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

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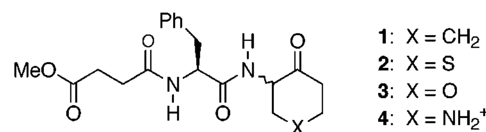
Received October 3, 1997



Introduction

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

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

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

Results and Discussion

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

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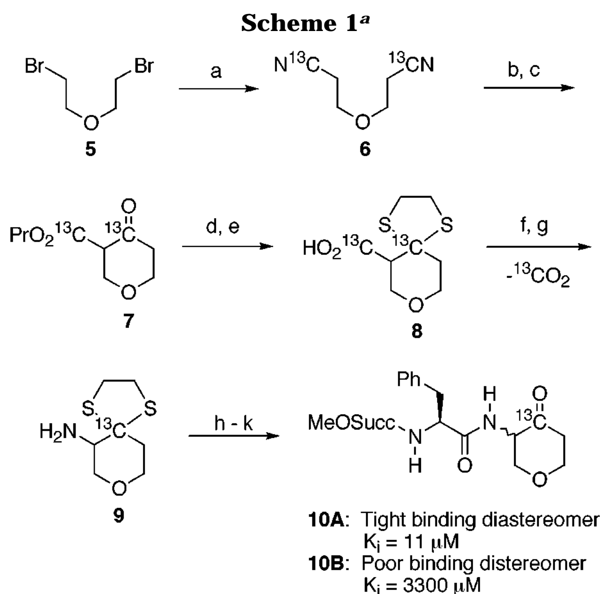
S0022-3263(97)01834-3 CCC: $15.00 © 1998 American Chemical Society
Published on Web 03/17/1998

Scheme 1[a]



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

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

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

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

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

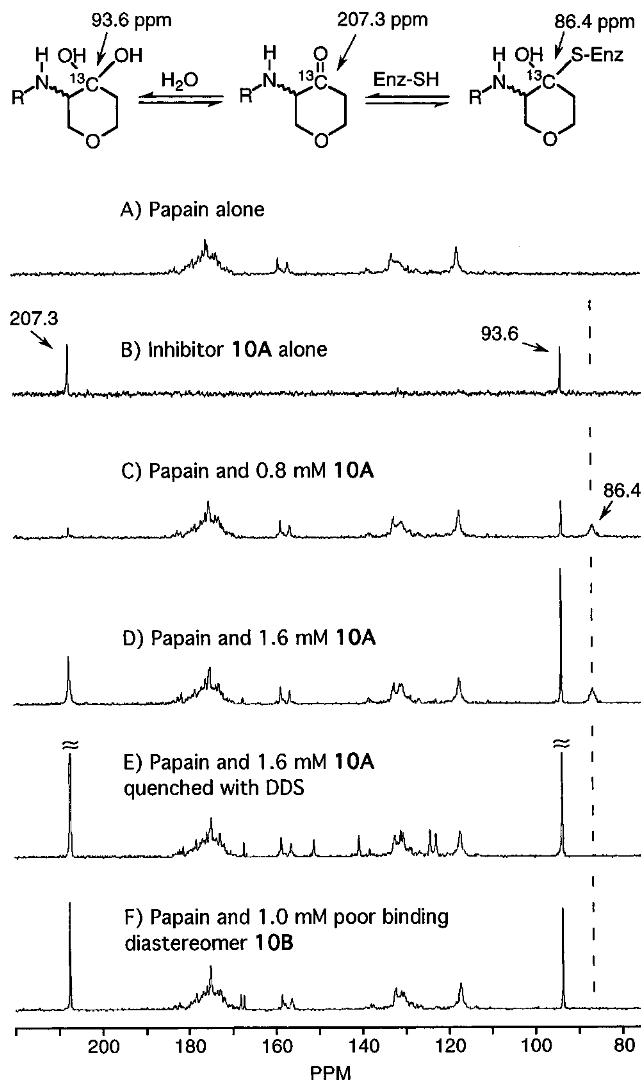


Figure 1. Partial ^{13}C NMR spectra of papain incubated with the ^{13}C-enriched inhibitor **10**. The concentration of enzyme in all spectra that contain papain is 0.9 mM.

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

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

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

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

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

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

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

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

Experimental Section

General Methods. NMR spectra were recorded on a Bruker AM-400 instrument. Spectra were calibrated using TMS (δ = 0.00 ppm) for ^1H NMR and $CDCl_3$ (δ = 77.0) or DMSO-d_6 (δ = 39.51) for ^{13}C NMR. Mass spectra were recorded on a Kratos MS 80 under electron impact (EI), chemical ionization (CI), or fast-atom bombardment (FAB) conditions. HPLC analyses were performed on a Rainin HPLC system with Rainin Microsorb silica or C18 columns and UV detection. Semipreparative HPLC was performed on the same system using a semipreparative column (21.4 × 250 mm). $K^{13}CN$ (99%) was obtained from Cambridge Isotope Laboratories. Details of the synthesis of unlabeled **10** from unlabeled **6** and experimental procedures for determining racemization rates have been reported previously.[4]

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

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

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

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

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

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

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

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Acknowledgment. This research was supported by the Petroleum Research Fund, administered by the American Chemical Society (Grant 30544-G4), the U.S. Army Medical Research and Materiel Command−Breast Cancer Research Initiative (Career Development Award to C.T.S., Grant DAMD17-96-1-6328), and Brown University (Salomon Faculty Research Award). J.L.C. was supported by a GAANN Fellowship from the Department of Education and by a University Fellowship from Brown University.

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

JO971834B