Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 1613–1622

BIOORGANIC & MEDICINAL CHEMISTRY

Design of Mechanism-Based Carboxypeptidase A Inactivators on the Basis of the X-ray Crystal Structure and Catalytic Reaction Pathway[1]

Kyung Joo Lee and Dong H. Kim*

Department of Chemistry and Center for Biofunctional Molecules, Pohang University of Science and Technology, San 31 Hyojadong, Pohang 790-784, Korea

Received 2 March 1998; accepted 10 April 1998

Abstract—The X-ray crystal structure of the complex of carboxypeptidase A (CPA) and Gly-Tyr, has been documented. The crystal structure reveals that both the amide carbonyl oxygen and the terminal amino nitrogen of Gly-Tyr coordinate to the active site zinc ion of CPA in a bidentate fashion, whereby the zinc-bound water molecule is displaced by the amino group. As to the catalytic mechanism of CPA, it is generally believed that while in the cases of ester substrates the carboxylate of Glu-270 functions as the nucleophile which attacks the scissile carbonyl carbon (anhydride pathway), in the case of peptide substrates the zinc-bound water molecule attacks the scissile peptide bond (general base pathway). In light of the X-ray crystal structure and the proposed catalytic mechanism for the enzyme, it is envisioned that the ester bond of *O*-(hydroxyacetyl)-L-β-phenyllactic acid (L-**1**) would be hydrolyzed by the attack of the carboxylate of Glu-270 to generate an anhydride intermediate. The latter intermediate would then undergo an intramolecular rearrangement initiated by the attack of the hydroxyl to result in to form an ester bond with the Glu-270 carboxylate. This ester formation impairs the catalytic activity of CPA. We have demonstrated using kinetic analysis that L-**1** is indeed an inactivator for the enzyme having the k_{inact}/K_I value of $0.057\,M^{-1}\,s^{-1}$. We have also demonstrated that *N*-(hydroxyacetyl)-L-phenylalanine (L-**2**) inactivates the enzyme with the k_{inact}/K_I value of $0.071\,M^{-1}\,s^{-1}$, suggesting that the carboxylate becomes to attack the peptide carbonyl carbon to generate the same anhydride intermediate as that formed in the inactivation of CPA by L-**1**. The formation of the anhydride intermediate rather than a tetrahedral transition state that is expected for peptide type substrates was envisioned to occur on the ground that the zinc-bound water molecule is displaced by the hydroxyl of L-**2** upon binding to the enzyme.

Introduction

Proteases are an important class of enzymes, playing key roles in a wide variety of biological processes such as the digestion of ingested food, clotting of blood, control of blood pressure and the production of hormones.[2] Their aberrant function, usually in the direction of excessive activity, leads to the development of physical and mental ailment. Accordingly, inhibitors of proteases are important as therapeutic agents, and development of design methodologies of inhibitors for proteases have received much attention.[3] Of these proteases, carboxypeptidase A (CPA)[4] bears a special importance. As a prototypical zinc containing proteolytic enzyme, it has served as a target enzyme in the development of new inhibitor design strategies[5] which can be useful for zinc proteases of medicinal interest.[6] In this report we wish to describe a new design strategy which we have developed for CPA on the bases of the X-ray crystallographic structure of the enzyme complexed with a slowly hydrolyzing substrate and a catalytic mechanism proposed for the enzymic reaction.

*Corresponding author. E-mail: dhkim@vision.postech.ac.kr

0968-0896/98/$—see front matter
PII: S0968-0896(98)00082-0

Results and Discussion

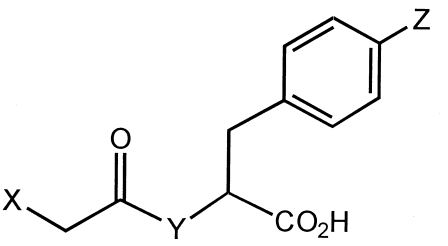
Design rationale

Carboxypeptidase A is a zinc containing proteolytic enzyme which selectively cleaves C-terminal amino acid residue having a hydrophobic side chain.[4] The active site of CPA consists of a hydrophobic pocket (primary substrate recognition site) which is primarily responsible for the substrate specificity, a guanidinium moiety of Arg-145 which forms hydrogen bonds to the carboxylate of substrate, and Glu-270 whose carboxylate plays a critical role functioning either as a nucleophile to attack the scissile carboxamide carbonyl carbon of the substrate or as a base to activate the zinc bound water molecule, which in turn attacks the scissile peptide bond. The active site zinc ion is coordinated to the backbone amino acid residues of His-69, Glu-72, His-196, and a molecule of water.[7] Upon binding a substrate to the enzyme, the scissile amide carbonyl oxygen is coordinated to the zinc ion, whereby the carbonyl carbon becomes an electrophilic center.

Gly-Tyr is a slowly hydrolyzing substrate for CPA with k_{cat} value of $0.9\,min^{-1}$.[8] The X-ray crystal structure of the CPA complexed with Gly-Tyr reveals that both the amide carbonyl oxygen and terminal amino nitrogen of Gly-Tyr bind to the active site zinc ion of CPA in a bidentate fashion.[9] The X-ray crystal structure also reveals that the terminal carboxylate is hydrogen bonded to the guanidinium moiety of Arg-145 and the benzyl group is anchored in the primary substrate recognition pocket at the active site of the enzyme. The zinc bound water molecule in the native CPA is displaced by the terminal amino group.

In general, proteases catalyze the hydrolysis of esters as well, often with a rate acceleration higher than that for peptides. The detailed catalytic mechanism of CPA remains to be established but it is generally believed that the hydrolysis of ester substrates by CPA takes place with an involvement of an anhydride intermediate which is formed by the nucleophilic attack of the carboxylate of Glu-270 on the ester carbonyl carbon (anhydride pathway).[10] On the other hand, in the hydrolysis of peptide substrate the zinc bound water molecule which is activated by the carboxylate of Glu-270 attacks on the scissile carboxamide carbonyl carbon with the generation of a transient tetrahedral intermediate that collapses to products (general base pathway).[11]

On the basis of the X-ray structural data of the CPA·Gly-Tyr complex and the catalytic mechanism proposed for the hydrolysis of ester substrates,[10] we have envisioned that *O*-(hydroxyacetyl)-L-β-phenyllactic acid (L-**1**) would be a potential mechanism-based inactivator for CPA. The compound is expected to bind to the active site of the enzyme in light of their structural similarity to Gly-Tyr: Expectedly, its terminal carboxylate forms hydrogen bonds with the guanidinium moiety of backbone Arg-145 residue and the benzyl aromatic ring fits in the primary substrate recognition pocket of the enzyme. There then would follow the coordination of both the carbonyl oxygen and the terminal hydroxyl to the active site zinc ion of the enzyme. In this complex, the catalytic carboxylate of Glu-270 would attack the scissile ester carbonyl carbon of the ligand to generate an anhydride intermediate with expulsion of the β-phenyllactic acid. The anhydride intermediate thus formed may then undergo an intramolecular rearrangement initiated by the attack of the hydroxyl group at the anhydride carbonyl carbon as depicted in Figure 1. The rearrangement reaction would result in to modify covalently the catalytically essential carboxylate of Glu-270 in the form of an ester, causing to an impairment of the enzymic activity of CPA (Fig. 1).[12]



$X = NH_2$, $Y = NH$, $Z = OH$; Gly-L-Tyr
$X = OH$, $Y = O$, $Z = H$; **1**
$X = OH$, $Y = NH$, $Z = H$; **2**
$X = OH$, $Y = NCH_3$, $Z = H$; **3**
$X = BnO$, $Y = O$, $Z = H$; **4**
$X = BnO$, $Y = NH$, $Z = H$; **5**

N-(Hydroxyacetyl)-L-phenylalanine (L-**2**) was thought to be a ligand of interest. As a peptide, the CPA-catalyzed hydrolysis of it may proceed by the general base mechanism, but since in the CPA–L-**2** complex the zinc-bound water molecule is expected to be replaced by the hydroxyl group of the ligand, there is present no longer nucleophilic water molecule in this complex, and as a consequence the carboxylate of Glu-270 may attack the scissile amide carbonyl carbon. If this would take place, then one can expect that the generation of the same anhydride intermediate as that postulated for L-**1**, leading to inactivation of CPA (Fig. 1). We have also evaluated the analogues of these two potential inactivators, in which the terminal hydroxyl is replaced with a benzyloxy group as an effort to verify the proposed design rationale.

Synthesis

Compound **1** was synthesized by allowing benzyloxyacetyl chloride to react with β-phenyllactic acid benzyl ester in the presence of pyridine in THF followed by hydrogenolysis to remove the benzyl moieties (Scheme 1). Compound **4** was prepared by the treatment of benzyloxyacetyl chloride with L-β-phenyllactic acid (Scheme 1).

Coupling of glycolic acid with phenylalanine methyl ester using DCC followed by alkaline hydrolysis afforded **2** (Scheme 2).

Benzyloxyacetyl chloride was allowed to react with phenylalanine methyl ester in the presence of pyridine to give **10** which was then treated with alkaline solution to afford **5**. Similarly, coupling of benzyloxyacetyl chloride with L-*N*-methylphenylalanine[13] gave the

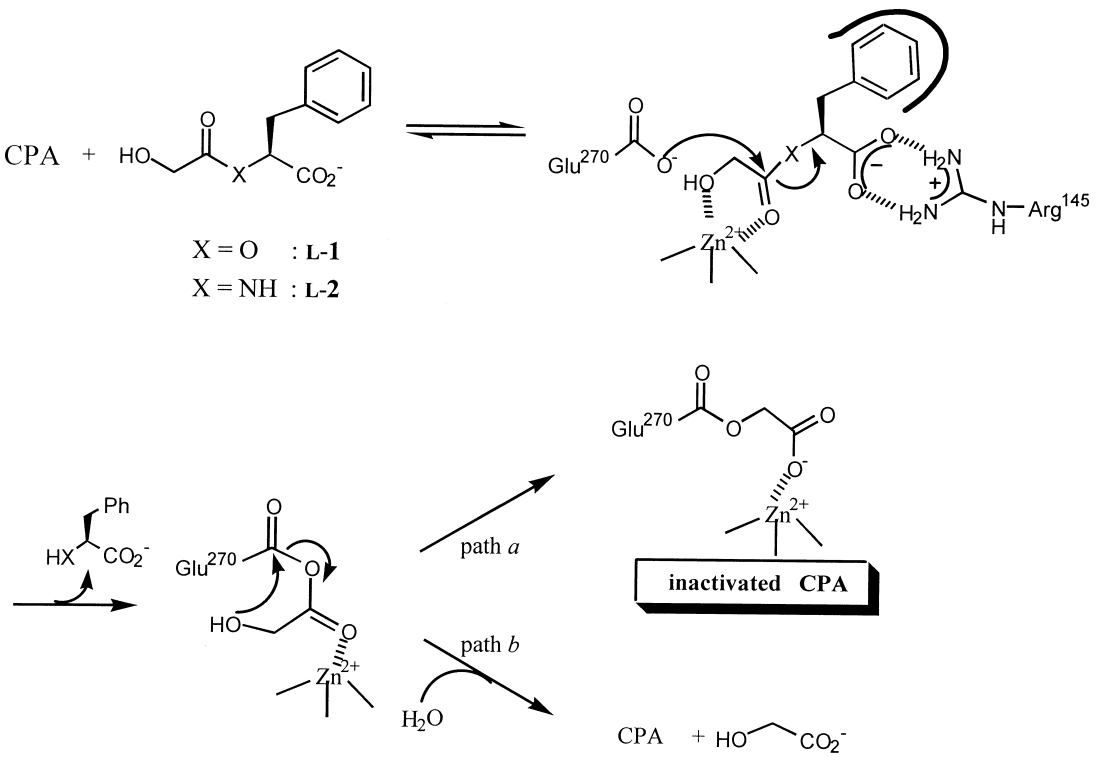
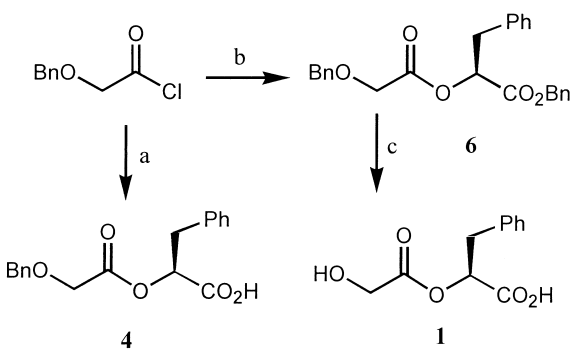


Figure 1. Schematic illustration of the rationale used for designing L-**1** and L-**2** as mechanism-based inactivators for CPA.

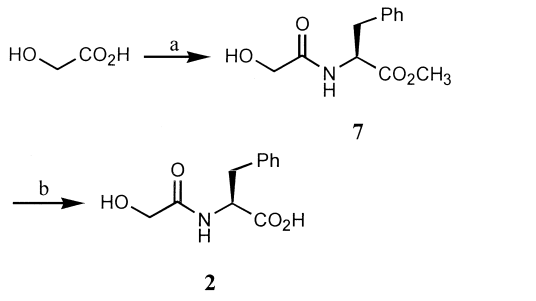


Scheme 1. Reagents and conditions: (a) L-β-Phenyllactic acid, pyridine, cat. DMAP, THF, 80%; (b) L-β-Phenyllactic acid benzyl ester, pyridine, THF, 81%; (c) H_2, Pd/C (1 atm), MeOH, quant.

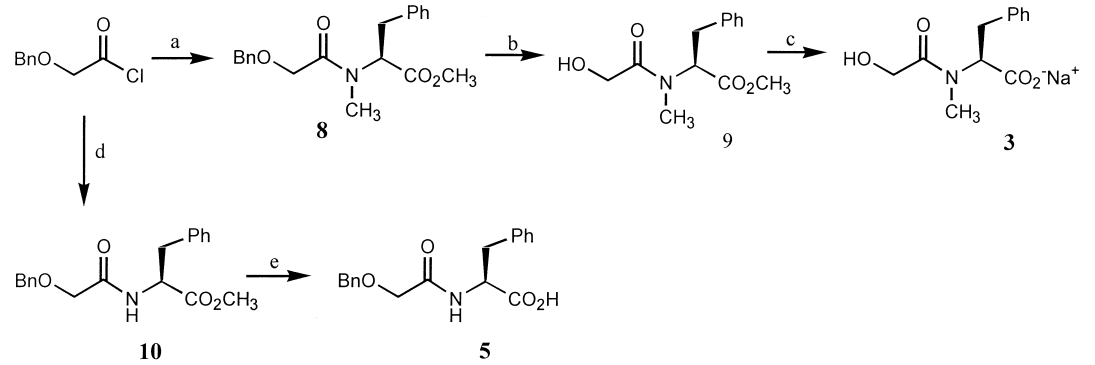
N-methylphenylalanine **8** which was debenzylated and hydrolyzed to give **3** as a sodium salt (Scheme 3).

Kinetics

Enzymic assay and inhibition kinetics were carried out at 25 °C in Tris buffer (0.05 M) of pH 7.5 using *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactic acid (Cl-CPL)[14] as the substrate. Semilogarithmic plot of the percent activity remaining versus time at different concentrations of inhibitor gave straight lines in the cases of L-**1** and L-**2** (Fig. 2), suggesting that the inhibition of CPA by these compounds occurs in an irreversible fashion. Their enantiomers failed to show the time-dependent loss of the enzymic activity. The rate of each inhibition was reduced in the presence of 2-benzylsuccinic acid,[15] a well known potent competitive inhibitor for CPA, suggesting that these inhibitors bind to the active site of CPA as 2-benzylsuccinic acid does (Fig. 3). Kinetic parameters for the irreversible inhibition, K_I and k_{inact} were estimated according to the method of Kitz and Wilson[16] as reported[17] previously (Fig. 4) and are listed in Table 1. The first order rate constant can be expressed as $k_{obs} = k'[\text{inhibitor}]^n$, where n is the order of inhibitor in its reaction with enzyme.[18] Thus, the slope in the plot of log k_{obs} versus log [inhibitor] corresponds to n. There were obtained slopes of 0.81 and 0.85 for L-**1** and L-**2**, respectively, to suggest that the inhibitions occur in a 1:1



Scheme 2. Reagents and conditions: (a) L-Phe-OCH_3, HCl, DCC, HOBt, NEM, THF, 45%; (b) 1N LiOH, MeOH, 90%.



Scheme 3. Reagents and conditions: (a) *N*-Methyl-L-Phe-OCH_3, Et_3N, THF, 84%; (b) H_2, Pd/C, MeOH, 90%; (c) 1N NaOH, MeOH, 90%; (d) L-Phe-OCH_3-HCl, pyridine, THF, 75%; (e) 1N LiOH, MeOH, 95%.

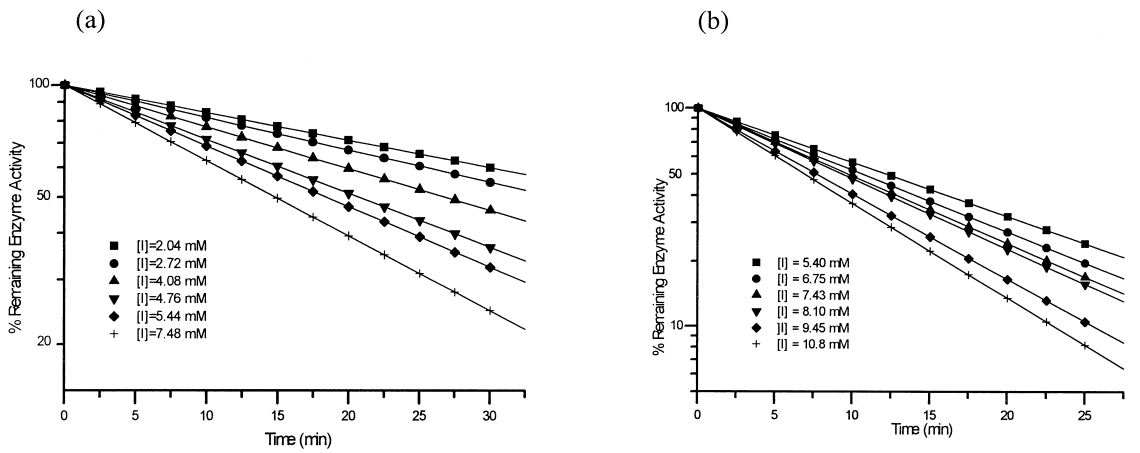


Figure 2. Loss of enzyme activity of CPA as a function of incubation time.

stoichiometry (Fig. 5). The involvement of the terminal hydroxyl group of the inhibitors in the irreversible inhibition of the enzyme was supported by the observation that *O*-(benzyloxyacetyl)-L-β-phenyllactic acid (**4**) and *O*-(benzyloxyacetyl)-L-Phe (**5**) failed to inactivate the enzyme. The inactivated enzyme failed to regain its enzymic activity upon dialysis for 2 days, confirming the irreversible nature of the inhibitions. The partition ratio of the inhibition, which is a measure of the efficiency of an inactivator was determined by the titration method[19] (Fig. 6) and included in Table 1. All the substrate analogues including **1** and **2** synthesized for the present study were evaluated as competitive inhibitors for CPA, and their reversible inhibitory constants (K_i) which reflect the affinity of the inhibitor for the enzyme were calculated from the respective Dixon plot[20] and listed in Table 1.

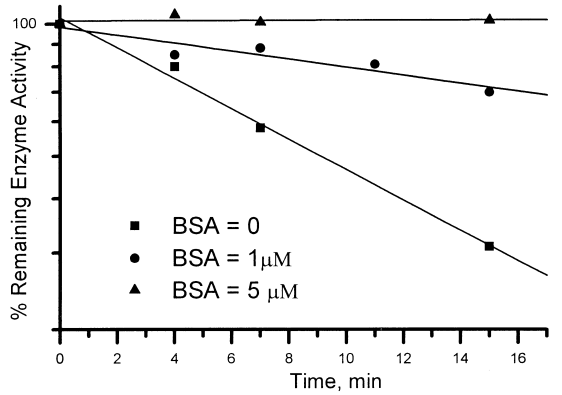


Figure 3. The rate of inactiviation by L-**1** was reduced by the presence of benzylsuccinic acid.

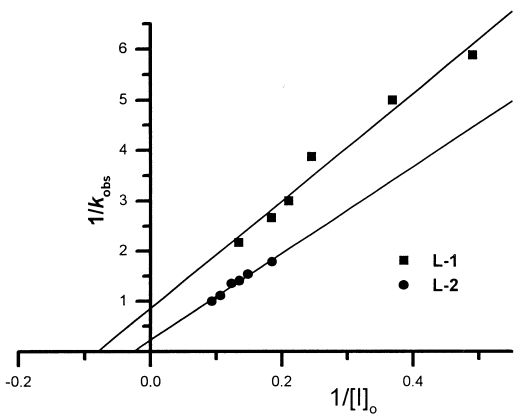


Figure 4. Double reciprocal plot of k_{obs} versus [inactivator]$_o$. Values of k_{inact} and K_I were calculated from y intercept and the slope, respectively.

Table 1. Kinetic parameters in the inhibition of CPA

Inhibitor	K_i (μM)	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I ($M^{-1}s^{-1}$)	Partition ratio
L-**1**	400	0.012	3.51	0.057	1246
D-**1**	2,000[a]	—	—	—	—
L-**2**	1,320	0.044	10.3	0.071	1234
D-**2**	1,800	—	—	—	—
L-**3**	7,000[a]	—	—	—	—
L-**4**	4.7	—	—	—	—
L-**5**	140	—	—	—	—

[a]Approximate values due to poor binding ability.

Discussion

As described above, substrate analogue L-**1** was designed as a mechanism-based inactivator for CPA on the basis of the X-ray crystal structure of the CPA complexed with Gly-Tyr, a slowly hydrolyzing substrate[9] and a proposed mechanism[10] of the catalytic action. The kinetic analyses (Table 1) together with experimental observations presented above suggest

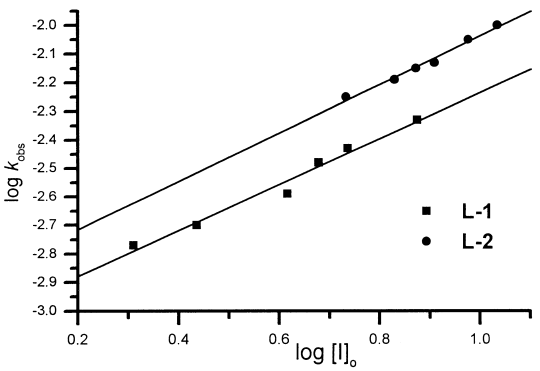


Figure 5. Determination of the reaction order in the inactivation of CPA by L-**1** and L-**2**.

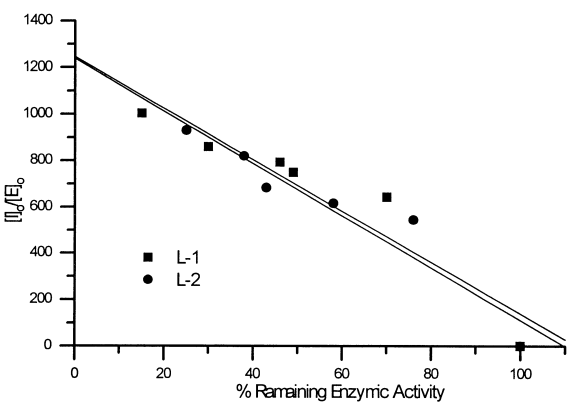


Figure 6. Determination of partition ratio by the titration method.

strongly that L-**1** is indeed a mechanism-based inactivator for CPA, suggesting that the design rationale employed is valid (Fig. 1). There may be a question of stability of the newly generated ester linkage which modifies the catalytic carboxylate of Glu-270 towards hydrolysis by bulk water. In this regard, it should be noted that our previous works[17,21] as well as a recent report of Massova et al.[22] which demonstrate that the covalently modified CPA at the carboxylate of Glu-270 in the form of an ester is highly stable and resists to be hydrolyzed at pH even at 10.

We found that peptide L-**2** is also an effective inactivator for CPA, suggesting that the carboxylate of Glu-270 functions as the nucleophile which attacks the peptide bond in the absence of the zinc-bound water molecule as discussed in the design rationale. This explanation is consistent with the conclusion derived from molecular dynamic calculations performed by Banci et al.[23] on a complex of CPA and Val-Leu-Phe-Phe. They reported that when the zinc-bound water molecule is displaced, the Glu-270 carboxylate becomes to attack the peptide carbonyl carbon to generate an anhydride intermediate.[23]

The high partition ratio exhibited by these inactivators suggests that the turn over process (path b) competes favorably with the inactivation, i.e. there appears to occur extensive hydrolytic cleavage of the anhydride intermediate by water with regeneration of the enzyme (Fig. 1). While it is generally desirable for mechanism-based inactivators to have a small partition ratio, there are present some important exceptions: the partition ratio[24] for sulbactam, a mechanism-based inactivator for β-lactamase is 1000–3000, yet it is currently being used clinically in conjunction with penicillins in treatment of bacterial infections. The order of inactivation reaction calculated from the plot of log k_{obs} versus log [I]o is 0.81 for L-**1** and 0.85 for L-**2**, indicating that CPA interacts with both inhibitors in a 1:1 stoichiometry in the inactivation. To confirm that the hydroxyl group in those inactivators is required for them to exhibit the inactivation property, compounds L-**4** and L-**5** in which the hydroxyl in L-**1** and L-**2** are replaced with a benzyloxy group were evaluated. Although their K_i values are almost 100 times lower than those for L-**1** and L-**2**, possibly due to anchoring of the second benzyl moiety in the hydrophobic S_2 pocket of CPA , they did not exhibit a time-dependent loss of enzymic activity up to the concentration of 15 mM. These observations are consistent with the proposition that the hydroxyl group in these inactivators is directly involved in the inactivation of CPA.[25] It has been known that CPA does not hydrolyze *N*-methylated peptides.[26] To gain an additional supportive evidence for the design rationale discussed above, compound **3**, a *N*-methylated analogue of **2** was prepared. Expectedly, **3** showed no time-dependent loss of CPA activity up to the concentration of 24 mM when incubated with CPA. The result supports the proposition that the hydrolytic cleavage of the amide or ester is the crucial step in the inactivation chemistry.

It is worthy noting that only **1** and **2** having the L-configuration are able to inactivate the enzyme and their mirror image forms in the D-series just competitive inhibitors. It appears that in the case of **2**, the stereochemistry is reflected in the first step of the inactivation, i.e. the cleavage of the amide bond because the K_i values of both enantiomers are comparable. On the other hand, in the case of **1**, one can not draw any conclusion because D-**1** is shown to bind the enzyme very poorly. We notice in Table 1 that unexpectedly the k_{inact} ($0.044\,min^{-1}$) for L-**2** is significantly larger than that ($0.012\,min^{-1}$) for L-**1**, but no explanation can be offered presently for the observation.

Conclusion

L-**1** and L-**2** are a new type of mechanism-based inactivators for CPA, which have been designed rationally on the basis of the X-ray crystal structure of the enzyme which is complexed with a slowly hydrolyzing substrate, Gly-Tyr and a catalytic mechanism proposed for the enzyme. In designing them we have exploited the subtle difference in physical and chemical properties between an amino and a hydroxyl group. When Gly-Tyr is bound to the enzyme, the nucleophilic carboxylate of Glu-270 becomes involved in an ionic interaction with the terminal amino group of Gly-Tyr, and as a result the carboxylate is no longer to function as a nucleophile. On the other hand, in the cases of **1** and **2**, the carboxylate is free to attack the electrophilic center of the ester or amide carbonyl carbon of the inactivators, leading to irreversible inhibition of the enzyme with a covalent modification. The design strategy demonstrated in this work may be of considerable interest to those who are engaged in the rational design of inhibitors for zinc-containing proteases.

Experimental

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and were uncorrected. ^1H NMR 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 BOMEM FT IR M100-C15 spectrometer. Mass spectra were obtained with KRATOS MS 25 RFA spectrometer. High resolution mass spectra were recorded on a JEOL JMX-HX110/110A by the Korea Basic Science Center, Taejon, Korea. Flash chromatography was performed on silica gel 60 (230–400 mesh) and thin layer

chromatography (TLC) was carried out on silica coated glass sheets (Merck silica gel 60 F-254). Elemental analyses were performed at the Basic Science Center, Kyungbook National University, Taegu, Korea.

***O*-(Benzyloxyacetyl)-L-β-phenyllactic acid benzyl ester (6).** To a solution of L-β-phenyllactic acid benzyl ester (0.5 g, 1.95 mmol) and pyridine (0.46 g, 6 mmol) in THF (10 mL) was added benzyloxyacetyl chloride (0.36 g, 2 mmol) dropwise at 0 °C and the mixture was stirred at room temperature for 8 h. The reaction mixture was diluted with ethyl acetate (30 mL), washed with 3 N HCl, saturated $NaHCO_3$ solution, and brine, then dried ($MgSO_4$) and evaporated in vacuo. The residue was purified by column chromatography to give the product as a colorless oil (0.64 g, 81%). IR (neat) 1755, 1725 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.10–3.27 (m, 2H, benzylic), 4.12 (d, $J = 7.2$ Hz, 2H), 4.54 (s, 2H), 5.17 (s, 2H), 5.40–5.43 (m, 1H, a-H), 7.18–7.36 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 38.3, 52.9, 67.7, 69.6, 73.8, 127.6–135.9, 170.0, 172.0.

***O*-(Hydroxyacetyl)-L-β-phenyllactic acid (L-1)**. Compound **6** (0.6 g) was dissolved in MeOH (5 mL) and was hydrogenated (1 atm) in the presence of 10% Pd-C (100 mg) at room temperature for 6 h. The mixture was filtered through a celite pad and washed with methanol. The filtrate was concentrated in vacuo to give L-**1** as a colorless oil in a quantitative yield. $[\alpha]_D = -23.0°$ (c 1, $CHCl_3$); IR (neat) 3000-3500 (br), 1750, 1720 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.10–3.32 (m, 2H), 4.16 (d, $J = 18$ Hz, 2H), 5.38 (m, 1H, α-H), 7.21–7.34 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 37.4, 60.8, 73.5, 127.8, 129.1, 129.6, 135.6, 167.3, 173.3; EI HRMS calcd for $C_{11}H_{12}O_5$: 224.0685, found: 224.0685.

***O*-(Hydroxyacetyl)-D-β-phenyllactic acid (D-1).** Compound D-**1** was prepared following the same procedure as for L-**1** starting with D-phenyllactic acid. $[\alpha]_D = +20.5°$ (c 1, $CHCl_3$)

***N*-(Hydroxyacetyl)-L-phenylalanine methyl ester (7).** To a stirred suspension of glycolic acid (2 g, 26.2 mmol), dicyclohexylcarbodiimide (5.4 g, 26.2 mmol), and hydroxybenzotriazole-hydrate (3.56 g, 26.2 mmol) in THF (30 mL) were added L-phenylalanine methylester hydrochloride (5.64 g, 26.2 mmol) and *N*-ethylmorpholine (3.4 mL, 26.2 mmol). The mixture was stirred at room temperature for 28 h, diluted with ethyl acetate (40 mL) and the precipitated dicyclohexylurea was removed by filtration. The filtrate was washed with 10% citric acid (20 mL×2), 10% sodium bicarbonate (20 mL×2) solution and finally with brine (20 mL×3). The organic extract was dried ($MgSO_4$) and evaporated in vacuo. The residue was purified by column chromatography (ethyl acetate/*n*-hexane = 1/1) to give the product as a yellow oil (2.79 g, 45%). IR (neat) 3200–3400, 1738, 1650, 1520 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.10–3.21 (m, 2H, benzylic), 3.73 (s, 3H), 4.08 (s, 2H, CH_2O), 4.89–4.95 (m, 1H, α-H), 6.81 (d, $J = 9$ Hz, amide NH), 7.10–7.32 (m, 5H).

***N*-(Hydroxyacetyl)-L-phenylalanine (L-2)**. A mixture of **7** (0.25 g, 1 mmol) and 1 N LiOH (2 mL) in methanol (10 mL) was heated at 40 °C for 1 h, then acidified with 3 N HCl to pH of 1. It was extracted with ethyl acetate (15 mL×3), washed with brine (10 mL×3), dried ($MgSO_4$), then concentrated in vacuo. The crude product was recrystallized from ethyl acetate (10% *n*-hexane) to give the product as a white solid (0.1 g, 45%). mp 116–118 °C; $[\alpha]_D = +31.2°$ (c 1.0, DMSO); ^1H NMR (DMSO-d_6) δ 2.96–3.11 (m, 2H, benzylic), 3.76 (s, 2H, CH_2O), 4.49–4.56 (m, 1H, α-H), 7.17–7.29 (m, 5H), 7.61 (d, $J = 8.1$ Hz, CON*H*); ^{13}C NMR (DMSO-d_6) δ 37.5, 53.0, 112, 127.9, 129.1, 130.0, 137, 172, 173; Anal. calcd for $C_{11}H_{13}NO_4$: C, 59.20; H, 5.87; N, 6.28. found: C, 59.63; H, 5.93; N, 6.74.

***N*-(Hydroxyacetyl)-D-phenylalanine (D-2).** Compound D-**2** was prepared following the same procedure as for L-**2** starting with D-phenylalanine. $[\alpha]_D = -34.0°$ (c 1, DMSO).

***N*-Benzyloxyacetyl-*N*-methyl-L-phenylalanine methyl ester (8).** To a solution of *N*-methyl-L-phenylalanine methyl ester (0.5 g, 2.6 mmol) and triethylamine (0.44 mL, 3.12 mmol) in THF (15 mL) was added benzyloxyacetyl chloride (0.41 mL, 2.6 mmol) dropwise at 0 °C and the mixture was stirred at room temperature for 5 h. The reaction mixture was diluted with ethyl acetate (30 mL), washed with 3 N HCl, saturated $NaHCO_3$ solution, and brine, then dried ($MgSO_4$) and evaporated in vacuo. The residue was purified by column chromatography (ethyl acetate/*n*-hexane = 1/2) to give the product as a colorless oil (0.6 g, 84%). This compound is a mixture of *cis* and *trans* amide (3:7 from the ^1H NMR). ^1H NMR ($CDCl_3$) δ 2.86 and 2.93 (s, 3H), 3.08 (m, 1H, benzylic), 3.39 (m, 1H, benzylic), 3.69–3.77 (s, 3H), 4.10 (d, $J = 3.0$ Hz, 2H), 4.44 (m, 2H), 4.90 (dd, $J = 11.2$, 5.3 Hz, α-H, 0.3H), 5.38 (dd, $J = 11.2$, 5.3 Hz, α-H, 0.7H).

***N*-Hydroxyacetyl-*N*-methyl-L-phenylalanine sodium salt (3).** A solution of **8** (0.5 g, 1.46 mmol) in methanol (10 mL) was hydrogenated (1 atm) in the presence of 10% Pd-C (100 mg) at room temperature for 6 h. The mixture was filtered through a Celite pad and washed with methanol. The filtrate was concentrated in vacuo to give *N*-hydroxyacetyl-*N*-methyl-L-phenylalanine methyl ester (**9**) in a quantitative yield. This product (**9**) was dissolved in methanol (10 mL) and 1 N NaOH (1.4 mmol) was added and stirred at room temperature

for 1 h. The mixture was concentrated in vacuo to give the product as a hygroscopic solid in a quantitative yield. This compound is also a mixture of *cis* and *trans* amide (4:6 from ^{1}H NMR). This product was isolated as a sodium salt. ^{1}H NMR (D_2O) δ 2.63 and 2.76 (s, 3H), 2.83–2.90 (m, 1H, benzylic), 3.08–3.25 (m, 1H, benzylic), 3.89 (t, J = 16 Hz, 1H), 4.09 (m, 1H), 4.65 (dd, J = 12.0, 4.5 Hz, α-H, 0.6 H), 4.65 (dd, J = 12.0, 4.5 Hz, α-H, 0.4 H); ^{13}C NMR (D_2O) δ 30.5 and 30.8, 35.1 and 35.6, 59.5 and 59.9, 61.6 and 63.8, 126.9, 127.3, 128.9, 129.2, 129.4, 138.7 and 138.9, 173.5 and 174.0, 176.5 and 177.7.

***O*-(Benzyloxyacetyl)-L-β-phenyllactic acid (4).** To a solution of L-β-phenyllactic acid (0.5 g, 3 mmol), *N*,*N*-dimethylaminopyridine (70 mg, 0.6 mmol) and pyridine (0.7 g, 9 mmol) in THF (10 mL) was added benzyloxyacetyl chloride (0.47 mL, 3 mmol) dropwise at 0 °C and the mixture was stirred at room temperature for 12 h. The reaction mixture was diluted with ethyl acetate (30 mL), washed with 3 N HCl, saturated $NaHCO_3$ solution, and brine, then dried ($MgSO_4$) and evaporated in vacuo. The residue was purified by column chromatography to give the product as a colorless oil (0.75 g, 80%). $[\alpha]_D = -18.2°$ (c 1.2, $CHCl_3$); IR (neat) 1755, 1722 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.13–3.34 (m, 2H, benzylic), 4.17 (d, J = 6.3 Hz, 2H), 4.55 (s, 2H), 5.41 (dd, J = 8.9, 4.0 Hz, 1H, α-H), 7.24–7.40 (m, 10H), 8.8 (br, 1H, CO_2H); ^{13}C NMR ($CDCl_3$) δ 37.5, 67.1, 73.1, 73.7, 127.7–137.2, 170.4, 174.9; EI MS 314 (M^+), 296, 208, 91.

***N*-(Benzyloxyacetyl)-L-phenylalanine (5).** To a solution of L-phenylalanine methyl ester (0.5 g, 2.3 mmol) and pyridine (0.55 g, 6.9 mmol) in THF (15 mL) was added benzyloxyacetyl chloride (0.44 g, 2.3 mmol) dropwise at 0 °C and the mixture was stirred at room temperature for 6 h. The reaction mixture was diluted with ethyl acetate (30 mL), washed with 3 N HCl, saturated $NaHCO_3$ solution, and brine, then dried ($MgSO_4$) and evaporated in vacuo to give *N*-(benzyloxyacetyl)-L-phenylalanine methyl ester (**10**) as a colorless oil. This product (**10**) was dissolved in MeOH (20 mL) and 1 N NaOH (5 mL) was added and the resulting mixture was stirred at room temperature for 2.5 h. The mixture was acidified with 3 N HCl, extracted with ethyl acetate (10 mL×2), washed with brine, dried ($MgSO_4$), then concentrated in vacuo. The crude product was recrystallized from ethyl acetate (30% *n*-hexane) to give the product as a white solid (0.36 g, 48%). mp 114–115 °C; $[\alpha]_D = +25.4°$ (c 1, $CHCl_3$); IR 1730, 1652 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.11–3.27 (m, 2H, benzylic), 3.98 (s, 2H), 4.49 (d, J = 5.8 Hz, 2H), 4.88 (m, 1H, α-H), 7.04 (d, J = 7.8 Hz, CON*H*), 7.15–7.38 (m, 10H); ^{13}C NMR ($CDCl_3$) δ 37.7, 52.9, 69.4, 73.8, 127.7–137.0, 170.8, 175.2. Anal. Calcd for $C_{18}H_{19}NO_4$: C, 69.00; H, 6.11; N, 4.47. Found: C, 69.48; H, 6.02; N, 4.66.

General remarks for kinetic experiments

All solutions were prepared by dissolving in doubly distilled and deionized water. Stock assay solutions were filtered before use. Carboxypeptidase A was purchased from Sigma Chemical Co. (Allan form, twice crystallized from bovine pancreas, aqueous suspension in toluene) and used without further purification. CPA stock solutions were prepared by dissolving the enzyme in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer solution and the concentrations were determined from the absorbance at 278 nm ($\varepsilon = 6.42\times10^4$ M^{-1} cm^{-1}). *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate (Cl-CPL) was synthesized according to the method reported by Suh et al.[14] and the decrease in the absorbance at 320 nm was followed at 25 °C. A Perkin-Elmer HP 8452 or HP8453 UV/VIS spectrometer was used in enzyme inhibition studies.

Determination of K_i

Initial velocities were calculated from the linear initial slopes of the change in absorbance where the amount of substrate consumed was less than 10%. The K_i values were then estimated from the semireciprocal plot of the initial velocity versus the concentration of the inhibitors according to the method of Dixon.[20] Two concentrations of the substrate (50 and 100 μM) of Cl-CPL were used. Typically, enzyme stock solution was added to various concentrations of inhibitors in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer (1 mL cuvette) , and the initial rates were measured immediately.

Determination of K_I and k_{inact}

Into the cuvette (1 mL) containing 595–635 μL of 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer solution, 300 μL of 1 mM solution of Cl-CPL in the same buffer (10% dimethyl sulfoxide, final concentration is 300 μM), 15–55 μL of 136 mM stock solution of inactivator in dimethyl sulfoxide (final concentrations are 2.0–7.5 mM for L-**1** and 5.4–10.8 mM for L-**2**) and 5–45 μL of dimethyl sulfoxide to make a total concentration of dimethyl sulfoxide to 10%, was added 50 μL of 8 nM solution of CPA in the Tris buffer (final concentration is 0.4 nM), and the change in absorbance at 320 nm was recorded over a time interval of 0–200 s. The values of k_{obs} were calculated from the progress curves using the computer assisted spectrophotometer and values of K_I and k_{inact} were calculated, respectively, from the slope and the y intercept of straight lines in Figure 4. In the calculation, the K_m values of 113 μM was used.

Active site protection test

To a preincubated solution of 1–10 μM benzylsuccinate and 1 μM of CPA in 0.05 M Tris/0.5 M NaCl, pH 7.5

buffer for 10 min was added an inactivator (final concentration is 15.6 mM for L-**1**, and 13.4 mM for L-**2**, dimethyl sulfoxide content: 10%) and the whole mixture was incubated at room temperature. At specified intervals, 50 μL samples of the incubation mixture were removed and added to a 950 μL assay mixture and the activity was monitored at 320 nm (Figure 3).

Determination of the partition ratios

A series of solutions (500 μL each) containing 14.5 μM of CPA and various molar equivalents of inactivators were prepared to give $[I]_o/[E]_o$ ratios of 0 to 1200 in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer, supplemented with 15% dimethyl sulfoxide and were stirred gently at 4 °C for 16 h. The incubation mixtures were then dialyzed in the same buffer solution at 4 °C for 28 h. Subsequently 50 μL samples of each solution were diluted into 450 μL of the same buffer. Then a 30 μL portion of each dilution was added to 970 μL of the assay mixture and the activity was measured immediately. Partition ratio was calculated from the plot of fraction of activity remaining versus $[I]_o/[E]_o$.

Acknowledgements

The authors wish to express their sincere thanks to the Pohang University of Science and Technology and the Korea Science and Engineering Foundation for the financial support of this work.

References and Notes

1. A preliminary account of this work has been published: Kim, D. H.; Lee, K. J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2607–2612.

2. (a) *The Enzymes*, Boyer, P. D., Academic Press, New York, 1971, Vol. III. (b) Fersht, A. *Enzyme Structure and Mechanism*, 2nd edn; W. H. Freeman Co.: Cambridge, England, 1984: pp 405–426.

3. (a) *Protease Inhibitors*; Barrett, A. J.; Salvesen, G. Eds.; Elsevier: Amsterdam, 1986. (b) Rich, D. H. In *Comprehensive Medicinal Chemistry*; Hansch, C.; Sammes, P. C.; Taylor, J. B. Eds.; Pergamon Press: Oxford, 1990; Vol. 2, pp 391–441. (c) Fischer, G. *Nat. Prod. Rep.* **1988**, 465–495. (d) Walpole, C. S. J.; Wrigglesworth, R. *Nat. Prod. Rep.* **1989**, 311–346.

4. (a) Quiocho, F. A.; Lipscomb, W. N. *Adv. Prot. Chem.* **1971**, *25*, 1–78. (b) Christianson, D. W.; Lipscomb, W. N. *Acc. Chem. Res.* **1989**, *22*, 62–69. (c) Lipscomb, W. N.; Sträter, N. *Chem. Rev.* **1996**, *96*, 2375–2433.

5. (a) Ondetti, M. A.; Rubin, R.; Cushman, D. W. *Science*, **1977**, *196*, 441–444. (b) Cushman, D. W.; Cheung, H. S.; Sabo, E. F.; Ondetti, M. A. *Biochemistry* **1977**, *16*, 5484–5941. (c) Patchett, A. A.; Harris, E.; Tristram, R. W.; Wyvratt, M. J.; Wu, M. T.; Taub, D.; Peterson, E. R.; Ikeler, T. J.; ten Broeke, J.; Payne, L. G.; Ondeyka, D. L.; Thorsett, E. D.; Greenlee, W. J.; Lohr, N. S.; Hoffsommer, R. D.; Joshua, H.; Ruyle, W. V.; Rothrock, J. W.; Aster, S. D.; Maycock, A. L.; Robinson, F. M.; Hirschman, R.; Sweet, C. S.; Ulm, E. H.; Gross, D. M.; Vassil, T. C.; Stone, C. A. *Nature* (London) **1980**, *288*, 280–283. (d) Gafford, J. T.; Skidgel, R. A.; Erdos, E. G.; Hersh, L. B. *Biochemistry* **1983**, *22*, 3265–3271. (e) Kim, D. H.; Guinosso, C. J.; Buzby, Jr., G. C.; Herbst, D. R.; McCaully, R. J.; Wicks, T. C. *J. Med. Chem.* **1983**, *26*, 394–403.

6. (a) Redshaw, S. In *Medicinal Chemistry*, 2nd edn, Roberts, S. M; Ganellin, C. R. Eds; Academic Press, San Diego, CA, 1993, pp 163–186. (b) Whal, R. C.; Dunlap, R. P. *Ann. Rep. Med. Chem.* **1989**, *25*, 177–184. (c) Hagman, W. K.; Lark, M. W.; Becker, J. *Ann. Rep. Med. Chem.* **1996**, *31*, 231–240.

7. Kim, D. H.; Kim, K. S.; Park, J. K. *Bull. Korean Chem. Soc.* **1994**, *15*, 805–807.

8. Izmiya, N.; Uchio, H. *J. Biochem.* **1959**, *46*, 235–245.

9. (a) Rees, D. C.; Lewis, M.; Honzatko, R. B.; Lipscomb, W. N.; Hardman, K. D. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 3408–3412. (b) Mackenzie, N. E.; Fagerness, P. E.; Scott, A. I. *J. Chem. Soc., Chem. Commun.* **1985**, 635–637. (c) Christianson, D. W.; Lipscomb, W. N. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 7568–7572.

10. (a) Makinen, M. W.; Yamamura, K.; Kaiser, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3882–3886. (b) Makinen, M. W.; Kuo, L. C.; Dymowski, J. J. *J. Biol. Chem.* **1979**, *254*, 356–366. (c) Kuo, L. C.; Makinen, M. W. *J. Biol. Chem.* **1982**, *257*, 24–27. (d) Suh, J.; Hong, S.-B.; Chung, S. *J. Biol. Chem.* **1986**, *261*, 7112–7114. (e) Suh, J.; Cho, W.; Chung, S. *J. Am. Chem. Soc.* **1985**, *107*, 4530–4535. (f) Sander, M. E.; Witzel, H. *Biochem. Biophys. Res. Commun.* **1985**, *132*, 681–687. (g) Britt, B. M.; Peticolas, W. L. *J. Am. Chem. Soc.* **1992**, *114*, 5295–5303.

11. (a) Breslow, R.; Wernick, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1303–1307. (b) Breslow, R.; Wernick, D. L. *J. Am. Chem. Soc.* **1976**, *98*, 259–261. (c) Nau, H.; Riordan, J. F. *Biochemistry* **1975**, *14*, 5285–5294. (d) Galdes, A.; Auld, D. S.; Vallee, B. L. *Biochemistry* **1983**, *22*, 1888–1893. (e) Galdes, A.; Auld, D. S.; Vallee, B. L. *Biochemistry* **1986**, *25*, 646–651. (f) Breslow, R.; Shepartz, A. *Chem. Lett.* **1987**, 1–4.

12. The slow hydrolysis of Gly-Tyr was attributed to the formation of a salt bridge between the carboxylate of Glu-270 and the terminal amino group of the ligand.[9]

13. Hass, G. M.; Neurath, H. *Biochemistry* **1971**, *10*, 3535–3540.

14. Suh, J.; Kaiser, E. T. *J. Am. Chem. Soc.* **1976**, *98*, 1940–1947.

15. (a) Beyers, L. D.; Wolfenden, R. *Biochemistry* **1973**, *12*, 2070–2078. (b) Beyers, L. D.; Wolfenden, R. *J. Biol. Chem.* **1972**, *247*, 606–608.

16. Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245–3249.

17. Lee, S. S.; Li, Z.-H.; Lee, D. H.; Kim, D. H. *J. Chem. Soc. Perkin Trans. 1*, **1995**, 2877–2882.

18. Levy, H. M.; Leber, P. D.; Ryan, E. M. *J. Biol. Chem.* **1963**, *238*, 3654–3659.

19. Silverman, R. *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC: Boca Raton, FL, 1988; Vol. I, pp 22–23.

20. Dixon, M. *Biochem. J.* **1953**, *55*, 170–171.

21. (a) Kim, D. H.; Kim, K. B. *J. Am. Chem. Soc.* **1991**, *113*, 3200–3202. (b) Yun, M.; Park, C.; Kim, S.; Nam, D.; Kim, S. C.; Kim, D. H. *J. Am. Chem. Soc.* **1992**, *114*, 2281–2282. (c) Ryu, S.-E.; Choi, H.-J.; Kim, D. H. *J. Am. Chem. Soc.* **1997**, *119*, 38–41.

22. Massova, I.; Martin, P.; Mel, S.; Tanaka, Y.; Edwards, B.; Mobashery, S. *J. Am. Chem. Soc.* **1996**, *118*, 12479–12480.

23. Banci. L.; Bertini, I.; La Penna, G. *Proteins: Structure, Funtions, Genetics* **1994**, *18*, 186–197.

24. Labia, R.; Lelievre, V.; Peduzzi, *J. Biochim. Biophys. Acta* **1980**, *611*, 351–357.

25. We observed that Gly-L-Phe and Gly-L-phenyllactic acid undergo exclusive hydrolytic cleavage when incubated with CPA, suggesting that they are not inhibitors but substrates for CPA.

26. Stahmann, M.; Fruton, J. S.; Bergmann, M. *J. Biol. Chem.* **1946**, *164*, 753–760.