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Cleavage of β-Lactone Ring by Serine Protease. Mechanistic Implications

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Abstract—Both enantiomers of 3-benzyl-2-oxetanone (**1**) were found to be slowly hydrolyzed substrates of α-chymotrypsin having k_{cat} values of 0.134 ± 0.008 and $0.105 \pm 0.004\,min^{-1}$ for (*R*)-**1** and (*S*)-**1**, respectively, revealing that α-CT is virtually unable to differentiate the enantiomers in the hydrolysis of **1**. The initial step to form the acyl-enzyme intermediate by the attack of Ser-195 hydroxyl on the β-lactone ring at the 2-position in the hydrolysis reaction may not be enzymatically driven, but the relief of high ring strain energy of β-lactone may constitute a major driving force. The deacylation step is also attenuated, which is possibly due to the hydrogen bond that would be formed between the imidazole nitrogen of His-57 and the hydroxyl group generated during the acylation in the case of (*R*)-**1**, but in the α-CT catalyzed hydrolysis of (*S*)-**1** the imidazole nitrogen may form a hydrogen bond with the ester carbonyl oxygen.

Introduction

Serine proteases that may be represented by α-chymotrypsin (α-CT)[1] have received an increasing attention in recent years because of their roles in the pathology of numerous diseases such as rheumatoid arthritis,[2] thrombosis,[3] and pulmonary emphysema.[4] These enzymes are characterized by having a catalytically essential serine residue at the active site. The hydroxyl group of the serine residue attacks in collaboration with His and Asp the scissile peptide or ester bond of substrates, generating an acyl-enzyme intermediate which is, in turn, hydrolyzed by the active site water molecule to yield the second product with regeneration of the free enzyme.[1] These three amino acid residues which are directly involved in the enzymic hydrolysis are generally referred to as the catalytic triad. In the case of α-CT, Ser-195 is the essential serine residue which together with His-57 and Asp-102 constitute the catalytic triad. The His-57 functions as a general base, activating the nucleophilic hydroxyl of Ser-195 and the water molecule that is required for the deacylation reaction. The transition states that are generated in the processes of the formation of acyl-enzyme intermediate and its subsequent hydrolysis are stabilized through hydrogen bonding interactions with two backbone NHs of Ser-195 and Gly-193, which together are referred to as the oxyanion hole.[1,5] At the S_1 subsite of α-CT there is present a large hydrophobic pocket that accommodates the P_1 amino acid residue having a bulky hydrophobic side chain such as Phe. As a prototypical enzyme for a large family of serine proteases, α-CT has been served as a model target for the development of inhibitor design strategies that can be of value in designing inhibitors for proteases of medicinal interest.[6]

Recently, we have reported that 3-benzyl-4-bromomethyloxetan-2-one is a fast acting alternate substrate inhibitor[7] for α-CT.[8] In a continued effort in this line of study, we have synthesized enantiomerically pure (*R*)- and (*S*)- forms of 3-benzyl-2-oxetanone (**1**) and investigated their chemistry at the active site of α-CT. We anticipated that (*R*)-**1** would be a substrate for α-CT as its stereochemistry corresponds to the stereochemistry of substrate, but its enantiomer, i.e., (*S*)-**1** may function as an irreversible inactivator for the enzyme. Because of its stereochemistry (*S*)-**1** may bind to α-CT at the active site with its lactone ring being rested in such a way that

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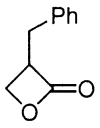

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the Ser-195 hydroxyl would attack the ring at the 4-position rather than the 2-position to result in the formation of an ether bond that resists to be cleaved. The postulated configuration-dependent reaction paths of **1** at the active site of α-CT are depicted schematically in Figure 1. The 2-oxetanone ring is an inherently reactive moiety by virtue of its high ring strain energy (22.8 kcal mol^{-1}), and is known to undergo a facile ring cleavage reaction by the attack either at the C-2 carbonyl carbon or at the C-4 position.[9]

Results and Discussion

The hydroxyl group in **2** that was prepared by the literature method[10] was oxidized to the corresponding carboxylate with Jones' reagent, and then treated with K_2CO_3 in aqueous methanolic solution to give **4**. Racemic **4** was converted into its methyl ester **5**, and then was subjected to enzymatic kinetic resolution using α-CT to obtain (*R*)-**4** and (*S*)-**5**. The optical purity of (*R*)-**4** determined as its methyl ester. Thus, (*R*)-**4** was converted into the corresponding methyl ester under the acidic conditions and its optical purity was determined by chiral HPLC to be 93.6% ee. The (*S*)-**5** from the kinetic resolution was treated again with α-CT to improve the optical purity of 98.6% ee. The (*S*)-**5** was hydrolyzed to give (*S*)-**4**. Both enantiomers of **1** were prepared by lactonization of the optically active **4** under the modified Mitsunobu conditions (Scheme 1).[11] Vederas et al.[11] reported that the lactonization proceeds without racemination under the reaction conditions. Racemic **1** was prepared by lactonization of **4**.

When α-CT was exposed to 50-fold Molar excess of racemic **1**, about 90% of the enzyme activity was lost within several minutes, followed by slow regaining of the enzymic activity, as shown by the upward swing of the activity curve in the plot of activity remaining versus time (Fig. 2). About 75% of the enzyme activity was recovered in 17 h. The experimental results suggest that compound **1** is a substrate for α-CT albeit poor, and furthermore that the cleavage of the lactone ring occurs possibly in two steps with formation of an intermediate that may be formed by the cleavage of the lactone ring of **1** by a nucleophile present at the active site of the enzyme. This has been confirmed by the electrospray ionization mass spectrometry (ESI-MS): The native α-CT and the intermediate formed by the interaction of α-CT with each enantiomer of **1** showed a peak at 25.234 and 25.397 Da, respectively (Fig. 3). The peak of 25.397 Da corresponds to α-CT + **1**, indicating that the intermediate is the addition product generated most likely by the nucleophilic attack of the catalytic Ser-195 hydroxyl group on the lactone ring as anticipated. The nucleophilic attack may take place either at the C-2 or C-4 position.[9] However, since both enantiomers were shown to be substrates for α-CT, the possibility that the intermediates may have an ether linkage by the attack at the C-4 of the lactone ring is unlikely, because if the latter intermediates are formed, they would resists undergoing further reactions, causing inactivation of the enzyme.[12,13] A positive active site protection shown in the competitive binding assay with an excess substrate established that both enantiomers of **1** bind the enzyme at the active site (Fig. 4). The K_M values[14] for binding of (*R*)-**1** and (*S*)-**1** to α-CT and the k_a for the formation of the intermediates were estimated from the respective Kitz–Wilson plot[15] constructed using the k_{obs} values obtained for the initial 150 and 250 s for (*S*)-**1** and (*R*)-**1**, respectively, after the addition of α-CT to a solution of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide[16] (Suc-AAPF-*p*-NA) and optically active **1** (Figs 5 and 6). Dissociation constants (K_S) of the Michaelis complexes were estimated from the respective Dixon plot (Fig. 7).[17] The stabilities of the acylated α-CT were determined by the proflavin displacement assay method:[18] A rapid decrease of absorbance at 466 nm due to the intermediate formation was followed by a slow return of the absorption for the each enantiomer as can be seen from Figure 8. The rate constant for the recovery of the enzymic activity (k_d) was estimated from the progress curves (Fig. 8). The kinetic data thus obtained are collected in Table 1.[19]

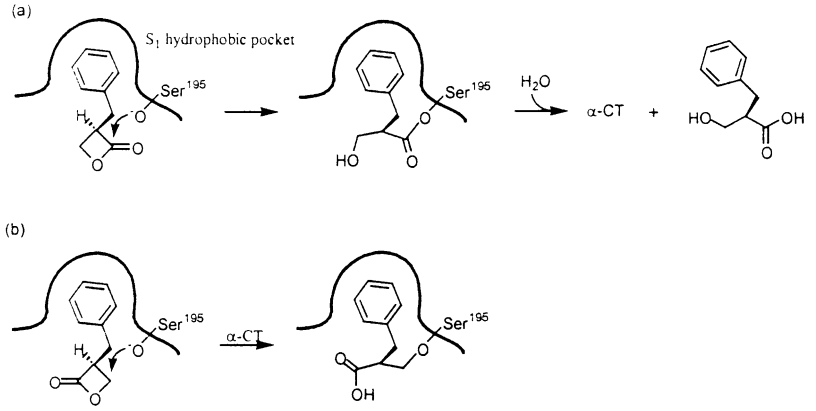
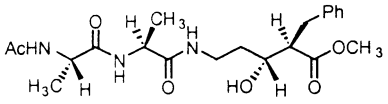


Figure 1. Postulated reaction paths of (a) (*R*)-**1** and (b) (*S*)-**1** at the active site of α-CT.

The above kinetic and mass spectral data together with well established catalytic mechanism[1] for α-CT suggest strongly that both enantiomers of **1** form acyl-enzyme intermediates with concurrent cleavage of the lactone ring by the Ser-195 hydroxyl upon exposing **1** to α-CT, and the intermediates thus formed hydrolyze at a much slower rate than that for the acylation reaction. Figure 9 shows computer generated models of α-CT that is acylated at the Ser-195 hydroxyl by each enantiomer of **1**. Both deacylation rate constants are drastically attenuated compared with those of normal substrates ($k_d = \sim 4000\,min^{-1}$).[20] The slow deacylation reaction may be ascribed to the reduced basicity of the imidazole moiety in His-57 as a result of hydrogen bonding interactions in the acyl-intermediates: As can be seen in Figure 9a, in the acyl-intermediate formed with (*R*)-**1** the hydroxyl group generated in the ring cleavage reaction is positioned within the distance of a hydrogen bonding (3.09 Å). On the other hand, when the Ser-195 hydroxyl is acylated by (*S*)-**1**, the imidazole nitrogen may form a hydrogen bond with the ester carbonyl oxygen; the two atoms are separated by 2.99 Å (Fig. 9b). Recently, Mobashery and associates exploited such hydrogen bonding to develop a novel type of α-CT inhibitor, **6**.[21] Their molecular modeling study suggested that the hydrogen bond between the imidazole of His-57 and the hydroxyl β to the ester carbonyl of the acyl-enzyme intermediate may reduce the basicity of the imidazole of His-57, leading to impair the catalytic machinery for the deacylation reaction.[21] The molecular modeling further suggested that the hydrogen bonding is not sensitive to the spatial orientation of the hydroxyl group.[21] Our kinetic results and the molecular models of the acyl-intermediates are in accordance with the proposition stipulated by Mobashery and associates.[212]

One of the distinctive features of enzymic reactions is the substrate specificity including specificity due to the spatial configuration of substrate molecule. The S_1 subsite hydrophobic pocket is situated in such a way that it can preferentially accommodate L-amino acid residue having a hydrophobic side chain, in forming α-CT substrate complex thus to manifest substrate stereochemistry in favor of the L-stereochemistry. Surprisingly, however, no noticeable stereoselectivity was observed in the α-CT catalyzed hydrolysis of the β-lactone of **1**. It appears that this may be the first example that α-CT fails to exhibit stereospecificity in its enzymic reactions, although there are precedences that some of the unnatural substrates and inhibitors for α-CT display reversed stereospecificity, i.e., the D-stereospecificity.[22] The k_a values obtained with the lactone substrates are drastically reduced, in spite of its inherent high chemical reactivity owing to the high ring strain, compared with that reported for structurally comparable acyclic ester substrates such as *N*-acetyltryptophan ethyl ester ($k_{cat} = 1620\,min^{-1}$),[23] suggesting that the tetrahedral transition states generated in the acylation reaction of α-CT with **1** may not be stabilized by the oxyanion hole, and thus the relief of high ring strain energy associated with the lactone ring cleavage may constitute the driving force for the acylation reaction. The k_{obs} for the hydrolysis of unsubstituted β-lactone in pH 7.5 buffer at room temperature was reported to be $2.3\times10^{-3}\,min^{-1}$.[24] This



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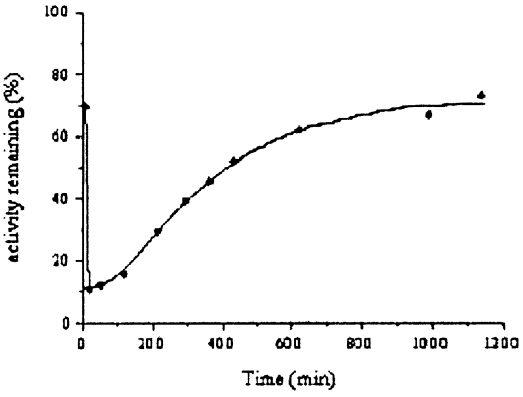
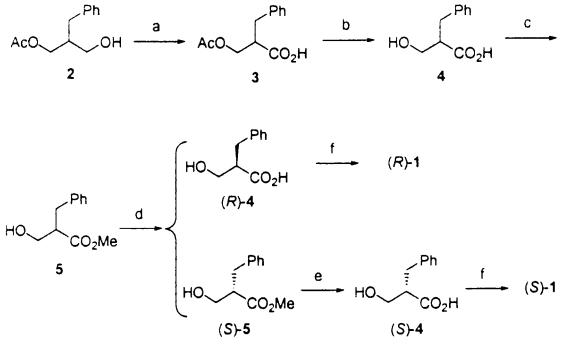


Figure 2. Spontaneous reactivation of α-CT inactivated by **1** at 25 °C in 0.05 M Tris buffer pH 7.5 containing 20% DMSO. The final concentrations of α-CT and **1** are 25 nM and 1.25 μM, respectively. The enzymic activity was assayed using Suc-AAPF-*p*NA (125 μM) as the substrate.



Scheme 1. Reagents, conditions, and (yields): (a) Jones' reagent, 0 °C (73%); (b) K_2CO_3, MeOH/H_2O (90%); (c) H^+, MeOH (86%); (d) α-CT, 0.05 M phosphate buffer (pH 7.5); (e) (i) α-CT, 0.05 M phosphate buffer (pH 7.5); (ii) LiOH, MeOH/H_2O (70%); (f) Ph_3P, DMAD, −78 °C (75%).

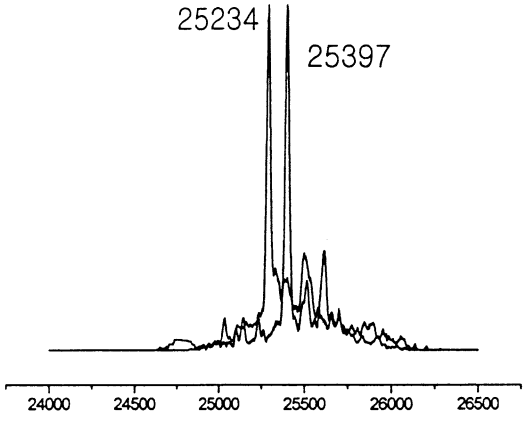


Figure 3. Superimposed electrospray mass spectra of α-CT (*m*/*z* 25234 Da) and α-CT complexed with (*R*)-**1** (*m*/*z* 25397 Da).

rate constant for the uncatalyzed β-lactone cleavage compares favorably with the k_a values obtained with **1** and tends to support the notion that the acylation reactions of the Ser-195 hydroxyl by **1** are not enzymatically driven.[25] It may thus be concluded that the unique chemical property of β-lactone in **1** is responsible, at least in part, for the lack of stereospecificity shown in the α-CT catalyzed hydrolysis of **1**.

It is interesting to note that the present observation that both enantiomers of **1** are hydrolyzed by α-CT is in contrast to the recent finding of Lall et al.[26] who reported that (*R*)-*N*-benzyloxycarbonylamino-2-oxetanone inactivates irreversibly the 3C cysteine protease of hepatitis A virus (HAV). The thiol of Cys-172 in the cysteine protease was shown to attack at the 4-position of the β-lactone of the compound to form a thioether linkage that resists hydrolysis.[26] Although serine and cysteine proteases are members of evolutionary distant proteases, they share mechanistic similarities.[27] Thus, both enzyme families bear similar active site geometries and form covalent acyl-enzyme intermediates.[27] The reason why the two mechanistically similar enzymes show such different reactivities toward β-lactone is not apparent to us.

Conclusion

We have synthesized both enantiomers of 3-benzyl-2-oxetanone (**1**) and investigated their chemistry at the active site of α-CT to find that contrary to our expectation both are slowly hydrolyzed substrates of α-CT having k_{cat} values of 0.134 ± 0.008 and $0.105 \pm 0.004\ min^{-1}$ for (*R*)-**1** and (*S*)-**1**, respectively. α-CT is virtually unable to differentiate the enantiomers in the catalytic hydrolysis of the β-lactone ring in **1**. It appears that the acylation step in the hydrolysis of the β-lactone is not enzymatically driven, and the relief of high ring strain energy of the β-lactone would constitute the driving force for the reaction. The deacylation step is attenuated, which is possibly due to the reduced basicity of His-57 imidazole ring as a consequence of the hydrogen bond that would be formed between the imidazole ring of His-57 and the hydroxyl group generated in the acylation step. The imidazole moiety of His-57 functions as a general base, activating the water molecule that attacks the acyl-enzyme intermediate. The results of present study may be of value in designing a novel type of inhibitors for serine proteases. We are currently exploiting the findings made in the present study to develop a new design strategy that can be useful for designing inhibitors effective against serine proteases.

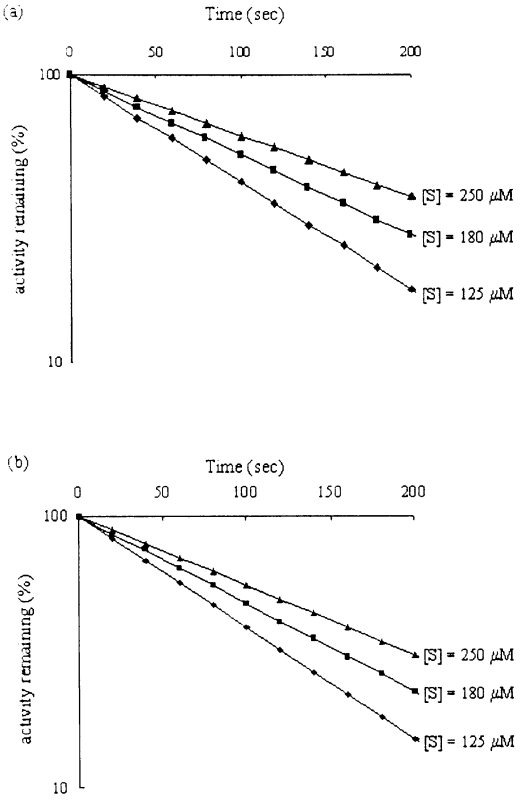


Figure 4. Active site protection tests for (a) (*R*)-**1** ([α-CT] = 25 nM, [(*R*)-**1**] = 500 μM, and [Succ-AAPF-*p*NA] = 125, 180, and 250 μM), and (b) (*S*)-**1** ([α-CT] = 25 nM, [(*S*)-1] = 200 μM, and [Succ-AAPF-*p*NA] = 125, 180, and 250 μM).

Experimental

General

Melting points were measured on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Bruker AM 300 (300 MHz) instrument using tetramethylsilane as the internal standard. IR spectra were recorded on a Bruker Equinox 55 FT-IR spectrophotometer. High

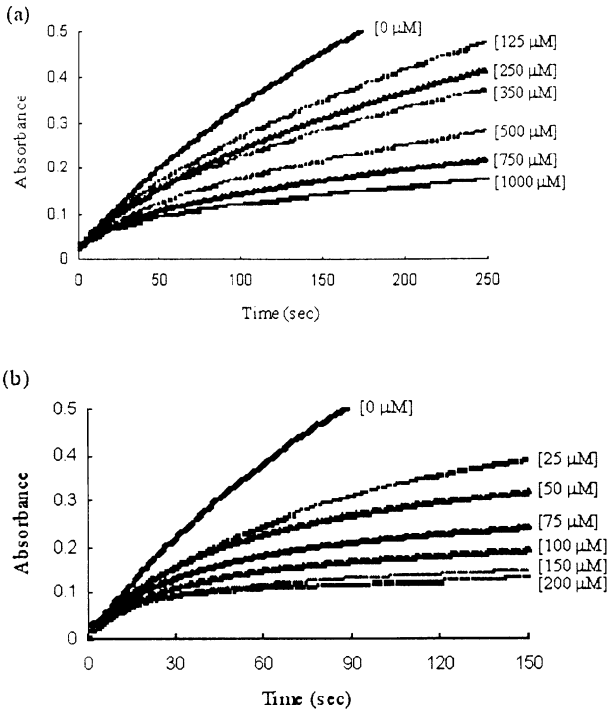


Figure 5. Progress curves showing the α-CT catalyzed hydrolysis of Succ-AAPF-pNA in the presence of varied concentrations of (a) (*R*)-**1** and (b) (*S*)-**1**. Assay conditions are described in Experimental.

resolution mass spectra were obtained at Korea Basic Science Center, Daejeon, Korea. ESI-MS spectrometric analysis was performed using a triple quadrupole mass spectrometer (Quattro, Micromass, Manchester, UK; 0.1% resolution, 0.01% accuracy) equipped with an ESI source. Flash chromatography was performed on Silica gel 60 (230–400 mesh) from Merck. Optical rotations were measured on a Rudolph Research Autopol III digital polarimeter. Elemental analyses were performed at the Center for Biofunctional Molecules, Pohang University of Science and Technology, Pohang, Korea. All chemicals were obtained from Aldrich Chemical Co. and solvents were purified before use.

3-Acetoxy-2-benzylpropanoic acid (3). To an ice-chilled solution of **2**[10] (3.1 g, 15 mmol) in 10 mL of acetone was added slowly the Jones' reagent until brownish color of the solution remains over 20 min, then 2-propanol was added until the solution became clear. The precipitate that separated was filtered using a Celite pad and the filtrate was evaporated under reduced pressure. The residue was diluted with 5 mL of 1 N HCl then extracted with ethyl acetate (30 mL×3). The extract was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to give a yellowish syrup. The residue was purified by flash column chromatography (silica gel, EtOAc:hexane = 3:1) to give **3**[28] as an oil (73%): IR (KBr) 1741, 1712 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.16–7.32 (m, 5H), 4.10–4.23 (m, 2H), 2.96–3.09 (m, 2H), 2.81–2.94 (m, 1H), 1.98 (s, 3H); MS (CI) m/z 223 $(M+1)^+$.

2-Benzyl-3-hydroxypropanoic acid (4). Compound **3** (1.8 g, 8 mmol) was dissolved in methanol (10 mL) containing K_2CO_3 (1.4 g) and several drops of water. After stirring for 2 h at room temperature, the reaction mixture was treated with several drops of acetic acid, and concentrated in vacuo. The residue was purified by flash column chromatography (silica gel, EtOAc:hexane = 1:3) to give **4** (90%): mp 58–59 °C; lit[29] mp 63–65°C: IR (KBr) 3300, 1710 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.16–7.31 (m, 5H), 4.8 (br, 2H), 3.68–3.80 (br, 2H), 3.00–3.07 (m, 1H), 2.80–2.92 (m, 2H).

2-Benzyl-3-hydroxypropanoic acid methyl ester (5). To an ice-chilled stirred solution of **4** (1.3 g, 7 mmol) in MeOH (20 mL) was added acetyl chloride (2 mL). The mixture was stirred for 24 h at room temperature, and then evaporated under reduced pressure. The residue was dissolved in EtOAc (30 mL) and aqueous $NaHCO_3$ solution added to neutralize the mixture. The organic layer was washed with copious water, and brine, dried over $MgSO_4$, and evaporated to give an oily residue, which was purified by column chromatography (silica gel, EtOAc:Hexane = 1:5) to yield the product **5**[30] as an oil (86%): IR (KBr) 3300, 1735 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.19–7.35 (m, 5H), 3.65–3.80 (m, 2H), 3.70 (s, 3H), 3.03 (dd, J = 16.9, 9.2 Hz, 1H), 2.88 (m, 2H), 2.18 (t, J = 7.7 Hz, 1H); MS (EI) m/z 195 $(M+1)^+$.

(*R*)-2-Benzyl-3-hydroxypropanoic acid ((*R*)-4). Racemic **5** (3.48 g, 18 mmol) was suspended in 50 mL of 0.01 M phosphate buffer solution. To the mixture was added α-CT (300 mg), and stirred slowly. The pH of the reaction mixture was maintained at pH 7.5 by addition of sodium hydroxide solution (0.2 N) using a pH-stat

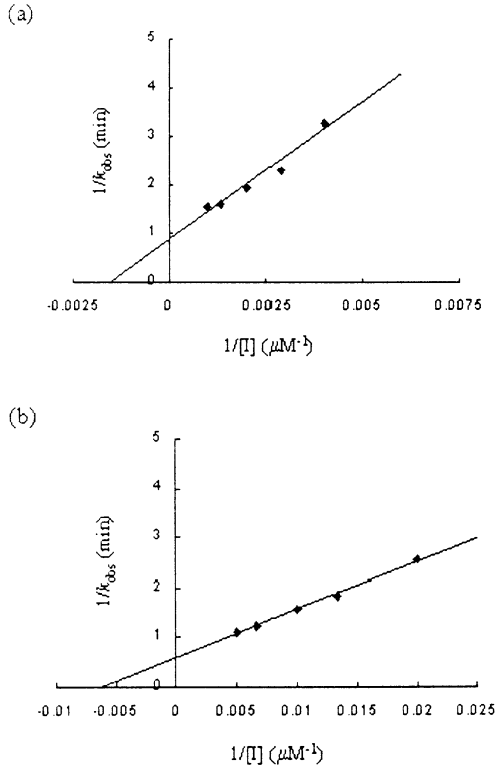


Figure 6. Kitz–Wilson plots for determinations of K_M and k_a values for (a) (*R*)-**1** and (b) (*S*)-**1**.

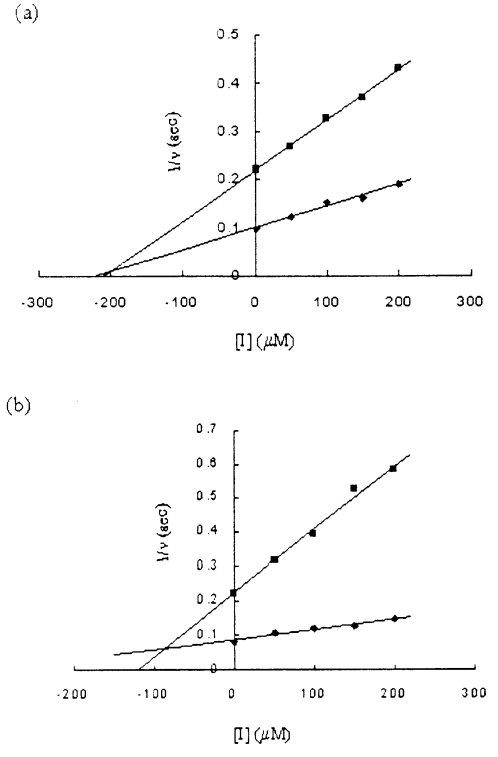


Figure 7. Dixon plots for determinations of K_S values in the acylation reactions of α-CT with (a) (*R*)-**1** and (b) (*S*)-**1**. Assay conditions are found in Experimental.

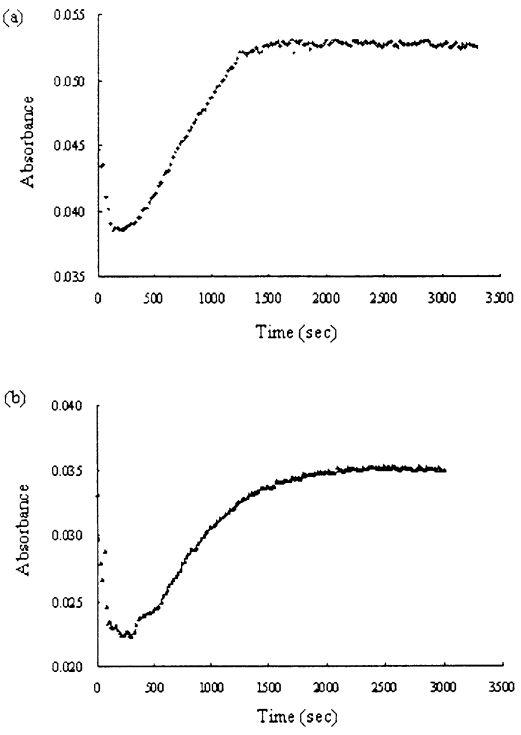


Figure 8. The determinations of k_d values for (a) (*R*)-**1** and (b) (*S*)-**1** by the proflavin displacement assay method.

potentiometer. When 35 mL of the alkaline solution was consumed (18 h), the reaction mixture was acidified with 1 N HCl solution and saturated with sodium chloride then extracted with ethyl acetate. The extract was dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, EtOAc:hexane = 1:4 to 1:1) to give (*R*)-**4**[29] and crude (*S*)-**5**. The spectral data of these compounds were identical with those of respective racemic compounds, i.e., **4** and **5**. (*R*)-**4**: $[\alpha]_D = +23.5°$ ($c = 1.00$, MeOH). In order to determine the optical purity of (*R*)-**4** thus obtained, the product was converted into the corresponding methyl ester under acidic conditions ($MeOH + SOCl_2$), and the product was analyzed by HPLC using a chiral column (Chiralcel OD, Diacel Chemical Ind.) to find that it has the optical purity of 93.6% ee. The HPLC was performed under the following conditions: eluent, *n*-hexane (98)/2-propanol (2); flow rate, 1 mL min^{-1}; detection at 217 nm.

(*S*)-2-Benzyl-3-hydroxypropanoic acid ((*S*)-4). The crude (*S*)-**5** from the enzymic resolution of **5** was treated again with α-CT until 15 mL of 0.2 N NaOH was consumed. The solution was extracted with ether several times. The combined organic layer was washed with 5% $NaHCO_3$, brine, dried over $MgSO_4$, and then evaporated under reduced pressure to give optically pure (*S*)-**5**[30] whose optical purity was determined by HPLC using a chiral column as described above for (*R*)-**5** to obtain the % ee value of 98.6. To the solution of (*S*)-**5** (0.9 g, 4.9 mmol) in tetrahydrofuran (5 mL) and MeOH (1.5 mL) at 0 °C, was added dropwise aqueous solution of LiOH (0.13 g, 5.4 mmol). The resulting solution was stirred at room temperature for 12 h. The solution was acidified to pH 2 with 1 N HCl, extracted with ether (3×20 mL), dried over $MgSO_4$, and evaporated under reduced pressure. The residue was recrystallized to give (*S*)-**4**[29] (0.6 g, 70%): mp 68.5 °C; $[\alpha]_D = -22.3°$ ($c = 1.5$, MeOH).

(*S*)-3-Benzyl-2-oxetanone ((*S*)-1). To a solution of triphenylphosphine (2.65 g, 10 mmol) in THF (30 mL) was added dropwise dimethyl azodicarboxylate (3.7 mL of 40% solution in toluene, 10 mmol) at −78 °C and stirred for 20 min. (*S*)-2-Benzyl-3-hydroxypropionic acid (1.3 g, 7.21 mmol) in THF (15 mL) was added. The resulting mixture was stirred at −78 °C for 1 h, and then allowed to warm to room temperature under stirring for 4 h. The mixture was evaporated under reduced pressure to give a reddish oil which was purified by column chromatography (hexane:ethyl acetate = 5:1) to yield (*S*)-**1** as an oil (880 mg, 75%): $[\alpha]_D$ +95.0° ($c = 0.88$, $CHCl_3$); IR (KBr) 1821 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) 7.18–7.36 (m, 5H), 4.32 (m, 1H), 3.98–4.08 (m, 2H), 3.08–3.21 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) 171.5, 137.2, 129.3, 129.0, 127.6, 64.8, 53.4, 34.1; HRMS-FAB *m/z*: $(M+1)^+$ calcd for $C_{10}H_{10}O_2$, 163.0759, found, 163.0761.

(*R*)-3-Benzyl-2-oxetanone ((*R*)-1). was prepared in a similar fashion starting with (*R*)-2-benzyl-3-hydroxypropionic acid and obtained in an overall yield of 78%: $[\alpha]_D$ −99.0° ($c = 0.58$, $CHCl_3$); HRMS-FAB *m/z*: $(M+1)^+$ calcd for $C_{10}H_{10}O_2$, 163.0759, found, 163.0754.

Kinetics

General. α-CT and Suc-Ala-Ala-Pro-Phe-*p*NA were purchased from Sigma Chemical Co. Tris buffer (0.05 M, pH 7.5) containing 0.05 M $CaCl_2$ was used for the enzyme assay. All solutions were prepared by dissolving in doubly distilled and deionized water. Stock assay solutions were filtered through a membrane filter before use. The enzyme activity was measured by monitoring the change in absorbance at 410 nm caused by *p*-nitroanilide that was generated by the enzymic hydrolysis of Suc-Ala-Ala-Pro-Phe-*p*NA (substrate). The stock solutions of the substrate (5 mM) and **1** were prepared in DMSO solution.

Competitive substrate assay for the determination of K_M and k_a values. α-CT (15 nM) was added to a 20% DMSO solution of the Tris buffer (pH 7.5) containing

Table 1. Kinetic parameters[a]

Compound	k_a (min^{-1})	K_M (μM)	K_S (μM)	k_a/K_M (M^{-1} min^{-1})	k_d (min^{-1})	k_{cat} (min^{-1})
(*R*)-**1**	1.15±0.05	167±32	196±11	6890	0.152±0.005	0.134±0.008
(*S*)-**1**	1.64±0.03	39.4±4.1	83±6.6	41,600	0.112±0.001	0.105±0.004

[a]Data represent the mean of three experiments.

the substrate (400 μM) and **1** of specified concentrations (112 to 672 μM). The change in absorbance at 410 nm was recorded on a UV spectrophotometer (HP 8453) for 150–250 s and plotted against time to obtain progress curves (Fig. 5). The pseudo-first order rate constants (k_{obs}) were calculated by fitting the progress curves to equation 1. Values of K_M, and k_a were obtained from the plots of $1/k_{obs}$ versus $1/[I]_0$ (Fig. 6) according to eq. 2:

$$A_{410\,nm} = v_S.t + (v_0 - v_S)\cdot\left(1 - e^{-k_{obs}\cdot t}\right)/k_{obs} + c \quad (1)$$

$$1/k_{obs} = 1/k_a + [K_M\cdot(1 + [S]_0/K_M)]/(k_a\cdot[I]_0) \quad (2)$$

Determination of k_d values

To a mixture of proflavin solution in the pH 7.5 Tris buffer (100 μL, 100 μM) and α-CT stock solution in the same Tris buffer (1.0 mL, 100 μM) was added a solution of **1** in DMSO (10 μL, 10 mM) at 25 °C (the final concentrations of proflavin, α-CT, and **1** were 9, 90, and 90 μM, respectively). The change in absorbance at 466 nm was followed and plotted against time to have progress curve (Fig. 8). Replot of the increasing absorbance versus time in a semilogarithmic manner gave a straight line the slope of which corresponds k_d.

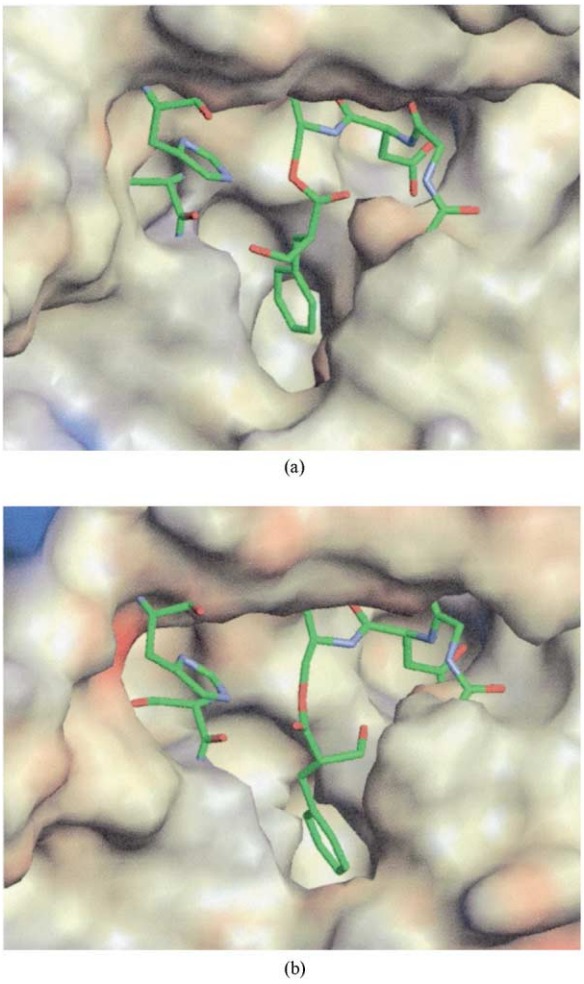


Figure 9. Computer generated models of the active site of α-CT whose Ser-195 hydroxyl is acylated by (a) (*R*)-**1** and (b) (*S*)-**1**.

Determination of K_S values

K_S values were estimated from the semireciprocal plot of the initial velocity versus the concentration of **1** (Fig. 7) according to the method of Dixon. Two concentrations of the substrate were used (100 and 200 μM). Typically, the enzyme stock solution was added to various concentrations of **1** (0 to 200 μM) in 20% DMSO solution of the Tris buffer (pH 7.5) (1 mL cuvette), and the initial rates were measured immediately using a microcomputer-interfaced UV spectrometer.

Active site protection test

To premixed solutions of **1** (500 and 200 μM for (*R*)-**1** and (*S*)-**1**, respectively) and varying concentrations of Suc-AAPF-*p*NA (125, 180, and 250 μM) in 20% DMSO solution of the Tris buffer (pH 7.5) was added α-CT (25 nM), and the remaining enzymic activity was monitored at 410 nm at 25 °C, and the activity remaining was plotted against time (Fig. 4). The rate of inactivation was decreased as the concentration of the substrate increased.

Electrospray ionization mass spectrometry

Electrospray ionization mass spectra were recorded on a VG Quattro quadrupole mass spectrometer equipped with an electrospray interface. α-CT (80 μM) was incubated in the Tris buffer (pH 7.5) with a 50-fold excess of **1** for 10 min, and then diluted with a 50% aqueous acetonitrile solution containing 0.5% formic acid to have 40 pmol L^{-1} of α-CT substrate covalent complex (an intermediate in the α-CT catalyzed hydrolysis of **1**). A sample solution (10 μL) thus obtained was introduced into the electrospray source using a loop injector. Mass spectra were typically obtained from 20 scans over the range of 900 to 1600 M_r. Mass spectra were acquired with a cone voltage of 31 V and a source temperature of 75 °C. The instrument was calibrated with horse heart myoglobin. Electrospray ionization mass spectra of both α-CT and **1** complexes in Figure 3 were calibrated with α-CT.

Molecular modeling

The initial geometries of the complexes were generated by superimposing hydrocinnamaldehyde on the X-ray crystal structure of L-[1-acetamido-2-(*p*-chlorophenyl) ethyl]boronic acid[31] bound to α-CT and a hydroxymethyl group was added to the hydrocinnamaldehyde at the 2-position. The hydroxyl group of Ser-195 and the carbonyl carbon of the hydrocinnamaldehyde were covalently bound to form an ester bond and then the L-[1-acetamido-2-(*p*-chlorophenyl)ethyl]boronic acid was removed from the complexes.

The simulations were performed with the Discover program, version 2.9.0 (MSI, San Diego, CA, USA) on a Silicon Graphics Indigo 2 computer, using the CVFF force field. A dielectric constant of 1 was used in all calculations. The energy of the system was minimized with respect to all $3N$ Cartesian coordinates until the maximum derivative of 0.1 kcal mol^{-1} Å^{-1} was reached. The resulting structure was used as the starting point for the molecular dynamics calculations. The complex was subjected to a simulated annealing in which the complex was heated from 0 to 300 K in 10 ps, equilibrated for 10 ps, and then cooled to 0 K in 10 ps. The selected structures were minimized using steepest descents until the maximum derivative was less than 5.0 kcal mol^{-1} Å^{-1}, and then using a conjugate gradient, until the maximum derivative was less than 0.1 kcal mol^{-1} Å^{-1}.

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References and Notes

1. Wharton, C. W. In *Comprehensive Biological Catalysis*; Sinnott, M. Ed.; Academic Press: New York, 1998; Vol. 1, Chapter 9.
2. Bernstein, P. R.; Edwards, P. E.; Williams, J. C. *Prog. Med. Chem.* **1994**, *31*, 59.
3. Ripka, W. C.; Vlasuk, G. R. *Ann. Rep. Med. Chem.* **1997**, *32*, 71.
4. Taylor, J. C.; Mittman, C., Eds; *Pulmonary Emphysema and Proteolysis: 1986*; Academic Press: New York, 1987; p 1.
5. Warshel, A.; Naray-Szabo, G.; Sussman, F.; Hwang, J.-K. *Biochemistry* **1989**, *28*, 3629.
6. For example: Silverman, R. B. *The Organic Chemistry of Drug Design and Drug Action*, Academic Press: New York, 1992; p 172.
7. (a) Kranz, A.; Spencer, R. W.; Tam, T. F.; Thomas, E.; Copp, L. J. *J. Med. Chem.* **1987**, *30*, 591. (b) Reed, P. E.; Katzenellenbogen, J. A. *J. Biol. Chem.* **1991**, *266*, 13.
8. Kim, D. H.; Ryoo, J. J. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1287.
9. Yang, H. W.; Romo, D. *Tetrahedron* **1999**, *55*, 6403.
10. Lee, J.; Lee, J.; Kim, J.; Kim, S. Y.; Chun, M. W.; Cho, H.; Hwang, S. W.; Oh, U.; Park, Y. H.; Marquez, V. C.; Beheshti, M.; Szabo, T.; Blumberg, P. M. *Bioorg. Med. Chem.* **2001**, *9*, 19.
11. Arnold, L.; Kalantar, T. H.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 7105.
12. It is highly unlikely that the intermediates detected by the ESI-MS would be Michaelis–Menten complexes considering the high K_S values estimated for the complexes (Table 1).
13. (a) One of the referees has called our attention to the possibility that the product generated by the C_4 attack may undergo a retro-aldol type reaction catalyzed by a base at the active site (possibly His-57) to form an α,β-unsaturated acid with regeneration of the catalytic activity of the α-CT. However, this possibility is thought to be unlikely, considering the precedents reported in the literature: Li, M.; Luo, W.; White, E. H. *Arch. Biochem. Biophys.* **1995**, *320*, 135. (b) Kim, D. H.; Li, Z.-H. *Bioorg. Med. Lett.* **1994**, *4*, 2297. (c) Kim, Y. J.; Li, Z.-H.; Kim, D. H.; Hahn, J. H. *Bioorg. Med. Chem. Lett.* **1996**, *13*, 1449.
14. K_M denotes the binding constant for the overall acylation process defined by $k_{+1}/(k_{-1}+k_a)$.
15. Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245.
16. DelMar, E. G.; Largman, C.; Brodrick, J. W.; Giokas, M. C. *Anal. Biochem.* **1979**, *99*, 316.
17. Dixon, M. *Biochem, J.* **1953**, *55*, 170.
18. Bernhard, S. A.; Lee, B. F.; Tashjian, Z. H. *J. Mol. Biol.* **1966**, *18*, 405.
19. The α-CT catalyzed β-lactone ring cleavage reaction may be represented by the Scheme 2, in which K_M represents the Michaelis–Menten constant $(k_{+1}/(k_{-1}+k_a))$, K_s the dissociation constant of the E·S complex (k_{+1}/k_{-1}), k_a the rate constant for acylation reaction, and k_d the rate constant for deacylation reaction. The k_{cat} denotes the second order rate constant for the overall reaction.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E{\cdot}S \xrightarrow[k_a]{} E\text{-}S \xrightarrow[k_d]{} E + P$$

Scheme 2.

20. Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, 1985; p 201.
21. Li, Z.-H.; Bulychev, A.; Kotra, L. P.; Massova, I.; Mobashery, S. *J. Am. Chem. Soc.* **1998**, *120*, 13003.
22. (a) Hein, G.; Niemann, C. *J. Am. Chem. Soc.* **1962**, *84*, 4495. (b) Lanonde, J. J.; Bergbreiter, D. E.; Wong, C.-H. *J. Org. Chem.* **1988**, *53*, 2323. (c) Baek, D.-J.; Reed, P. E.; Daniels, S. B.; Katzenellenbogen, J. A. *Biochemistry* **1990**, *29*, 4305.
23. Zerner, B.; Bond, R. P. M.; Bender, M. L. *J. Am. Chem. Soc.* **1964**, *86*, 3674.
24. Blackburn, G. M.; Dodds, H. L. H. *J. C. S. Perkin 2* **1974**, 377.
25. The β-lactone ring cleavage reaction that takes place at the active site of α-CT is faster by three orders of magnitude than that occurring in aqueous medium, but this may not be unreasonable considering that reactions at the enzyme active site might be treated as an intramolecular in nature (Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, 1985; p 56).
26. Lall, M. S.; Karvellas, C.; Vederas, J. C. *Org. Lett.* **1999**, *1*, 803.
27. Otto, H.-H.; Schirmeister, T. *Chem. Rev.* **1997**, *97*, 133.
28. Fincham, C. I.; Higginbottom, M.; Hill, D. R.; Horwell, D. C.; O'Toole, J. C.; Ratcliffe, G. S.; Rees, D. C.; Roberts, E. *J. Med. Chem.* **1992**, *35*, 1472.
29. Monteil, T.; Danvoy, D.; Plaquevent, J.-C.; Duhamel, L.; Duhamel, P.; Gros, C.; Schwartz, J.-C.; Lecomte, J.-M. *Synth. Commun.* **2001**, *31*, 211.
30. Peet, N. P.; Lentz, N. L.; Dudley, M. W.; Ogden, A. M. L.; McCarty, D. R.; Racke, M. M. *J. Med. Chem.* **1993**, *36*, 4015.
31. Stoll, V. S.; Eger, B. T.; Hynes, R. C.; Martichonok, V.; Jones, J. B.; Pai, E. F. *Biochemistry* **1998**, *37*, 451.