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

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

Introduction

The Field Effect. The physical-organic literature contains many examples of chemical systems that use through-space electronic interactions to control equilibria or regio- and stereospecificity of organic reactions.\(^1\)\(^2\) Molecules such as 4-substituted bicyclo[2.2.2]octane-1-carboxylic acid have been developed to investigate the Coulombic interaction between a polar substituent and a carboxylic acid.\(^3\) The through-space electrostatic interaction between these groups perturbs the \(pK_a\) of the acid. More recently, Siegel and co-workers examined through-space polar \(\pi\) interactions in \(\pi\)-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.

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,\(^10\) \(\alpha\)-keto carboxyl 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.\(^13\)

\(^{10}\) Abstract published in Advance ACS Abstracts, May 1, 1997.


Chart 1. Structures of Cysteine Protease Inhibitors

\[ X = \text{CH}_2, \text{S}, \text{O}, \text{NH}_2^+ \]

This mechanism is also likely to be operative in the \( \alpha \)-keto carbonyl 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 \( N \)-methoxysuccinylphenylalanine side chain. We have chosen papain for our initial studies because its structure and mechanism have been thoroughly characterized. In addition, it provides a good model for evaluating the design of new inhibitors and for comparing them to previously reported compounds. The inhibitors include a phenylalanine residue because papain has a high specificity for this amino acid at the P2 position.\(^{14} \) The methoxysuccinyl group was attached in order to increase solubility of the compounds in aqueous solution.

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

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

We have synthesized a series of inhibitors that incorporate increasingly electronegative functional groups at the 1-position 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 \( \text{D}_2 \text{O} \), shows resonances for the ketone (a and b) and the hydrate (c). The middle spectrum, taken in acetone-\( \text{d}_6 \), shows resonances that correspond to tetrahydropyranone. The middle spectrum, taken in \( \text{D}_2 \text{O} \), shows resonances for the both the ketone (a and b) and the hydrate (c and d). These two species are in slow exchange on the NMR time scale. Integration of the resonances can be used to determine the hydration equilibrium constant. The top spectrum shows a mixture of tetrahydropyranone and 3-mercaptopropionic acid. The bottom spectrum, taken in \( \text{D}_2 \text{O} \), shows resonances for the both the ketone (a and b) and the hydrate (c and d). These two species are in slow exchange on the NMR time scale. Integration of the resonances can be used to determine the hydration equilibrium constant. The top spectrum shows a mixture of tetrahydropyranone and 3-mercaptopropionic acid in \( \text{D}_2 \text{O} \). We observe resonances for ketone, hydrate, hemithioketal (e–h), and free thiol (i and j).

Table 1 shows equilibrium constants for addition of water and thiol to selected ketones. The equilibrium constants were determined using \(^1\text{H} \) NMR spectroscopy according to the procedure of Burkey and Fahey.\(^{18,23} \) Table 1 shows equilibrium constants for addition of water and thiol to selected ketones. The equilibrium constants were determined using \(^1\text{H} \) 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-\( \text{d}_6 \), shows resonances that correspond to tetrahydropyranone. The middle spectrum, taken in \( \text{D}_2 \text{O} \), shows resonances for the both the ketone (a and b) and the hydrate (c and d). These two species are in slow exchange on the NMR time scale. Integration of the resonances can be used to determine the hydration equilibrium constant. The top spectrum shows a mixture of tetrahydropyranone and 3-mercaptopropionic acid in \( \text{D}_2 \text{O} \). We observe resonances for ketone, hydrate, hemithioketal (e–h), and free thiol (i and j). Equilibrium constants for several of the ketones listed in Table 1 have been measured previously under different reaction conditions.\(^{18,24} \) Our equilibrium constants are in reasonable agreement with the

<table>
<thead>
<tr>
<th>X</th>
<th>( K_{\text{H}_2\text{O}} ) (M(^{-1}))</th>
<th>( K_{\text{RSH}} ) (M(^{-1}))</th>
<th>( K_{\text{RSH,lag}} ) (M(^{-1}))</th>
</tr>
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<tr>
<td>CH(_2)</td>
<td>8.1 × 10(^{-4})</td>
<td>0.22</td>
<td>0.21</td>
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<tr>
<td>S</td>
<td>9.0 × 10(^{-3})</td>
<td>1.5</td>
<td>0.99</td>
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<tr>
<td>O</td>
<td>8.0 × 10(^{-3})</td>
<td>1.8</td>
<td>1.3</td>
</tr>
<tr>
<td>NH(_2^+)</td>
<td>0.18</td>
<td>27.6</td>
<td>2.7</td>
</tr>
<tr>
<td>SO</td>
<td>0.068</td>
<td>11.7</td>
<td>2.5</td>
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<tr>
<td>SO(_2)</td>
<td>0.30</td>
<td>60.2</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Other Ketones

CH\(_3\)COCH\(_2\)\(_3\) | 2.3 × 10\(^{-5}\) | 0.0052 | 0.0052 |
CH\(_3\)COCO\(_2\)H\(^+\) | 0.031 | 58 | 22 |
CH\(_3\)COCO\(_2\)CH\(_3\) | 0.045 | 71 | 20 |

\(^{a} \text{RSH} = \text{HO}-\text{CCH}_2\text{SH}. \) Data taken from reference 23. 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 heteroatom influences the reactivity of the ketone in these compounds. We have thus measured the equilibrium constants for addition of water and thiol nucleophiles to simple 4-heterocyclohexanones. These nucleophilic additions serve as a model for reaction of the enzyme active site nucleophile with the inhibitors.

Table 1 shows equilibrium constants for addition of water and 3-mercaptopropionic acid to a variety of ketones. The equilibrium constants were determined using \(^1\text{H} \) 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-\( \text{d}_6 \), shows resonances that correspond to tetrahydropyranone. The middle spectrum, taken in \( \text{D}_2 \text{O} \), shows resonances for the both the ketone (a and b) and the hydrate (c and d). These two species are in slow exchange on the NMR time scale. Integration of the resonances can be used to determine the hydration equilibrium constant. The top spectrum shows a mixture of tetrahydropyranone and 3-mercaptopropionic acid in \( \text{D}_2 \text{O} \). We observe resonances for ketone, hydrate, hemithioketal (e–h), and free thiol (i and j). Equilibrium constants for several of the ketones listed in Table 1 have been measured previously under different reaction conditions.\(^{18,24} \) Our equilibrium constants are in reasonable agreement with the
previously reported values. Equilibrium constants for acetone, pyruvic acid, and methyl pyruvate are taken from the literature. 23

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

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

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

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

**Synthesis of Inhibitors.** We have developed a generalized strategy for the synthesis of our papain inhibitors (Scheme 1).

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.

(previous text continues)
preparation of each of the four target compounds. Dieckmann condensation of diesters 1 and 2 gives keto esters 4 and 5. Compounds 3 and 6 are commercially available. The yield for cyclization of 2 is only 31%, presumably because of competing β-elimination. However, this represents a significant improvement over the previously reported synthesis of methyl tetrahydropyran-4-one-3-carboxylate, which proceeded in 8% yield. 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. Curtius rearrangement followed by trapping of the isocyanates with t-BuOK gives carbamates 16–19. The Boc protecting groups are removed with trifluoroacetic acid, and the resulting amines are coupled with an N-protected groups in compounds 30 and 32 are separated by HPLC. The Cbz protecting group in compound 33 is removed by catalytic hydrogenation to give inhibitor 34, which is evaluated as a mixture of diastereomers. The diastereomers of 27 can be separated by flash chromatography, and each are then treated with acetone and p-toluenesulfonic acid to give the separate diastereomers of inhibitor 31.

Racemization of Inhibitors. Papain is assayed in 100 mM phosphate buffer at pH 6.5. These conditions may catalyze the enolization of the ketone in our inhibitors and thus lead to their racemization. We have monitored this reaction using HPLC or $^1$H NMR spectroscopy. The cyclohexanone-based inhibitor 30 was very stable under the assay conditions, showing less that 5% racemization after 24 h. Tetrahydropyranone 32 was somewhat less stable, with a half-time for racemization of 5.25 h. However, this reaction is slow enough so that over the time period of a typical enzyme assay, the compound racemizes less than 1%. We were unable to separate the diastereomers of piperidone inhibitor 34 or its precursor 33 by standard chromatographic techniques. However, the diastereomers of compound 35, which has an acetyl group on its N-terminus rather than a methoxysuccinyl group, were readily separated by HPLC. We therefore chose to study racemization of compound 36 by $^1$H NMR spectroscopy. Over the course of the 10 min required to prepare the sample and acquire the spectrum, this compound was completely racemized. Therefore, we measured the inhibition constant for compound 34 as a mixture of diastereomers. We have not examined racemization of the tetrahydrothiopyranone-based inhibitor 31, but observed reactivity trends and chemical intuition both suggest that it should have a racemization rate that falls between that of compounds 30 and 32.

Inhibition Studies. The 4-heterocyclohexanone-based inhibitors 30–32 and 34 are all reversible competitive inhibitors of papain (Table 2). 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.

The cyclohexanone-based inhibitor (X = CH$_2$) is 20 times more potent than the noncyclic acetone-based inhibitor. This is a reflection of the ring strain in cyclohexanone that destabilizes the ketone relative to the hemithioketal that is formed by reaction of the inhibitor with the active site nucleophile. Substituting electronegative functionality into the ring (X = S, O) leads to even better inhibitors. This trend in inhibition constants mirrors the differences that we observe for reaction of the parent ketones with water and thiol nucleophiles. The only compound that does not fit the trend is the piperidone-based inhibitor 34. This compound is protonated under the assay conditions (pH 6.5), and its low potency is likely caused by the unfavorability of placing this positive charge into the enzyme active site.

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

<table>
<thead>
<tr>
<th>X</th>
<th>more-potent diastereomer $K_i$ (µM)</th>
<th>less-potent diastereomer $K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_2$</td>
<td>78</td>
<td>3200</td>
</tr>
<tr>
<td>S</td>
<td>26</td>
<td>2400</td>
</tr>
<tr>
<td>O</td>
<td>11</td>
<td>3300</td>
</tr>
<tr>
<td>NH$_3^+$</td>
<td>121*</td>
<td></td>
</tr>
</tbody>
</table>

Other Ketone-Based Inhibitors

- **AcPh-NHCH$_2$COOMe**: 1550µM
- **ZPhe-NHCH$_2$COOH**: 7µM
- **ZPhe-NHCH$_2$COOC$_2$H$_5$**: 1³µM

* Assayed as a mixture of diastereomers. This compound racemizes under the assay conditions.

### 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 have constructed a modified Hammett equation (eq 2) in which they describe the electronic characteristics of substituents in terms of two parameters; a field substituent constant $F$, and a resonance substituent constant $R$.

$$
\log(K_i/K_{i1}) = \rho(F + rR) \quad (2)
$$

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

Enzyme assays were performed according to the procedures of ref 11. None of these compounds showed evidence of slow-binding inhibition. Lineweaver–Burk plots are available in the Supporting Information.


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

(32) A good correlation also exists between \( \log K_{RS\text{H}} \) and \( F \) and between \( \log K_{RS\text{H,app}} \) and \( F \). However, there is a poor correlation between \( \log K_{RS\text{H,app}} \) and the resonance substitution constant \( R \) (correlation coefficient = 0.41).

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

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 \( \log K_{RS\text{H,app}} \) has a slope of 0.8. This slope, which is less than unity, indicates that the enzymatic addition reaction responds less efficiently to the electrophilicity of the ketone than does the model system. The difference in reactivity is likely caused by the differences in steric, electronic, solvation, and orientational requirements of the enzymatic reaction compared to the reaction in solution.

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

**Conclusions.** The results presented in this paper demonstrate that through-space electrostatic interactions can be useful and predictable design elements for construction of bioactive molecules. The physical-organic correlations point the way toward synthesis of more potent inhibitors. This goal can be achieved by choosing functionality that further increase the electrostatic repulsion between the heteroatom and the ketone in 4-heterocyclohexanones, such as a sulfoxide or sulfone. In addition, potency and specificity can be increased by functionalizing both the 3- and 5-positions of the heterocyclohexane 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 conjunction 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 \(^1\)H 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. Semipreparative HPLC was performed on the same system using a semipreparative column (21.4 \( \times \) 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 CaH2. 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 MgSO4 unless otherwise noted. Solvent removal was performed by rotary evaporation at water aspirator pressure.

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

Di-n-propyl 4-Oxa-1,7-heptanediol 2. A solution of 3,3’-oxydipropionitrile (189.1 g, 512 mmol) and p-toluenesulfonic acid (p-TsOH) monohydrate (115.8 g, 608 mmol) in n-propanol (200 mL) was refluxed for 24 h. The solution was cooled and concentrated to approximately 150 mL. The resulting solution was partitioned between 350 mL of water and 350 mL of chloroform. The organic layer was separated and washed with saturated NaHCO3 (200 mL), water (300 mL), and brine (150 mL). The solution was dried, filtered, and concentrated, and the crude material was purified by flash chromatography (1:3 EtOAc/hexanes) to yield 2 (21.2 g, 57%) as a colorless liquid. The product can also be purified by vacuum distillation (bp 158°C, 6 mm) in somewhat lower yields (45%).

-3-ethylcarbodiimide hydrochloride (EDC, 114 mg, 0.60 mmol), and diisopropylethylamine (DIEA, 1.9 g, 1.50 mmol), and diphenylphosphoryl azide (DPPA, 28.0 g, 0.1 mmol) in benzene (10 mL) was refluxed overnight. Aliquots of the reaction mixture were monitored for disappearance of the acyl azide peak at 2168 cm⁻¹ and appearance of the isocyanate peak at 2245 cm⁻¹ by FT-IR. After the Curtius rearrangement was judged complete by IR, the solution was cooled in an ice bath and slowly added to an ice-cold solution of potassium tert-butoxide (0.34 g, 3.0 mmol) in THF (10 mL). The reaction was stirred for 15 min and then partitioned between 15 mL of 1 N HCl and 15 mL of EtOAc. The organic layer was separated and washed with 1:1 EtOAc/hexanes (10 mL), and the aqueous layer was washed with 1:1 EtOAc/hexanes (10 mL), and then cooled and diluted with 0.2 N NaOH (10 mL). The solution was stirred at 30°C for 15 min, and then the reaction was quenched by the addition of 25 mL of H2O. The solution was partitioned between 200 mL of 1 N HCl and 200 mL of hexanes. The resulting aqueous layer was extracted with EtOAc (150 mL), and the combined organic layers were washed with brine (300 mL). The solution was dried, filtered, and concentrated, and the crude material purified by flash chromatography (1:4 EtOAc/hexanes) to yield 5 (1.19 g, 31%) as a mixture of keto and enol tautomers: Rf = 0.54 (1:1 EtOAc/hexanes); 1H NMR (400 MHz, CDCl3) δ 0.95 (t, J = 6.3 Hz, 2H), 1.27–1.31 (m, 13H), 1.61–1.65 (m, 2H), 2.37–2.41 (m, 3H), 2.52–2.59 (m, 2H), 2.66–2.73 (m, 3H), 3.46–3.50 (m, 3H), 3.71–3.75 (m, 3H), 3.85 (t, J = 5.7 Hz, 2H), 3.98–4.10 (m, 4H, J = 6.6 Hz, 2H), 4.16–4.25 (m, 4H, J = 1.7 Hz, 2H), 11.85 (s, 1H); 13C NMR (100 MHz, CDCl3) δ 10.2, 21.9, 86.4, 57.8, 63.9, 65.8, 66.3, 67.0, 68.1, 69.6, 97.4, 127.8, 129.7, 168.1, 201.4; HRMS-El (M+) calc'd for C11H12O4: 186.0892, found 186.0894.

Phenyllalanyltetrahydropyranone 24. To a solution of 21 (250 mg, 0.82 mmol) and DIEA (529 mg, 4.1 mmol) in CH2Cl2 (10 mL) that was cooled in an ice bath. The solution was stirred at 0°C for 1 h, concentrated, redissolved in CH2Cl2, and then concentrated again to remove excess TFA. The crude oil was then triturated with ether to yield 21 (0.18 g, 95%) as a white solid: 1H NMR (400 MHz, CDCl3) 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); 13C NMR (100 MHz, CDCl3) δ 39.9, 40.1, 41.5, 56.7, 66.1, 68.5, 68.6, 118.2 (q), 162.9 (q); HRMS-El (M+) calc'd for C14H12N2O4: 241.0963, found 241.0967.
results. For 36a: 1 H NMR (400 MHz, CDCl 3 ) δ 1.98 (brs, 3H), 2.45–
2.54 (brm, 3H), 3.06 (brs, 1H), 4.38–4.45 (brm, 2H), 4.75–4.82 (brm,
2.5), 5.17 (brm, 2H), 6.34 (brs, 1H), 6.79 (brs, 1H), 7.18–7.38 (brm,
10H); 13C 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, 128.7, 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 3 
ClO 4 Na + : 459.1522, found 459.1521.

Papain Assays. Papain (recrystallized two times) and l-BAPNA
(Nε-benzoyl-l-arginine p-nitroanilide hydrochloride) were used as
received from Sigma Chemical Co. Reaction progress was monitored
with a Perkin-Elmer 4542A diode array UV-vis spectrometer. Papain
was assayed at 25 °C in 100 mM phosphate buffer (pH6.5) containing
5 mM EDTA and 5 mM cysteine. BAPNA and inhibitor stock solutions
were prepared in buffer (5–100 mM), and all assay mixtures contained
a final DMSO concentration of 10%. Papain stock solutions (0.5–1 mg/mL)
were prepared in buffer (5x), and the enzyme was activated for 1 h
before the assays were run. Initial rates were determined by monitoring
the change in absorbance at 412 nm from 60 to 120 s after mixing.
None of the inhibitors showed evidence of slow binding. The most
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, 5.5, 27.4, 55, 110 μM; 32a 0, 2, 25, 50,
75, 100 μM; 34a 0, 13.9, 69.5, 139, 209, 417 mM) at least five substrate
concentrations were used (30a 0.37, 0.53, 0.75, 1.5, 7.5 μM; 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 determina-
tions at each concentration. K m was measured to be 4.89 mM. The
background hydrolysis rate was less than 1% of the slowest rate measure
and thus ignored. K i values were determined by nonlinear fit to the
Michaelis–Menten equation for competitive inhibition using simple
weighing. Competitive inhibition was confirmed by Lineweaver–Burk
analysis using robust statistical weighting to the linear fit of I/V vs
I/[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 0.1, 0.5,
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 (Eritacus Software Ltd).

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

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