Inhibition of Carboxypeptidase A with β-Lactone-bearing Phenylalanine. Design, Synthesis, and Stereochemistry-dependent Inhibition Mode

Mijoon Lee and Dong H. Kim*

Department of Chemistry, Division of Molecular and Life Sciences Pohang University of Science and Technology, San 31 Hyoja-dong, Pohang 790-784, Korea
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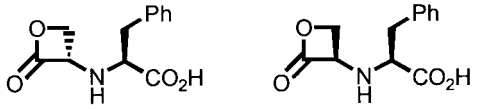
(3*S*,1'*S*)-3-(1'-Carboxy-2'-phenyl)ethylamino-2-oxetanone (**1a**) and (3*R*,1'*S*)-3-(1'-carboxy-2'-phenyl)ethylamino-2-oxetanone (**1b**) were designed, synthesized, and evaluated as inhibitors for carboxypeptidase A, a prototypical zinc protease that removes the C-terminal amino acid having an aromatic side chain from oligopeptide substrate. It was concluded from the analysis of inhibition kinetics that while **1a** inactivates CPA irreversibly, its diastereoisomer, **1b** is a weak competitive inhibitor for CPA. A possible explanation for the observed difference in inhibition mode that is dependent on the inhibitor stereochemistry is offered.

Keywords : Inhibition stereochemistry, Inhibitor design strategy, β-Lactone, Carboxypeptidase A.

Introduction

Zinc proteases have received an increasing attention in recent years as the roles that these enzymes play in physiological processes and pathological conditions become unraveled. Inhibitors of certain zinc proteases such as angiotensin converting enzyme and matrix metalloproteases are valuable therapeutic agents useful for the treatment of heart diseases, malignant tumors and arthritis.[1] These enzymes, are characterized by having at the active site a zinc ion that is essential for the catalytic activity. Of these enzymes, carboxypeptidase A (CPA) isolated from the pancreas is the most extensively studied and serves as a prototypical enzyme for the zinc proteases.[2] CPA has been used as a model enzyme for developing inhibitor design strategies that may be useful for designing inhibitors of zinc proteases of medicinal interest. The enzyme removes the C-terminal amino acid having an aromatic side chain from oligopeptide and esters structurally reminiscent to the peptide substrates.[3] The zinc ion at the active site of CPA is coordinated to His-69, His-196, Glu-72, and a water molecule as the fourth ligand.[2] The water molecule is loosely bound and directly involved in the hydrolysis of peptide substrate. At the active site of CPA there is present a carboxylate of Glu-270, which functions as a general base, activating the zinc bound water molecule for nucleophilic attack on the scissile peptide bond of substrate.[2,4,5] It has been known that in the hydrolysis of ester substrate the Glu-270 carboxylate attacks directly on the carbonyl carbon to generate an anhydride intermediate.[6] In binding the substrate to CPA, the C-terminal carboxylate forms a hydrogen bond with the guanidinium moiety of Arg-145 and the aromatic ring in the P_1' residue is accommodated in a hydrophobic pocket (the primary substrate recognition pocket) at the active site.

We have designed compound **1** as a potential irreversible inactivator for CPA. It is expected that upon binding of **1** to CPA, the carbonyl oxygen of the lactone ring would rest in the close vicinity to the active site zinc ion to form a coordinative bond so that the lactone ring may become further activated for the ring cleavage reaction with the carboxylate nucleophile present at the active site. Such a reaction would result in tethering **1** to CPA with concurrent ring cleavage of the heterocycle. This covalent modification of the enzyme would impair the catalytic activity of CPA permanently. In this study we have restricted the inhibitor having the (*S*)-configuration at the 1' position since the (*S*)-stereochemistry corresponds to the stereochemistry of substrate, *i.e.*, the L-series.

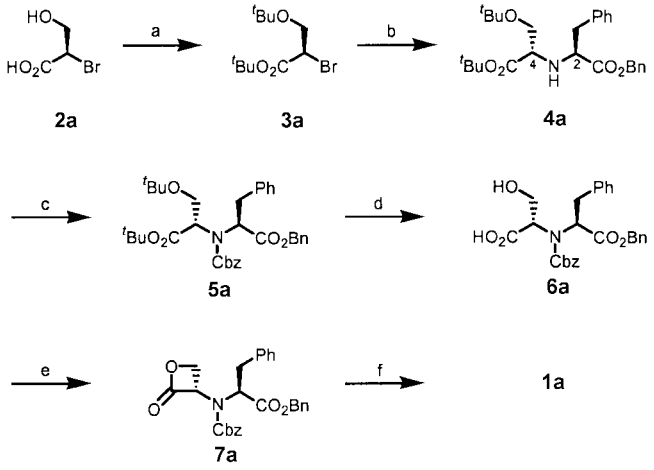


1a **1b**

Structures

Results

Chemistry. Both the carboxylate and hydroxyl groups in (*R*)-2-bromo-3-hydroxypropanoic acid (**2a**) that was prepared by the literature method[7] were protected to give **3a**. The latter compound was then allowed to react with L-phenylalanine benzyl ester in the presence of sodium bicarbonate under reflux conditions to obtain a mixture of **4a** and its diastereomer (**4b**) in the ratio of 5 : 1. The mixture was separated by column chromatography.[8] The minor product (**4b**) was shown to be the diastereoisomer of **4a** having the (2*S*,4*R*)-configuration. Intermediate **4a** could also be prepared by the reductive amination of phenylpyruvic acid with *O*-*tert*-butyl L-serine *tert*-butyl ester[9] followed by esterification with benzyl bromide, but in a lower yield. It appears that **4b** is formed from (*S*)-**2** generated in the preparation of **2a** from D-Ser[11] and present in **2a** as an impurity. The amino group in **4a** was protected with CbzCl in the presence of 2,6-lutidine in dichloromethane to obtain



Scheme 1. (a) H_2SO_4, $MgSO_4$, ^tBuOH, CH_2Cl_2; (b) L-Phe-OBnHCl, $NaHCO_3$, CH_3CN, rt to reflux; (c) CbzCl, 2,6-lutidine, THF; (d) TFA, CH_2Cl_2, rt to 30 °C; (e) Ph_3P, DMAD, THF, -78 °C to rt; (f) H_2, Pd/C, MeOH.

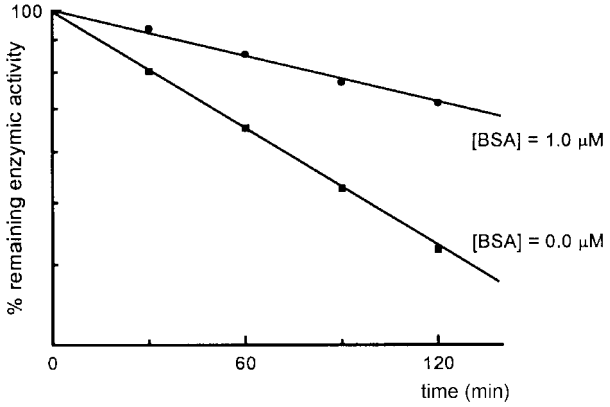


Figure 2. Time dependent loss of CPA activity caused by **1a** in the absence and presence of 2-benzylsuccinic acid, a CPA inhibitor known to bind CPA at the active site (substrate, Hip-L-Phe; [S]=250 μM; [E]=1.3 μM; Tris buffer of pH 7.5; temperature, 25 °C)

5a in 85% yield.[8] Both *tert*-butyl groups in **5a** were removed by the treatment of trifluoroacetic acid in dichloromethane to afford **6a** which was then cyclized to give **7a** in 45% yield under the modified Mistunobu conditions as reported by Arnold *et al.*[10] The hydrogenolysis of **7a** gave the target molecule **1a**. The synthesis of **1b** was accomplished by the same sequences of reactions as those used for the preparation of **1a** starting with (*S*)-2-bromo-3-hydroxypropanoic acid (**2b**).

Inhibition Assay. The inhibitors thus synthesized were assayed for their inhibitory activities against CPA. The enzyme was incubated with excess inhibitor, and the loss of enzymic activity was followed by withdrawing aliquots at 30 min intervals and assayed for the remaining activity by measuring the rate of the CPA catalyzed hydrolysis of the chromophoric substrate, hippuryl-L-phenylalanine (Hip-L-Phe) at 25 °C in Tris buffer (0.05 M) of pH 7.5. Semi-logarithmic plot of the activity remaining *versus* incubation time at different concentrations of the inhibitor gave straight lines when CPA was treated with **1a** (Figure 1a), suggesting that the inhibition occurs in an irreversible fashion. The inactivated CPA by **1a** failed to gain its enzymic activity upon dialysis against the assay medium for 2 days, supporting the irreversible nature of the inhibition.

Kinetic parameters for the irreversible inhibition, K_I and k_{inact} were estimated from the double reciprocal plot of k_{obs} *versus* inhibitor concentration according to the method of Kitz and Wilson[11] as exemplified by Figure 1b, and are listed in Table 1. A protection of the CPA inhibition by **1a** was observed when CPA was preincubated with 2-benzylsuccinic acid, a known active site directed competitive inhibitor of CPA,[12] indicating that the inactivation takes place at the active site of the enzyme (Figure 2).

On the other hand, **1b** failed to show the time dependent loss of the enzymic activity, indicating that **1b** is devoid of the CPA inactivating property. It, however, inhibited the enzyme competitively with the K_i value of 246.5 μM as

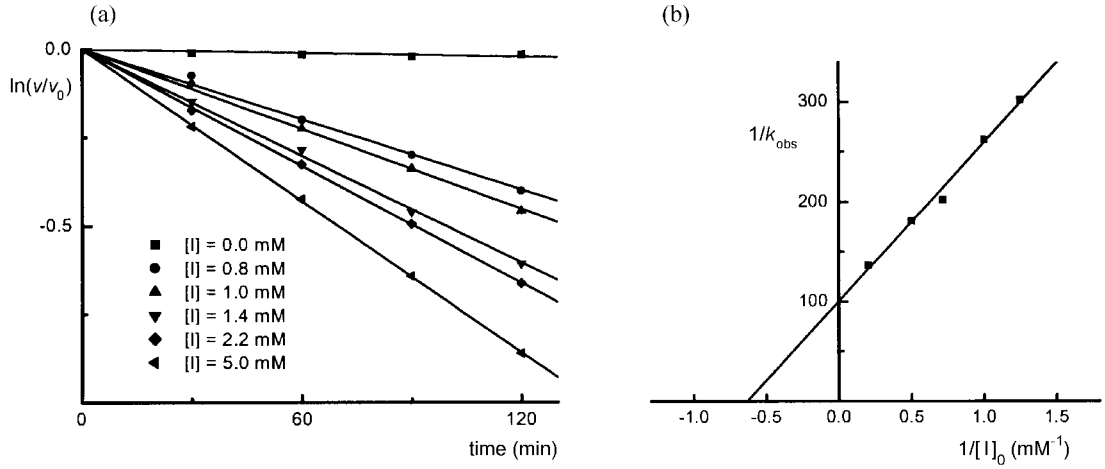


Figure 1. (a) Loss of enzymic activity of CPA as a function of time of the incubation of CPA with **1a** at different concentrations (substrate, Hip-L-Phe; [S]=250 μM; [E]=1.3 μM; Tris buffer of pH 7.5; temperature, 25 °C); (b) The double reciprocal plot of k_{obs} *versus* concentration of **1a**, $[I]_0$ for the inactivation of CPA with **1a**.

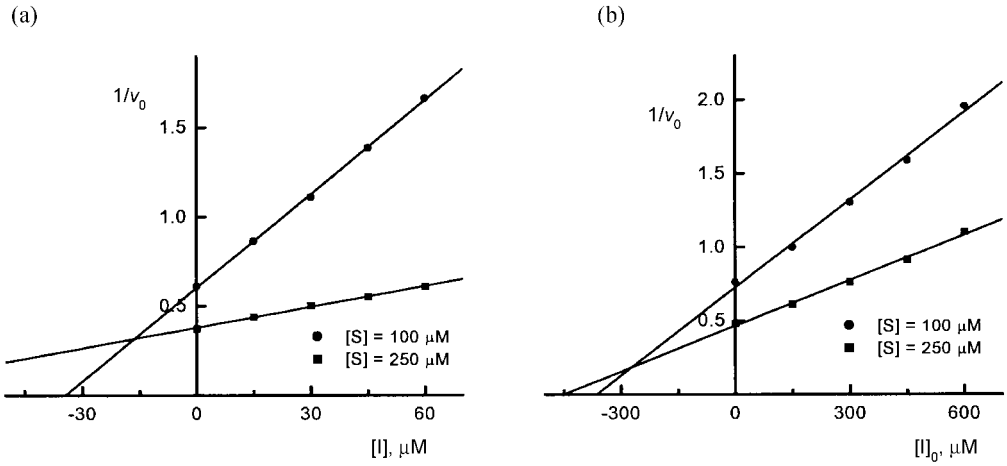


Figure 3. The Dixon plot for the reversible inhibition of CPA with **1a** (a) and **1b** (b) (substrate, Hipp-L-Phe; Tris buffer of pH 7.5; temperature, 25 °C)

Table 1. Kinetic parameters for the inactivation of CPA by lactone derivatives

Inhibitor	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I ($M^{-1}min^{-1}$)	K_i (μM)
1a	0.0099±0.0007	1.6±0.09	6.3±0.4	16.7±1.1
1b	NI [a]			246.5±29.2

[a]NI means no irreversible inhibition.

determined by the Dixon plot (Figure 3b and Table 1).[13]

Discussion

We and others have demonstrated that inactivators of CPA that impair the enzymic activity of CPA by covalently modifying the catalytically essential nucleophile of the Glu-270 carboxylate may be obtained by incorporating a latent electrophile into a molecular scaffold reminiscence to the P_1' residue of CPA substrate.[3,14] Small heterocycles having high ring strain energy such as oxirane,[15] thiirane,[16] 3-oxoisoxazolidine[17] have been served effectively as the latent electrophile. 2-Oxetanone is a much studied heterocycle having a high ring strain energy and susceptible to ring cleavage reaction by a variety of nucleophiles.[18] Thus, the compounds derived by tethering 2-oxetanone to Phe that is most frequently found P_1' residue in the substrate of CPA are anticipated to yield a highly potent inactivating activity for CPA. This strategy has not been explored previously for the

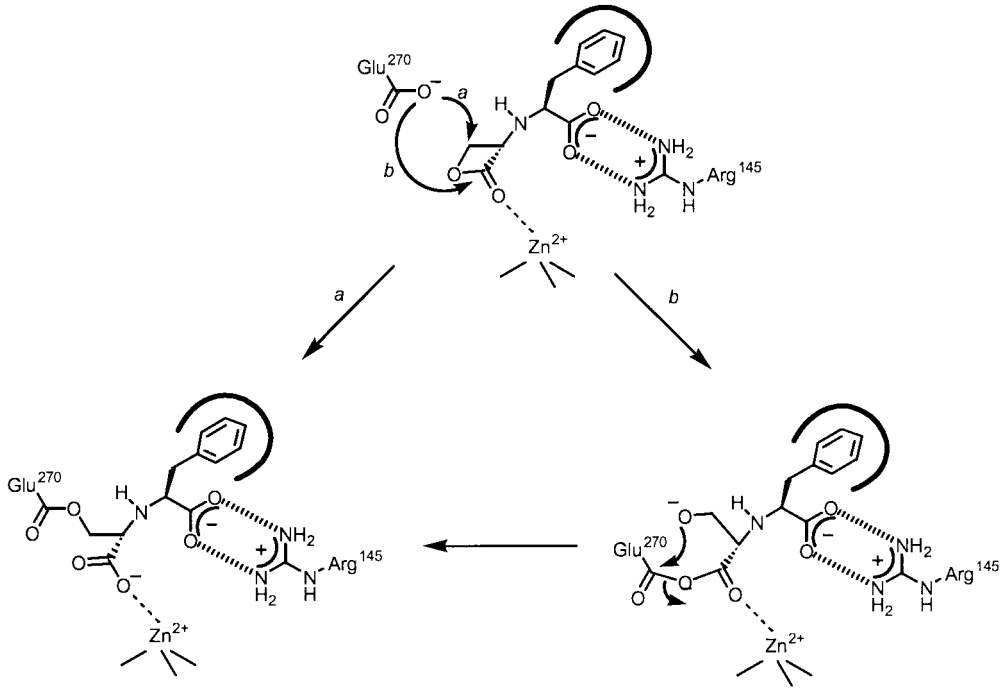


Figure 4. A proposed inactivation mechanism of CPA by **1a**.

design of irreversible inhibitors for zinc proteases. The stereochemistry associated with inactivation of proteases, CPA in particular, has been the subject of our long lasting interest,[19] and in this study we are interested in examining the stereochemistry associated with the inhibition of CPA by the novel type of inhibitors. Accordingly, inhibitors, **1a** and **1b** in a diastereoisomeric relationship were synthesized.

The kinetic analysis for the inhibition of CPA by the inhibitors showed that **1a** is an effective irreversible inactivator for the enzyme with the k_{inact}/K_I value (the second order inhibitory rate constant) of 6.3 ± 0.4 M^{-1} min^{-1}. Its diastereoisomer **1b** failed to inactivate the enzyme but is a weak reversible inhibitor for the enzyme. A possible mechanism for the inactivation of CPA by **1a** is depicted in Figure 4. Expectedly, the carboxylate of **1a** forms a hydrogen bond with the guanidinium moiety of Arg-145 and the benzene ring anchors in the S_1 subsite pocket. This binding mode would place the β-lactone ring in the vicinity of the zinc ion and there may form a coordinative bond between the carbonyl oxygen of the β-lactone and the zinc ion, resulting in the activation of the heterocycle for the electrophilic reaction with the carboxylate of Glu-270, the catalytically essential nucleophile present at the active site.[20]

2-Oxetanone ring is known to undergo a ring cleavage reaction by the nucleophilic attack at either the C-2 or C-4 position of the ring.[21] Accordingly, there are two possible routes, *i.e*., *a* and *b* for the cleavage of the β-lactone ring by the carboxylate in the inactivation of CPA by **1a**. In reaction path *a* that involves the S_N2 type nucleophilic attack at the C-4 position of the ring, the carboxylate nucleophile is required to approach the electrophilic center in the direction of 180 to the scissile CO bond. On the other hand, in reaction path *b*, the ring cleavage reaction occurs by the nucleophilic addition and elimination mechanism. It has been known that such a reaction does not require the stringent directionality for the attacking nucleophile.[22] This has been demonstrated in the inactivation of CPA by α-benzyl-2-oxo-1,3-oxazolidine-4-carboxylic acid.[23] In this case all four diastereoisomers of the inhibitor were effective irreversible inactivators for CPA.[23] On the basis of the foregoing discussion on the stereochemistry in the inactivation of CPA, it may be concluded that the present covalent modification reaction takes more likely *via* path *a*, the Glu-270 carboxylate attacking at the 4-position of the 2-oxetanone moiety in **1a**. The lack of CPA inactivation activity shown by **1b** may be explained on the following ground: If **1b** would bind to the enzyme in the way that its diastereoisomer **1a** binds, the electrophilic center (the C-4) in the 2-oxetanone ring would not rest at the position amenable for the S_N2 type ring opening reaction to take place with the Glu-270 carboxylate. In this regard, it is worth noting the report of Kim *et al*. who showed that 2-benzyl-3,4-epoxybutanoic acid is a highly stereospecific CPA inactivator with the stereoisomer having the (2*R*,3*S*)-configuration being effective.[15,24] They rationalized the lack of inactivation property shown by its diastereoisomer with the proposition that in the CPA·inhibitor complex the C_4-O bond in the inhibitor bearing the (2*R*,3*R*)-configuration is not properly aligned with respect to the carboxylate nucleophile for the S_N2 type ring cleavage reaction.[24]

In conclusion, we have demonstrated that the 2-oxetanone ring is a viable latent electrophile that can be used in the design of irreversible inhibitor for CPA and the inactivation reaction leading to covalent modification of the Glu-270 carboxylate occurs in a stereospecific fashion that only the inhibitor having the (*S*,*S*)-configuration is effective. The design strategy reported here may find applications in designing inhibitors of other zinc proteases, should one be able to modify judiciously the molecule so that it can bind the active site of the target enzyme and the lactone carbonyl achieves coordination with the active site zinc.

Experimental Section

All chemicals were of reagent grade obtained from Aldrich Chemical Co. and solvents were purified before use. 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). All melting points were determined on a Thomas-Hoover capillary melting point apparatus and were not corrected. ^{1}H NMR and ^{13}C NMR spectra were measured 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 spectrometer. Optical rotations were measured on a Rudolp Research Autopol III digital polarimeter. Mass spectra and elemental analyses were performed at Central Machine and Facilities Shop, Pohang University of Science and Technology, Pohang, Korea. High resolution mass spectra were performed by Mass Spectrometry Analysis Team, Korea Basic Science Institute, Taegu, Korea.

(*R*)-2-Bromo-3-(*tert*-butoxy)propanoic acid *tert*-butyl ester (3a). Concentrated sulfuric acid (2.7 mL, 50 mmol) was added to a vigorously stirred suspension of anhydrous magnesium sulfate (47.8 g, 0.4 mol) in 400 mL of CH_2Cl_2 and the mixture was stirred for 15 min. To this solution, (*R*)-2-bromo-3-hydroxypropanoic acid (**2a**)[7] (8.45 g, 50 mmol) was added and stirring was continued for 15 min followed by addition of *tert*-butanol (95.6 mL, 0.5 mol). The flask containing the mixture was stoppered tightly and stirred for 36 h at room temperature. The reaction mixture was then quenched with 200 mL of saturated sodium bicarbonate solution and the stirring was continued until all magnesium sulfate was dissolved. The organic phase was separated, washed with water, and brine, dried over $MgSO_4$, and concentrated to afford the crude product which was purified by column chromatography (yellowish oil, 11.9 g, 85%). $[\alpha]_D^{25}$ = +3.9° (*c* 1.3, $CHCl_3$); ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.45 (s, 9H), 1.44 (s, 9H), 3.59-3.63 (m, 1H), 3.80 (t, *J* = 9.0 Hz, 1H), 4.07-4.12 (m, 1H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 27.8, 28.1, 44.9, 64.0, 74.4, 82.6, 168.2.

(*S*)-2-Bromo-3-(*tert*-butoxy)propanoic acid *tert*-butyl ester (3b). This compound was obtained as a yellowish oil in 80% yield by the same procedure described above starting with (*S*)-2-bromo-3-hydroxypropanoic acid (**2b**). $[\alpha]_D^{25}$ = –4.0° (*c*

1.0, $CHCl_3$).

(2*S*,4*S*)- and (2*S*,4*R*)-2-Benzyl-5-*tert*-butoxy-4-*tert*-butoxycarbonyl-3-azapentanoic acid benzyl ester (4a and 4b). To a solution of L-phenylalanine benzyl ester hydrochloride salt (10.2 g, 35.0 mmol) in 80 mL of acetonilrile was added 8.82 g (105.0 mmol) of $NaHCO_3$. After stirring for 1 h, **3a** (10.6 g, 37.8 mmol) was added dropwise to the mixture and stirred at room temperature for 1 day, then under reflux for 1 day. The reaction mixture was cooled and filtered through Celite and the filtrate was concentrated *in vacuo*. The residue was dissolved in ethyl acetate and the solution was washed with water and brine, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel to give **4a** (colorless oil, 11.3 g, 70.8%) and **4b** (colorless oil, 2.26 g, 14.2%). **4a**: $[\alpha]_D^{25}$ = –9.0° (*c* 1.0, $CHCl_3$); EI-MS *m/z* 455 (M^+); FAB HRMS calcd for $C_{27}H_{38}NO_5$ (MH^+) 456.2750, found 456.2755; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.12 (s, 9H), 1.40 (s, 9H), 3.00 (d, *J* = 6.6 Hz, 2H), 3.32 (t, *J* = 5.3 Hz, 1H), 3.44-3.54 (m, 2H), 3.74 (t, *J* = 7.1 Hz, 1H), 5.05 (s, 2H), 7.15-7.37 (m, 10H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 22.8, 28.5, 40.3, 60.9, 61.7, 63.8, 66.8, 73.4, 81.5, 126.3, 127.1, 128.6, 128.9, 129.0, 129.7, 136.0, 137.4, 172.2, 174.1. **4b**: $[\alpha]_D^{25}$ = +4.9° (*c* 1.0, $CHCl_3$); EI-MS *m/z* 455 (M^+); FAB HRMS calcd for $C_{27}H_{38}NO_5$ (MH^+) 456.2750, found 456.2763; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.12 (s, 9H), 1.43 (s, 9H), 2.97 (d, *J* = 6.2 Hz, 2H), 3.25 (t, *J* = 4.8 Hz, 1H), 3.47 (d, *J* = 4.8 Hz, 2H), 3.74 (t, *J* = 6.7 Hz, 1H), 5.07 (d, *J* = 4.1 Hz, 2H), 7.18-7.31 (m, 10H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 27.7, 28.5, 40.2, 60.9, 61.2, 61.9, 63.6, 66.9, 73.3, 81.5, 126.9, 127.1, 128.6, 128.7, 128.8, 129.8, 136.1, 137.9, 172.3, 174.2.

(2*S*, 4*R*)-2-Benzyl-5-*tert*-butoxy-4-*tert*-butoxycarbonyl-3-azapentanoic acid benzyl ester (4b). This compound was obtained as the major product (73% yield) from **3b** by the same procedure as that used for the preparation of **4a**.

(2*S*,4*S*)- and (2*R*,4*S*)-2-Benzyl-5-*tert*-butoxy-4-*tert*-butoxycarbonyl-3-azapentanoic acid benzyl ester (4a and 4c). A solution of *O*-*tert*-butyl L-serine *tert*-butyl ester[25] (6.52 g, 30.0 mmol), phenylpyruvic acid (5.91 g, 36.0 mmol), and 4.92 g (60.0 mmol) of anhydrous NaOAc in 750 mL of absolute ethanol was cooled under stirring in an ice-water to which was added slowly $NaBH_3CN$ (3.77 g, 60.0 mmol) in 75 mL of EtOH. The reaction mixture was stirred for 1 h at 0 °C, then at room temperature for 48 h. The solvent was evaporated under reduced pressure and the residue was suspended in anhydrous MeOH (200 mL). Cs_2CO_3 (7.17 g, 22.0 mmol) was added to the mixture and stirred for 30 min, then concentrated under reduced pressure. The residue was suspended in DMF (200 mL). To the mixture was added dropwise benzylbromide (2.3 mL, 20.0 mmol) and stirred for 12 h. The reaction mixture was diluted with water (300 mL) and extracted with ethyl acetate (3 × 200 mL). The combined extracts were washed with water and brine, dried over $MgSO_4$, and evaporated under reduced pressure. The residue (a mixture of two diastereomers) was separated into the (*R*,*S*)- and (*S*,*S*)-isomers by column chromatography on silica gel to give **4a** (colorless oil, 3.83 g, 28%) and **4c** (colorless oil, 3.42 g, 25%). **4a**: $[\alpha]_D^{25}$ = –9.0° (*c* 1.0, $CHCl_3$); EI-MS *m/z* 455 (M^+); ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.12 (s, 9H), 1.40 (s, 9H), 3.00 (d, *J* = 6.6 Hz, 2H), 3.32 (t, *J* = 5.3 Hz, 1H), 3.44-3.54 (m, 2H), 3.74 (t, *J* = 7.1 Hz, 1H), 5.05 (s, 2H), 7.15-7.37 (m, 10H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 22.8, 28.5, 40.3, 60.9, 61.7, 63.8, 66.8, 73.4, 81.5, 126.3, 127.1, 128.6, 128.9, 129.0, 129.7, 136.0, 137.4, 172.2, 174.1. **4c**: $[\alpha]_D^{25}$ = –5.0° (*c* 1.0, $CHCl_3$); EI-MS *m/z* 455 (M^+); ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.12 (s, 9H), 1.43 (s, 9H), 2.97 (d, *J* = 6.2 Hz, 2H), 3.25 (t, *J* = 4.8 Hz, 1H), 3.47 (d, *J* = 4.8 Hz, 2H), 3.74 (t, *J* = 6.7 Hz, 1H), 5.07 (d, *J* = 4.1 Hz, 2H), 7.18-7.31 (m, 10H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 27.7, 28.5, 40.2, 60.9, 61.2, 61.9, 63.6, 66.9, 73.3, 81.5, 126.9, 127.1, 128.6, 128.7, 128.8, 129.8, 136.1, 137.9, 172.3, 174.2.

(2*S*,4*S*)-*N*-Benzyloxycarbonyl-2-benzyl-5-*tert*-butoxy-4-*tert*-butoxycarbonyl-3-azapentanoic acid benzyl ester (5a). To an ice chilled THF solution (30 mL) of **4a** (8.66 g, 19.0 mmol) and 2,6-lutidine (4.2 mL, 36.1 mmol) was added dropwise benzyl chloroformate (3.8 mL, 26.6 mmol). The reaction mixture was stirred for 48 h at room temperature then evaporated under reduced pressure. The resulting mixture was dissolved in ethyl acetate (50 mL), and the solution was washed with 2 N HCl, water and brine and evaporated *in vacuo*. The residue was purified by column chromatography on silica gel to give **5a** as a colorless oil (9.5 g, 85%). $[\alpha]_D^{25}$ = –78.5° (*c* 2.0, $CHCl_3$); IR (neat) 1720, 1684 cm^{-1}; EI-MS *m/z* 589 (M^+); FAB HRMS calcd for $C_{35}H_{44}NO_7$ (MH^+) 590.3118, found 590.3121; ^{1}H NMR 300 MHz ($CDCl_3$) δ 0.99 (d, *J* = 15.0 Hz, 9H), 1.33 (d, *J* = 6.5 Hz, 9H), 3.11-3.18 (m, 2H), 3.27-3.37 (m, 2H), 4.46-4.54 (m, 1H), 4.80 (t, *J* = 7.6 Hz, 1H), 4.89-5.27 (m, 4H), 7.13-7.35 (m, 15H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 27.7, 28.2, 36.6, 37.7, 59.1, 60.4, 67.2, 67.9, 73.6, 81.9, 82.0, 127.1, 128.4, 128.5, 128.6, 128.8, 130.0, 130.1, 135.9, 138.3, 138.5, 155.6, 169.2, 170.0, 172.8.

(2*S*,4*R*)-*N*-Benzyloxycarbonyl-2-benzyl-5-*tert*-butoxy-4-*tert*-butoxycarbonyl-3-azapentanoic acid benzyl ester (5b). This compound was prepared as a colorless oil in 81% yield from **4b** by the same procedure as that used for the preparation of **5a**. $[\alpha]_D^{25}$ = –4.1° (*c* 1.2, $CHCl_3$); IR (neat) 1800, 1725 cm^{-1}; EI-MS *m/z* 589 (M^+); FAB HRMS calcd for $C_{35}H_{44}NO_7$ (MH^+) 590.3118, found 590.3126; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.01 (d, *J* = 14.0 Hz, 9H), 1.43 (d, *J* = 6.0 Hz, 9H), 2.76-2.92 (m, 2H), 3.29-3.40 (m, 2H), 4.50-4.54 (m, 1H), 4.82 (t, *J* = 7.6 Hz, 1H), 4.89-5.27 (m, 4H), 7.13-7.35 (m, 15H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 27.7, 29.8, 36.6, 38.9, 59.1, 60.4, 67.2, 67.9, 73.6, 81.9, 82.0, 127.1, 128.4, 128.5, 128.6, 128.8, 130.0, 130.1, 135.9, 138.3, 138.5, 155.6, 169.2, 170.0, 173.9.

(2*S*, 4*S*)-*N*-Benzyloxycarbonyl-2-benzyl-4-carboxy-5-hydroxy-3-azapentanoic acid benzyl ester (6a). A solution of **5a** (6.7 g, 11.4 mmol) and trifluoroacetic acid (10 mL) dissolved in CH_2Cl_2 (100 mL) was stirred for 1 h at room temperature then 3 h at 30 °C. Evaporation of the solvent and excess trifluoroacetic acid yielded **6a** which was

purified by column chromatography (yellowish resin, 3.32 g, 61%). $[\alpha]_D^{25}$ = –19.0° (*c* 1.0, MeOH); IR (neat) 3400, 1730, 1720 cm^{-1}; EI-MS *m/z* 477 (M^+); FAB HRMS calcd for $C_{27}H_{28}NO_7$ (MH^+) 478.1866, found 478.1871; ^{1}H NMR 300 MHz ($CDCl_3$) δ 3.11-3.18 (m, 2H), 3.27-3.37 (m, 2H), 4.46-4.54 (m, 1H), 4.80 (t, *J* = 7.6 Hz, 1H), 4.89-5.27 (m, 4H), 7.13-7.35 (m, 15H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 36.6, 37.7, 59.1, 60.4, 73.6, 81.9, 82.0, 127.1, 128.4, 128.5, 128.6, 128.8, 130.0, 130.1, 135.9, 138.3, 138.5, 155.6, 169.2, 172.9, 178.0.

(2*S*, 4*R*)-*N*-Benzyloxycarbonyl-2-benzyl-4-carboxy-5-hydroxy-3-azapentanoic acid benzyl ester (6b). This compound was obtained as a yellowish resin in 80% yield from **5b** by the same procedure as that used for the preparation of **6a**. $[\alpha]_D^{25}$ = –10.0° (*c* 1.0, MeOH); IR (neat) 3400, 1735, 1719 cm^{-1}; EI-MS *m/z* 477 (M^+); FAB HRMS calcd for $C_{27}H_{28}NO_7$ (MH^+) 478.1866, found 478.1870; ^{1}H NMR 300 MHz ($CDCl_3$) δ 2.76-2.92 (m, 2H), 3.29-3.40 (m, 2H), 4.50-4.54 (m, 1H), 4.82 (t, *J* = 7.6 Hz, 1H), 4.89-5.27 (m, 4H), 7.13-7.35 (m, 15H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 36.6, 38.9, 59.1, 60.4, 67.2, 67.9, 73.6, 81.9, 82.0, 127.1, 128.4, 128.5, 128.6, 128.8, 130.0, 130.1, 135.9, 138.3, 138.5, 155.6, 169.2, 173.9, 178.0.

(3*S*,1'*S*)-*N*-Benzyloxycarbonyl-3-(1'-benzyloxycarbonyl-2'-phenyl)ethylamino-2-oxetanone (7a). A solution of triphenylphosphine (2.39 g, 9.1 mmol) in anhydrous THF (30 mL) was stirred for 15 min at 78 °C. Dimethyl azocarboxylate (40% solution in toluene, 3.44 g, 9.4 mmol) was added dropwise to the mixture over 30 min and the stirring was continued for 30 min, at which time was added dropwise **6a** (3.0 g, 6.29 mmol) in THF (5 mL) in 20 min and the resulting mixture was continued to stir for an additional 1.5 h at 78 °C. It was allowed to warm slowly to room temperature, and evaporated *in vacuo*. The residue was purified by chromatography on silica gel to give **7a** as a colorless oil (1.3 g, 45%). $[\alpha]_D^{25}$ = –28.4° (*c* 0.5, $CHCl_3$); IR (neat) 1805, 1720 cm^{-1}; EI-MS *m/z* 459 (M^+); FAB HRMS calcd for $C_{27}H_{26}NO_6$ (MH^+) 460.1760, found 460.1765; ^{1}H NMR 300 MHz ($CDCl_3$) δ 2.89-3.06 (m, 1H), 3.37 (td, *J* = 5.4, 14.7 Hz, 1H), 3.88-3.95 (m, 1H), 4.00-4.04 (m, 1H), 4.83-4.89 (m, 2H), 4.99-5.24 (m, 4H), 7.01-7.33 (m, 15H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 36.5, 36.8, 60.1, 60.6, 63.3, 64.1, 65.3, 66.5, 68.0, 68.9, 69.1, 127.7, 127.7, 128.7, 128.8, 129.1, 135.5, 135.6, 135.7, 136.2, 155.0, 155.4, 170.4, 170.7.

(3*R*,1'*S*)-*N*-Benzyloxycarbonyl-3-(1'-benzyloxycarbonyl-2'-phenyl)ethylamino-2-oxetanone (7b). This compound was obtained as a colorless oil in 40% yield from **6b** by the same procedure as that used for the preparation of **7a**. $[\alpha]_D^{25}$ = –2.0° (*c* 1.0, $CHCl_3$); IR (neat) 1810, 1740 cm^{-1}; EI-MS *m/z* 459 (M^+); FAB HRMS calcd for $C_{27}H_{26}NO_6$ (MH^+) 460.1760, found 460.1764; ^{1}H NMR 300 MHz ($CDCl_3$) δ 2.96-3.01 (m, 1H), 3.26-3.32 (m, 1H), 4.02-4.12 (m, 1H), 4.68-4.72 (m, 1H), 4.82-4.86 (m, 1H), 4.98-5.20 (m, 5H), 7.08-7.37 (m, 15H); ^{13}C-NMR 300 MHz ($CDCl_3$) δ 36.5, 38.0, 60.3, 61.6, 64.4, 64.5, 65.5, 66.8, 68.3, 69.5, 127.7, 128.0, 128.9, 129.2, 129.3, 129.5, 129.8, 135.4, 135.9, 136.2, 168.0, 170.1, 171.5.

(3*S*,1'*S*)-3-(1'-Carboxy-2'-phenyl)ethylamino-2-oxetanone (1a). To a methanol solution (20 mL) containing **7a** (1.5 g, 3.26 mmol) and a catalytic amount of Pd/C was stirred for 3 h at room temperature. The product was precipitated out as the reaction proceeded. After completion of the reaction, the reaction mixture was heated at 60 °C to dissolve the product. The hot solution was filtered and the filter residue was washed with hot MeOH. The combined filtrates were evaporated under reduced pressure to give **1a** which was recrystallized from MeOH (white solid, 0.65 g, 85%). mp = 178-180 °C (dec.); $[\alpha]_D^{25}$ = –20.0° (*c* 0.5, DMSO); IR (KBr) 3200, 1800, 1740 cm^{-1}; EI-MS *m/z* 235 (M^+); ^{1}H NMR 300 MHz (DMSO-d6) δ 2.89-3.06 (m, 1H), 3.37 (td, *J* = 5.4, 14.7 Hz, 1H), 3.88-3.95 (m, 1H), 4.00-4.04 (m, 1H), 4.83-4.89 (m, 2H), 7.01-7.30 (m, 5H); ^{13}C-NMR 300 MHz (DMSO-d6) δ 38.0, 36.5, 60.3, 61.6, 64.4, 64.5, 65.5, 68.3, 127.7, 128.9, 129.2, 135.4, 135.9, 136.2, 170.1, 174.5. Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; N, 5.95; H, 5.57. Found: C, 61.53; N, 6.14; H, 5.75.

(3*R*,1'*S*)-3-(1'-Carboxy-2'-phenyl)ethylamino-2-oxetanone (1b). This compound was obtained as a white solid in 80% yield from **7b** by the same procedure as that used for the preparation of **1a**. mp = 182-184 °C (dec.); $[\alpha]_D^{25}$ = –10.2° (*c* 1.0, DMSO); IR (KBr) 3300, 1805, 1740 cm^{-1}; EI-MS *m/z* 235 (M^+); ^{1}H NMR 300 MHz (DMSO-d6) δ 2.90-3.20 (m, 2H), 3.90-3.92 (m, 1H), 4.02-4.04 (m, 1H), 4.81-4.83 (m, 2H), 7.15-7.35 (m, 5H); ^{13}C-NMR 300 MHz (DMSO-d6) δ 36.5, 36.8, 60.1, 60.6, 63.3, 64.1, 65.3, 66.5, 68.0, 127.7, 128.7, 129.1, 135.6, 135.7, 136.2, 170.4, 174.1. Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; N, 5.95; H, 5.57. Found: C, 61.45; N, 6.15; H, 5.84.

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 pancrease, 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. Enzyme concentrations were estimated from the absorbance at 278 nm (ε_{278} = 64,200). Hippuryl-L-phenylalanine (Hip-L-Phe) was purchased from Sigma Chemical Co. A Perkin-Elmer HP 8453 UV-vis spectrometer was used for UV absorbance measurement in kinetic experiments.

Determination of k_{inact} and K_I. Inactivation experiments were performed with concentrations of **1a** to be within the 0.8 - 4.0 mM range. A solution of the inactivator obtained by dissolving in a 1 : 1 mixture of DMSO and 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer was added to an enzyme solution in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer to afford a final concentration of 1.3 μM of CPA in 5% DMSO. The solutions were inacubated at 25 °C. At 30 min interval, 50 μL aliquots of the inactivation mixture were taken and added to a 950 μL of the assay mixture containing 50 μL of 5 mM solution of Hip-L-Phe, and the increase in the absorbance at 254 nm was monitored for the first 40 sec. Semilogarithmic plots of the

residual enzymic activity *versus* incubation time gave straight lines with a slope of k_{obs} (Figure 1a). The values of K_I and k_{inact} were calculated from the double reciprocal plot of the k_{obs} *versus* concentration of inhibitors according to the method of Kitz-Wilson. (Figure 1b).[11]

Active site protection test.[12] CPA (1.3 μM) was incubated with 2-benzylsuccinic acid (1 μM) for 10 min at 25 °C. Inhibitor (**1a)** was added to the mixture to give a final inactivator concentration of 5 mM. At 30 min intervals, 50 μL aliquots of the incubation mixture were removed and added to a 950 μL assay mixture containing 250 μM of Hip-L-Phe and the remaining enzymic activity was monitored at 254 nm at 25 °C. Figure 2 was obtained from the data.

Determination of K_i. A series of assay mixtures containing both the substrate (Hip-L-Phe, 100 and 250 μM) and various concentrations of the inhibitor (in the range 15-60 μM for **1a** and 150-600 μM for **1b**) were prepared in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer. Enzyme stock solution was added to the assay mixture to afford a final enzyme concentration of 20 nM in a total volume of 1,000 μL. The initial rates of enzymic reaction were measured immediately using a microcomputer-interfaced UV spectrometer. 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 (Figure 3).[13]

Dialysis. Solutions of the inactivators (1-10 mM) and CPA (1 μM) in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer were incubated at 4 °C for 48 h. The mixture was then dialyzed for 24 h at room temperature against 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer. The buffer was changed every 6 h. After dialysis, 50 μL aliquots of inactivation mixture were removed and added to 950 μL of assay mixture. The remaining enzymic activity was monitored at 254 nm. The enzyme failed to show the proteolytic activity.

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