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Peptidyl cyclopropenones: Reversible inhibitors, irreversible inhibitors, or substrates of cysteine proteases?

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Abstract: Peptidyl cyclopropenones were previously introduced as selective cysteine protease reversible inhibitors. In the present study we synthesized one such peptidyl cyclopropenone and investigated its interaction with papain, a prototype cysteine protease. A set of kinetics, biochemical, HPLC, MS, and ^{13}C-NMR experiments revealed that the peptidyl cyclopropenone was an irreversible inhibitor of the enzyme, alkylating the catalytic cysteine. In parallel, this cyclopropenone also behaved as an alternative substrate of the enzyme, providing a product that was tentatively suggested to be either a spiroepoxy cyclopropanone or a gamma-lactone. Thus, a single family of compounds exhibits an unusual variety of activities, being reversible inhibitors, irreversible inhibitors and alternative substrates towards enzymes of the same family.

Keywords: cyclopropenone; cysteine proteases; enzyme inhibition; irreversible inhibition; alternative substrate

Introduction

Peptidyl cyclopropenones [Fig. 1(a)] were introduced as selective cysteine protease inhibitors.[1,2] They exhibited amino acid sequence selectivity and stereoselectivity in the inhibition of a few enzymes of this family. They did not inhibit serine- and aspartic proteases. Detailed mechanistic analysis of the inhibition process of the cysteine protease m-calpain revealed fully reversible competitive inhibition.[1]

Cyclopropenones are intriguing chemical entities, as they are both electrophilic and basic. They may undergo nucleophilic attack at either of the olefinic carbons or at the carbonyl carbon. On the other hand, protonation of the carbonyl oxygen will yield a 2π aromatic hydroxycyclopropenyl cation [Fig. 1(b)].[3–5]

These reactions may all be manifested in the active site of cysteine proteases: the catalytic cysteine can attack the cyclopropenone carbonyl to form a thiohemiacetal or add across the C=C double bond. Experimental[6–8] and computational[9–12] studies suggest that protonation of a substrate in the active site of cysteine proteases leads to the formation of a neutral (protonated) tetrahedral intermediate. Thus, a peptidyl cyclopropenone inhibitor may become protonated in the enzyme active site, forming the aromatic hydroxycyclopropenyl cation. All of the above reactions are reversible and therefore fit the described inhibition mechanism.[1] Alternatively, peptidyl cyclopropenones may behave as classical competitive inhibitors, occupying the enzyme active site without undergoing any covalent modification.

In this article we describe our studies of the inhibition of the prototype cysteine protease papain by

Abbreviations: Boc, *t*-butoxycarbonyl; BSA, bovine serum albumin; Cbz, benzyloxy carbonyl; COSY, correlated spectroscopy; DEPT, Distortionless Enhancement by Polarization Transfer; DMSO, dimethyl sulfoxide; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HMBC, Heteronuclear Multiple Bond Correlation; HMQC, Heteronuclear Multiple-Quantum Correlation; Me, methyl; NOSEY, Nuclear Overhauser effect spectroscopy; THF, tetrahydrofuran; TMEDA, *N,N,N',N'*-tetramethylethylenediamine; TMS, tetramethylsilane.

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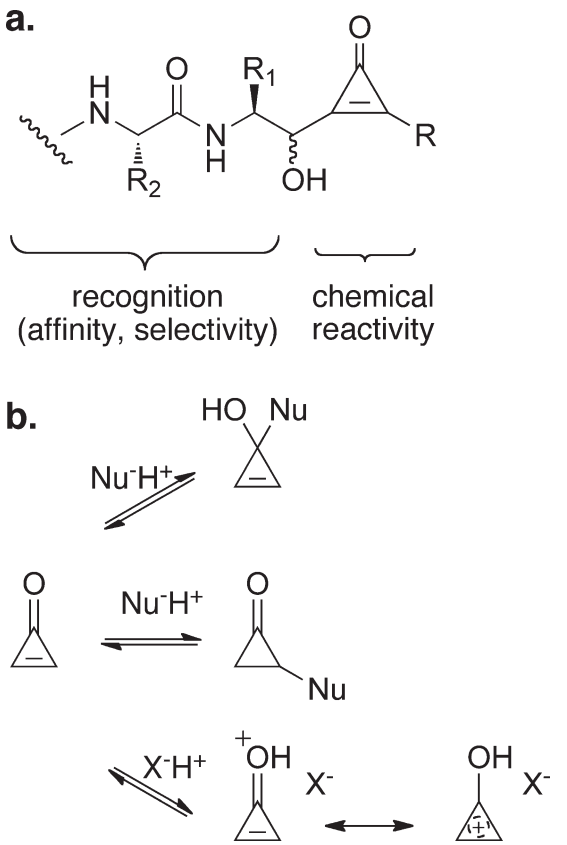


Figure 1. Peptidyl cyclopropenones. (a) General structure of peptidyl cyclopropenones. (b) Possible electrophilic and basic modes of reactivity of cyclopropenones.

a peptidyl cyclopropenone, in order to identify the specific mode of reversible inhibition, and the many surprises we encountered.

Results

Inhibitor synthesis

The synthesis of the peptidyl cyclopropenone inhibitor **9** follows the synthesis developed by Ando et al.[1] with some modifications (Scheme 1).

The reaction to form the cyclopropenone acetal **5** is very sensitive to the stoichiometric ratio of the base and the dihalide substrate **4**, and therefore required a very clean substrate and a carefully titrated base. A ratio < 2 afforded a mixture of the product **5** and a chlorocyclopropanone acetal intermediate, whereas a ratio > 2 led to the formation of *t*-butoxycyclopropanone acetal, the product of addition of the base across the C=C double bond of the required product. A recent publication describes the elimination of *t*-butanol from this product, to regenerate the cyclopropenone acetal.[13] The cyclopropenone acetal was extremely acid labile: it was not stable upon silica chromatography, and therefore it was purified on a deactivated alumina column. Furthermore, the acetal decomposed in $CDCl_3$, used for the NMR analysis, due to the usual slight acidity of this solvent. Therefore, its spectrum could be taken only in $CDCl_3$ that was pre-treated with a base (K_2CO_3) or in CD_3CN. Indeed, removal of the acetal protecting group from **6** was achieved by simply dissolving it in $CHCl_3$. Condensation of the anion of **5** with Boc-valinal[14] afforded compound **6** as a 3:1 diastereomeric mixture (*SS*:*SR*, respectively). The rest of the synthesis was carried out on the diastereomeric mixture, and the two diastereomers of cyclopropenone inhibitor **9** were separated by chromatography.

^{13}C-labeled inhibitor

Inhibitor **9** was also prepared with selective ^{13}C labeling on its cyclopropenone carbonyl carbon, starting from labeled phenyl acetone **1**. We tried a few approaches to prepare the latter, the best of which was activation of ^{13}C-labeled phenylacetic acid as a Weinreb amide,[15] followed by condensation with MeLi (Scheme 2).

Enzyme inhibition

Time- and concentration-dependent inhibition was observed upon incubation of the cysteine protease papain with the S isomer of peptidyl cyclopropenone **9** (Fig. 2). The corresponding kinetic parameters are $K_i = 46\ \mu M$ and $k_i = 0.021\ \text{min}^{-1}$. The R isomer was about two fold less reactive ($K_i = 98\ \mu M$ and $k_i = 0.020\ \text{min}^{-1}$).

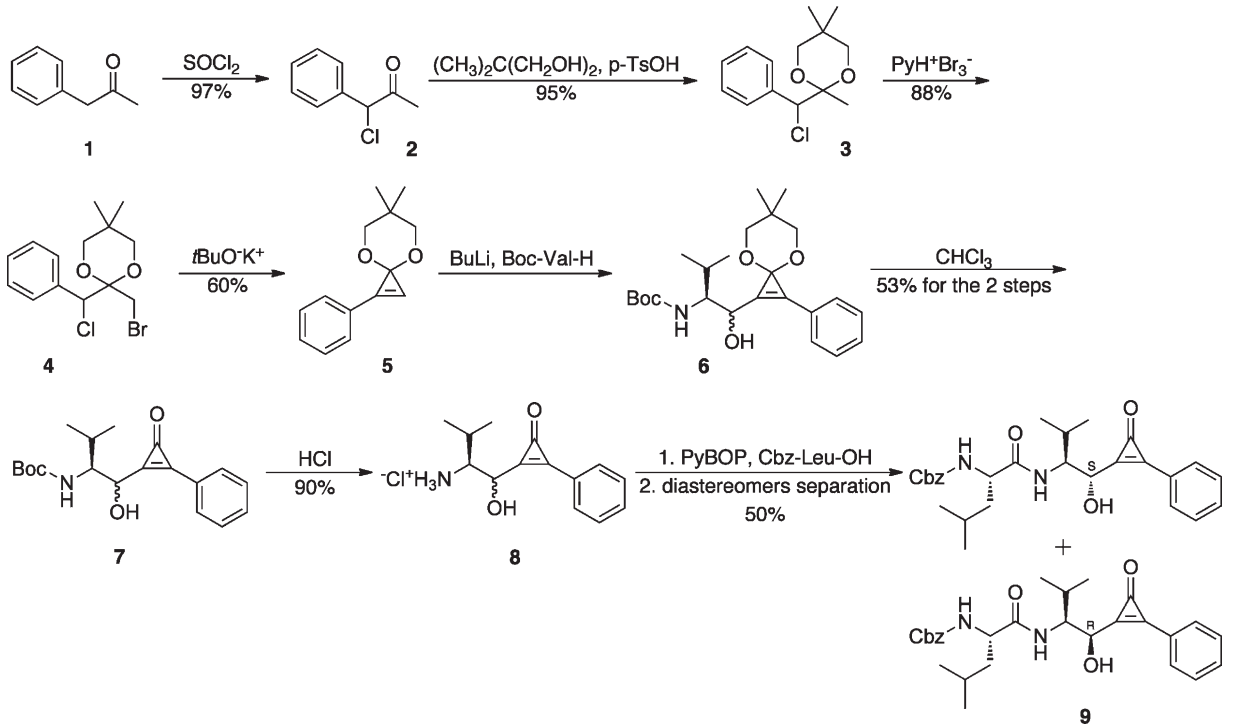
In order to confirm the irreversibility of papain inhibition by peptidyl cyclopropenone **9**, the enzyme-inhibitor complex (less than 30% residual enzymatic activity) was dialyzed extensively (2×4 h) against phosphate buffer solution containing DTT. No regeneration of enzymatic activity was observed. As a control, active papain that was similarly dialyzed retained about 90% of its enzymatic activity.

Peptidyl cyclopropenone **9** (the S isomer) was also tested as an inhibitor of cathepsin B, another cysteine protease that was previously reported to be inhibited reversibly by this family of inhibitors.[1] Concentration-dependent and time-independent inhibition indicated reversible inhibition, with $IC_{50} = 85\ \mu M$ under the specific experimental conditions. The reversible nature of the inhibition of cathepsin B was further validated by full recovery of enzymatic activity upon dialysis, as compared with active enzyme control.

Alkylation site

The alkylation site on papain by the irreversible peptidyl cyclopropenone inhibitor **9** was identified as follows:

(a) Activated papain was separated from excess DTT by gel filtration. Complete separation (3 fractions) was achieved, as determined by measuring the absorption (A_{280}) of each fraction for enzyme concentration and by interacting a sample of



Scheme 1. Synthesis of peptidyl cyclopropenone **9**.

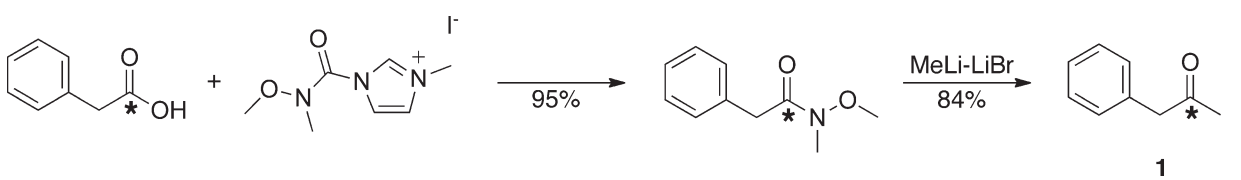
each fraction with DTNB (Ellman's reagent)[16] and measuring the absorption (A_{412}) of the reaction solution for free thiol concentration. The active enzyme was then incubated with inhibitor **9**. Samples from the incubation solution were removed at time points along the inhibition process, and measured for both residual enzymatic activity and free thiol concentration. Very good correlation between these two values of each sample was demonstrated (Fig. 3).

(b) Activated papain was separated from excess DTT by gel filtration, as above. The enzyme fraction was split into two parts, one serving as a control (active enzyme) and one incubated with inhibitor **9** till 70% inhibition was measured. Each of the two samples, that of the inhibited enzyme and the control active enzyme, was split again. One part was treated with DTNB to determine the concentration of free active-site thiol. The second part was first denatured with 6N guanidinium chloride and then treated with DTNB. The measurements show that both samples of the inhibited enzyme, the native and the denatured, had practically identical concentration of thiols, correlated with the residual 30% enzymatic activity. The control samples of the free enzymes, both the native and the denatured, exhibited thiol concentration correlated with above 90% enzymatic activity.

The above two experiments confirm that peptidyl cyclopropenone **9** alkylates papain on its active-site cysteine.

Fate of the ^{13}C Labeling

Papain was inhibited with the specifically ^{13}C labeled inhibitor **9**, in an attempt to identify the structure of the inhibitor's carbonyl carbon in the inhibition complex. The process involved activation of the enzyme, separation of the active enzyme from various inactive forms by mercury affinity chromatography,[17] inhibition of the active enzyme by labeled inhibitor **9**, and measuring of the ^{13}C-NMR spectrum of the inhibition complex [Fig. 4(c)]. The same process was also carried out with an unlabeled inhibitor **9** [Fig. 4(b)]. A difference spectrum of these two spectra should present only species associated with the labeled inhibitor and the products of its



Scheme 2. Synthesis of ^{13}C-specifically labeled starting material phenyl acetone **1**.

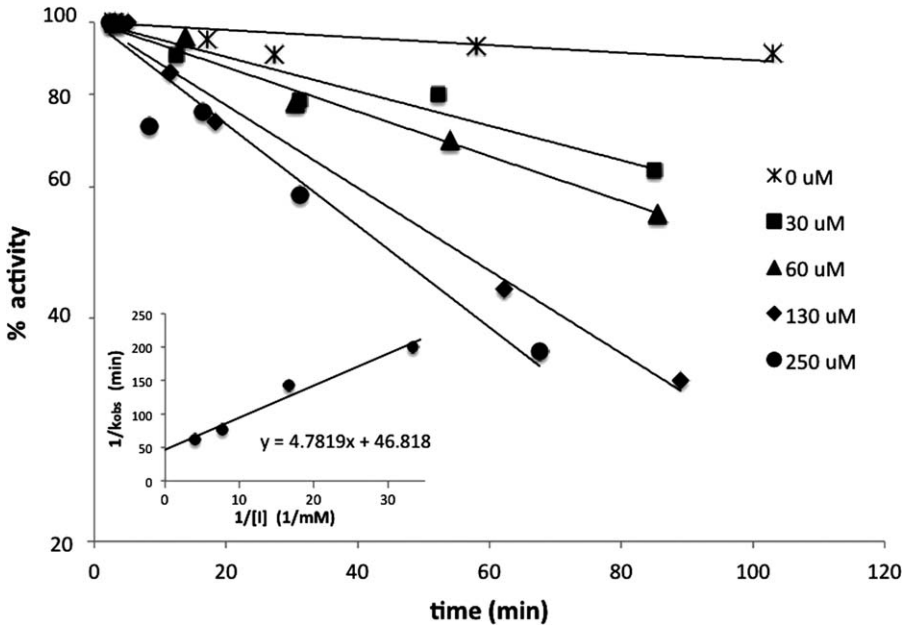


Figure 2. Time- and concentration-dependent inhibition of papain by peptidyl cyclopropenone **9**. Papain was activated in KPi buffer (100 mM, pH 7.0) containing EDTA (1 mM) and DTT (0.5 mM). The active enzyme solution (200 μL) was incubated with various concentrations of inhibitor (100 μL in DMSO) in KP_i buffer solution (700 μL). At time points, aliquots from the inactivation solution (100 μL) were treated with substrate solution (20 μL of Cbz-Gly-ONp 5 mM in CH_3CN) in KP_i buffer (880 μL). The hydrolysis rate was assayed spectrophotometrically at 404 nm. The insert is a replot of $1/k_{obs}$ vs 1/[I], from which the inhibition parameters K_i and k_i were determined.

interaction with the enzyme. The difference spectrum [Fig. 4(d)] has two signals: a resonance at 159 ppm, which corresponds to the free inhibitor **9** [Fig. 4(a)], and a new resonance at 176 ppm. Both resonances are relatively narrow, about 19 Hz, indicating that they are not associated with the protein. Formation of the 176 ppm signal was enzyme-dependent; it was not observed in the spectrum of ^{13}C-labeled inhibitor **9** incubated in either buffer alone (with EDTA and DTT, but without the enzyme), buffer with BSA, or buffer with denatured papain.

The enzyme-inhibitor incubation solution from the NMR experiment, which includes the excess labeled inhibitor and the new compound represented by the 176 ppm signal, was chromatographed by HPLC. The chromatogram [Fig. 5(a)] is characterized by a peak of the free inhibitor, eluting at 33.7 min, and another peak at 38.2 min. The formation of the new peak at 38.2 min was time-dependent, growing in on the expense of the free inhibitor peak at 33.7 min [Fig. 5(b)]. As controls, active enzyme in buffer, which contained EDTA and DTT, [Fig. 5(c)] and a solution of the inhibitor in buffer (including EDTA and DTT) and BSA [Fig. 5(d)] were also chromatographed. The 38.2 min peak is clearly the product of some enzyme – inhibitor **9** interaction, as it does not appear in either the solution of the buffer and the activating agents or a solution of the inhibitor with a nonrelevant protein.

Small organic molecules tend to precipitate from aqueous solutions. Indeed, a precipitate formed in the NMR experiment solution was separated from the soluble enzyme by centrifugation. HPLC analysis of the precipitate revealed the two peaks at 33

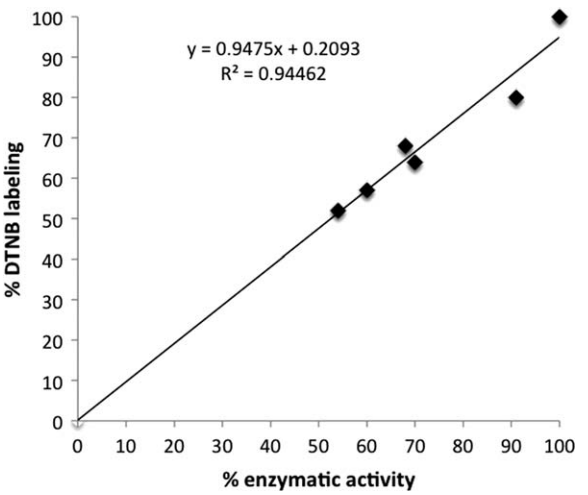


Figure 3. Linear correlation between residual enzymatic activity and free active-site thiol upon partial inhibition of the enzyme papain by peptidyl cyclopropenone **9**. Activated papain (16 mg) was purified from small molecules (DTT and EDTA) by Sephadex G-15 chromatography, and was assayed for both activity and free thiol concentration (by titration with DTNB). The enzyme was then incubated with inhibitor **9**. Aliquots from the inactivation solution were removed at various time points, and assayed for their residual enzymatic activity and for the remaining free thiol concentration. An active enzyme solution was similarly treated and assayed as a control.

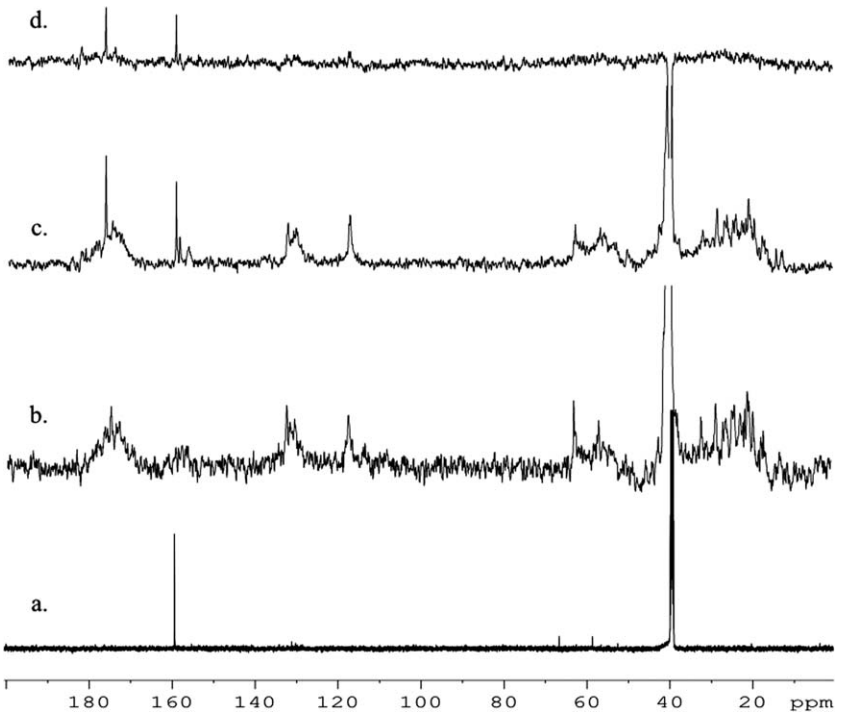


Figure 4. ^{13}C-NMR spectra of specifically labeled cyclopropenene **9** and papain at 150 MHz. (a) Peptidyl cyclopropenone **9** in DMSO-d_6 and KPi buffer with DTT and EDTA (0.5 mM and 1mM respectively). (b) Papain inhibited by unlabeled peptidyl cyclopropenone **9**. (c) Papain inhibited by ^{13}C-labeled peptidyl cyclopropenone **9**. (d) Difference spectrum (c-b).

and 38 min whereas the corresponding ^{13}C-NMR spectrum in DMSO-d_6 was characterized by two resonances, at 156 and 173 ppm. (The 3 ppm shift from the original spectrum stems from the change in solvents, from 10% DMSO in water to DMSO alone). No enzyme signals were observed. This experiment confirms that the new 176 ppm resonance in the ^{13}C-NMR spectrum belongs to a small molecule, separated from the enzyme. Furthermore, it correlates this resonance with the HPLC peak at 38 min. Finally, the 38 min peak from the HPLC was collected and submitted to mass spectroscopy analysis. HRMS of the compound was characterized by a mass of 479.254, which corresponds to a molecular formula of $C_{28}H_{35}N_2O_5$ (protonated compound, MH^+, calculated mass of 479.251). Interestingly, this mass is identical to the mass of peptidyl cyclopropenone **9**. A second peak in the mass spectrum corresponds to a fragment with a mass of 435.265 (MH^+-CO_2, calculated mass of 435.261). The loss of 44 Da in the MS analysis is typical to carbamate-protected peptides and amino acids.

Discussion

The irreversible mode of inhibition of papain by peptidyl cyclopropenone **9**, observed in the present study, is markedly different from the reversible competitive inhibition pattern previously observed for this type of inhibitors.[1,2] It should be noted that the previous detailed mechanistic study was carried out on m-calpain (though the same inhibition pattern was also observed for papain and a few other cysteine proteases), whereas our detailed study was carried out on papain. Furthermore, the two studies also differ in the exact identity of the papain inhibitor: both studies were carried out on the same peptide sequence and cyclopropenone substituent, but the previous study employed a cyclohexyl methoxycarbonyl protecting group whereas inhibitor **9** in this study is Cbz-protected. This difference also affected the inhibitor concentrations used.

It is also interesting to note that, in contrast to its irreversible covalent inhibition of papain, peptidyl cyclopropenone **9** was a reversible inhibitor of cathepsin B (IC_{50} = 85 μM), another cysteine protease, as was also observed in the previous study.[1] Thus, the same compound exhibits two very different inhibitory activities towards two enzymes belonging to the same family of proteases.

Time-dependent loss of enzymatic activity is indeed reminiscent of irreversible covalent inhibition, but it could also represent reversible slow tight binding inhibition.[18] The fact that enzymatic activity was not recovered upon dialysis of the enzyme-inhibitor complex indicates that indeed the inhibition is irreversible. It may still be argued that a very low off-rate (k_{off}) would leave most of the enzyme in its complex with the inhibitor even under a few hours of dialysis. The DTNB titration experiments resolve the reversibility issue. It clearly shows that the inhibitor alkylates the catalytic thiol (the only free thiol in papain), and that this bond is stable even under denaturation conditions.

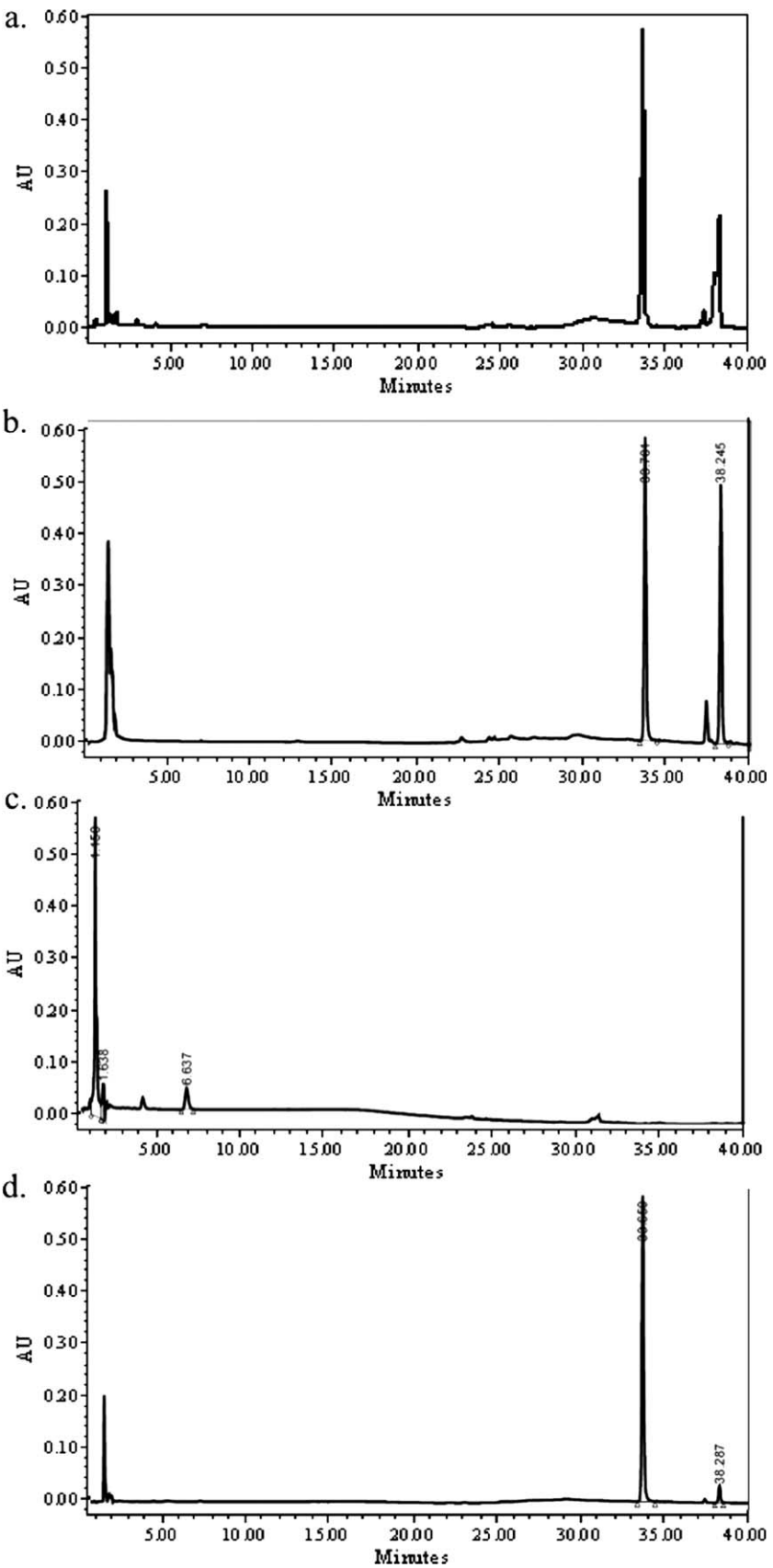


Figure 5. HPLC analysis of the papain – inhibitor **9** inhibition process. HPLC was carried out on Nucleosil 120×5 C18 column, at a flow rate of 1 mL/min, applying linear gradients of 100% to 20% water-acetonitrile in 40 min. (a) The reaction mixture after short incubation of papain with inhibitor **9**. (b) The reaction mixture after 12 h incubation of papain with inhibitor **9**. (c) Papain in buffer (100 mM KPi) and EDTA (1 mM) and DTT (0.5 mM). (d) Ppetidyl cyclopeopenone **9** incubated in buffer (with EDTA and DTT) and BSA for 12 h.

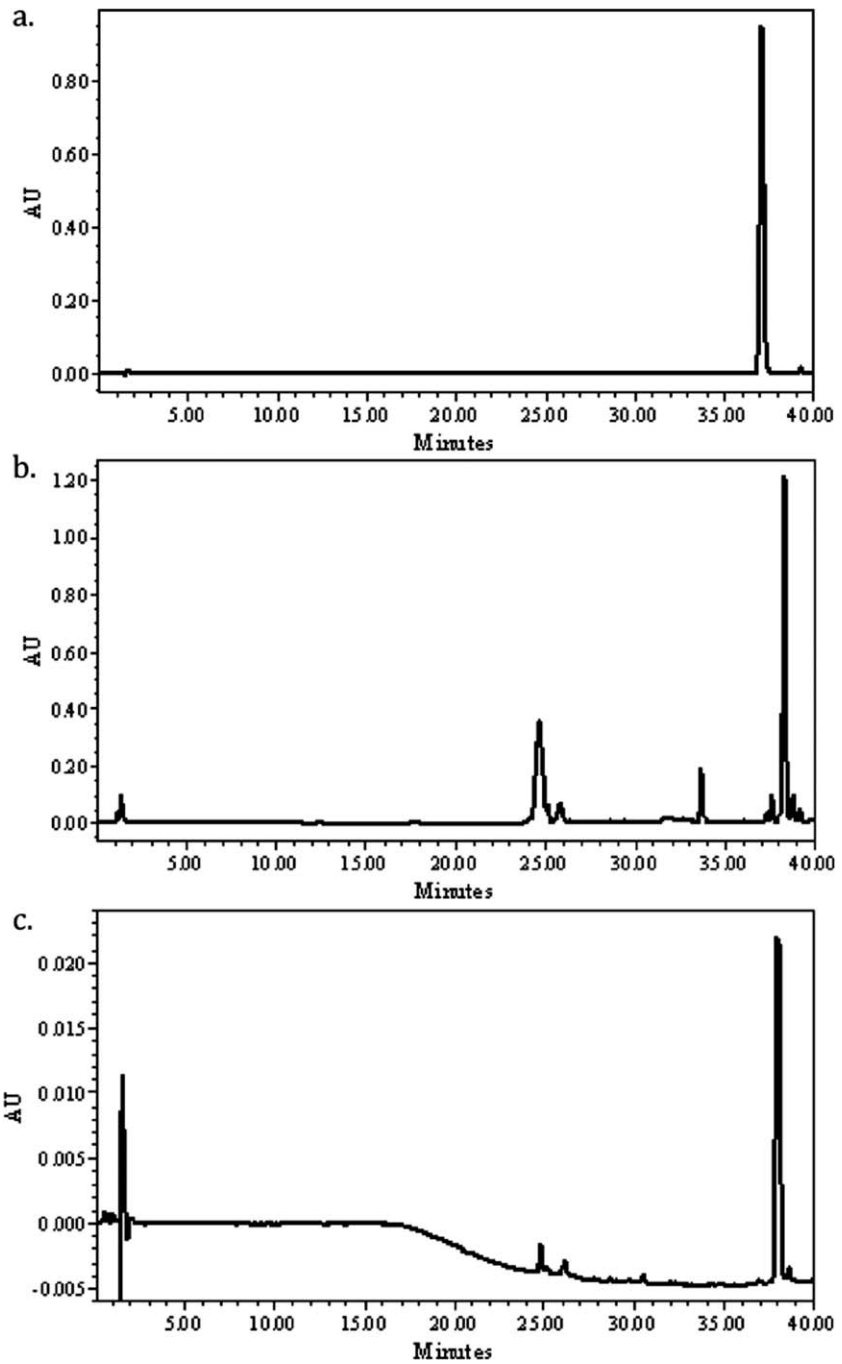
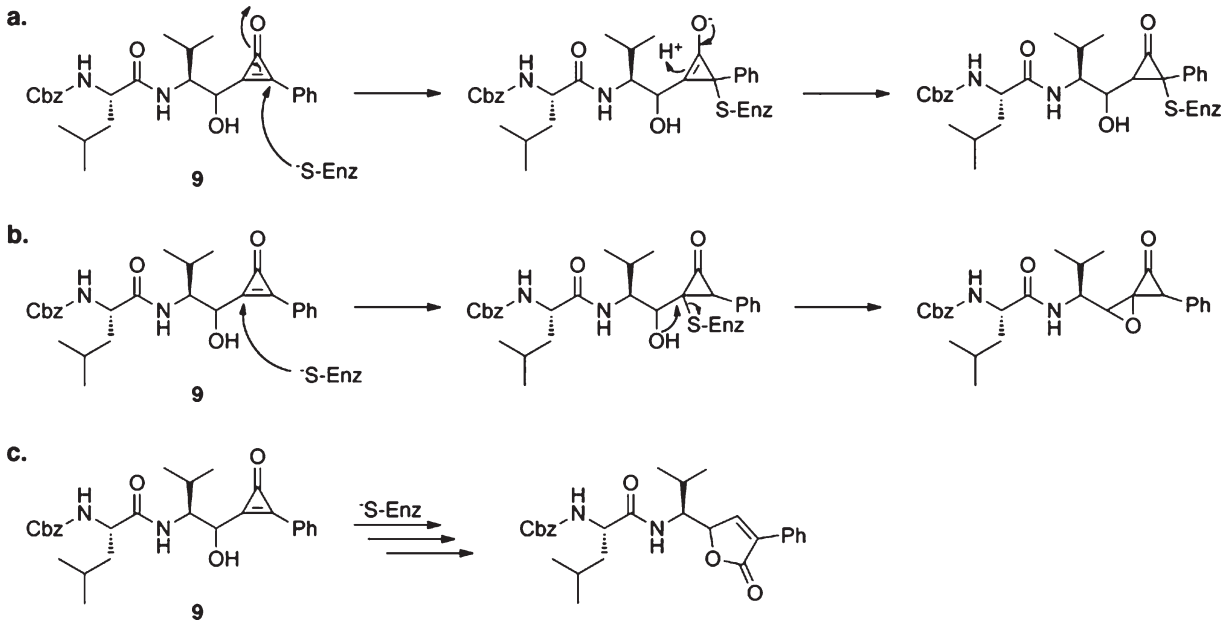


Figure 6. HPLC analysis of the reaction product of inhibitor **9** with NaOH. HPLC was carried out on Nucleosil 120×5 C18 column, at a flow rate of 1 mL/min, applying linear gradients of 100% to 20% water-acetonitrile in 40 min. (a) Purified product from the interaction of papain with inhibitor **9**. (b) The product mixture arising from treatment of **9** with one eq. of NaOH. (c) Co-injection of the two products, from the enzymatic- and the base treatment reactions.

From the mechanistic point of view, the above results rule out the simple reversible binding of the cyclopropenone inhibitor **9** in the enzyme active-site and the possibility of inhibitor protonation in the active-site to form a 2π aromatic hydroxy cyclopropenium species. It probably also eliminates the option of a nucleophilic attack of the catalytic thiol at the inhibitors cyclopropenone carbonyl, to yield a thiohemiacetal. Such a covalent complex is expected to undergo hydrolysis under the denaturation conditions, which would be manifested in a titratable thiol. A reasonable mechanism then is the addition of the nucleophilic catalytic thiol across the cyclopropenone C=C double bond.

^{13}C-NMR had previously been employed to study covalent interactions between proteases and various inhibitors.[19–24] In this study we employed this approach in order to identify the structure of



Scheme 3. Suggested mechanisms for the interaction of cyclopropenone **9** with papain. (a) Irreversible inhibition of papain by peptidyl cyclopropenone **9**. (b) Peptidyl cyclopropenone **9** as an alternative substrate of the enzyme. (c) An alternative product resulting from the interaction of papain with cyclopropenone **9**.

cyclopropenone inhibitor **9** in its complex with the enzyme papain. Unfortunately, the ^{13}C-NMR spectrum of the complex did not reveal any clear new signal that could be assigned to the complex. This may be attributed to the fact that the labeled carbon does not carry any protons, and therefore it is expected to be small (despite the 100% labeling). Furthermore, it is expected to be a very wide peak (in the order of 50 Hz) due to its association with a protein bound carbon.

On the other hand, the ^{13}C-NMR experiment provided a surprise in the form of a new signal at 176 ppm. This signal belongs to a small molecule, not attached to the enzyme. This small molecule is only formed when both cyclopropenone **9** and active papain are present, but not in the presence of other proteins (BSA) or denatured papain. Thus, peptidyl cyclopropenone **9** is not only an irreversible inhibitor of the cysteine protease papain, but also a substrate of the enzyme. HPLC and MS analyses provide some insight into the structure of the product of this interaction: this product elutes slower than cyclopropenone **9** from the reversed-phase HPLC column, indicating that it is more hydrophobic. Its mass spectrum (both HRMS molecular peak and fragmentation pattern) is practically identical to that of cyclopropenone **9**. This new product could not be fully characterized due to the small amount of its production by an enzyme that is being irreversibly inhibited at the same time.

On the basis of the above experimental data, we can suggest possible mechanisms for the inhibition of papain by peptidyl cyclopropenone **9** and for the process in which cyclopropenone **9** is an alternative substrate of papain (Scheme 3). We suggest that the catalytic cysteine can attack both carbons of the C=C double bond of the cyclopropenone inhibitor. It is not unusual for the catalytic cysteine of cysteine proteases to attack the carbon adjacent to the would-be scissile bond carbon. This was previously observed with peptidyl chloromethyl ketones, peptidyl diazomethyl ketones, peptidyl acyloxymethyl ketones,[25] and peptidyl epoxides.[19] Thus, Michael addition at the carbon more distant from the peptide provides a stable covalent enzyme-inhibitor complex (Scheme 3a). On the other hand, addition of the catalytic cysteine across the inhibitor's C=C double bond by attack of the thiol on the carbon closer to the peptide and protonation of the other C=C carbon would yield an α-hydroxy sulfide intermediate [Scheme 3(b)]. This intermediate could then undergo intramolecular nucleophilic substitution reaction, in which the hydroxyl group displaces the thiol to form a spiroepoxide product that can leave the enzyme's active site. This product is more hydrophobic than the starting material, peptidyl cyclopropenone **9**, but they both have the same molecular weight, as was observed in our HPLC and MS experiments.

This product is formally the result of an intramolecular addition of the hydroxyl across the cyclopropenone double bond. Indeed, interaction of cyclopropenone **9** with one equivalent of NaOH gave a mixture of products, one of which comigrated with the enzyme product on an HPLC reversed-phase column in two different eluting systems (water-acetonitrile and water-methanol) (Fig. 6).

Alternatively, the enzymatic product could be the result of some rearrangement/ring expansion to yield a γ-lactone product [Scheme 3(c)].

Materials and Methods

Synthesis

General. Anhydrous THF and TMEDA were dried and freshly distilled from sodium/benzophenone. CH_2Cl_2 was dried on molecular sieves and used without distillation. Chromatography refers to flash column chromatography,[26] carried out on silica gel 60 (230–400 mesh ASTM, E. Merck), or aluminum oxide (Brockmann activity 3, Fluka) when indicated. TLC was performed on E. Merck 0.2 mm percolated silica gel F-254 plates. Compounds were detected by UV light (254 nm) and/or by staining with vanillin, Cl_2/KI-tolidine.[27] ^{1}H- and ^{13}C-NMR spectra were recorded at 600, 300, or 200 MHz and 150 or 75 MHz, respectively, in $CDCl_3$. Chemical shifts are reported relative to internal TMS. Some ^{1}H-NMR assignments were supported by 2D homonuclear COSY and NOSEY experiments. ^{13}C-NMR assignments were supported by DEPT or 2D HMQC and HMBC experiments. Mass spectra were recorded in DCI mode with methane as the reagent gas.

Phenylacetic acid N,O-dimethylhydroxamate. To a suspension of carbamoylimidazolium salt (1-{[methoxy(methyl)amino]-carbonyl}-3-methyl-1H-imidazol-3-ium iodide)[15] (2.38 g, 8.07 mmol) in acetonitrile (48 mL) were added phenyl acetic acid (1.09 g, 8.07 mmol) and Et_3N (1.1 mL, 8.07 mmol). The reaction was stirred at room temperature overnight. The solvent was removed in vacuo and the residue was dissolved in CH_2Cl_2 (50 mL) and 0.2 N HCl (50 mL) was added. The aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic layers were washed with 0.2 N HCl (100 mL), 0.5 M K_2CO_3 (100 mL), and brine, dried over anhydrous $MgSO_4$, filtered and evaporated to give clean hydroxamate as colorless oil (1.38 g, 95% yield). ^{1}H-NMR: δ 7.6-7.3 (m, 5H); 3.77 (s, 2H); 3.59 (s, 3H); 3.19 (s, 3H). ^{13}C-NMR: δ 135.0, 129.4, 128.6, 126.8, 61.4, 39.5, 36.2. MS: m/z 180 (MH^+, 60), 118 (67), 91 (100). HRMS: m/z $C_{10}H_{14}NO_2$ (MH^+) calcd. 180.1025 found 180.0989.

Phenyl acetone (1). To dry THF (20 mL) under argon atmosphere at –78°C, was added a solution of MeLi-LiBr (1.5*M* in ether, 16.5 mL) and ^{13}C-labeled phenylacetic acid *N,O*-dimethylhydroxamate (1.14 g, 6.36 mmol) dissolved in dry THF (10 mL). After 3 h the solution was warmed to 0°C in an ice bath. After additional 3 h, the resulting mixture was quenched by adding water (30 mL). The aqueous layer was extracted with ether (2 × 50 mL) and the combined organic layer was washed with 0.5*N* HCl (30 mL), saturated $NaHCO_3$ solution (50 mL) and brine (50 mL). Drying over $MgSO_4$, filtration and evaporation gave the clean phenyl acetone **1** (0.71 g, 84% yield). ^{1}H-NMR: δ 7.3-7.2 (m, 5H); 3.69 (d, J=6.6 Hz, 2H); 2.15 (d, J=5.7 Hz, 3H). ^{13}C-NMR: δ 206.5, 134.4, 129.5, 128.9, 127.2, 51.2 (d, J=38 Hz), 29.4 (d, J= 41 Hz). MS: m/z 136 (MH^+, 7), 123 (16), 91(40). HRMS: m/z for $^{12}C_8{}^{13}CH_{11}O$ (MH^+): calcd. 136.0843, found 136.0812.

α-Chlorophenyl acetone (2). Phenyl acetone **1** (5 mL, 36.7 mmol) was dissolved in CH_2Cl_2 (50 mL), and sulfuryl chloride (3.6 mL, 44.8 mmol) was added slowly at 0°C. After stirring at room temperature for 5 h, water (50 mL) was added, and the separated aqueous layer was extracted with CH_2Cl_2 (2 × 50 mL). The combined organic layer was washed with brine and dried over anhydrous $MgSO_4$. Filtration and evaporation afforded the α-chloroketone **2** as slight yellow oil, which was used without purification (6.04 g, 97% yield). ^{1}H-NMR: δ 7.43-7.36 (m, 5H); 5.35 (s, 1H); 2.21 (s, 3H). ^{13}C-NMR: δ 200.2, 135.2, 129.3, 129.2, 127.9, 66.7, 25.9. MS: m/z 170 (M^+, 4), 168 (M^+, 14), 118 (17), 127 (60), 125 (100), 90 (31). HRMS: m/z for $C_9H_9O^{37}Cl$ (M^+): calcd. 170.0312, found 170.0328, for $C_9H_9O^{35}Cl$ (M^+): calcd. 168.0342, found 168.0345, for $^{12}C_8{}^{13}CH_9O^{35}Cl$ (M^+): calcd. 169.0375, found 169.0374.

2-Chlorobenzyl-2,5,5-trimethyl-1,3-dioxane (3). To a solution of α-chloroketone **2** (6.04 g, 35.8 mmol) in dry toluene (50 mL) were added neopentyl glycol (6.71 g, 64.4 mmol) and *p*-toluensulfonic acid (136 mg, 0.72 mmol). Then the mixture was refluxed over night in a Dean-Stark apparatus. After cooling to room temperature, water (50 mL) was added. The layers were separated and the aqueous layer was re-extracted with hexane (2 × 50 mL). Then the combined organic layers were washed with saturated $NaHCO_3$ solution and brine successively and dried over anhydrous $MgSO_4$. Filtration and evaporation afforded crude acetal **3**, which was used without purification (8.67 g, 95% yield). ^{1}H-NMR: δ 7.6-7.5 (m, 2H); 7.37-7.25 (m, 3H); 4.96 (s, 1H); 3.61 (d, J=11.7 Hz, 1H); 3.57 (d, J=11.7 Hz, 1H); 3.49 (dd, J=11.7, 2.1 Hz, 1H); 3.47 (dd, J=11.7, 2.1 Hz, 1H); 1.40 (s, 3H); 0.98 (s, 3H); 0.79 (s, 3H). ^{13}C-NMR: 137.7, 128.4, 128.2, 127.8, 99.3, 71.0, 70.1, 66.8, 30.1, 22.7, 22.3, 16.2. MS: m/z 257 (MH^+, 3) 255 (MH^+, 7), 129 (100). HRMS: m/z for $C_{14}H_{20}O_2{}^{35}Cl$ (MH^+): calcd. 255.1109, found 255.1152; for $^{12}C_{13}{}^{13}CH_{20}O_2{}^{37}Cl$ (MH^+): calcd. 258.1156, found 258.1202; for $^{12}C_{13}{}^{13}CH_{20}O_2{}^{35}Cl$ (MH^+): calcd. 256.1185, found 256.1167.

2-Bromomethyl-2-(chlorobenzyl)-5,5-dimethyl-1,3-dioxane (4). Acetal **3** (0.54 g, 2.17 mmol) was dissolved in dried chloroform (30 mL), and pyridinium hydrobromide perbromide (0.66 g, 2.07 mmol)

was added. After refluxing for 2 h, it was cooled to room temperature, and then water (50 mL) was added. The aqueous layer was extracted with chloroform, and the combined organic layer was washed with water, saturated $NaHCO_3$ solution, and brine successively and dried over anhydrous $MgSO_4$. Filtration and evaporation afforded crude dihalide **4**, which was used without purification. The crude can be recrystallized from hexane to give the desired dihalide **4** (0.62 g, 88% yield). ^{1}H-NMR: δ 7.6-7.5 (m, 2H); 7.3-7.3 (m, 3H); 5.29 (s, 1H); 3.94 (d, J=11.7 Hz, 1H); 3.62 (d, J=11.4 Hz, 1H); 3.57 (d, J=11.7 Hz, 1H); 3.56 (d, J=11.1 Hz, 1H); 3.52 (dd, J=11.7, 2.4 Hz, 1H); 3.43 (dd, J=11.7, 2.4 Hz, 1H); 0.80 (s, 3H); 0.76 (s, 3H). ^{13}C-NMR: δ 136.4, 129.9, 128.6, 127.8, 97.9, 71.2, 71.0, 63.6, 29.7, 27.8, 22.3. MS: m/z (M-Cl) 299 (5) 297 (5), 209 (90), 207 (90), 69 (100). HRMS: m/z for $^{12}C_{13}{}^{13}CH_{19}O_2BrCl$ (MH^+): calcd. 334.0290 found 334.0291; for $^{12}C_{13}{}^{13}CH_{18}O_2{}^{81}Br$ (M-Cl): calcd. 300.0503 found 300.0408; for $^{12}C_{13}{}^{13}CH_{18}O_2{}^{79}Br$ (M-Cl): calcd. 298.0524 found 298.0442; for $C_{14}H_{18}O_2{}^{79}Br$ (M-Cl): calcd. 297.0490 found 297.0468.

Phenyl cyclopropenone Acetal (5). To solution of potassium tert-butoxide (0.59 g, 5.23 mmol) in THF (5 mL) and HMPA (1.37 mL, 7.83 mmol) was added via cannula dihalide **4** (0.87g, 2.61 mmol) in THF (5 mL). The reaction was carried under argon atmosphere. After stirring for 4 h at 0°C the reaction was allowed to warm up to room temperature and stirred overnight. Then water (20 mL) was added, and the aqueous solution was extracted with hexane (3 × 20 mL). The combined organic layer was washed with brine and dried over anhydrous $MgSO_4$. Filtration and evaporation gave a crude product, which was purified by deactivated (brockmann 3) aluminum oxide chromatography (hexane: EtOAc 30:1) to afford compound **5**. (0.39 g, 60% yield). The $CDCl_3$ for NMR was passed through K_2CO_3 (Alternatively CD_3CN was used as solvent). ^{1}H-NMR: δ 7.68 (s, 1H); 7.64-7.61 (m, 2H); 7.50-7.35 (m, 3H); 3.74 (s, 4H); 1.14 (s, 3H); 1.06 (s, 3H). ^{13}C-NMR: δ 135.6, 130.1, 129.7, 128.9, 125.9, 114.7, 83.0, 77.8, 30.5, 22.6, 22.4. MS: m/z 217 (M^+, 100). HRMS: m/z for $^{12}C_{13}{}^{13}CH_{17}O_2$ (MH^+): calcd. 218.1262 found 218.1251.

2-{(2S)-2-Bocamine-1-hydroxy-3-methyl-butyl}-3-phenylcyclopeopenone (7). To a solution of **5** (0.82 g, 3.8 mmol) and dry *N,N,N',N'*-tetramethylethylenediamine (1.14 mL, 7.8 mmol) in dry THF (8 mL), was added *n*-BuLi (1.2 M in THF, 3.16 mL) at -78°C over 5 min. After the mixture was stirred for 20 min, a solution of N-Boc-valinal[14] (0.42 g, 2.11 mmol) in dry THF (4 mL) was added via cannula. The mixture was stirred for 2 h at -78°C. After addition of 1:4 water:THF (4 mL) the mixture was diluted with ether and extracted with water (×3). The aqueous solution was extracted with ether (3×30 mL). The combined organic layer was washed with brine and dried over anhydrous $MgSO_4$. Filtration and evaporation gave a crude product, which was purified by aluminum oxide (brockmann 3) column chromatography (hexane:EtOAc 6:1) to give a mixture of the diastereomers **6** and recovered **5**. The two diastereomers **6** were dissolved in $CHCl_3$ to afford the desired diastereomers **7** (0.36 g, 53% yield) and the diol, which was separated by chromatography (hexane:EtOAc 3:1). ^{1}H-NMR: δ 8.0-7.9 (m, 2H); 7.6-7.4 (m, 3H); 5.22 (d, J=7. 2 Hz, 1H mi); 5.11 (d, J=2.4 1H ma); 5.10 (s, 1H mi); 4.78 (d, J=6.6 Hz, 1H ma); 3.75 (ddd, J=8.1, 7.2, 2.4 Hz, 1H ma); 3.41 (m, 1H mi); 2.39 (m, 1H mi); 2.03 (dsept, J= 8.1, 6.6 Hz, 1H ma); 1.31 (s, 9H ma); 1.26 (s, 9H mi); 1.15 (d, J=6.6 Hz, 3H); 1.09 (d, J=6.6 Hz, 3H); 1.087 (d, J=6.6 Hz, 3H); 1.03 (d, J=6.6 Hz, 3H). ^{13}C-NMR: δ 158.5, 157.6, 156.5, 156.0, 155.7, 154.7, 132.9, 132.8, 132.6, 122.9, 81.1 (mi), 80.8 (ma), 73.2 (mi), 71.4 (ma), 62.4 (mi), 61.3 (ma), 29.8 (ma, mi), 28.1, 28.0 (ma, mi), 20.1 (ma), 20.0 (ma), 19.7 (mi), 19.1 (mi). MS: m/z 332 (3), 276 (100), 232 (14). HRMS: m/z for (MH^+) $C_{19}H_{26}NO_4$: calcd. 332.1862, found 332.1834; for (MH^+-C_4H_8) $C_{15}H_{18}NO_4$: calcd. 276.1236, found 276.1175; for (MH^+) $^{12}C_{18}{}^{13}CH_{26}NO_4$: calcd. 333.1895, found 333.1926; for (MH^+-C_4H_8) $^{12}C_{14}{}^{13}CH_{18}NO_4$: calcd. 277.1269, found 277.1219.

2-{(2S)-2-amino-1-hydroxy-3-methylbutyl}-3-phenylcyclopropenone hydrochloride (8). To solution of **7** (87 mg, 0.26 mmol) in 1,4-dioxane (0.25 mL) and water (5.6 mL) 4*N* HCl in 1,4-dioxane (0.8 mL) was added at room temperature. After stirring for 30 min, the solution was evaporated in warm bath to afford compound **8** as highly hygroscopic salt. (54 mg, 90% yield). ^{1}H-NMR: δ 8.1-8.0 (m, 2H); 7.7-7.5 (m, 3H); 5.41 (d, J=4.5 Hz, 1H mi); 5.19 (d, J=6.6 Hz, 1H ma); 3.42 (dd, J=6.3, 5.4 Hz, 1H ma); 3.33 (dd, J=9.6, 4.5 Hz, 1H mi); 2.28 (septd, J=6.9, 5.4 Hz, 1H ma); 2.08 (dsept, J=9.6, 6.9 Hz, 1H mi); 1.21 (d, J=6.9 Hz, 1H mi); 1.19 (d, J=6.9, 1H ma); 1.16 (d, J=6.9 Hz, 1H mi); 1.158 (d, J=6.9 Hz, 1H ma). ^{13}C-NMR: δ 157.5, 157.1, 154.4, 134.8, 133.9, 130.5, 123.7, 68.6 (mi), 68.0 (ma); 62.6 (mi), 60.8 (ma), 30.1 (mi), 29.3 (ma), 19.9 (mi), 19.7 (ma), 17.4 (ma, mi). MS: m/z 232 (100), 214 (50).

2-{(2S)-2-(Cbz-Leucyl-amino)-1-hydroxyl-3-methylbutyl}-3-phenyl-cyclopropenone (9). Cbz-Leu-OH (77 mg, 0.29 mmol), PyBOP (154 mg, 0.29 mmol) and the hydrochloride salt **8** (60 mg, 0.26 mmol) were dissolved in CH_2Cl_2 (2 mL) and Et_3N (0.13 mL, 9.5 mmol) was added. After 15 min of stirring at room temperature, the pH checked for basic condition and the mixture was stirred for additional 2.5 h. It was then quenched with an aq $KHSO_4$

solution (0.5 g, 5 mL). The aqueous phase was extracted with CH_2Cl_2 (3 × 20 mL), the combined organic layers were washed with brine, dried over $MgSO_4$ and evaporated to give a crude product, which was purified by chromatography (hexane:EtOAc 1:4) to afford the compound **9** as two separated diastereomers. (61 mg, 49% yield, **9a**:**9b** 5:1).

2-{(1S,2S)-2-(Cbz-Leucyl-amino)-1-hydroxyl-3-methylbutyl}-3-phenyl-cyclopropenone (**9a**). ^{1}H-NMR: δ 8.0−7.9 (m, 2H); 7.62 (d, J=6.9, 1H); 7.6−7.4 (m, 3H); 7.3−7.2 (m, 5H); 5.74 (d, J=7.5 Hz, 1H); 5.07 (d, J=2.9 Hz, 1H); 5.02 (d, J=12.3 Hz, 1H); 4.91 (d, J=12.3 Hz, 1H); 4.17 (brq, J=8 Hz, 1H); 3.69 (br.dt, J=8.1, 7.2, 2.4 Hz, 1H); 2.33 (d.sept, J=9.6, 6.6 Hz, 1H); 1.50 (nonet, J=6.6 Hz, 1H); 1.31 (m, 1H); 1.06 (d, J=6.6, 3H); 0.98 (d, J=6.6 Hz, 3H); 0.78−0.71 (m, 6H). ^{13}C-NMR: δ 174.9, 156.4, 156.2, 156.0, 154.9, 136.1, 133.0, 132.6, 129.2, 128.5, 128.2, 127.9, 122.8, 70.5, 67.2, 61.5, 53.9, 41.1, 28.0, 24.7, 22.8, 21.6, 19.7, 19.5. MS: m/z 479 (MH^+, 87), 451 (97), 433 (100), 319 (35), 248 (52), 91 (76). HRMS: m/z for (MH^+) $C_{28}H_{35}N_2O_5$: calcd. 479.2546, found 479.2560; for $^{12}C_{27}{}^{13}CH_{35}N_2O_5$: calcd. 480.2580, found 480.2567.

2-{(1R,2S)-2-(Cbz-Leucyl-amino)-1-hydroxyl-3-methylbutyl}-3-phenyl-cyclopropenone (**9b**). ^{1}H-NMR: δ 8.1−8.0 (m, 2H); 7.6−7.5 (m, 3H); 7.4−7.3 (m, 5H); 6.95 (d, J=7.2, 1H); 5.34 (d, J=7.2 Hz, 1H); 5.16 (bd, J=2.9 Hz, 1H); 5.04 (d, J=12 Hz, 1H); 4.94 (d, J=12 Hz, 1H); 4.17 (dt, J=7.8, 6.6 Hz, 1H); 4.08 (ddd, J=9.6, 7.2, 2.4 Hz, 1H); 2.16 (d.sept, J=9, 6.6 Hz, 1H); 1.55−1.34 (m, 3H); 1.16 (d, J=6.6, 3H); 1.09 (d, J=6.6Hz, 3H); 0.72 (d, J=6.3, 6H). ^{13}C-NMR: δ 175.5, 156.4, 155.7, 154.4, 136.2, 133.1, 132.8, 129.3, 128.6, 128.3, 128.2, 123.0, 72.7, 67.3, 61.5, 53.9, 41.1, 29.4, 24.8, 22.6, 22.1, 20.2, 19.8. MS: m/z 479 (MH^+, 100), 433 (60), 319 (37), 176 (53), 91 (87). HRMS: m/z for (MH^+) $C_{28}H_{35}N_2O_5$: calcd. 479.2546, found 479.2570.

Biochemistry

Enzyme kinetics. Papain (EC 3.4.22.2) and cathepsin B (EC 3.4.22.1) were activated, assayed, and inhibited as previously described.[7]

Dialysis. A 1.2 mL solution of inhibited enzyme (whose residual activity determined) was dialyzed in a 3,500 Da cut-off dialysis bag against 400 mL of phosphate buffer (100 m*M* KPi, pH 7.0, containing 0.5 m*M* DTT). The buffer solution was replaced after 4 h, and dialysis continued for additional 4 h, after which the enzyme activity was measured again. As a control, active enzyme underwent the same protocol.

Free thiol determination. Activated papain (16 mg in 100 mL) was concentrated to about 3 mL in a 10,000 cutoff Amicon tube. The concentrated enzyme solution was purified from small molecules (DTT and EDTA) by Sephadex G-15 chromatography (15 × 1 cm^2 column, degassed 100 m*M* KPi buffer pH 7.0). Fractions of 1.5 mL were collected and their A_{280} was measured. The enzyme-containing fractions were pooled and the enzyme activity assayed. Concentration of free thiol in the enzyme fraction, as well as the other fractions was determined by addition of aq. DTNB solution (30 μL, 10 m*M*) and measuring A_{412} (ε = 13,600 M^{-1} cm^{-1})[16] after 5 min of incubation.

To correlate residual enzyme activity with free thiol concentration, aliquots (1.5 mL) from the inactivation solution were removed at various time points. Totally, 10 μL of the aliquot was used for enzymatic activity assay (immediate dilution into the assay buffer solution practically stopped the inactivation reaction), and the rest was treated with DTNB as above to determine the remaining free thiol concentration. An active enzyme solution was similarly treated and assayed as a control.

Denaturation

Heat denaturation was carried out by boiling an enzyme solution for 3 min. Chemical denaturation was achieved by addition of guanidinium chloride (at 6*M* final concentration). Residual enzyme activity and free thiol concentration were determined in both cases.

Mercury affinity chromatography

Activated papain was separated from inactive protein by mercury affinity chromatography as previously described.[17]

^{13}C-NMR analysis

Papain (100 mg) was activated and the active enzyme was purified by mercury affinity chromatography. The active enzyme solution was concentrated to 10 mL through a 10,000 cutoff filter (Amicon), diluted in potassium phosphate buffer (90 mL, 100 m*M*, pH 7.0), and reconcentrated to 2 mL by Amicon and further concentrated into 0.5 mL by a stream of nitrogen. To this solution was added a solution of inhibitor **9** in DMSO-d_6 (0.1 mL, 12 m*M*), or DMSO-d_6 alone (0.1 mL) in the control.

HPLC analysis

Nucleosil 120×5 C18 column (Macherey-Nagel) was used at a flow rate of 1 mL/min, applying linear gradients of either 100% to 20% water-acetonitrile in 40 min or 100% to 20% water-methanol in 50 min.

Conclusions

Peptidyl cyclopropenones were previously introduced as competitive reversible inhibitors of cysteine proteases. Their specific mechanism of interaction with the enzyme active site was not clarified. In this

study we show that a peptidyl cyclopropenone behaves as an irreversible covalent inhibitor of a cysteine protease, alkylating the catalytic cysteine of papain. In parallel, it also behaves as an alternative substrate of the enzyme. A detailed mechanism for these two reactions was suggested. Thus, this study draws attention to the reality that a family of compounds may exhibit various modes of interaction with different enzymes sharing the same catalytic mechanism, and even a dual mode of interaction with a single enzyme.

References

1. Ando R, Sakaki T, Morinaka Y, Takahashi C, Tamao Y, Yoshii N, Katayama S, Saito K, Tokuyama H, Isaka M, Nakamura E (1999) Cyclopropenone-containing cysteine proteinase inhibitors. synthesis and enzyme inhibitory activities. Bioorg Med Chem 7:571–579.
2. Ando R, Morinaka Y, Tokuyama H, Isaka M, Nakamura E (1993) A new class of proteinase inhibitor. Cyclopropenone-containing inhibitor of papain. J Am Chem Soc 115:1174–1175.
3. Staley SW, Norden TD, Taylor WH, Harmony MD (1987) Electronic structure of cyclopropenone and its relationship to methylenecyclopropene: Evaluation of criteria for aromaticity. J Am Chem Soc 109: 7641–7647.
4. Eicher T, Weber JL (1975) Structure and reactivity of cyclopropenones and triafulvenes. Topics Current Chem 57:1–109.
5. Potts KT, Baum JS (1974) Chemistry of cyclopropenones. Chem Rev 74:189–213.
6. Albeck A, Kliper S (1997) Mechanism of cysteine protease inactivation by peptidyl epoxides. Biochem J 322:879–884.
7. Albeck A, Fluss S, Persky R (1996) Peptidyl epoxides: Novel selective inactivators of cysteine proteases. J Am Chem Soc 118:3591–3596.
8. Frankfater A, Kuppy T (1981) Mechanism of association of N-acetyl-L-phenylalanylglycinal to papain. Biochemistry 20:5517–5524.
9. Shokhen M, Khazanov N, Albeck A (2011) The mechanism of papain inhibition by peptidyl aldehydes. Proteins 79:975–985.
10. Shokhen M, Traube T, Vijayakumar S, Hirsch M, Uritsky N, Albeck A (2011) Differentiating serine and cysteine protease mechanisms by new covalent QSAR descriptors. ChemBioChem 12:1023–1026.
11. Shokhen M, Arad D (1996) The source for the difference between sulfhydryl and hydroxyl anions in their nucleophilic addition reaction to a carbonyl group: A DFT approach. J Mol Model 2:399–409.
12. Howard AE, Kollman PA (1988) OH- versus SH- nucleophilic attack on amides: Dramatically different gas-phase and solvation energetics. J Am Chem Soc 110:7195–7200.
13. Sakaki T, Ando R (2007) 2-tert-Butoxy-3-phenylcyclopropanone acetal, a stable precursor of lithiated 2-phenylcyclopropenone acetal. Tetrahedron Lett 48: 7011–7014.
14. Mirilashvili S, Chasid-Rubinstein N, Albeck A (2010) Optically Active N- and C-terminal building blocks for the synthesis of peptidyl olefin peptidomimetics. Eur J Org Chem 4671–4686.
15. Grzyb JA, Shen M, Yoshina-Ishii C, Chi W, Brown RS, Batey RA (2005) Carbamoylimidazolium and thiocarbamoylimidazolium salts: Novel reagents for the synthesis of ureas, thioureas, carbamates, thiocarbamates and amides. Tetrahedron 61:7153–7175.
16. Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77.
17. Sluyterman LAE, Wijdenes J (1970) An agarose mercurial column for separation of mercaptopapain and nonmercaptopapain. Biochim Biophys Acta 200:593–595.
18. Silverman RB (2000) The Organic Chemistry of Enzyme-Catalyzed Reactions. Academic Press, San Diego, pp 570–587.
19. Albeck A, Kliper S (2000) Inactivation of cysteine proteases by peptidyl epoxides: Characterization of the alkylation sites on the enzyme and the inactivator. Biochem J 346:71–76.
20. Liang T-C, Abeles RH (1987) Complex of alpha-chymotrypsin and n-acetyl-l-leucyl-l-phenylalanyl trifluoromethyl ketone—Structural studies with nmr-spectroscopy. Biochemistry 26:7603–7608.
21. Finucane MD, Hudson EA, Malthouse JPG (1989) A C-13-NMR investigation of the ionizations within an inhibitor-alpha-chymotrypsin complex—Evidence that both alpha-chymotrypsin and trypsin stabilize a hemiketal oxyanion by similar mechanisms. Biochem J 258:853–859.
22. Yabe Y, Guillaume D, Rich DH (1988) Irreversible inhibition of papain by epoxysuccinyl peptides—C-13-NMR characterization of the site of alkylation. J Am Chem Soc 110:4043–4044.
23. Liang T-C, Abeles RH (1987) Inhibition of papain by nitriles—Mechanistic studies using nmr and kinetic measurements. Arch Biochem Biophys 252:626–634.
24. Moon JB, Coleman RS, Hanzlik RP (1986) Reversible covalent inhibition of papain by a peptide nitrile—C-13 NMR evidence for a thioimidate ester adduct. J Am Chem Soc 108:1350–1351.
25. Powers JC, Asgian JL, Ekici OD, James KE (2002) Irreversible inhibitors of serine, cysteine, and threonine proteases. Chem Rev 102:4639–4750.
26. Still WC, Kahn M, Mitra A (1978) Rapid chromatographic technique for preparative separations with moderate resolution. J Org Chem 43:2923–2925.
27. Krebs KG, Heusser D, Wimmer H. In: Stahl E, Ed. (1969) Thin Layer Chromatography, 2nd ed. New York: Springer Verlag, pp 862.