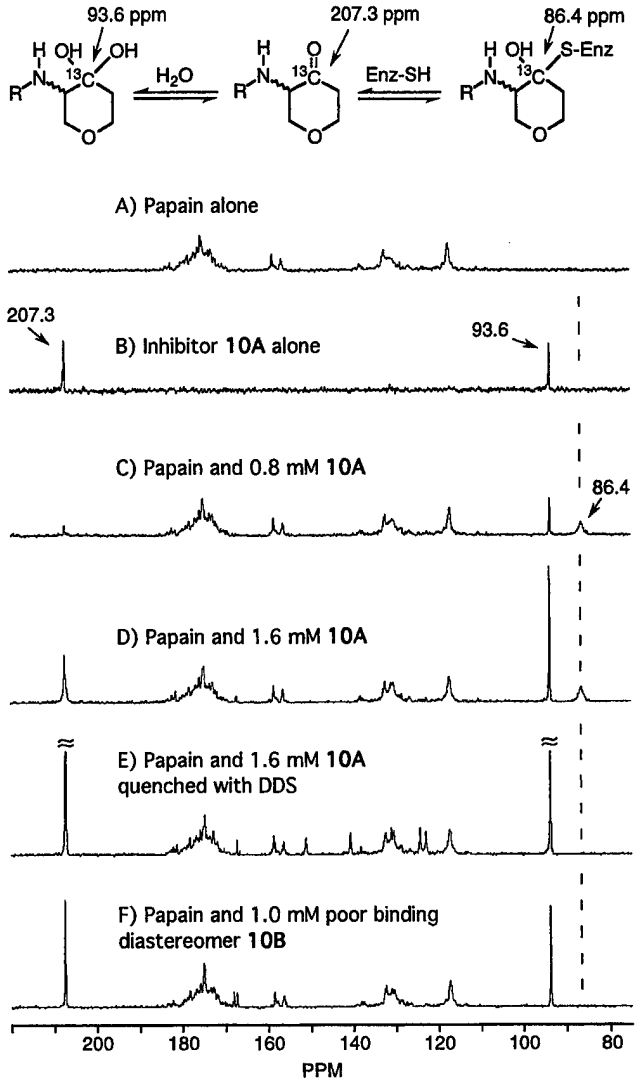


Figure 1. Partial ^{13}C NMR spectra of papain incubated with the ^{13}C-enriched inhibitor **10**. The concentration of enzyme in all spectra that contain papain is 0.9 mM.

against papain. The tight binding diastereomer **10A** has a K_i value of 11 μM, in contrast with the poor binding diastereomer **10B**, which has a K_i of 3300 μM. We have not determined the absolute configuration of these diastereomers. Figure 1 shows the ^{13}C NMR spectrum of each of these diastereomers in the presence of papain.

Figure 1A shows the ^{13}C NMR spectrum of papain alone. Figure 1B shows the spectrum of inhibitor **10A** alone. There are two major resonances in this spectrum. The resonance at 207.3 ppm corresponds to the ^{13}C-labeled ketone, and the resonance at 93.6 ppm corresponds to the hydrate. The similar intensities of these two resonances are consistent with the reported hydration equilibrium constant for tetrahydropyranone of 8.0×10^{-3} M^{-1}.[4] In $CDCl_3$ solution, inhibitor **10A** has a single major resonance for the ketone at 202.2 ppm. Figure 1C shows papain in the presence of slightly less than 1 equiv of **10A**. There are resonances for a small amount of both free ketone and hydrate. Importantly, a new resonance at 86.4 ppm appears that is not present in either Figure 1A or B. We assign this new resonance as the ^{13}C atom of a covalent hemithioketal adduct between the enzyme active-site nucleophile and the ketone of the inhibitor.

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Three lines of evidence support this structural assignment. First, the chemical shift of this peak clearly indicates that it corresponds to an sp^3-hybridized rather than an sp^2-hybridized carbon. This observation demonstrates that the new resonance cannot correspond to a simple noncovalent complex between the enzyme and the ketone form of the inhibitor. Second, the line width of this resonance, which is approximately 100 Hz, is fully consistent with an enzyme-bound species that is tumbling slowly on the NMR time scale.[13] Finally, reaction of inhibitor **10A** with the small molecule thiol, 3-thiopropionic acid, yields two diastereomeric hemithioketal adducts with resonances in the ^{13}C NMR spectrum at 82.6 and 83.7 ppm. These chemical shifts are similar to the 86.4 ppm that is found for the hemithioketal between **10A** and the enzyme-active-site cysteine residue.[14,15]

The resonances for free ketone and hydrate in Figure 1C are more pronounced than one would expect on the basis of the inhibition constant for compound **10A** and the enzyme and inhibitor concentrations in the sample. Using these values, we calculate that approximately 6% of the inhibitor should be in the free form. However, integration of the resonances suggests that the ratio of free inhibitor (ketone plus hydrate) to enzyme-bound inhibitor is approximately 1:2. Two factors are likely to contribute to this discrepancy. First, the sample may be contaminated with a small amount of the poor binding diastereomer **10B** due to incomplete separation of diastereomers during the HPLC purification. However, on the basis of the ^1H NMR spectrum of purified **10A**, we estimate that the sample was contaminated with not more than 5% of **10B** before the start of the experiment. A second factor, which we believe to be more important, is the differential saturation of the ^{13}C label in the free and enzyme-bound species. The ^{13}C label in the enzyme bound inhibitor will have a much longer correlation time and, likely, a longer relaxation time than the ^{13}C label in the free inhibitor. If the recycle time is shorter than either of these relaxation times, then the difference in the relaxation times will cause the integration for the enzyme-bound species to be smaller than expected on the basis of the true ratio of free to enzyme-bound inhibitor.

Addition of excess inhibitor to the enzyme (Figure 1D) simply results in an increase in the intensities of the resonances for free inhibitor. However, quenching the enzyme with DDS (Figure 1E), which forms a disulfide with the active-site cysteine residue and thus displaces the inhibitor from the active site, results in the disappearance of the resonance for hemithioketal. There is also a corresponding increase in the intensity of signals for free ketone and hydrate. These results show that inhibitor **10A** is bound at the enzyme active site through formation of a reversible covalent bond and that the inhibitor and papain are in equilibrium. The additional peaks in Figure 1E that appear between 120 and 160 ppm correspond to DDS and 2-thiopyridone.

Figure 1F shows 0.9 mM papain incubated with 1.0 mM of the poor binding diastereomer, **10B**. The absence of a broad resonance in the vicinity of 86.4 ppm shows that this diastereomer does not form a hemithioketal adduct. On the basis of the inhibition constant for compound **10B**, which is 3300 μM,[4] approximately 20% of the inhibitor should be bound to the enzyme at these concentrations.

It is noteworthy that the tight binding diastereomers of inhibitors **1**, **2**, and **3** have a range of inhibition constants against papain (78, 26, and 11 μM, respectively) and that these values correlate with both the electronic properties of the heteroatom in the 4-heterocyclohexanone ring and with the electrophilicity of the ketone moiety.[4] These data are consistent with a mechanism of inhibition that involves formation of a hemithioketal adduct. In addition, the NMR results shown above clearly demonstrate that the ^{13}C-labeled derivative of inhibitor **3** (compound **10A**) does indeed form such an adduct. We believe that these two observations, taken together, make it likely that the tight binding diastereomers of inhibitors **1** and **2** also form covalent adducts with the enzyme-active-site nucleophile.

In contrast, the poor-binding diastereomers of **1**–**3** all bind to papain with similar affinities (3.2, 2.4, and 3.3 mM, respectively), and there is no correlation between inhibition constants and ketone electrophilicity.[4] These observations, together with the fact that the poor binding diastereomer of ^{13}C-labeled **3** (compound **10B**) does not give a hemithioketal when incubated with papain, suggest that the poor-binding diastereomers of **1**–**3** all bind similarly in the active site and that none of these compounds form a reversible covalent bond with the active site cysteine residue.

In conclusion, we have demonstrated that the mechanism by which 4-heterocyclohexanone derivatives inhibit cysteine proteases involves nucleophilic attack by the active-site thiol on the reactive ketone. This attack results in reversible formation of a hemithioketal adduct that mimics the tetrahedral intermediate formed during enzyme-catalyzed hydrolysis of amide bonds. Future work will be aimed toward exploring the potential of 4-heterocyclohexanones as inhibitors for serine proteases and the hydrates of these compounds as inhibitors of metalloproteases and aspartic proteases.

Experimental Section

General Methods. NMR spectra were recorded on a Bruker AM-400 instrument. Spectra were calibrated using TMS (δ = 0.00 ppm) for ^1H NMR and $CDCl_3$ (δ = 77.0) or DMSO-d_6 (δ = 39.51) for ^{13}C NMR. 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. Semipreparative HPLC was performed on the same system using a semipreparative column (21.4 × 250 mm). $K^{13}CN$ (99%) was obtained from Cambridge Isotope Laboratories. Details of the synthesis of unlabeled **10** from unlabeled **6** and experimental procedures for determining racemization rates have been reported previously.[4]

[Bis-^{13}CN]-3-oxa-1,5-pentanedinitrile (6). A solution of tetraethylammonium [^{13}C]cyanide (19.9 g, 126 mmol) in 60 mL of dry CH_2Cl_2 was cooled in an ice bath. To the solution was added 2-bromoethyl ether (13.97 g, 60 mmol) via syringe, and the reaction was stirred under an N_2 atmosphere and allowed to warm to room temperature overnight. The reaction mixture

(13) A line width of 88 Hz has been reported for the covalent complex between a peptide aldehyde inhibitor and papain (see ref 5).

(14) For comparison, reaction between papain and several ^{13}C-labeled nitrile-based inhibitors gave covalent thioimidate adducts with resonances in the ^{13}C NMR spectra in the range of 182.1–194.2 ppm. The thioimidate carbons of several model compounds are in the range of 193.0–198.5 ppm (see ref 8b–8d). Reaction between papain and a ^{13}C-labeled aldehyde-based inhibitor gave a hemithioacetal adduct with a chemical shift for the hemithioacetal carbon of 74.9 ppm. A model hemithioacetal had a chemical shift of 73.3 ppm (see ref 5).

(15) Addition of 3-thiopropionic acid to inhibitor **10B** also gives two diastereomeric hemithioketals with resonances in the ^{13}C NMR spectrum at 82.7 and 83.8 ppm.

was filtered through a plug of silica gel and eluted with ethyl acetate to remove the salts. The resulting solution was concentrated by rotary evaporation, and the crude product was purified by flash chromatography (1:1 EtOAc/hexanes) to yield compound **6** as a clear oil (5.72 g, 75%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.66 (dt, J = 21.6, 6.2 Hz, 4H), 3.74 (dt, J = 6.3, 6.2 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 18.4 (d, J = 57.8 Hz), 65.4 (d, J = 3.1 Hz), 117.5 (s); HRMS-CI (M + H$^+$) calcd for $^{13}C_2{}^{12}C_4H_8N_2O$ 127.0782, found 127.0788.

Purification of Papain. Papain (twice crystallized) from Sigma was purified by affinity chromatography on an agarose–mercurial column according to the procedure of Sluyterman and Wijdenes.[11] Mercurial papain was eluted from the column using 10% DMSO, 0.5 mM $HgCl_2$, 1.0 mM EDTA, 100 mM KCl, and 50 mM NaOAc buffer at pH 5.0. The resulting solution of mercurial papain was concentrated using an Amicon Diaflow ultrafiltration apparatus with a YM-10 membrane. Mercurial papain can be stored at this stage in 0.5 mM $HgCl_2$ at a concentration of 3 mg/mL for over 1 month without loss of activity. Active papain was regenerated by washing the enzyme in the Amicon Diaflow apparatus with 1.0 mM cysteine, 1.0 mM EDTA, and 10 mM phosphate buffer at pH 6.5. The concentration of papain was determined by UV spectroscopy at 280 nm assuming an A_{280} of 25 absorbance units for a 1% solution and a molecular weight of 23, 000.[16] The activity of the enzyme preparations was determined by titrating the active-site cysteine nucleophile with 2,2′-dipyridyl disulfide according to the procedure of Brocklehurst and Little.[12] The samples were found to be greater than 95% active by this method.

^{13}C NMR Experiments. NMR samples of 2.0 mL were prepared in 10 mm NMR tubes. All samples contained 10 mM phosphate buffer at pH 6.5, 1 mM cysteine, 1 mM EDTA, and 5–10% DMSO-d_6. In addition, samples A–F (Figure 1) contained the following: (A) 0.9 mM papain; (B) inhibitor **10A**; (C) 0.9 mM papain and 0.8 mM **10A**; (D) 0.9 mM papain and 1.6 mM **10A**; (E) 0.9 mM papain, 1.6 mM **10A**, and 4.5 mM 2,2′-dipyridyl disulfide; and (F) 0.9 mM papain and 1.0 mM inhibitor **10B**. Inhibitor stock solutions were prepared in DMSO-d_6 to avoid racemization. Spectra were acquired on a Bruker AM-400 spectrometer operating at 100 MHz and were broad-band ^{1}H decoupled. A file size of 64K, a pulse width of 30°, and a receiver delay of 0.0 s was used to give a total acquisition time of 1.25 s. An exponential line broadening of 10 Hz was used during processing. Approximately 32, 000 scans were acquired for samples that contained protein.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for compound **6** (2 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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