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A new class of competitive inhibitors for serine and cysteine proteases is described. These compounds are potential anticancer agents that would act by inhibiting metastasis and angiogenesis. The first part of this report describes inhibitors that 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.

In the second part, ^{13}C Nuclear Magnetic Resonance spectroscopy is used to investigate the mechanism of inhibition of the cysteine protease papain by 4-heterocyclohexanones. A tetrahydropyranone-based inhibitor has been synthesized that incorporates a ^{13}C label at the electrophilic ketone moiety. Reaction of this inhibitor with a stoichiometric amount of papain can be monitored by ^{13}C NMR spectroscopy, which shows a new resonance at 86.4 ppm. This new resonance is identified as a covalent hemithioketal adduct between the ketone of the inhibitor and the active site cysteine nucleophile. The tetrahedral geometry of this reversibly formed hemithioketal adduct mimics the intermediate that occurs during enzyme catalyzed hydrolysis of peptides.

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July 28, 1997

PI - Signature Date

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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.

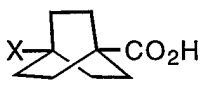
The report is organized in two sections. Part 1 describes our work to validate the design of our protease inhibitors, which are based around a 4-heterocyclohexanone framework. This part corresponds to the completion of Task 1 from the proposal Statement of Work. The results from these studies have been recently published as a full paper in the Journal of the American Chemical Society, Volume 119, pages 4285 to 4291 (1997).

Part 2 describes our efforts to probe the mechanism of protease inhibition by these compounds. This work corresponds to completion of tasks 8 and 9 from the statement of work. These studies will be submitted for publication in the near future.

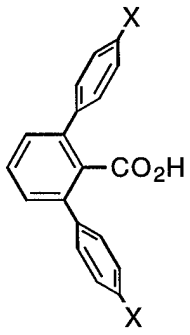
Part 1. Using the Electrostatic Field Effect to Design a New Class of Inhibitors for Cysteine Proteases

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 carboxylic acid. More recently, Siegel and coworkers 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

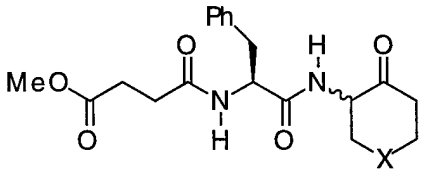


2,6-diphenylbenzoic acids

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-ketocarbonyl 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] This mechanism is also likely to be operative in the α-ketocarbonyl[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.

Chart 1. Structure of Cysteine Protease Inhibitors



X = CH_2, S, O, NH_2^+

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 substitute them with electron withdrawing substituents. This strategy, that 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.

Results

Model System. Before we undertook the multistep synthesis of our cysteine protease inhibitors, we first wanted to investigate the degree to which the heteratom 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 ^{1}H 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 gives 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, f, g, and 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 previously reported values. Equilibrium constants for acetone, pyruvic acid, and methyl pyruvate are taken from the literature.[23]

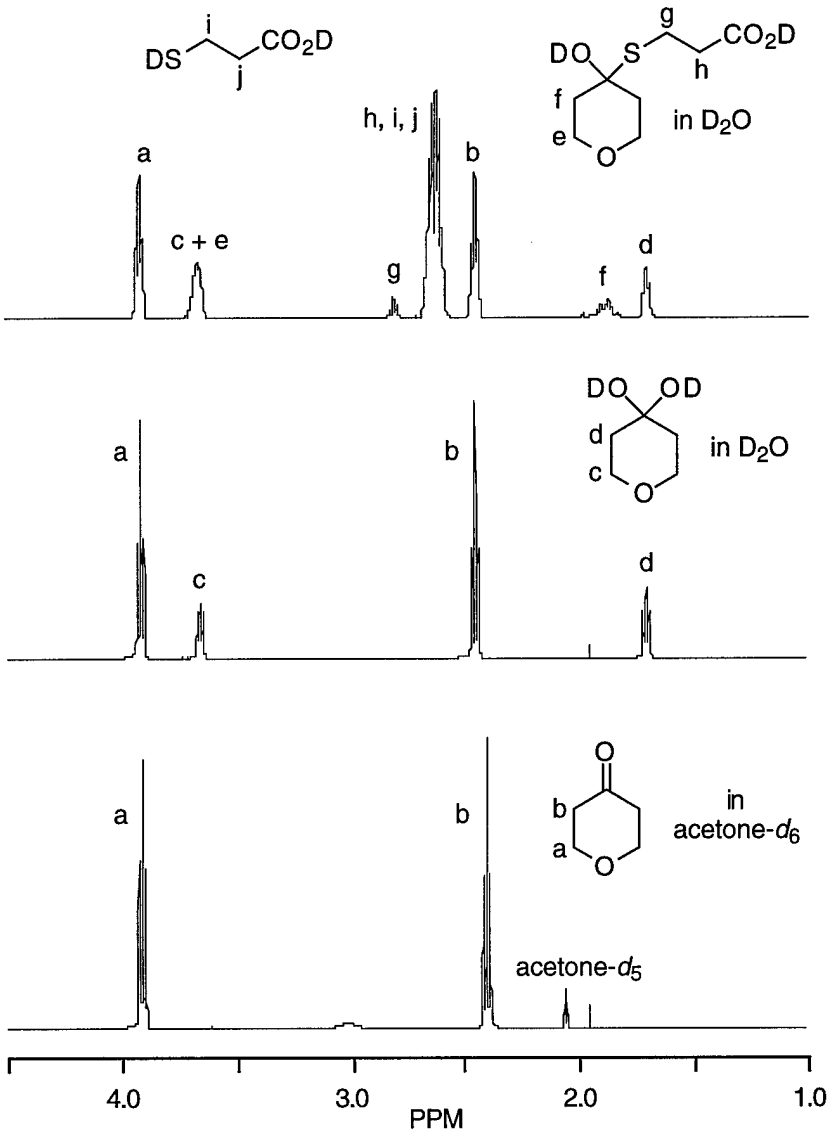
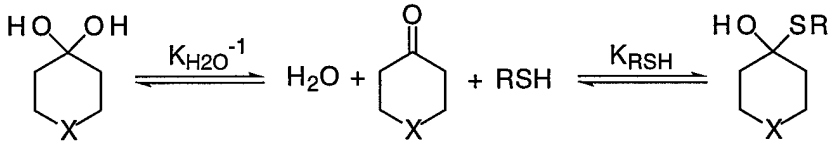


Figure 1. ^{1}H NMR spectra of the ketone, hydrate, and hemithioketal of tetrahydropyranone. The bottom spectrum shows the ketone in acetone-d_6 solution. The middle spectrum shows a mixture of ketone and hydrate in D_2O solution. The top spectrum shows a mixture of ketone, hydrate, hemithioketal, and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

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



X	K_{H2O} (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.

The hydration equilibrium constant for cyclohexanone is thirty-five 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

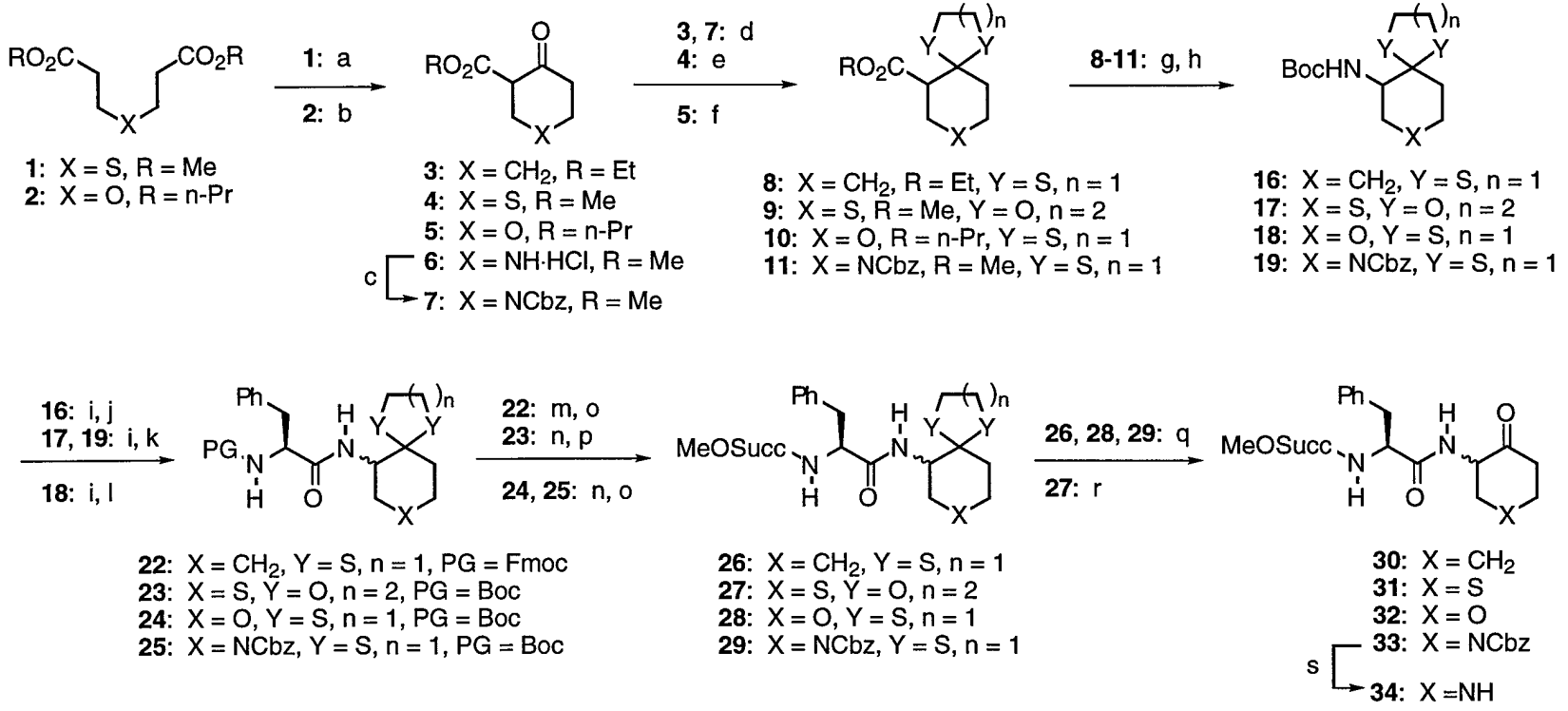
competition will lower the effective concentration of the inhibitor. We have calculated an *apparent* equilibrium constant for addition of thiol to ketone ($K_{RSH,app}$), first described by Jencks,[25] that accounts for the fact that the inhibitor will be present as a mixture of both ketone and hydrate in aqueous solution.

$$K_{RSH,app} = [\text{hemithioketal}]/[\text{ketone} + \text{hydrate}][\text{thiol}] = K_{RSH}/(1 + K_{H2O}[H_2O]) \quad (1)$$

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 preparation of each of the four target compounds. Dieckmann condensation of diesters **1** and **2** gives ketoesters **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 in THF 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]

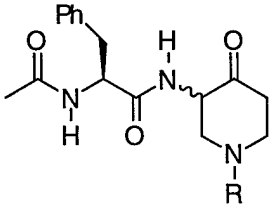
Scheme 1[a]



[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, $BF_3 \cdot Et_2O$, 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%.

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**.

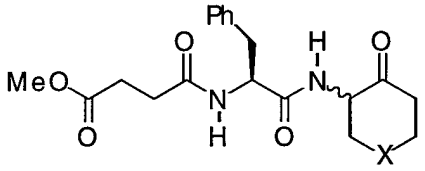
Racemization of Inhibitors. Papain is assayed in 100 mM phosphate buffer at pH 6.5. These conditions may catalyze the enolization of the ketone in our inhibitors, and thus lead to their racemization. We have monitored this reaction using HPLC or ^{1}H NMR spectroscopy. The cyclohexanone-based inhibitor **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**, that 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 that are required to prepare the sample and acquire the spectrum, this compound was completely racemized. Therefore, we measured 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**.



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

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



	K_i (μM)	
X	More Potent Diastereomer	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 reference 15. [c]Data from reference 11.

The cyclohexanone-based inhibitor (X = CH_2) is twenty 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 it's 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 (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**.

$$\log(K_X/K_H) = \rho(f\mathbf{F} + r\mathbf{R}) \qquad (2)$$

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

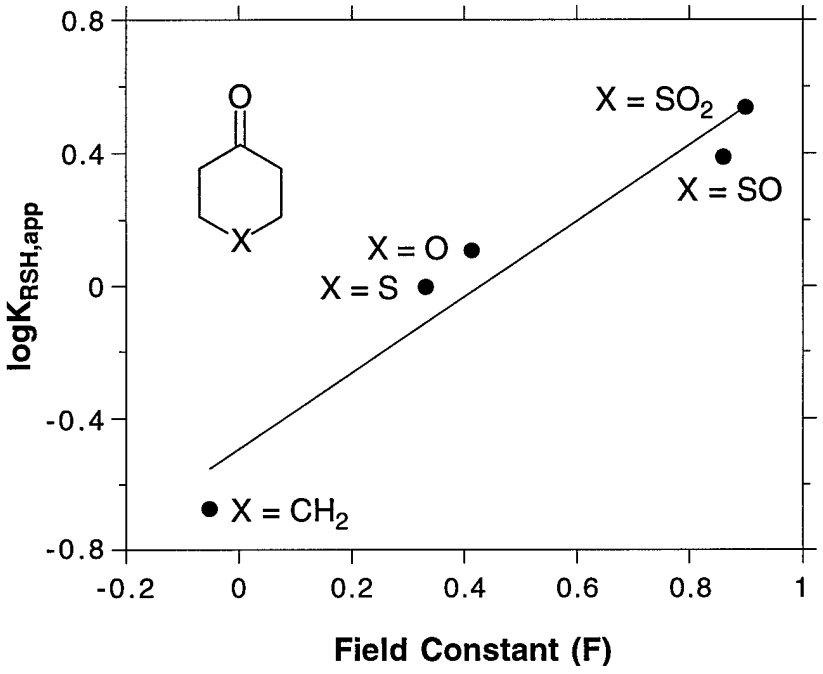


Figure 2. Correlation between the logarithm of the apparent equilibrium constant for addition of thiol to 4-heterocyclohexanones and the field substituent constant **F** ($\log K_{RSH,app} = 1.1\mathbf{F} - 0.5$; correlation coefficient = 0.97).

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 twice as 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 much closer together than they are in 4-substituted benzoic acids.

Correlation Between Ketone Reactivity and Enzyme Inhibition. We have designed our cysteine protease inhibitors based upon 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]

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.

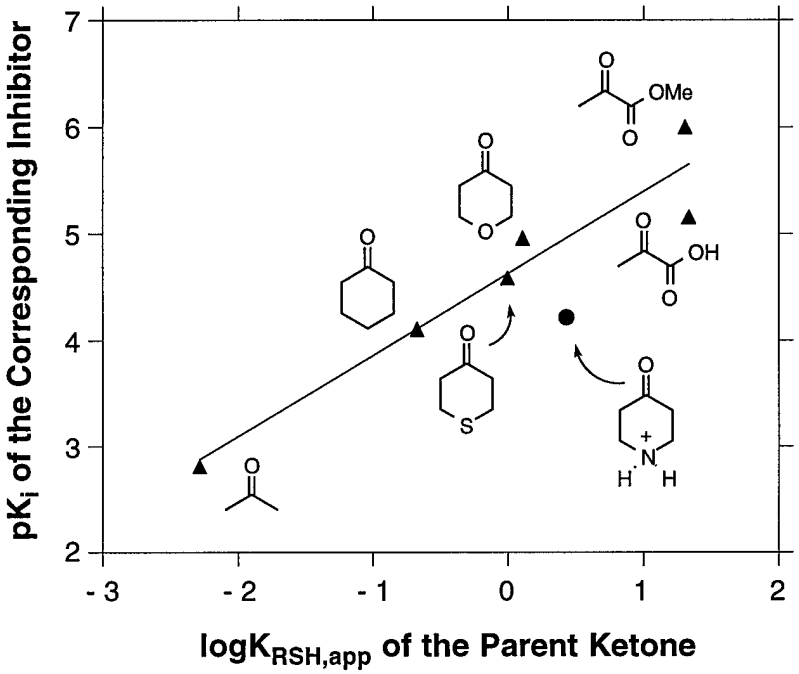


Figure 3. Correlation between inhibition constant (pK_i) of the ketone-based inhibitors and the logarithm of the apparent equilibrium constant for addition of thiol to the parent ketones ($pK_i = 0.8\log K_{RSH,app} + 4.6$; correlation coefficient = 0.96).

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 ($\delta = 0.00$ ppm) for ^1H NMR and $CDCl_3$ ($\delta =$ 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. Semi-preparative HPLC was performed on the same system using a semi-preparative column (21.4 x 250 mm).

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

Cyclohexanone thioketal (8). A solution of ethyl-2-cyclohexanonecarboxylate (5.00 g, 29.3 mmol), 1,2-ethanedithiol (3.04 g, 32.3 mmol), and *p*-toluenesulfonic acid monohydrate (*p*-TsOH) (0.20 g, 1.05 mmol) in benzene (100 mL) was refluxed with a Dean-Stark trap overnight. The solution was cooled and washed with 10% aqueous NaOH (2 x 150 mL), water (2 x150 mL), and brine (100 mL). The solution was dried, filtered, concentrated, and purified by flash chromatography (1:19 EtOAc/hexanes) to yield **8** (6.76 g, 94%) as a colorless oil: ^1H NMR (400 MHz, $CDCl_3$) δ 1.28 (t, $J = 7.1$ Hz, 3H), 1.42-1.49 (m, 2H), 1.53-1.61 (m, 1H), 1.69-1.74 (m, 1H), 1.93-1.99 (m, 3H), 2.57-2.64 (m, 1H), 2.94-2.97 (m, 1H), 3.19-3.30 (m, 4H), 4.12-4.19 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.2, 21.4, 25.7, 29.2, 38.4, 38.9, 40.2, 54.2, 60.2, 68.8, 173.0; HRMS-EI (M^+) calcd for $C_{11}H_{18}O_2S_4$ 246.0748, found 246.0746.

Cyclohexanone carboxylic acid (12). To a solution of ester **8** (6.00 g, 24.4 mmol) in MeOH (100 mL) was added 1 N aqueous NaOH (125 mL). The solution was refluxed for 14 h. The solution was then cooled and washed with 1:1 EtOAc/hexanes (200 mL). The aqueous layer was acidified with 1 N HCl to pH 2 and extracted with EtOAc (2 x 100 mL). These two EtOAc portions were combined and washed with brine (200 mL), dried, treated with decolorizing carbon, filtered, and concentrated to yield **12** (4.81 g, 90%) as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.26-1.36 (m, 1H), 1.41-1.48 (m, 2H), 1.63-1.67 (m, 1H), 1.79-1.91 (m, 3H), 2.55-2.62 (m, 1H), 2.84 (t, J = 4.4 Hz, 1H), 3.19-3.26 (m, 4H), 12.29 (br s, 1H); ^{13}C NMR (100 MHz, MeOH-d_4) δ 22.4, 26.9, 30.4, 39.2, 39.8, 41.0, 69.7, 176.7; HRMS-EI (M^+) calcd for $C_9H_{14}O_2S_2$ 218.04352, found 218.0442.

Cyclohexanone carbamate (16). A solution of acid **12** (1.09 g, 5.0 mmol), diisopropylethylamine (DIEA) (1.31 mL, 7.5 mmol), and diphenylphosphoryl azide (DPPA) (1.08 mL, 5.0 mmol) in benzene (35 mL) was refluxed overnight. Aliquots of the reaction mixture were monitored for disappearance of the acyl azide peak (2168 cm^{-1}) and appearance of the isocyanate peak (2249 cm^{-1}) by FT-IR. After the Curtius rearrangement was judged complete by IR, a solution of potassium *t*-butoxide (1.68 g, 15.0 mmol) in THF (75 mL) was prepared and cooled in an ice bath. The isocyanate solution was added dropwise to the cold alkoxide solution and the mixture was stirred for 15 min. The solution was washed with 1 N HCl, saturated $NaHCO_3$, brine, and dried. The dried solution was concentrated and purified by flash chromatography (1:9 EtOAc/hexanes) to yield **16** (0.97g, 67%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.18-1.52 (m, 3H), 1.45 (s, 9H), 1.61-1.72 (m, 2H), 1.92-1.99 (m, 2H), 2.20-2.25 (m, 1H), 3.22-3.30 (m, 4H), 3.77-3.82 (m, 1H), 5.01-5.03 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.4, 25.3, 28.4, 33.8, 38.8, 39.4, 43.6, 56.8, 74.3, 79.1, 155.5; HRMS-EI (M^+) calcd for $C_{13}H_{23}NO_2S_2$ 289.1172, found 289.1166.

Aminocyclohexanone·trifluoroacetic acid salt (20). Trifluoroacetic acid (TFA) (9 mL) was added to a solution of **16** (0.96 g, 3.3 mmol) in CH_2Cl_2 (30 mL) cooled in an ice bath, and the solution was stirred for 30 min. The solution was then concentrated, redissolved in CH_2Cl_2, and concentrated again. The residue was triturated with Et_2O to give a white solid. This material was collected by suction filtration and washed with hexanes to yield **20** (0.96 g, 96%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31-1.41 (m, 1H), 1.47-1.60 (m, 2H), 1.72-1.80 (m, 2H), 1.93-2.01 (m, 1H), 2.21-2.32 (m, 2H), 3.23-3.43 (m, 5H), 8.06 (br s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.5, 24.8, 30.3, 38.9, 39.5, 43.4, 57.7, 71.1, 162.4 (q); HRMS-EI (M^+) calcd for $C_8H_{15}NS_2$ 189.0646, found 189.0642.

Phenylalanylcyclohexanone (22). To a solution of **20** (1.79 g, 5.9 mmol) and DIEA (5.14 mL, 29.5 mmol) in CH_2Cl_2 (25 mL) was added solid N-Fmoc-phenylalanyl fluoride[28] (2.76 g, 7.0 mmol). The solution was stirred for 1 h, then washed with 1 N HCl, saturated $NaHCO_3$, and brine. The solution was dried, concentrated, and the crude material was purified by flash chromatography (2:3 EtOAc/hexanes) to yield a mixture of diastereomers of **22** (3.15 g, 96%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88-1.01 (m, 1H), 1.11-1.52 (m, 6H), 1.64-1.75 (m, 4H), 1.86-1.97 (m, 3H), 2.14-2.21 (m, 2H), 2.93-3.03 (m, 4H), 3.06-3.15 (m, 8H), 4.01-4.15 (m, 2H), 4.16-4.22 (m, 2H), 4.29-4.47 (m, 6H), 5.22 (br m, 1H), 5.52 (br m, 1H), 5.99 (br d, J = 9.3 Hz, 1H), 6.42 (br d, J = 8.4 Hz, 1H), 7.21-7.32 (m, 14H), 7.40 (t, J = 7.4 Hz, 3H), 7.52-7.57 (m, 4H), 7.76 (d, J = 7.5 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.0, 24.1, 25.0, 25.1, 29.7, 33.2, 33.5, 38.4, 38.7, 38.8, 39.3, 43.8, 44.0, 47.06, 47.09, 54.8, 55.0, 56.2, 56.5, 67.1, 67.2, 73.2, 120.0, 125.00, 125.03, 125.1, 126.9, 127.1, 127.7, 128.6, 128.7, 129.3, 129.5, 136.4, 136.6, 141.3, 143.6, 143.7, 143.8, 155.8, 169.5, 169.9; HRMS-FAB (M + Na^+) calcd for $C_{32}H_{34}N_2NaO_3S_2$ 581.1909, found 581.1923.

Methoxysuccinylcyclohexanone (26). A solution of **22** (2.72 g, 4.9 mmol) and tris(2-aminoethyl)amine (TAEA) (20 mL) in CH_2Cl_2 (50 mL) was stirred at 25 °C for 30 min. The

solution was diluted with an additional 50 mL of CH_2Cl_2, washed with brine (3 x 100 mL) and phosphate buffer (1.0 M, pH 5.5, 3 x 100 mL), and dried over Na_2CO_3. This solution was concentrated to 25 mL and added to a solution of methylsuccinate (0.65 g, 5.0 mmol), 1-hydroxybenzotriazole (HOBT) (0.78 g, 5.0 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (1.23 g, 6.4 mmol) and N-methylmorpholine (0.55 mL, 5.0 mmol) in 25 mL CH_2Cl_2. This solution was stirred overnight, and extracted with water, 1 M $KHSO_4$, saturated Na_2CO_3 (50 mL), and dried over Na_2CO_3. The dried solution was concentrated and the crude material purified by flash chromatography (1:1 EtOAc/hexanes) to yield a mixture of diastereomers of **26** (1.57 g, 70%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88-0.99 (m, 1H), 1.14-1.52 (m, 6H), 1.57-1.70 (m, 4H), 1.86-1.98 (m, 3H), 2.14-2.21 (m, 2H), 2.44-2.47 (m, 2H), 2.50-2.52 (m, 2H), 2.56-2.68 (m, 4H), 2.91-2.99 (m, 2H), 30.2-3.22 (m, 10H), 3.67 (s, 3H), 3.68 (s, 3H), 3.98-4.05 (m, 1H), 4.06-4.13 (m, 1H), 4.58-4.63 (m, 1H), 4.69-4.74 (m, 1H), 5.93 (br d, J = 9.3 Hz, 1H), 6.30 (br d, J = 8.2 Hz, 1H), 6.42 (br d, J = 9.4 Hz, 1H), 6.59 (br d, J = 7.7 Hz, 1H), 7.19-7.32 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.9, 24.0, 25.0, 25.1, 29.17, 29.2, 30.9, 33.0, 33.4, 38.0, 38.6, 38.7, 39.3, 43.6, 43.8, 73.07, 73.08, 126.7, 126.9, 128.5, 128.6, 129.3, 129.5, 136.6, 136.8, 169.6, 169.9, 171.0, 171.2, 173.00, 173.04; HRMS-FAB (M + Na^+) calcd for $C_{22}H_{30}N_2NaO_4S_2$ 473.1545, found 473.1537.

Cyclohexanone inhibitors (30a and 30b). A solution of N-bromosuccinimide (NBS) (3.90 g, 21.9 mmol) in 80% aqueous acetonitrile (60 mL) was cooled in an ice bath. To this solution was added compound **26** (1.56 g, 3.47 mmol) dissolved in acetonitrile (40 mL). The reaction mixture was stirred for 15 min, during which time it was allowed to warm to room temperature. The reaction mixture was partitioned between 1:1 CH_2Cl_2/EtOAc (100 mL) and saturated Na_2SO_3 (100 mL). The organic layer was separated and washed with saturated $NaHCO_3$ and brine (100 mL), and dried. The solution was then concentrated, and the crude material purified and the diastereomers separated by RPHPLC (C18) with 25% MeCN/H_2O as the mobile

phase. The retention times for diastereomers **30a** and **30b** were 33.0 and 37.0 min, respectively. Lyopholization of the appropriate fractions yielded **30a** (0.50 g, 38%) and **30b** (0.55 g, 42%) as white solids: **30a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.30 (qd, J = 13, 4.0 Hz, 1H), 1.59 (qt, J = 13.0, 4.2 Hz, 1H), 1.77 (qt, J = 14.0, 3.5 Hz, 1H), 1.82-1.88 (m, 1H), 2.09-2.16 (m, 1H), 2.31-2.40 (m, 1H), 2.46-2.51 (m, 1H), 2.49 (t, J = 6.7 Hz, 2H), 2.55-2.62 (m, 1H), 2.62-2.69 (m, 2H), 3.00-3.14 (m, 2H), 3.66 (s, 3H), 4.32-4.38 (m, 1H), 4.67-4.73 (m, 1H), 6.30 (d, J = 7.7 Hz, 1H), 6.64 (d, J = 6.1 Hz, 1H), 7.16-7.20 (m, 2H), 7.21-7.25 (m, 1H), 7.27-7.31 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.9, 27.9, 29.1, 30.9, 35.1, 38.4, 40.9, 51.8, 54.3, 58.1, 127.0, 128.6, 129.2, 136.3, 170.3, 171.1, 173.2, 206.5; HRMS-FAB (M + Na^+) calcd for $C_{20}H_{26}N_2NaO_5$ 397.1740, found 397.1734. **30b**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.17 (qd, J = 13.0, 4.0 Hz, 1H), 1.57 (qt, J = 13.0, 4.2 Hz, 1H), 1.74 (qt, J = 13.0, 3.5 Hz, 1H), 1.83-1.87 (m, 1H), 2.07-2.13 (m, 1H), 2.30-2.39 (m, 1H), 2.41-2.49 (m, 4H), 2.58-2.72 (m, 2H), 3.01-3.14 (m, 2H), 3.66 (s, 3H), 4.37-4.44 (m, 1H), 4.68-4.74 (m, 1H), 6.38 (d, J = 7.7 Hz, 1H), 6.61 (d, J = 6.5 Hz, 1H), 7.16-7.30 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.0, 27.8, 29.1, 30.9, 34.7, 38.5, 41.0, 51.8, 54.4, 57.9, 126.9, 128.6, 129.3, 136.5, 170.1, 171.1, 173.3, 206.7; HRMS-FAB (M + Na^+) calcd for $C_{20}H_{26}N_2NaO_5$ 397.1740, found 397.1744.

Dimethyl 3,3'-thiodipropionate (1). To a stirred solution of 3,3'-thiodipropionic acid (6.0 g, 34 mmol) in 200 mL methanol was added 1 mL concentrated sulfuric acid. The solution was heated at reflux for 3.5 h. It was then concentrated to 20% of the original volume and diluted with 200 mL of ethyl acetate. The organic layer was washed twice with 100 mL of 1:1 H_2O/saturated $NaHCO_3$ solution, and once with brine. The solution was dried and concentrated, and the clear oily liquid was purified by flash chromatography (1:4 EtOAc/hexanes) to give 6.9 g (33 mmol, 99%) of compound **1**: ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.63 (td, J = 7.1, 1.1 Hz, 4H), 2.81 (td, J = 7.8, 1.1 Hz, 4H), 3.71 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.8, 34.3, 51.6, 172.0; HRMS-EI (M^+) calcd for $C_8H_{14}O_4S$ 206.0613, found 206.0617.

Methyl tetrahydrothiopyran-4-one-3-carboxylate (4). To a suspension of NaH (4.6 g, 60% dispersion in mineral oil, 110 mmol) in 200 mL dry ether was added 10 drops of methanol. Diester **1** (16 g, 78 mmol) was added to the mixture as a solution in 20 mL of ether. After heating at reflux for 8 h, the reaction was quenched with 100 mL of 3.1 N aqueous AcOH. The aqueous layer was extracted three times with ether. The combined organic layers were washed with saturated $NaHCO_3$ solution and dried. The oily liquid resulting from the concentration of the organic layer was purified by flash chromatography (1:4 EtOAc/hexanes) to give 11 g (27 mmol, 81%) of compound **4** as a 3:1 mixture of keto and enol tautomers: ^{1}H NMR (250 MHz, $CDCl_3$) δ enol: 2.60 (m, 2H), 2.79 (m, 2H), 3.35 (s, 2H), 3.78 (s, 3H), 12.5 (s, 1H); keto: 2.84-3.08 (m), 3.25 (m), 3.67 (m); ^{13}C NMR (100 MHz, $CDCl_3$) δ 23.4, 24.6, 30.3, 30.8, 32.4, 43.5, 51.7, 52.4, 58.6, 97.2, 171.8, 172.4; HRMS-EI (M^+) calcd for $C_7H_{10}O_3S$ 174.0351, found 174.0348.

Tetrahydrothiopyranone ketal (9). To a flask fitted with a Dean-Stark trap was added 250 mL of benzene, ketone **4** (6.3 g, 36 mmol), 1,3-propanediol (5.1 mL, 70 mmol), and *p*-TsOH (1.3 g, 7.0 mmol) and the solution was heated at reflux for 11 h. The reaction mixture was then cooled and the acid was neutralized with saturated $NaHCO_3$ solution. The organic layer was dried, filtered, and concentrated and the crude material was purified by flash chromatography (2:1 hexanes/EtOAc) affording 6.0 g (28 mmol, 77%) of ketal **9**: ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.43-1.51 (dquintet, J = 3.2, 13.4 Hz, 1H), 1.86-1.97 (m, 2H), 2.62-2.70 (m, 2H), 2.79-2.95 (m, 2H), 3.02-3.13 (m, 2H), 3.73 (s, 3H), 3.81-4.08 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.06, 25.11, 27.8, 29.4, 51.4, 51.7, 59.0, 59.1, 96.4, 170.9; HRMS-EI (M^+) calcd for $C_{10}H_{16}O_4S$ 232.0769, found 232.0764.

Tetrahydrothiopyranone carboxylic acid (13). A methanol solution (40 mL) that contained 40 mL of 1 N aqueous NaOH and ester **9** (8.1 g, 37 mmol) was heated at reflux for 6.5 h. The reaction was cooled, diluted with 50 mL of H_2O, and the MeOH was removed by rotary

evaporation. The unreacted ester was extracted out of the solution with EtOAc. The aqueous layer was then acidified with 1 N HCl and extracted with EtOAc to remove the carboxylic acid. The separate organic layers were dried and concentrated via rotary evaporation to give 3.8 g (17 mmol, 46%) of recovered ester and 4.0 g (20 mmol, 54%) of the desired carboxylic acid **13**. Compound **13** was used in the next reaction without further purification: ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.49 (m, 1H), 1.75 (ddd, J = 3.5, 11.9, 14.3 Hz, 1H), 2.01-2.21 (m, 1H), 2.49-3.06 (m, 6H), 3.98 (m, 3H), 4.14-4.25 (dt, J = 4.5, 12.4 Hz, 1H), 10.5 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.6, 24.7, 26.9, 29.6, 58.8, 59.5, 59.6, 98.0, 171.1; HRMS-EI (M^+) calcd for $C_9H_{14}O_4S$ 218.0613, found 218.0621.

Tetrahydrothiopyranone carbamate (17). A solution of carboxylic acid **13** (2.1 g, 10 mmol), triethylamine (TEA) (1.7 mL, 12 mmol), and DPPA (2.5 mL, 11 mmol) in 200 mL of benzene was heated at reflux for 6 h. Aliquots of the reaction mixture were monitored for the disappearance of the acyl azide peak (2170 cm^{-1}) and the appearance of the isocyanate peak (2250 cm^{-1}) by FT-IR. After the Curtius rearrangement was judged complete by IR, the solution was cooled to room temperature, placed in an addition funnel, and added dropwise to a stirred solution of potassium *t*-butoxide (2.3 g, 21 mmol) in 100 mL of THF that was cooled in an ice bath. After addition was complete the solution was stirred for 1 h. The reaction was washed with 3.1 N aqueous AcOH solution, saturated $NaHCO_3$, dried, and concentrated by rotary evaporation. The crude material was purified by flash chromatography (1:1 EtOAc/hexanes) affording 2.1 g (7.1 mmol, 69%) of carbamate **17**: ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.46 (s, 9H), 1.70-1.88 (m, 3H), 2.44- 2.71 (m, 4H), 2.90 (d, J = 12.8 Hz, 1H), 3.79-4.10 (m, 4H), 4.26 (m, 1H), 5.31 (d, J = 8.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.6, 25.0, 28.4, 30.9, 32.1, 49.6, 59.06, 59.14, 79.3, 96.2, 129.3, 155.5; HRMS-FAB ($M + Na^+$) calcd for $C_{13}H_{23}NNaO_4S$ 312.1246, found 312.1257.

Phenylalanyltetrahydrothiopyranone (23). A CH_2Cl_2 solution (30 mL) of the carbamate **17** (0.62 g, 2.1 mmol) was treated with 3.1 mL (40 mmol) of TFA. After 1 h the solvent and excess TFA was removed by rotary evaporation. The resultant oily liquid was diluted with 5 mL CH_2Cl_2 and added to a solution containing HOBT (0.29 g, 2.1 mmol), EDC (0.53 g, 2.8 mmol), N-methylmorpholine (0.70 mL, 6.4 mmol), and N-Boc-phenylalanine (0.57 g, 2.1 mmol) in 30 mL of CH_2Cl_2 which was cooled in an ice bath. The reaction mixture was stirred at 0° C for 1.5 h, then allowed to warm to room temperature overnight. The reaction was washed with H_2O, 1 M $KHSO_4$, saturated Na_2CO_3 solution, then dried and concentrated by rotary evaporation. The crude material was purified by flash chromatography (4:1 EtOAc/hexanes) to yield 0.78 g (1.8 mmol, 84%) of **23** as a mixture of diastereomers: ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.42 (m, 9H), 1.60-1.82 (m, 3H), 2.28-3.20 (m, 7H), 3.72-4.07 (m, 4H), 4.36-4.48 (m, 2H), 5.09-5.20 (m, 1H), 6.40 (s, 0.5H), 6.56 (d, J = 8.5 Hz, 0.5H), 7.19-7.33 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.47, 24.50, 24.89, 24.94, 28.21, 28.25, 30.2, 30.3, 31.7, 32.0, 38.4, 38.8, 59.0, 59.06, 59.12, 59.14, 79.9, 95.88, 95.93, 126.7, 126.9, 128.5, 128.6, 129.3, 129.4, 136.6, 136.7, 155.2; HRMS-FAB (M + $Na^{+)}$ calcd for $C_{22}H_{32}N_2NaO_5S$ 459.2030, found 459.1945.

Methoxysuccinyltetrahydrothiopyranones (27a and 27b). TFA (6.8 mL, 88 mmol) was added to a solution of compound **27** (2.0 g, 4.4 mmol) in CH_2Cl_2 (100 mL). After 3.5 h the solvent and TFA were removed by rotary evaporation. The resulting oily liquid was dissolved in 100 mL of CH_2Cl_2, and DIEA (2.3 mL, 13 mmol) and methyl(N-hydroxysuccinimidyl) succinate (2.0 g, 5.7 mmol) were added. The reaction was stirred overnight, then quenched with 50 mL of 2.6 N aqueous AcOH. The organic layer was washed with 50 mL of saturated $NaHCO_3$, dried, and concentrated by rotary evaporation. The crude material was purified and the diastereomers separated by flash chromatography (4:1 EtOAc/hexanes) to give 0.71 g (1.6 mmol, 36%) of **27a** and 0.59 g (1.3 mmol, 30%) of **27b**: **27a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.57-1.79 (m, 3H), 2.44-2.65 (m, 8H), 2.81 (d, J = 12.3

Hz, 1H), 3.04-3.16 (m, 2H) 3.67 (s, 3H), 3.78-3.98 (m, 4H), 4.42 (br m, 1H), 4.73 (q, J = 7.3 Hz, 1H), 6.48-6.50 (m, 2H), 7.20-7.30 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.5, 24.9, 29.1, 30.2, 30.8, 31.4, 38.4, 51.8, 54.4, 59.0, 59.1, 96.0, 126.8, 128.4, 129.5, 136.5, 170.1, 171.1, 173.1; HRMS-FAB (M + Na^+) calcd for $C_{22}H_{30}N_2NaO_6S$ 473.1723, found 473.1714. **27b**: ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.6-1.8 (m, 3H), 2.4 (dd, J = 8.0, 13.2 Hz, 1H), 2.5-2.8 (m, 8H), 3.0 (dd, J = 10.8, 13.5 Hz, 1H), 3.3 (dd, J = 5.8, 13.5 Hz, 1H), 3.8 (s, 3H), 3.9-4.0 (m, 4H), 4.5 (br m, 1H), 4.8 (m, 1H), 6.3 (d, J = 8.3 Hz, 1H), 6.7 (d, J = 7.5 Hz, 1H), 7.3-7.4 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.5, 24.9, 29.2, 30.1, 30.9, 31.6, 38.9, 51.8, 54.8, 59.1, 59.2, 95.9, 127.0, 128.7, 129.3, 136.7, 169.9, 171.0, 173.2; HRMS-FAB (M + Na^+) calcd for $C_{22}H_{30}N_2NaO_6S$ 473.1723, found 473.1734.

Tetrahydrothiopyranone inhibitors (31a and 31b). This procedure was performed separately on each of the two diastereomers of compound **27**. *p*-TsOH (for **27a**: 0.055 g, 0.29 mmol; for **27b**: 0.073 g, 0.38 mmol) which had been dried by heating to 100 °C under vacuum for 4 h was added to a solution of ketal **27** (for **27a**: 0.13 g, 0.29 mmol; for **27b**: 0.17 g, 0.38 mmol) dissolved in acetone-d_6 (for **27a**: 15 mL; for **27b**: 10 mL). The reaction was monitored by observing the disappearance of the ketal multiplet at 3.7-4.0 ppm and appearance of the 1,3-propanediol triplet at 3.8 ppm by ^{1}H NMR spectroscopy. After heating at reflux (for **27a**: 16.5 h; for **27b**: 7 h) the reaction was quenched with saturated aqueous $NaHCO_3$. The acetone was removed in vacuo and the resultant slurry was partitioned between EtOAc and H_2O. The organic layer was dried and concentrated by rotary evaporation, and the crude material was purified by flash chromatography (2:1 EtOAc/hexanes) to give ketone **31** (starting from **27a**: 0.076 g, 0.2 mmol, 67%; starting from **27b**: 0.12 g, 0.31 mmol, 79%): **31a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.47-2.91 (m, 9H), 3.02-3.12 (m, 2H), 3.40 (m, 1H), 3.67 (s, 1H), 4.50-4.71 (m, 2H), 6.33 (br m, 1H), 6.89 (br m, 1H), 7.17-7.31 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.1, 30.9, 31.1, 36.3, 38.3, 38.5, 44.17, 44.20, 51.9, 54.42, 54.45, 59.5, 127.1, 127.2, 128.71, 128.74, 129.2, 129.3, 136.2, 170.5, 171.3, 173.3, 204.0; HRMS-

CI (M + H^+) calcd for $C_{19}H_{25}N_2O_5S$ 393.1493, found 393.1489. **31b**: ^1H NMR (250 MHz, $CDCl_3$) δ 2.30-2.40 (dd, *J* = 11.4, 13.1 Hz, 1H), 2.46-2.91 (m, 8H), 2.97-3.14 (m, 2H), 3.18-3.25 (dd, *J* = 5.2, 13.6 Hz, 1H), 3.67 (s, 3H), 4.61-4.76 (m, 2H), 6.39-6.42 (d, *J* = 7.8 Hz, 1H), 6.83-6.86 (d, *J* = 6.2 Hz, 1H), 7.18-7.33 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.2, 30.9, 31.1, 36.0, 38.5, 44.2, 51.8, 54.5, 59.3, 127.1, 128.7, 129.2, 129.3, 136.4, 170.4, 171.3, 173.3, 204.2; HRMS-FAB (M + Na^+) calcd for $C_{19}H_{24}N_2NaO_5S$ 415.1304, found 415.1296.

Di-n-propyl 4-oxa-1,7-heptanedioate (2). A solution of 3,3'-oxydipropionitrile (18.9 g, 152 mmol) and *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, concentrated, and the crude material 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); ^1H 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, then quenched by the addition of 25 mL of H_2O. The solution was partitioned between 200 mL of 1 N HCl and 200 mL 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, 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); ^1H 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 $BF_3 \cdot Et_2O$ (1.04 mL, 8.5 mmol). The solution was stirred at 0 °C for 4 h, then it was washed with 10% aqueous NaOH solution, water, and brine (20 mL). The organic layer was dried, 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: ^1H 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 separated and acidified with 1 N HCl. The acidic aqueous solution was extracted with EtOAc (2 x 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), DIEA (0.19 g, 1.50 mmol), and 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 *t*-butoxide (0.34 g, 3.0 mmol) in THF (10 mL). The reaction was stirred for 15 min, 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, concentrated, and crude material 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 (br d, *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). 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, then concentrated, redissolved in CH_2Cl_2, and 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, then washed with 1 N HCl, saturated $NaHCO_3$, and brine (10 mL). The solution was dried over Na_2CO_3, concentrated, and the crude material purified by flash chromatography (2:3 EtOAc/hexanes) to yield a mixture of diastereomers of **24** (218 mg, 61%) as a white solid: ^1H NMR (400 MHz, $CDCl_3$) δ 1.31 (s, 9H), 1.34 (s, 9H), 2.03-2.15 (m, 4H), 2.82 (br s, 1H), 2.94-3.16 (m, 13H), 3.46-3.51 (m, 2H), 3.57 (br m, 1H), 3.67-3.77 (m, 3H), 4.14 (br m, 1H), 4.19-4.27 (m, 2H), 4.37 (br m, 1H), 5.14 (br m, 1H), 5.31 (br m, 1H), 6.18 (br m, 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.

Methoxysuccinyltetrahydropyranone (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 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 methylsuccinate (61 mg, 0.46 mmol), HOBT (72 mg, 0.46 mmol), 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, 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 purified by flash chromatography (7:3 EtOAc/hexanes) to yield a mixture of diastereomers of **28** (144 mg, 70%) as a white solid: ^1H 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 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 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% isopropanol in CH_2Cl_2 as the mobile phase. The retention times for diastereomers **32a** and **32b** were 13.1 and 14.1 min, respectively: **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 (br m, 1H), 4.42 (br m, 1H), 4.61 (br m, 1H) 4.72 (br m, 1H), 6.28 (br m, 1H), 6.59 (br m, 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. **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 (br m, 1H),

6.32 (br m, 1H), 6.66 (br m, 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.

Methyl N-carbobenzyloxy-4-piperidone-3-carboxylate (7). Methyl 4-piperidone-3-carboxylate hydrochloride (10 g, 52 mmol) and TEA (14 mL, 100 mmol) were combined in 400 mL of CH_2Cl_2 and cooled in an ice bath. Benzylchloroformate (8.8 mL, 61.9 mmol) was added and the mixture was stirred at 0 °C for 2 h. The solution was then poured into 3.1 N aqueous AcOH and the aqueous layer was extracted twice with CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$ solution, dried, and concentrated, and the crude material was purified by flash chromatography (4:1 hexanes/EtOAc) to give 14.4 g (49.4 mmol, 95%) of compound **7**: ^1H NMR (400 MHz, $CDCl_3$,) δ 2.38 (br s, 2H), 3.64 (t, J = 5.8 Hz, 2H), 3.77 (s, 3H), 4.12 (s, 2H), 5.16 (s, 2H), 7.36 (m, 5H), 12.00 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.8, 40.0, 40.5, 43.6, 45.7, 51.5, 52.4, 56.2, 67.3, 67.8, 96.0, 126.9, 128.0, 128.1, 128.3, 128.5, 128.6, 136.1, 136.6, 155.2, 168.2, 169.5, 170.1, 170.9, 202.0, 207.6; HRMS-CI (M + H^+) calcd for $C_{15}H_{18}NO_5$ 292.1185, found 292.1190.

Piperidone thioketal (11). A solution containing ketone **7** (7.1 g, 24 mmol), ethanedithiol (2.5 g, 26 mmol) and *p*-TsOH (0.23 g, 1.2 mmol) in 150 mL of benzene was heated at reflux with a Dean Stark apparatus. After 12 h, an additional 1.4 g (7.4 mmol) of *p*-TsOH and 4.0 mL (48 mmol) of ethanedithiol were added and heating was continued for an additional 12 h. The reaction was then cooled to room temperature, and washed with 10% aqueous NaOH solution (3 x 75 mL), 150 mL H_2O, and 100 mL brine. The solution was dried and concentrated by rotary evaporation, and the crude material was purified by flash chromatography (35% Et_2O/hexanes) to give 6.6 g (18 mmol, 74%) of compound **11**: ^1H NMR (400 MHz, $CDCl_3$) δ 1.89-1.95 (br d, J = 13.5 Hz, 1H), 2.79-2.90 (m, 2H), 3.12-3.33 (m, 5H), 3.50-3.73 (br m, 4H), 3.95-4.40 (br m, 2H), 5.11 (br m, 2H), 7.35 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 38.4, 38.9, 44.0, 46.0,

51.6, 53.6, 66.5, 67.1, 127.7, 127.9, 128.3, 136.5, 154.6, 171.2; HRMS-FAB (M + Na^+) calcd for $C_{17}H_{21}NNaO_4S_2$ 390.0810, found 390.0815.

Piperidone carboxylic acid (15). A solution of ketal **11** (6.2 g, 16 mmol), 1 N aqueous NaOH (50 mL), and 100 mL of MeOH was heated at reflux for 2 h. The reaction was then concentrated to remove MeOH, and the resulting aqueous solution was washed twice with 150 mL portions of CH_2Cl_2. The aqueous layer was then acidified with concentrated HCl and extracted twice with 150 mL portions of CH_2Cl_2. These CH_2Cl_2 extracts were dried and concentrated by rotary evaporation. The crude material was used in the next reaction without further purification: ^1H NMR (400 MHz, $CDCl_3$) δ 1.92 (br m, 1H), 2.77 (br m, 1H), 2.95 (br m, 1H), 3.19-3.28 (br m, 5H), 3.56 (br m, 1H), 4.00 (br m, 1H), 4.21-4.32 (br m, 1H), 5.11 (br m, 2H), 7.28 (m, 5H), 9.47 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 38.6, 39.0, 44.0, 46.0, 53.4, 66.4, 67.6, 77.2, 127.9, 128.0, 128.4, 136.4, 155.2, 175.5; HRMS-FAB (M + Na^+) calcd for $C_{16}H_{19}NNaO_4S_2$ 376.0654, found 376.0652.

Piperidone carbamate (19). A solution of carboxylic acid **15** (5.9 g, 16 mmol), DPPA (6.0 g, 21 mmol), and DIEA (3.2 g, 25 mmol) in 80 mL benzene was heated at reflux for 2.5 h. Aliquots of the reaction mixture were monitored for disappearance of the acyl azide peak (2170 cm^{-1}) and appearance of the isocyanate peak (2250 cm^{-1}) by FT-IR. After the Curtius rearrangement was judged complete by IR, the solution was cooled to room temperature, placed in an addition funnel, and added dropwise to a stirred solution of potassium *t*-butoxide (11 g, 100 mmol) in 100 mL of THF that was cooled in an ice bath. After the addition was complete, the solution was allowed to warm to room temperature over 30 min. The reaction was diluted with 150 mL hexanes, washed with H_2O (2 x 400 mL), 200 mL of 1 N aqueous HCl, 200 mL of 1 N aqueous NaOH, 200 mL of H_2O and 100 mL of brine. The organic layer was dried and concentrated, and the crude material was purified by flash chromatography (1:4 EtOAc/hexanes) to give 5.0 g (11 mmol, 71% for 2 steps) of compound **19**: ^1H NMR (400 MHz, $CDCl_3$) δ 1.40 (m,

9H), 2.10 (br s, 2H), 2.40 (br m, 0.5H), 2.64 (br t, J = 11.9 Hz, 0.5H), 2.93-3.19 (m, 6H), 4.02-4.30 (br m, 2H), 4.40 (m, 0.5H), 4.85 (br s, 0.5H), 5.06-5.16 (br m, 2H), 5.99 (br s, 0.5H), 6.50 (br d, J = 9.3 Hz, 0.5H), 7.19-7.36 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.2, 28.3, 37.7, 38.5, 38.96, 38.99, 39.3, 41.8, 43.1, 43.2, 47.8, 48.0, 52.7, 53.0, 55.6, 56.3, 67.4, 70.8, 80.3, 126.9, 127.0, 128.0, 128.1, 128.5, 128.6, 128.7, 129.3, 129.4, 136.4, 154.9, 155.4, 170.5, 170.8; HRMS-FAB (M + Na^+) calcd for $C_{20}H_{28}N_2NaO_4S_2$ 447.1389, found 447.1382.

Phenylalanylpiperidone (25). A CH_2Cl_2 solution (35 mL) of carbamate **19** (3.3 g, 7.7 mmol) was treated with 5 mL (65 mmol) of TFA and reaction was stirred at room temperature. After 45 min an addition 5 mL of TFA was added, and stirring was continued for an additional 2 h. The solvent and excess TFA were removed by rotary evaporation and the resultant oily liquid was partitioned between 150 mL of EtOAc and 100 mL of 1 N aqueous NaOH solution. The organic layer was dried and concentrated, and resulting material was dissolved in 10 mL of CH_2Cl_2. This solution was added to a mixture of HOBT (1.2 g, 7.6 mmol), EDC (1.9 g, 9.9 mmol), N-methylmorpholine (1.14 mL, 10.4 mmol), and N-Boc-phenylalanine (2.0 g, 7.6 mmol) in 50 mL of CH_2Cl_2 which had been pre-stirred at 0 °C for 1.5 h. The reaction mixture was warmed to room temperature overnight, then the solvent was removed by rotary evaporation. The resulting material was partitioned between 200 mL of EtOAc and 100 mL of 1 N aqueous HCl solution. The organic layer was washed with 150 mL of H_2O, 100 mL of saturated Na_2CO_3 solution, 100 mL of H_2O, and 100 mL of brine. It was then dried and concentrated, and the crude material was purified by flash chromatography (35% EtOAc/hexanes) to give 3.57 g (6.25 mmol, 81%) of compound **25** as a mixture of diastereomers: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (d, J = 10.1 Hz, 9H), 2.10 (br s, 2H), 2.39 (br m, 0.5H), 2.59-2.68 (dd, J = 10.3, 13.1 Hz, 0.5H), 2.91-3.18 (br m, 7H), 4.03-4.32 (br m, 3H), 4.39-4.42 (q, J = 7.7 Hz, 0.5H), 4.83 (br s, 0.5H), 5.05-5.20 (br m, 2H), 6.00 (br s, 1H), 6.50 (br d, J = 9.3 Hz, 1H), 7.20-7.37 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.2, 28.3, 37.7, 38.5, 38.9, 39.0, 39.3, 41.8, 43.1, 43.2, 47.7,

48.0, 52.7, 52.9, 55.6, 56.3, 67.4, 70.7, 70.8, 80.3, 126.85, 126.93, 127.98, 128.00, 128.1, 128.5, 128.6, 128.7, 129.2, 129.4, 136.4, 136.7, 154.8, 155.4, 170.5, 170.8; HRMS-FAB (M + Na^+) calcd for $C_{29}H_{37}N_3NaO_5S_2$ 594.2073, found 594.2069.

Methoxysuccinylpiperidone ketal (29). A mixture of compound **25** (1.27 g, 2.21 mmol), TFA (5 mL) and CH_2Cl_2 (15 mL) was stirred at room temperature for 45 min. The reaction mixture was diluted with 100 mL of EtOAc, washed with saturated aqueous $NaHCO_3$ solution (2 x 100 mL), dried, and concentrated. The crude amine was then dissolved in 10 mL of CH_2Cl_2 and added to a mixture of HOBT (0.37 g, 2.4 mmol), EDC (0.61 g, 3.2 mmol), monomethyl succinate (0.32 g, 2.4 mmol), and N-methylmorpholine (0.40 mL, 3.7 mmol) in 15 mL of CH_2Cl_2 which had been pre-stirred for 1 h at room temperature. After 1 h the solvent was evaporated and the residue dissolved in 100 mL of EtOAc. This organic solution was washed with 50 mL of 1 N HCl, 50 mL of H_2O, 50 mL of saturated Na_2CO_3, 50 mL of H_2O, 50 mL of brine, dried, and concentrated by rotary evaporation. The crude material was purified by flash chromatography (2:1 EtOAc/hexanes) to give 1.1 g (1.9 mmol, 89%) of compound **29** as a mixture of diastereomers: ^1H NMR (400 MHz, $CDCl_3$) δ 2.10 (br s, 2H), 2.43–2.67 (m, 4H), 2.78-3.23 (m, 8H), 3.65 (m, 3H), 3.95-4.22 (m, 3H), 4.60 (q, J = 6.1 Hz, 0.5H), 4.75 (q, J = 7.6 Hz, 0.5H), 5.09 (m, 2H), 6.03-6.68 (m, 2H), 7.19-7.36 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.8, 28.9, 29.1, 29.2, 30.8, 37.7, 38.4, 38.9, 39.0, 39.2, 39.3, 41.5, 41.7, 43.1, 43.2, 47.7, 47.9, 51.8, 52.7, 53.1, 54.3, 54.9, 67.39, 67.44, 70.55, 70.63, 126.9, 127.0, 127.95, 128.00, 128.08, 128.13, 128.5, 128.7, 129.2, 129.4, 136.4, 136.6, 154.8, 154.9, 170.2, 170.5, 171.3, 171.6, 173.07, 173.15; HRMS-FAB (M + Na^+) calcd for $C_{29}H_{35}N_3NaO_6S_2$ 608.1865, found 608.1874.

Methoxysuccinylpiperidone (33). An acetone (10 mL) solution containing the ketal **29** (0.20 g, 0.34 mmol) was added to a solution of NBS (0.43 g, 2.4 mmol) in 2 mL of water and 8 mL of acetone. The dark orange solution became colorless after 15 minutes. The solution was

then quenched with 30 mL of saturated Na_2CO_3 solution and extracted with 30 mL of EtOAc. The organic layer was washed with saturated $NaHCO_3$ solution, brine, dried, concentrated, and the crude material was purified by flash chromatography (4:1 EtOAc/hexanes) to give 0.12 g (0.23 mmol, 68%) of ketone **33** as a mixture of diastereomers: ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.46-2.76 (m, 8H), 3.01-3.16 (m, 3H), 3.64 (m, 3H), 4.38-4.56 (m, 2H), 4.70-4.84 (m, 2H), 5.30 (m, 2H), 6.48 (m, 1H), 6.80 (br d, J = 5.7 Hz, 2H), 7.17-7.38 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.1, 29.6, 30.7, 30.8, 38.1, 38.2, 40.3, 44.0, 48.3, 48.5, 51.8, 54.2, 54.3, 56.2, 56.4, 67.8, 126.98, 127.02, 128.0, 128.2, 128.56, 128.61, 129.2, 136.2, 136.4, 154.8, 170.8, 170.9, 171.3, 171.4, 173.3, 173.4, 202.9, 203.3; HRMS-FAB (M + Na^+) calcd for $C_{27}H_{31}N_3NaO_7$ 532.2060, found 532.2065.

Piperidone inhibitor (34). An ethanol solution (5 mL) containing compound **33** (0.025 g, 0.049 mmol) and a catalytic amount of 5% palladium on carbon (Degussa Type) was stirred under a hydrogen atmosphere for 3 days. The solution was then filtered through a plug of silica gel and concentrated. The resulting oily yellow liquid was purified by HPLC (silica, 5% MeOH/ CH_2Cl_2, retention time 31.4 min) leaving 0.015 g (0.039 mmol, 79%) of **34** as a mixture of diastereomers: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.24 (dt, J = 10.8, 12.5 Hz, 1H), 2.41-2.68 (m, 7H), 2.74-2.88 (m, 1H), 3.00-3.12 (m, 2H), 3.68 (m, 1H), 3.66-3.70 (m, 3.5H), 3.79-3.84 (ddd, J = 2.0, 6.3, 12.2 Hz, 0.5H), 4.43 (m, 1H), 4.77 (m, 1H), 6.54 (d, J = 7.8 Hz, 0.5H), 6.64 (d, J = 7.9 Hz, 0.5H), 6.78 (d, J = 6.4 Hz, 0.5H), 6.93 (d, J = 6.1 Hz, 0.5H), 7.16-7.30 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.16, 29.17, 30.81, 30.85, 38.6, 38.7, 43.3, 43.4, 48.1, 51.8, 53.2, 53.4, 54.3, 54.4, 59.1, 59.2, 127.0, 127.1, 128.6, 129.2, 129.3, 136.3, 136.4, 170.7, 170.9, 171.2, 171.3, 173.3, 173.4, 204.3, 204.5; HRMS-FAB (M + Na^+) calcd for $C_{19}H_{25}N_3NaO_5$ 398.1692, found 398.1685.

Tetrahydrothiopyran-4-one 1-oxide. Tetrahydrothiopyran-4-one (0.20 g, 1.73 mmol) was dissolved in 3 mL of methanol and cooled in an ice bath. A 0.5 M aqueous solution of

$NaIO_4$ (3.63 mL, 1.82 mmol) was added and the reaction was stirred at 0 °C for 1 h. Precipitated sodium iodate was removed by filtration and the solution was saturated with NaCl. The aqueous phase was extracted with EtOAc (3 x 5 mL) and acetone (3 x 5 mL) and the combined organic extracts were dried, filtered, and concentrated. The crude material was purified by flash chromatography (eluted with 10% MeOH in EtOAc; loaded in 1:1 CH_2Cl_2:EtOAc for solubility) to give 0.13 g (0.94 mmol, 57%) of the product as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6, mixture of ketone and hydrate) δ 1.68 (d, J = 14.3 Hz, 2H), 2.10 (dt, J = 3.2, 12.8 Hz, 2H), 2.65-2.85 (m, 4H), 5.57 (s, 1H), 5.70 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6, mixture of ketone and hydrate) δ 29.4, 32.1, 44.5, 45.8, 90.7, 205.4.

Tetrahydrothiopyran-4-one 1,1-dioxide. Tetrahydrothiopyran-4-one (0.25 g, 2.18 mmol) was dissolved in 15 mL of CH_2Cl_2 and cooled in an ice bath. To the reaction was added a solution of *3*-chloroperoxybenzoic acid (*m*-CPBA) (1.26 g of a 60% preparation, 4.37 mmol) dissolved in 25 mL of CH_2Cl_2. The mixture was warmed to room temperature and stirred for 1 h, during which time a white precipitate formed. The reaction mixture was concentrated and the material was purified by flash chromatography. The column was loaded in 1:1 CH_2Cl_2/EtOAc for solubility, and eluted first with 1:3 hexanes/EtOAc to remove benzoic acid and residual *m*-CPBA, followed by EtOAc to recover 0.31 g (2.11 mmol, 97%) of the sulfone as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.77 (t, J = 13.4 Hz, 4H), 3.51 (t, J = 13.5 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 37.8, 48.2, 203.2.

Measurement of K_{H2O} 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 ^1H 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 $k_{obs} = 2.2 \pm 0.5 \times 10^{-3}$ 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 above for the preparation of compound **34**, which yielded compounds **36a** and **36b**. ^1H 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 1.0 M phosphate buffer (pH 6.5) that was prepared using D_2O. The ^1H 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. ^1H NMR of this sample showed relatively slow reaction, with complete racemization after approximately 22 h. Diastereomer **36b** gave similar results. **36a**: ^1H NMR (400 MHz, $CDCl_3$) δ 1.98 (br s, 3H), 2.45-2.54 (br m, 3H), 3.06 (br s, 1H), 4.38-4.45 (br m, 2H), 4.75-4.82 (br m, 2H), 5.17 (br m,

2H), 6.34 (br s, 1H), 6.79 (br s, 1H), 7.18-7.38 (br m, 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; **36b**: ^1H NMR (400 MHz, $CDCl_3$) δ 1.98 (br s, 3H), 2.44 (br m, 3H), 3.01 (br m, 3H), 4.46 (br s, 2H), 4.67-4.76 (br m, 2H), 5.18 (br m, 2H), 6.38 (br s, 1H), 6.68 (br s, 1H), 7.19-7.40 (br m, 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 (3x's recrystallized) and L-BAPNA was 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 to 1 mg/mL) were prepared in 5x's buffer 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- 120 sec 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 Michalis-Menton 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).

References

(1) (a) Winstein, S.; Shatavsky, M.; Norton, C.; Woodward, R. B. *J. Am. Chem. Soc.* **1955**, *77*, 4183. (b) Winstein, S.; Shatavsky, M. *J. Am. Chem. Soc.* **1956**, *78*, 592. (c) For a recent review, see Bowden, K.; Grubbs, E. J. *Chem. Soc. Rev.* **1996**, *25*, 171.

(2) Lowry, T. H.; Richardson, K. S. *Mechanism and Theory in Organic Chemistry*, 3rd edition; Harper and Row: New York, 1987.

(3) (a) Roberts, J. D.; Moreland Jr., W. T. *J. Am. Chem. Soc.* **1953**, *75*, 2167. (b) Holtz, H. D.; Stock, L. M. *J. Am. Chem. Soc.* **1964**, *86*, 5188.

(4) Chen, C.-T.; Siegel, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 5959.

(5) For an example taken from synthetic organic chemistry, see: Boeckman, Jr., R. K.; Connell, B. T. *J. Am. Chem. Soc.* **1995**, *117*, 12368. For a discussion of field and resonance effects in benzoxazinone inhibitors of human leukocyte elastase, see: Krantz, A.; Spencer, R. W.; Tam, T. F.; Liak, T. J.; Copp, L. J.; Thomas, E. M.; Rafferty, S. P. *J. Med. Chem.* **1990**, *33*, 464.

(6) Van Noorden, C. F.; Smith, R. E.; Rasnick, D. *J. Rheumatol.* **1988**, *115*, 1525.

(7) Prous, J. R., Ed. *Drugs Future* **1986**, *11*, 927 - 943.

(8) (a) Liotta, L. A.; Steeg, P. S.; Stetler-Stevenson, J. G. *Cell* **1991**, *64*, 327. (b) Baricos, W. H.; Zhou, Y.; Mason, R. W.; Barrett, A. J. *Biochem. J.* **1988**, *252*, 301.

(9) (a) Hanzlik, R. P.; Jacober, S. P.; Zygmunt, J. *Biochim. Biophys. Acta* **1991**, *1073*, 33. (b) Cheng, H.; Keitz, P.; Jones, J. B. *J. Org. Chem.* **1994**, *59*, 7671.

(10) Hanzlik, R. P.; Zygmunt, J.; Moon, J. B. *Biochem. Biophys. Acta* **1990**, *1035*, 62.

(11) Hu, L.-Y.; Abeles, R. H. *Arch. Biochem. Biophys.* **1990**, *281*, 271.

(12) Ando, R.; Morinaka, Y.; Tokuyama, H.; Isaka, M.; Nakamura, E. *J. Am. Chem. Soc.* **1993**, *115*, 1174.

(13) (a) Moon, J. B.; Coleman, R. S.; Hanzlik, R. P. *J. Am. Chem. Soc.* **1986**, *108*, 1350. (b) Brisson, J.-R.; Carey, P. R.; Storer, A. C. *J. Biol. Chem.* **1986**, *261*, 9087.

(c) Gamcsik, M. P.; Malthous, J. P. G.; Primrose, W. U.; Mackenzie, N. E.; Boyd, A. S. F.; Russell, R. A.; Scott, A. I. *J. Am. Chem. Soc.* **1983**, *105*, 6324. (d) Liang, T.-C.; Abeles, R. H. *Arch. Biochem. Biophys.* **1987**, *252*, 626.

(14) (a) Hanzlik, R. P.; Jacober, S. P.; Zygmunt, J. *Biochim. Biophys. Acta* **1991**, *1073*, 33. (b) Berti, P. J.; Faerman, C. H.; Storer, A. C. *Biochemistry* **1991**, *30*, 1394.

(15) Bendall, M. R.; Cartwright, I. L.; Clart, P. I.; Lowe, G.; Nurse, D. *Eur. J. Biochem.* **1977**, *79*, 201.

(16) Geneste, P.; Durand, R.; Hugon, I.; Reminiac, C. *J. Org. Chem.* **1979**, *44*, 1971.

(17) Das, G.; Thornton, E. R. *J. Am. Chem. Soc.* **1993**, *115*, 1302.

(18) Burkey, T. J.; Fahey, R. C. *J. Org. Chem.* **1985**, *50*, 1304.

(19) (a) Allinger, N. L.; Tribgle, M. T.; Miller, M. A. *Tetrahedron*, **1972**, *28*, 1173. (b) Gung, B. W.; Wolf, M. A.; Mareska, D. A.; Karipides, A. *J. Org. Chem.* **1994**, *59*, 4899.

(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.

(21) (a) Imperiali, B.; Abeles, R. H. *Biochemistry*, **1986**, *25*, 3760. (b) Brady, K.; Abeles, R. H. *Biochemistry*, **1990**, *29*, 7608. (c) Govardhan, C. P.; Abeles, R. H. *Arch. Biochem. Biophys.* **1990**, *280*, 137.

(22) (a) Smith, R. A.; Copp, L. J.; Donnelly, S. L.; Spencer, R. W.; Krantz, A. *Biochemistry* **1988**, *27*, 6568. Peptide monofluoromethyl ketones have been shown to be selective irreversible inhibitors of cysteine proteases: (b) Rasnick, D. *Anal. Biochem.* **1985**, *149*, 461. (c) Rauber, P.; Angliker, H.; Walker, B.; Shaw, E. *Biochem. J.* **1986**, *239*, 633.

(23) Burkey, T. J.; Fahey, R. C. *J. Am. Chem. Soc.* **1983**, *105*, 868.

(24) (a) Wiberg, K. B.; Morgan, K. M.; Maltz, H. *J. Am. Chem. Soc.* **1994**, *116*, 11067. (b)Van Luppen, J. J.; Lepoivre, J. A.; Dommissee, R. A.; Alderweireldt, F. C. *Org. Mag. Res.* **1979**, *12*, 399.

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

(26) Dowd, P.; Choi, S.-C. *Tetrahedron* **1991**, *47*, 4847.

(27) Shioiri, T.; Ninomiya, K.; Yamada, S. *J. Am. Chem. Soc.* **1972**, *94*, 6203.

(28) (a) Carpino, L. A.; Sadat-Aalaee, D.; Chao, H. G.; DeSelms, R. H. *J. Am. Chem Soc.* **1990**, *112*, 9651. (b) Carpino, L. A.; Mansour, E. M. E.; Sadat-Aalaee, D. *J. Org. Chem.* **1991**, *56*, 2611.

(29) Corey, E. J.; Erickson, B. W. *J. Org. Chem.* **1971**, *36*, 3553.

(30) Enzyme assays were performed according to the procedures of reference 11. None of these compounds showed evidence of slow-binding inhibition. Lineweaver-Burke plots are available in the supplementary material.

(31) (a) Swain, C. G.; Lupton Jr., E. C. *J. Am. Chem. Soc.* **1968**, *90*, 4328. (b) Swain, C. G.; Unger, S. H.; Rosenquist, N. R.; Swain, M. S. *J. Am. Chem. Soc.* **1983**, *105*, 492.

(32) A good correlation also exists between $\log K_{RSH}$ and **F**, and between $\log K_{H2O}$ and **F**. However, there is a poor correlation between $\log K_{RSH,app}$ and the resonance substituent constant **R** (correlation coeffiecient = 0.41)

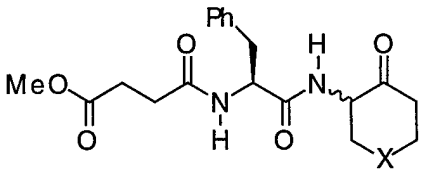
(33) For a similar analysis involving inhibitors of cathepsin B, see reference 22a.

Part 2. ^{13}C NMR Studies Demonstrate that Tetrahydropyranone-Based Inhibitors Bind to Cysteine Proteases by Reversible Formation of a Hemithioketal Adduct

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. These new proteases, which 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.[3] 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. Because cysteine proteases are involved in so many diseases, there is a strong motivation for developing potent and specific inhibitors of these enzymes. Such compounds could 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]



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

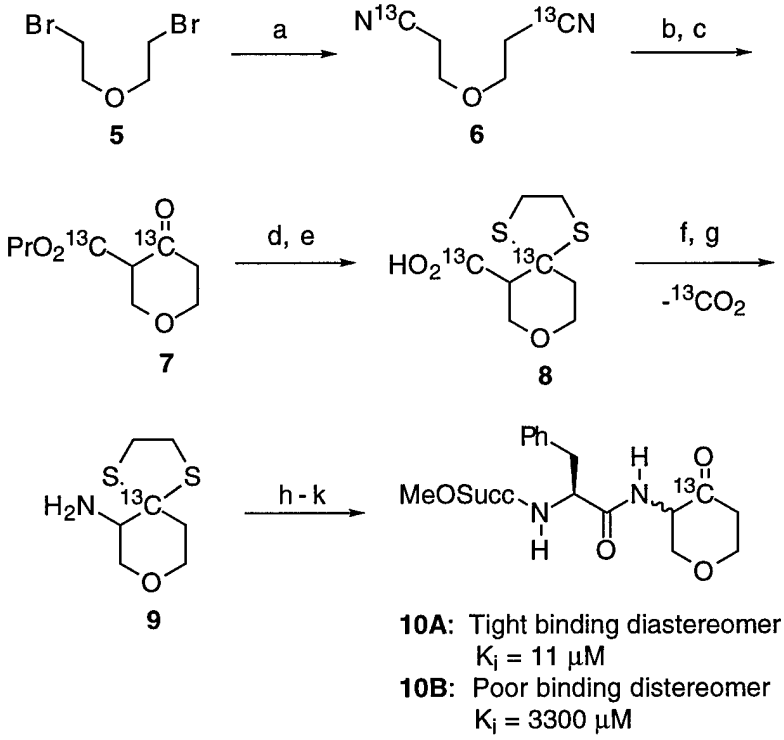
Our interpretation of these inhibition studies was based upon the assumption that a hemithioketal does indeed form between the inhibitors and the active site cysteine residue. This assumption is reasonable based upon comparison of 4-heterocyclohexanone inhibitors with other inhibitors, such as peptide aldehydes, that are known to give this type of covalent adduct.[5] 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.[6] 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. In order to determine unambiguously the mechanism of action of these 4-heterocyclohexanone-based inhibitors, we have synthesized 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 can be monitored by ^{13}C-NMR spectroscopy, allowing us to observe directly formation of the hemithioketal adduct between enzyme and inhibitor.[5,7]

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**.[8] The labeled reagent can be conveniently prepared from $K^{13}CN$ and Et_4NBF_4.[9] Alcoholysis of **6** followed by base promoted cyclization of the resulting diester gave ketoester **7**. After protection of the ketone and saponification of the ester, compound

8 was treated with diphenylphosphoryl azide to induce a Curtius rearrangement. Trapping of the resulting isocyanate with *t*-BuOK yielded the corresponding Boc-protected amine. Removing the Boc group with TFA resulted in loss of one 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.

Scheme 1[a]



10A: Tight binding diastereomer
K_i = 11 μM
10B: Poor binding distereomer
K_i = 3300 μM

[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.

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. 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 strongly dependent on 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 these 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 interference from racemization.

Enzyme Purification. Commercial preparations of papain are contaminated with a large amount of inactive enzyme. We have thus purified papain by affinity chromatography on a mercurial agarose column.[10] The enzyme is eluted from the column as a solution of mercurial papain, and the active enzyme is regenerated by washing with a buffered solution of cysteine and EDTA. Enzyme purified in this manner is greater that 95% active as judged by titration of the active site cysteine-25 thiolate with the reagent 2,2'-dipyridyl disulfide (DDS).[11] Protein concentrations were determined by UV spectroscopy.[12]

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

Spectrum 1 (Figure 1) shows papain on its own. Spectrum 2 shows two major resonances for inhibitor **10A** alone. The peak at 207.3 ppm corresponds to the ^{13}C-labeled ketone, and the peak at 93.6 ppm corresponds to the hydrate. The observation that these two resonances have similar intensities is 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.

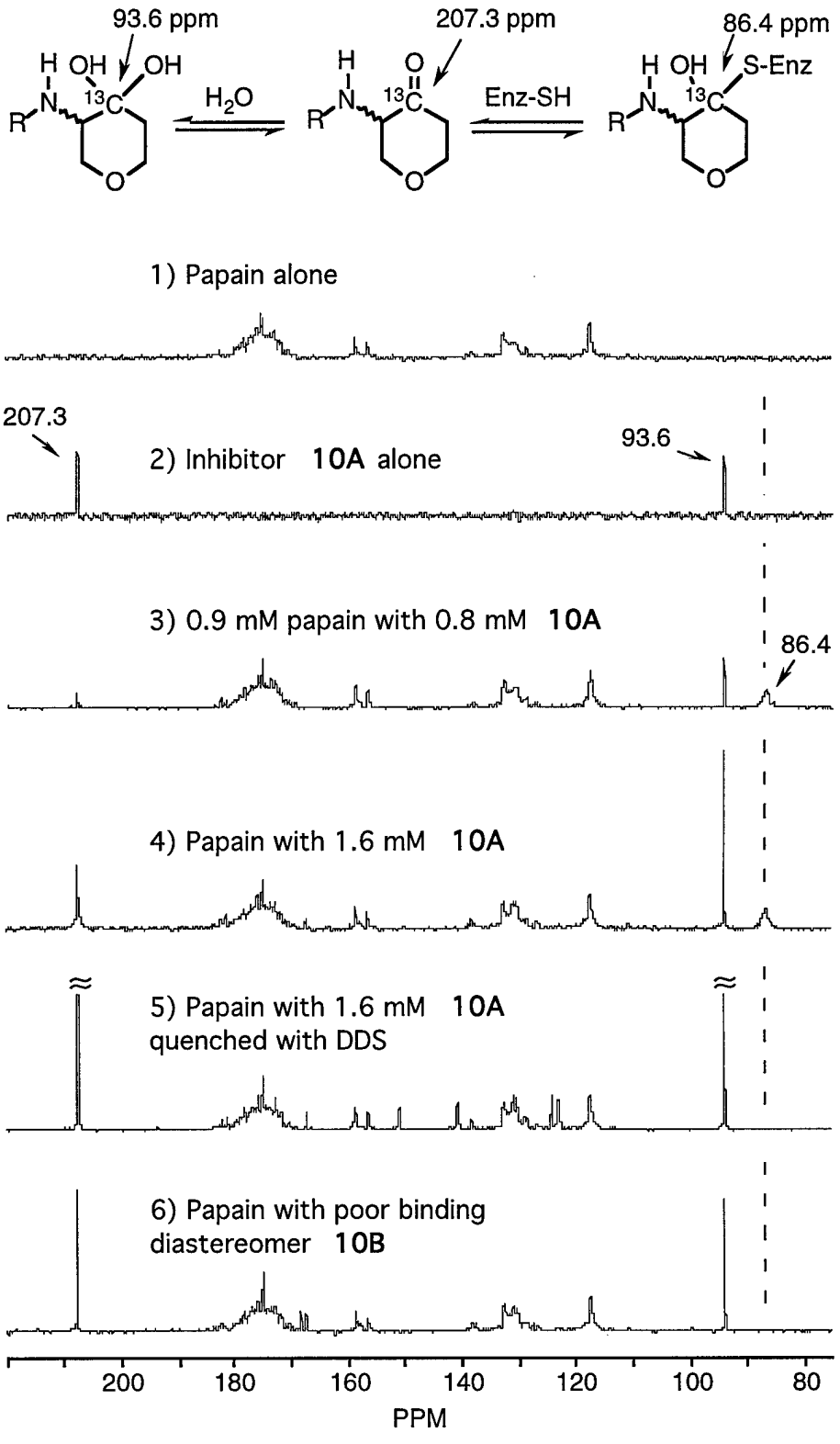


Figure 1. Partial ^{13}C NMR spectra of papain incubated with the ^{13}C enriched inhibitor **10**. The concentration of enzyme in all spectra that contain papain is 0.9 mM.

Spectrum 3 shows papain in the presence of slightly less than one equivalent of **10A**. There are resonances for a small amount of free ketone, and also hydrate. Importantly, a new resonance at 86.4 ppm appears that is not present in either spectra 1 or 2. We assign this new resonance as the covalent hemithioketal adduct between the enzyme active site nucleophile and the ketone of the inhibitor.

Three lines of evidence support this structural assignment. First, the chemical shift of this peak clearly indicates that it corresponds to an sp^3 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 a small molecule thiol such as 3-thiopropionic acid yields a hemithioketal adduct with two resonances in the ^{13}C NMR spectrum at 83.7 and 83.8 ppm. These chemical shifts are similar to the 86.4 ppm which is found for the hemithioketal between **10A** and the enzyme. In the reaction with thiopropionic acid, the two observed resonance correspond to addition of the thiol to the two diastereotopic faces of the ketone. In analogy, it is theoretically possible for inhibitor **10A** to bind in the enzyme active site in two different conformations in which the active site nucleophile has added to either face of the tetrahydropyranone ring. However, molecular modeling studies indicate that addition to only one of the two diastereotopic faces is allowed within the steric confines of the active site. These modeling studies do not give a clear indication as to which diastereomer of **10** is the better binding inhibitor.

Addition of excess inhibitor to the enzyme (spectrum 4) simply results in an increase in the intensities of the resonances for free inhibitor. However quenching the enzyme with DDS (spectrum 5), which forms a disulfide with the active site cysteine residue and thus displaces the inhibitor from the active site, results in a 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 spectrum 5 that appear between 120 and 160 ppm correspond to DDS and 2-thiopyridone.

Spectrum 6 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, even though 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] In contrast, the poor binding diastereomers of **1** - **3** all bind with similar affinities (3.2, 2.4, and 3.3 mM), and there is no correlation between inhibition constants and ketone electrophilicity.[4] These observations suggest that the poor binding diastereomers are all binding similarly in the active site, and that none of these compounds are forming a reversible covalent bond with the active site cysteine residue. Conversely, they also suggest that all of the tight binding diastereomers are to some extent forming a covalent adduct.

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 one of the diastereotopic faces of 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 Brucker AM-400 instrument. Spectra were calibrated using TMS ($\delta = 0.00$ ppm) for ^1H NMR and $CDCl_3$ ($\delta = 77.0$) or DMSO-d_6 ($\delta = 39.51$)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. Semi-preparative HPLC was performed on the same system using a semi-preparative column (21.4 x 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 a N_2 atmosphere and allowed to warm to room temperature overnight. The reaction mixture 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%): ^1H 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.[10] 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 a 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 using an extinction coefficient of $\varepsilon = 25$ absorbance units for 1% solution, and assuming a molecular weight of 23,000.[12] The activity of these enzyme preparations was determined by titrating the active site cysteine nucleophile with 2,2'-dipyridyl disulfide according to the procedure of Brocklehurst and Little.[11] 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 1 - 6 (Figure 1) contained: 1) 0.9 mM papain; 2) inhibitor **10A**; 3) 0.9 mM papain and 0.8 mM **10A**; 4) 0.9 mM papain and 1.6 mM **10A**; 5) 0.9 mM papain, 1.6 mM **10A**, and 4.5 mM 2,2'-dipyridyl disulfide; and 6) 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 64 k, a pulse width of 30 degrees, and a receiver delay of 0.0 sec was used to give a total acquisition time of 1.25 seconds. An exponential line broadening of 10 Hz was used during processing. Approximately 32,000 scans were acquired for samples that contained protein.

Computer Modeling. Modeling studies were performed on a Silicon Graphics Indy computer using the program QUANTA 4.0. The coordinates of the X-ray crystal structure of papain were obtained from the Brookhaven Protein Data Bank (structure 6Pad). This crystal structure has a chloromethylketone inhibitor (CbzPheGlyCH_2Cl) bound at the active site. The

chloromethyl ketone was removed from the active site and replaced by tetrahydropyranone inhibitor **10**. The tetrahydropyranone inhibitor was linked to the active site nucleophile by formation of a hemithioketal. The enzyme was then frozen in place and the structure of the inhibitor minimized in the active site using the Steepest Descent function in the CHARMm force field.

References

[1] For a recent review of cysteine proteases and their inhibitors, see: Otto, H. H.; Schirmeister, T. *Chem. Rev.* **1997**, *97*, 133.

[2] Wang, K.; Yuen, P.-W. *Trends Pharmacol. Sci.* **1994**, *15*, 412.

[3] (a) Miller, D. K. *Ann. Rep. Med. Chem.* **1996**, *31*, 249. (b) Schwartz, L. M.; Milligan, C. E. *Trends Neurosci.* **1996**, *19*, 555. (c) Nicholson, D. W.; Ali, A.; Thornberry, N. A.; Vaillancourt, J. P.; Ding, C. K.; Gallant, M.; Gareau, Y.; Griffin, P. R.; Labelle, M.; Lazebnik, Y. A.; Munday, N. A.; Raju, S. M.; Smulson, M. E.; Yamin, T.-T.; Yu, V. L.; Miller, D. K. *Nature* **1995**, *376*, 37. (d) Nicholson, D. W. *Nature Biotech.* **14**, *297*, 1996.

[4] Conroy, J. L.; Sanders, T. C.; Seto, C. T. *J. Am. Chem. Soc.* **1997**, *119*, 4285.

[5] Gamcsik, M. P.; Malthouse, J. P. G.; Primrose, W. U.; Mackenzie, N. E.; Boyd, A. S. F.; Russell, R. A.; Scott, A. I. *J. Am. Chem. Soc.* **1983**, *105*, 6324.

[6] (a) Gelb, M. H.; Svaren, J. P.; Abeles, R. H. *Biochemistry* **1985**, *24*, 1813. (b) Patel, D. V.; Rielly-Gauvin, K.; Ryono, D. E.; Free, C. A.; Smith, S. A.; Petrillo, E. W. *J. Med. Chem.* **1993**, *36*, 2431.

[7] For previous examples of this methodology, see: (a) Rich, D. H.; Bernatowicz, M. S.; Schmidt, P. G. *J. Am. Chem. Soc.* **1982**, *104*, 3535. (b) Moon, J. B.; Coleman, R. S.; Hanzlik, R. P. *J. Am. Chem. Soc.* **1986**, *108*, 1350. (c) Brisson, J. R.; Carey, P. R.; Storer, A. C. *J. Biol. Chem.* **1986**, *261*, 9087. (d) Liang, T. C.; Abeles, R. H. *Arch. Biochem. Biophys.* **1987**, *252*, 626. (e) Malthouse, J. P. G.; Mackenzie, N. E.; Boyd, A. S. F.; Scott, A. I. *J. Am. Chem. Soc.* **1983**, *105*, 1685.

[8] Simchen, G.; Kobler. H. *Synthesis* **1975**, 605.

[9] Kobler, H.; Munz, R.; Gasser, G. A.; Simchen, G. *Liebigs Ann. Chem.* **1978**, 1937.

[10] Sluyterman, L. A.; Wijdenes, J. *Methods Enzymol.* **1974**, *34*, 544.

[11] Brocklehurst, K.; Little, G. *Biochem. J.* **1973**, *133*, 67.

[12] Glaser, A. N.; Smith, E. L. *J. Biol. Chem.* **1965**, *240*, 201.

[13] A line width of 88 Hz has been reported for the covalent complex between a peptide aldehyde inhibitor and papain. See reference 5.