Mechanistic Insight into the Inactivation of Carboxypeptidase A by α-Benzyl-2-oxo-1,3-oxazolidine-4-acetic Acid, a Novel Type of Irreversible Inhibitor for Carboxypeptidase A with No Stereospecificity

Sang J. Chung, Suhman Chung, Hyun Soo Lee, Eun-Jung Kim, Kyung Seok Oh, Hyuk Soon Choi, Kwang S. Kim, Yeoun Jin Kim, Jong Hoon Hahn, and Dong H. Kim*

Center for Biofunctional Molecules and Division of Molecular and Life Sciences, Pohang University of Science and Technology, San 31 Hyojadong, Pohang 790-784, Korea

dhkim@ postech.ac.kr

Received April 23, 2001

On the basis of the active site topology and enzymic catalytic mechanism of carboxypeptidase A (CPA), a prototypical zinc-containing proteolytic enzyme, α-benzyl-2-oxo-1,3-oxazolidine-4-acetic acid (**1**), was designed as a novel type of mechanism-based inactivator of the enzyme. All four possible stereoisomers of the inhibitor were synthesized in an enantiomerically pure form starting with optically active aspartic acid, and their CPA inhibitory activities were evaluated to find that surprisingly all of the four stereoisomers inhibit CPA in a time dependent manner. The inhibited enzyme did not regain its enzymic activity upon dialysis. The inactivations were prevented by 2-benzylsuccinic acid, a competitive inhibitor that is known to bind the active site of the enzyme. These kinetic results strongly support that the inactivators attach covalently to the enzyme at the active site. The analysis of ESI mass spectral data of the inactivated CPA ascertained the conclusion from the kinetic results. The values of second-order inhibitory rate constants ($k_{obs}/[I]_o$) fall in the range of 1.7−3.6 M^{-1} min^{-1}. The lack of stereospecificity shown in the inactivation led us to propose that the ring cleavage occurs by the nucleophilic attack at the 2-position rather than at the 5-position and the ring opening takes place in an addition−elimination mechanism. The tetrahedral transition state that would be generated in this pathway is thought to be stabilized by the active site zinc ion, which was supported by the PM3 semiemprical calculations. In addition, α-benzyl-2-oxo-1,3-oxazolidine-5-acetic acid (**18**), a structural isomer of **1** was also found to inactivate CPA in an irreversible manner, reinforcing the nucleophilic addition−elimination mechanism. The present study demonstrates that the transition state for the inactivation pathway plays a critical role in determining stereochemistry of the inactivation.

Introduction

Carboxypeptidase A (CPA, EC 3.4.17.1) is a prototypical zinc-containing proteolytic enzyme that cleaves off the C-terminal amino acid residue having a hydrophobic side chain.[1] The enzyme also catalyzes the hydrolysis of esters having structural feature similar to that of peptide substrate.[1] The X-ray crystal structure of the enzyme has been known to the resolution of 1.54 Å.[2] There are numerous zinc proteases of phathological importance such as angiotensin-converting enzyme,[3] enkephalinase,[4] and matrix metalloproteases[5] whose active site structures and catalytic mechanisms are thought to resemble those of CPA. The enzyme inhibitor design principles developed using CPA as a model enzyme[6] are of importance because the principles may be transferred to the zinc proteases of medicinal interest, leading to generation of lead compounds for new drug development.[7] We wish to report herein a novel inhibitor design concept that exploits the

(1) (a) Hartsuck, J. A.; Lipscomb, W. N. In *The Enzymes*, 3rd ed.; Boyer, P., Ed.; Academic: New York, 1971; Vol. 3, pp 1−56. (b) Christianson, D. W.; Lipscomb, W. N. *Acc. Chem. Res.* **1989**, *22*, 62−69.

(2) Rees, D. C.; Lewis, M.; Lipscomb, W. N. *J. Mol. Biol.* **1983**, *168*, 367−387.

(3) (a) Erdös, E. G. *Circul. Res.* **1975**, *36*, 247−255. (b) Bünning, P.; Holmquist, B.; Riordan, J. F. In *Biological Functions of Proteinases*; Hotzer, Tachesche Eds.; Springer-Verlag: Heidelberg, 1979; pp 269−275. (c) Cushman, D. W.; Cheung, H. S.; Sabo, E. F.; Ondetti, M. A. *Biochemistry* **1977**, *16*, 5484−5491.

(4) Grafford, J. T.; Skidgel, R. A.; Erdös, E. G.; Hersh, L. B. *Biochemistry* **1983**, *22*, 3265−3271.

(5) Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. J. H. *Chem. Rev.* **1999**, *99*, 2735−2776.

(6) For example: (a) Ner, S. K.; Suckling, C. J.; Bell, A. R.; Wriggleswoarth, R. *J. Chem. Soc. Chem. Commun.* **1987**, 480−482. (b) Kemp, A.; Tedford, M. C.; Suckling, C. J. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 557−562. (c) Kemp, A.; Ner, S. K.; Rees, L.; Suckling, C. J.; Tedford, M. C.; Bell, A. R.; Wrigglesworth, R. *J. Chem. Soc., Perkin Trans. 2* **1993**, 741−748. (d) Husbands, S.; Suckling, C. A.; Suckling, C. J. *Tetrahedron* **1994**, *50*, 9729−9742. (e) Mobashery, S.; Ghosh, S. S.; Tamura, Y.; Kaiser, E. T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1578−1582. (f) Tanaka, Y.; Grapsas, I.; Dakoji, S.; Cho, Y. J.; Mobashery, S. *J. Am. Chem. Soc.* **1994**, *116*, 7475−7480. (g) Massova, I.; Martin, P.; de Mel, S.; Tanaka, Y.; Edwards, B.; Mobashery, S. *J. Am. Chem. Soc.* **1996**, *118*, 12479−12480. (h) Lee, K. J.; Kim, D. H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2431−2436. (i) Lee, K. J.; Kim, D. H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2431−2436. (j) Kim, D. H.; Lee, K. J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2607−2612. (k) Yun, M.; Park, C.; Kim, S.; Nam, D.; Kim, S. C.; Kim, D. H. *J. Am. Chem Soc.* **1992**, *114*, 2281−2282. (l) Ryu, S.-E.; Choi, H.-J.; Kim, D. H. *J. Am. Chem Soc.* **1997**, *119*, 38−41.

(7) For example, (a) Ondetti, M. A.; Rubin, B.; Cushman, D. W. *Science* **1977**, *196*, 441−444. (b) Chorev, M.; Shavitz, R.; Goodman, M.; Minick, S.; Guillemen, R. *Science* **1979**, *204*, 1210−1212. (c) Kim, D. H.; Guinosso, C. J.; Buzby, G. C., Jr.; Herbst, D. R.; McCaully, R. J. *J. Med. Chem.* **1983**, *26*, 394−403. (d) Gaffard, J. T.; Skidgel, R. A.; Erdos, E. G.; Hersh, L. B. *Biochemistry* **1983**, *22*, 3265−3271.

10.1021/jo010421e CCC: $20.00
Published on Web 08/22/2001

unique ring cleavage reaction of oxazolidinone heterocycle, and synthesis of optically active α-benzyl-2-oxo-1,3-oxazolidine-4-acetic acid as a CPA inactivator.[8] Surprisingly, all four possible stereoisomers of the inhibitor were found to inactivate irreversiblly the enzyme with comparable potency. The lack of stereospecificity shown in the inactivation has been probed to envision that the inactivation reaction occurs via the nucleophilic addition−elimination mechanism initiated by the attack of the carboxylate of Glu-270 at the 2-position of the oxazolidinone in the enzyme bound inhibitor.

Results and Discussion

The active site of CPA has been well characterized.[1] The most important residues at the active site are Glu-270 and Arg-145. The former is directly involved in the catalytic hydrolysis of substrate, and the latter forms hydrogen bonds with the C-terminal carboxylate of substrate. In addition, there is present a hydrophobic pocket the primary function of which is to recognize substrate by accommodating the aromatic side chain in the P_1' residue of substrate. The catalytically essential zinc ion is held by His-69, Glu-72, His-195, and a molecule of water.

In the CPA-catalyzed hydrolysis of ester substrate, the scissile bond of the substrate becomes activated by the coordination of its carbonyl oxygen to the zinc ion at the active site of the enzyme. The activated carbonyl carbon is subjected to a nucleophilic attack by the carboxylate of Glu-270 to form an anhydride intermediate that hydrolyzes to products with regeneration of the enzyme. This pathway is referred to as the anhydride mechanism.[9] There has also been proposed an alternative pathway in which the carboxylate serves as a general base, activating the zinc bound water molecule which, in turn, attacks at the activated carbonyl carbon of the substrate to generate a tetrahedral transition state.[10] It is generally believed that the latter mechanism is in operation for the hydrolysis of peptide substrate.

Oxazolidinones are relatively stable chemical entities under ordinary conditions as evidenced by the fact that the heterocycle constitutes the molecular framework of most widely used chiral auxiliary in asymmetric synthesis of chiral organic compounds.[11] Nevertheless, the heterocycle reportedly undergoes ring cleavage reaction under certain conditions: As early as in 1885, Nemirowsky reported that treatment of oxazolidinone with hydrochloric acid affords 2-chloroethylamine as a hydrochloride salt.[12] Recently, Hsieh documented that

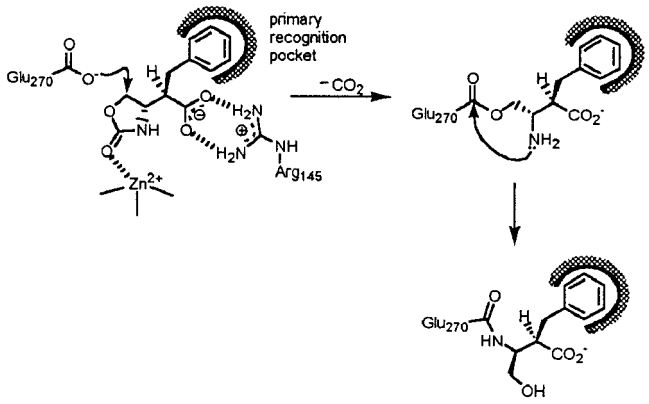
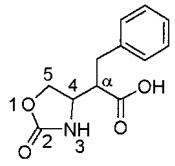


Figure 1. Rationale for designing **1** as a mechanism-based inactivator of CPA.

the cleavage of oxazolidinone also occurs by the treatment of carboxylic acids, generating esters of 2-aminoethanol.[13] These observations suggest that under the acidic conditions the 5-position of the ring becomes an electrophilic center presumably as a result of the protonation on the carbamoyl moiety in the ring. We envisioned that a metallic Lewis acid such as the zinc ion present at the active site of CPA might also be able to activate oxazolidinone for a nucleophile attack.

On the basis of the topology of the active site of CPA, the proposed catalytic mechanism, and the unique property of oxazolidinone, we have designed α-benzyl-2-oxo-1,3-oxazolidine-4-acetic acid (**1**) as an enzyme-activated irreversible inhibitor for CPA.



1

Due to its structural similarity to substrates of CPA, **1** is expected to bind the enzyme to form a Michaelis complex. In the complex, the carboxylate group of the inhibitor would form hydrogen bonds to the guanidinium moiety of Arg-145 and the phenyl ring anchors in the hydrophobic S_1' pocket of the enzyme (Figure 1).[14] This binding mode was thought to pose the oxazolidinone carbonyl oxygen of the inhibitor in close proximity to the active site zinc ion to form a coordinative bond with the zinc ion, resulting in the activation of the 5-position for a nucleophilic attack. An S_N2-type nucleophilic attack by the carboxylate of Glu-270 at the activated center may ensue. The unstable carbamic acid moiety that is generated in the process is expected to lose carbon dioxide to form an amino functionality which may undergo an intramolecular rearrangement, converting the ester linkage into a thermodynamically more stable amide bond. In the end, the inhibitor becomes covalently attached to the carboxylate and impairs the enzymic power of CPA permanently (Figure 1). To examine stereospecificity associated with the inhibition, we have synthesized all

(8) A preliminary account of the present work has been published: Kim, D. H.; Chung, S. J.; Kim, E.-J.; Tian, G. R. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 859−864.

(9) (a) Makinen, M. W.; Kuo, L. C.; Dymowski, J. D.; Jaffer, S. J. *J. Biol. Chem.* **1979**, *254*, 356−366. (b) Makinen, M. W.; Fukuyama, J. M.; Kuo, L. C. *J. Am. Chem. Soc.* **1982**, *104*, 2667−2669. (c) Sander, M. E.; Witzel, H. *Biochem. Biophys. Res. Commun.* **1985**, *132*, 681−687. (d) Suh, J.; Park, T. H.; Hwang, B. K. *J. Am. Chem. Soc.* **1992**, *114*, 5141−5146. (e) Britt, B. M.; Peticolas, W. L. *J. Am. Chem. Soc.* **1992**, *114*, 5295−5303.

(10) (a) Breslow, R.; Schepartz, A. *Chem. Lett.* **1987**, 1−4. (b) Breslow, R.; Wernick, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *107*, 1303−1307. (c) Galdes, A.; Auld, D. S.; Vallee, B. L. *Biochemistry* **1986**, *25*, 646−651. (d) Kim, H.; Lipscomb, W. N. *Biochemistry* **1990**, *29*, 5546−5555.

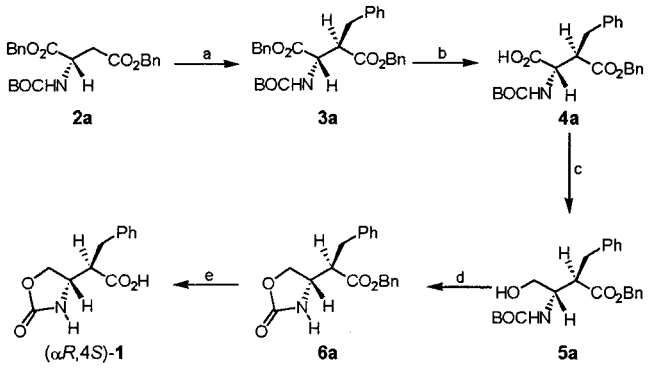
(11) (a) Seyden-Penne, J. *Chiral Auxiliaries and Ligands in Asymmetric Synthesis*, John Wiley: New York, 1995; pp 72−74. (b) Ager, D. J.; Prakash, I.; Schaad, D. R. *Aldrichimica Acta* **1997**, *30*, 3−12.

(12) Nemirowsky, J. J. *J. Prakt. Chem.* **1885**, *31*, 179.

(13) Hsieh, H. H. U.S. Patent **1984**, 4,444,694.

(14) Kim, D. H.; Kim, K. S.; Park, J. K. *Bull. Korean Chem. Soc.* **1994**, *15*, 805−807.

Scheme 1[a]



[a] Reagents, conditions, and (yields): (a) LHMDS (2 equiv), BnBr, −78 °C (46%); (b) 1 N NaOH, dioxane−water (5:1), rt, 24 h (90%); (c) isobutyl chloroformate, 1-ethylmorpholine, DME, −10 °C, 5 min, and then $NaBH_4$, −10 °C, 5 min; (d) TsCl, pyridine, rt, 7 d, (two-step yield: 33%); (e) H_2/Pd−C (80%).

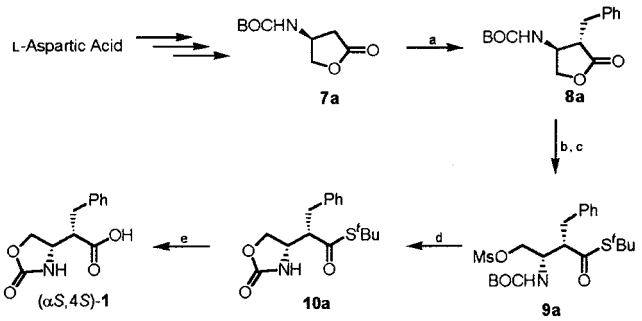
four possible stereoisomers of the inhibitor in an enantiomerically pure form and evaluated each of them for the CPA inactivating property.

Synthetic pathway for (α*S*,4*R*)-**1** is shown in Scheme 1. D-Aspartic acid was converted to N-protected dibenzyl ester (**2a**) which was then treated with benzyl bromide under Baldwin's conditions[15] to give **3a**. Trans-benzylated product was obtained exclusively. Regioselective hydrolysis to yield **4a** was achieved by the treatment of the dioxane solution of **3a** with 1 N NaOH solution according to the method reported by Berger and Katchalski.[16] The acid moiety was then reduced to the corresponding alcohol using the method described by Rodriguez et al.,[17] and the treatment of the N-protected amino alcohol (**5a**) thus obtained with tosyl chloride in pyridine afforded (α*S*,4*R*)-**1** as a benzyl ester **(6a)**.[18] Hydrogenolysis of the latter in the presence of Pd/C afforded (α*S*,4*R*)-**1**. In an analogous fashion, (α*R*,4*S*)-**1** was synthesized starting with L-aspartic acid.

Scheme 2 outlines synthetic path for the preparation of (α*S*,4*S*)-**1**. Lactone **7** obtained from L-aspartaic acid by the route reported by Yoda et al.[19] was benzylated as described by Takahashi et al.[20] to afford exclusively trans-benzylated product **8**. Treatment of **8** with the Weinreb's reagent[21] and subsequent methanesulfonylation of the resulted alcohol moiety gave **9** having the erythro configuration. Simple warming of **9** in pyridine produced **10** in 64% yield. Hydrolysis of the thioester moiety in **10** to give (α*S*,4*S*)-**1** was achieved by the method described by Masamune et al.[22]

The compounds thus synthesized were assayed for inhibitory activity against CPA using (*S*)-hippurylPhe

Scheme 2[a]



[a] Reagents, conditions, and (yields): (a) LDA (2.2 equiv), HMPA, and then BnBr, −78 °C, THF (73%); (b) ^{t}BuSAlMe$_2$, CH_2Cl_2, 0 °C to rt; (c) MsCl, Et_3N, CH_2Cl_2, 0 to 5 °C (two-step yield: 88%); (d) pyridine, 70 °C (64%); (e) $Hg(OCOCF_3)_2$, dioxane−water, 50 °C (64%).

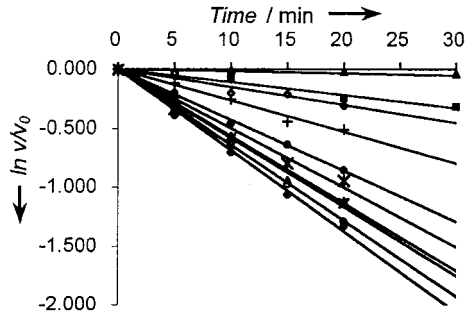


Figure 2. Plot of $-\ln(v/v_o)$ vs incubation time, showing time-dependent loss of CPA activity by (α*S*,4*R*)-**1** (◆, $[I]_0 = 30$ mM; ○, $[I]_0 = 28$ mM; △, $[I]_0 = 26$ mM; ×, $[I]_0 = 24$ mM; *, $[I]_0 = 22$mM; ●, $[I]_0 = 20$mM; +, $[I]_0 = 18$ mM; ◇, $[I]_0 = 16$ mM; ■, $[I]_0 = 14$ mM; ▲, $[I]_0 = 0$).

(Hipp-L-Phe) as substrate. CPA was incubated with an excess of the inhibitor and the loss of enzymic activity was monitored at different time intervals. Figure 2 is an exemplary plot for the inhibition of CPA with (α*S*,4*R*)-**1**.

All four diastereoisomers of **1** inhibited CPA in a time-dependent manner, suggesting that the inhibitions occur in an irreversible fashion.[23] The irreversible nature of the inhibitions was ascertained by dialysis experiment: The inactivated enzyme failed to regain the enzymic activity after dialysis against the buffered solution for 2 days. Furthermore, the analysis of the electrospray ionization (ESI) mass spectrum of the inactivated CPA in comparison with that of the native CPA confirmed the conclusion derived from the kinetic analysis. The native CPA showed peaks at 34070, 34103, 34127, 34154, 34181 and 34244 Da arising from allotypes of CPA[24] and the inactivated CPA by (α*S*,4*R*)-**1** at 34268, 34301, 34322, 34346, and 34436 Da. The molecular weight differences (192 Da) between the two spectra correspond to (CPA + **1**) − CPA − CO_2.[25]

The inactivation reaction of CPA by **1** may be represented by eq 1, in which E•I represents noncovalently bound complex of the enzyme with the inhibitor

(15) Baldwin, J. E.; Moloney, M. G.; North, M. *Tetrahedron* **1989**, *45*, 6309−6318.

(16) Berger, A.; Katchalski, E. *J. Am. Chem. Soc.* **1951**, *73*, 4084−4088.

(17) Rodriguez, M.; Llinares, M.; Doulut, S.; Heitz, A.; Martinez, J. *Tetrahedron Lett.* **1991**, *32*, 923−926.

(18) Curran, T. P.; Pollastri, M. P.; Abelleira, S. M.; Messier, R. J.; McCollum, T. A.; Rowe, C. G. *Tetrahedron Lett.* **1994**, *35*, 5409−5412.

(19) Yoda, H.; Nakagami, Y.; Tanabe, K. *Tetrahedron*: *Asymmetry* **1994**, *5*, 169−172.

(20) (a) McGarvey, G. J.; Williams, J. M.; Hiner, R. N.; Matsubara, Y.; Oh, T. *J. Am. Chem. Soc.* **1986**, *108*, 4943−4952. (b) Takahashi, Y.; Hasegawa, S.; Izawa, T.; Kobayashi, S.; Ohno, M. *Chem. Pharm. Bull.* **1986**, *34*, 3020−3024.

(21) Hatch, R. P.; Weinreb, S. M. *J. Org. Chem.* **1977**, *42*, 3960−3961.

(22) Masamune, S.; Kamata, S.; Schilling, W. *J. Am. Chem. Soc.* **1975**, *97*, 3515−3516.

(23) Silvermann, R. B. *Mechanism-Based Enzyme Inactivation*: *Chemistry and Enzymology*; CRC: Boca Raton, 1988; Vol. I, Chapter 1.

(24) (a) Bradshaw, R. A.; Ericsson, L. H.; Walsh, K. A.; Neurath, H. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *63*, 1389−1394. (b) Pétra, P. H.; Neurath, H. *Biochemistry* **1969**, *8*, 2466−2475. (c) Burkrinsky, J. T.; Bjerrum, J.; MKadziola, A. *Biochemistry* **1899**, *37*, 16555−16564.

$$E + I = E\bullet I \rightarrow E{-}I' \quad (1)$$

and E−I′ is covalently modified CPA by the inhibitor. In the inhibition assay, if the inhibitor concentration is sufficiently greater than the total concentration of the enzyme, k_{obs} is related to the total inhibitor concentration ($[I]_o$) by eq 2, in which K_I represents the dissociation constant of the E•I complex, and k_{inact} represents inactivation rate constant.[26]

$$\frac{1}{k_{obs}} = \frac{1}{k_{inact}} + \frac{K_I}{k_{inact}}\frac{1}{[I]_o} \quad (2)$$

Equation 2 may be further reduced to give eq 3, if the K_I value is much greater than the $[I]_o$ value,[27] showing that the second-order inhibitory rate constant can be expressed by $k_{obs}/[I]_o$. The irreversible inhibitory activities of all four diastereoisomers of **1** toward CPA were evaluated by the incubation method and their second-order rate constants expressed in $k_{obs}/[I]_o$ are listed in Table 1. The values of k_{obs} were calculated from plots of ln v/v_o vs incubation time.

$$\frac{k_{inact}}{K_I} = \frac{k_{obs}}{[I]_o} \quad (3)$$

Poor solubility of these compounds in the kinetic medium prohibited the determination of the K_i values. The protection from the inactivation by 2-benzylsuccinic acid, a potent active site directed reversible inhibitor for CPA[28] was observed for all cases, indicating that the inactivations take place at the active site (Figure 3). The partition ratio (k_{cat}/k_{inact}) that reflects the efficiency of an inactivator was determined for all four inhibitors by the titration method,[29] and these kinetic parameters are included in Table 1.

Enzyme catalyzes chemical transformations stereospecifically, and the stereospecificity has its origin in the chiral nature of the enzyme active site where the binding and chemical transformations of substrate take place. It was surprising to learn that all four diastereoisomers of **1** inactivate CPA. The lack of stereospecificity shown in the inactivation of CPA by **1** can hardly be accounted for in terms of the design rationale. The S_N2 type ring cleavage reaction caused by the attacking at the 5-position of the oxazolidinone requires the carboxylate nucleophile to approach the electrophilic center in the 180°direction to the leaving group, and only two out of the four diastereomers of **1** are thought to satisfy the requirement, thus leading to inactivation of CPA. This expectation is inferred from the inactivation of CPA by

Table 1. Kinetic Parameters for CPA Inactivation

inhibitor	k_{obs}[a] (min^{-1})	$k_{obs}/[I]_0$ (M^{-1} min^{-1})	partition ratio
(α*S*,4*R*)-**1**	0.0333	1.665	94
(α*R*,4*S*)-**1**	0.0453	2.265	120
(α*R*,4*R*)-**1**	0.0607	3.035	133
(α*S*,4*S*)-**1**	0.0723	3.615	121

[a] Apparent first-order rate constant at inhibitor concentration of 20 mM.

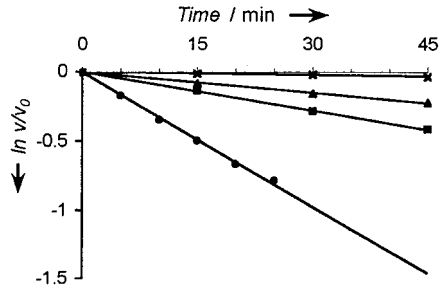


Figure 3. Active site protection of (α*S*,4*R*)-**1** caused CPA inactivation by (*R*)-2-benzylsuccinic acid (BSA): (×, no inhibitor; ▲, (α*S*,4*R*)-**1** (20 mM) and BSA (20 μM); ■, (α*S*,4*R*)-**1** (20 μM) and BSA (10 μM); ●, (α*S*,4*R*)-**1** (20 mM).

2-benzyl-3,4-epoxybutanoic acid[30] whose oxirane ring is cleaved by the S_N2 type nucleophilic attack at the 3-position of the ring by the Glu-270 carboxylate. Out of four stereoisomers of 2-benzyl-3,4-epoxybutanoic acid only two diastereoisomers having the (2*S*,3*R*)- and (2*R*,3*S*)-configurations that meet the stereochemical requirement for the S_N2 type ring cleavage reaction were active.[30]

We became to realize that the proposed binding mode of the oxazolidinone moiety in the complex of **1** with CPA is incongruous with the topology of the CPA active site crevice. The distance between the zinc ion and the carboxylate of Glu-270 is estimated to be 4.1−4.6 Å from the X-ray crystal structure of CPA.[2] On the other hand, the distance between the carbonyl oxygen and a hydrogen atom at the 5-position of oxazolidinone ring was learned to be 4.09 Å from the X-ray crystal structure,[31] indicating that the heterocycle is too big to fit in the space between the carboxylate and the zinc ion with its carbonyl oxygen forming a coordinative bond to the metal ion. Accordingly, a likely mode of resting of the heterocycle in the complex involves that the oxazolidinone ring resides horizontally between the carboxylate and the zinc ion. In this binding mode, the nucleophilic attack at the 5-position of the ring would be improbable. Instead, the carboxylate nucleophile would attack at the 2-position of the heterocycle. A recent report of Najer et al. is noteworthy in this regard. They reported that treatment of oxazolidinone with alkylamine affords N,N′-disubstituted urea,[32] indicating that in the absence of Lewis acid the nucleophilic attack takes place not at the 5-position but at the 2-position of the heterocycle and there occurs a ring cleavage by the addition−elimination mechanism. On the basis of the foregoing consideration, an alternative CPA inactivation pathway that can account for the observed lack of stereospecificity is proposed as depicted in Figure 4. The

(25) Suh and Kaiser reported that (+)-(*R*)-(*trans*-cinnamoyl)-α-mercapto-β-phenylpropinate acylates the phenolic hydroxyly of Tyr-248 and proposed that the inhibitor binds CPA in an inversed fashion with its carboxylate binding to the active site zinc ion and the hydroxyl of Tyr-248 attacks on the thioester moiety (Suh, J.; Kaiser, E. T. *Biochem. Biphys. Res. Commun.* **1975**, *64*, 863−869). An analogous binding of **1** to CPA may be feasible, but the possibility that the cleavage of the unactivated oxazolidinone ring by the Tyr hydroxyl is ruled out on the ground of the mass spectral data: if the hydroxyl group is modified, it would be in the form of carbamate, and the molecular weight difference would be 236 Da rather than 192 Da.

(26) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245- 3249.

(27) The K_I value of 576 mM was obtained for (α*S*,4*R*)-**1** from the Kitz-Wilson plot at high concentration (>20 mM) of the inactivator.

(28) Byers, L. D.; Wolfenden, R. *Biochemistry* **1973**, *12*, 2070−2078.

(29) Silvermann, R. B. *Mechanism-Based Enzyme Inactivation*: *Chemistry and Enzymology*; CRC: Boca Raton, 1988; Vol. I, pp 22−23.

(30) Lee, S. S.; Li, Z.-H.; Lee, D. H.; Kim, D. H. *J. Chem. Soc., Perkin Trans. 1* **1995**, 2877−2882

(31) Wouters, J.; Ooms, F.; Durant, F. *Acta Crystallogr.* **1997**, *C57*, 895−897.

(32) (a) Najer, H.; Chabrier, P.; Giudicelli, R.; Mabille, P. *Bull. Soc. Chim. Fr.* **1957**, 1069−1072. (b) Najer, H.; Chabrier, P.; Giudicelli, R.; Menin, J.; Duchemin, J. *Bull. Soc. Chim. Fr.* **1959**, 1841−1844.

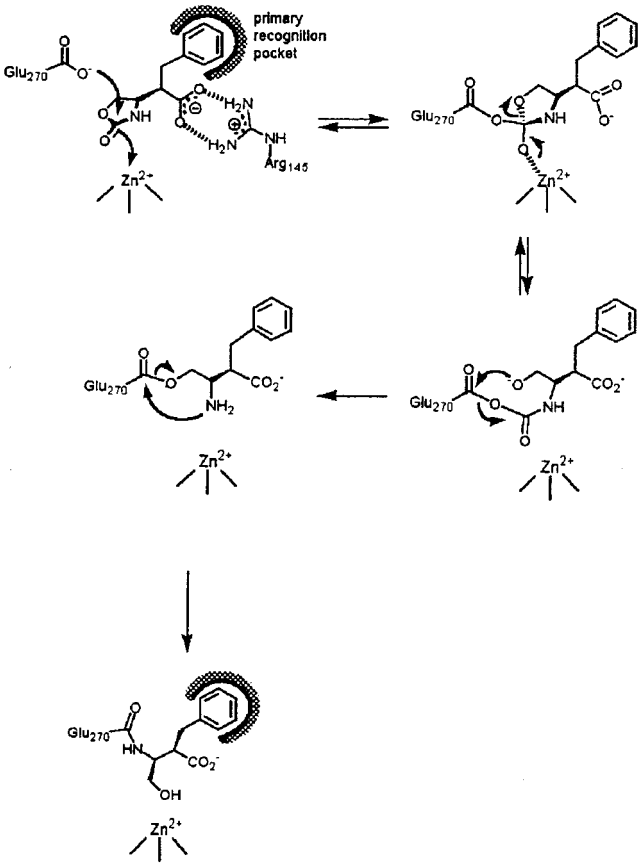


Figure 4. Proposed reaction pathway for the inactivation of CPA by **1**.

Glu-270 carboxylate attacks at the 2-position of the oxazolidinone of the CPA-bound **1** and opens the ring via a tetrahedral transition state. Such a reaction does not require the stringent directionality of the attacking nucleophile. The approach of a nucleophile on an ester carbonyl carbon is known to take place within a cone of 30° with the axis being 15° off of the normal to the carbonyl plane.[33]

Being different from chemical reactions taken place in solution, the chemical transformations that occur at the active site of an enzyme can proceed only when the two interacting species are poised within a critical distance with proper stereochemical orientation.[34] The lack of stereospecificity observed in the inhibition of CPA by **1** may now be envisioned: Since the oxazolidinone ring is not coordinated to the active site zinc ion, the heterocycle in the CPA-bound **1** may be poised in close proximity to the nucleophilic carboxylate and the tetrahedral transition states that are generated by the nucleophilic attack are conceivably stabilized by the active site zinc ion (Figure 4).

Wipff et al. suggested that the stereospecificity in enzymic reactions is determined in the transition states rather than in the initial Michaelis complex formation.[35] Transition state that is thought to be involved in the oxazolidinone ring cleavage by the nucleophilic attack

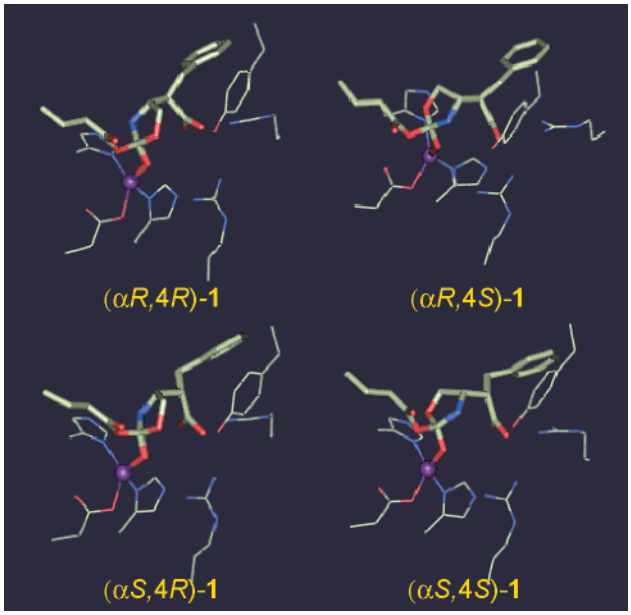


Figure 5. Transition states that are generated by the PM3 semiemprical calculations for the inactivation of CPA by **1**. Each of the putative tetrahedral transition states formed by the attack of Glu-270 carboxylate at the C-2 of oxazolidinone of the CPA bound **1** is stabilized by the active site zinc ion. Thin line represents key amino acid residues involved in the transition state formation and thick line denotes inhibitor **1**. A: Transition state formed with (α*R*,4*R*)-**1**. B: Transition state formed with (α*R*,4*S*)-**1**. C: Transition state formed with (α*S*,4*R*)-**1**. D: Transition state formed with (αS,4*S*)-**1**.

was examined by the PM3 semiempirical calculations.[36] Each of the diastereoisomers of **1** was docked to the X-ray crystal structure of the CPA active site that consists of Zn^{2+}, His-69, Glu-72, His-196, Arg-127, Tyr-248 Arg-145, and Glu-270, and the putative tetrahedral transition states were optimized (Figure 5).[37]

The transition state formed with (α*S*,4*S*)-**1** is found to be most stable. The calculated energies for transition states in the reaction with (α*R*,4*R*)-, (αR,4*S*)-, and (α*R*,4*S*)-**1** are higher compared with that of the most stable complex[38] by 1.6, 4.0, and 5.3 kcal mol^{-1}, respectively.[39] The plot of the relative energy against -ln(k_{obs}/$k_{obs[(aS,4S)-1]}$)[40] for the four inactivation reactions yielded a straight line, suggesting that all four diastereoisomers inactivate CPA along the similar pathway that involves the tetrahedral transition state which is stabilized by the active site zinc ion and by hydrogen bondings with Arg-127, Arg-147, and Tyr-248.

Compound **18** is an isomer of **1**, in which oxazolidinone ring is linked at its 5-position to the α-carbon of 3-phenylpropionic acid. It was thought to be worthwhile to evaluate **18** as an inactivator of CPA because if indeed the inactivation of CPA by **1** takes place with the attack

(33) (a) Storm, D. R.; Koshland, D. E., Jr. *J. Am. Chem. Soc.* **1972**, *94*, 5815−5825. (b) Bürgi, H. B.; Dunitz, J. D.; Shefter, E. *Acta Crystallogr* **1974**, *B30*, 1517−1527. (c) Lightstone, F. C.; Bruice, T. C. *J. Am. Chem. Soc.* **1996**, *118*, 2595−2605.

(34) Menger, F. M. *Acc. Chem. Res.* 1985, *18*, 128−134.

(35) Wipff, G.; Dearing, A.; Weiner, P. K.; Blaney, J. M.; Kollman, P. A. *J. Am. Chem. Soc.* **1983**, *105*, 997−1005.

(36) (a) Stewart, J. J. P. *J. Comput. Chem.* **1989**, *10*, 209−220. (b) Stewart, J. J. P. *J. Comput. Chem.* **1989**, *10*, 221−264.

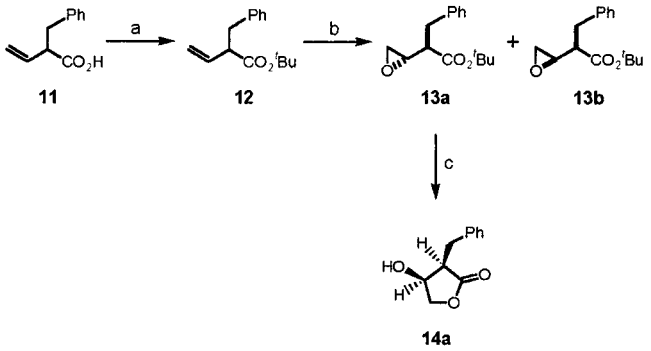
(37) In the optimization by the PM3 method, the active site structure of CPA was fixed to the geometry of the X-ray crystal structure of CPA.

(38) The energy of the most stable transition state was set to zero.

(39) The difference in the energy state shown for the four transition state may be much reduced in the real inactivation pathway because most of the strains imposed for the calculations by using the rigid X-ray structure would be expected to disappear in the real enzyme system, i.e., most of the strain energies would be absorbed in conformational changes of the enzyme.

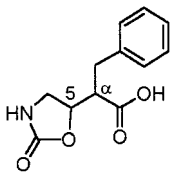
(40) (k_{obs}/$k_{obs[(\alpha S,4S)-1]}$) denotes the ratio of the k_{cat} value for a diastereoisomer of **1** vs that for (α*S*,4*S*)-**1**.

Scheme 3[a]



[a] Reagents, conditions, and (yields): (a) *t*-BuOH, H_2SO_4, $MgSO_4$, CH_2Cl_2 (70%); (b) *m*CPBA, K_2HPO_4, CH_2Cl_2 (81%); (c) TFA, CH_2Cl_2.

of the carboxylate at the 2-posistion of the oxazolidinone, **18** should also be equally effective as a CPA inactivator. Due to the ring nitrogen being a poor leaving group, it is highly unlikely that the oxazolidinone undergoes ring cleavage by the nucleophilic attack at the 4-positon of the ring.



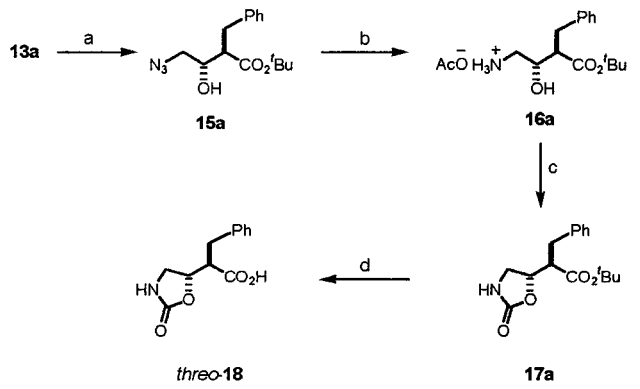
18

Synthesis of **18** commenced with vinylacetic acid. 2-Benzylvinylacetic acid that was obtained by benzylation on vinylacetic acid was esterified following the method reported by Wright et al.[41] to give **12**. Epoxidation of **12** with *m*-CPBA gave the diastereoisomeric mixture of **13a** and **13b** in the ratio of 2:1 (Scheme 3). The mixture was readily separated by column chromatography. Stereochemistry of **13a** and its conversion products including *threo*-**18** was ascertained by the 2D ^{1}H NMR NOE spectrum obtained with **14a**[42] that was prepared from **13a** by the treatment with TFA: A positive NOE was observed between the C_3-H at 2.84 (ddd, J = 11.5, 4.0, 4.0 Hz) and C_4−H at 4.41 (dd, J = 4.0, 3.0 Hz), indicating that the benzyl and hydroxyl groups in **14a** are on the same side of the lactone ring, from which it was inferred that the oxirane ring in **13a** is oriented trans to the benzyl moiety.

Treatment of **13a** with sodium azide in the presence of ammonium chloride in 60% ethanol solution afforded **15a**. Hydrogenation of **15a** in the presence of Pd/C converted the azido into amine to give **16a** which was treated with carbonyldiimidazole in methylene chloride to give **17a**. The ester moiety in **17a** was hydrolyzed by treatment with TFA to yield *threo*-**18** as a racemic mixture (Scheme 4). The erythro form of **18** was synthesized in a similar fashion from **13b**.

Compound **18** thus prepared in racemic threo and erythro forms were assayed for CPA inactivating activity

Scheme 4[a]



[a] Reagents, conditions, and (yields): (a) NaN_3, NH_4Cl, EtOH−H_2O (96%); (b) H_2, Pd/C, AcOH, MeOH−H_2O (60%); (c) carbonyldiimidazole, CH_2Cl_2 (74%); (d) TFA, CH_2Cl_2 (73%)

Table 2. Kinetic Parameters for CPA Inactivation

inhibitor	k_{obs}[a] (min^{-1})	$k_{obs}/[I]_0$ (M^{-1} min^{-1})
threo-**11**	0.0351	1.755
erythro-**11**	0.0773	3.685

[a] Apparent first-order rate constant at inhibitor concentration of 20 mM.

to find that both forms inhibit CPA in a time-dependent and competitive manner. The inactivated enzyme did not regenerate the catalytic activity upon dialysis against the kinetic buffer, suggesting strongly that they are effective as inactivators for CPA. Kinetic parameters for the inactivation of CPA by **18** are listed in Table 2. It can be seen from Table 2 that they inactivate CPA with potency comparable to those of **1**. These results provide an additional supportive evidence for the proposed inactivation pathway that involves the attack of the Glu-270 carboxylate at the C-2 site of oxazolidinone of the CPA bound **1** as depicted in Figure 4.

Conclusion

Inhibitor **1** represents a new type of mechanism-based inactivator for CPA designed rationally on the basis of the established active site topology of the enzyme and a proposed catalytic mechanism for the enzymic reaction. In the designing, the unique property of the oxazolidinone heterocycle was exploited as a latent inactivating species. Surprisingly, all four diastereoisomers of **1** inactivated CPA. The unexpected lack of stereospecificity shown by the inhibitor may be envisioned on the ground that the Glu-270 carboxylate attacks at the carbamate carbonyl carbon rather than the 5-position of the oxazolidinone of CPA bound **1**, and each of tetrahedral transition state thus generated is stabilized by the active site zinc ion. This stabilization of the transition state may also explain the facile cleavage of the relatively stable oxazolidinone by the enzyme under the ambient conditions, leading to covalently modify the nucleophilic carboxylate. The new design concept may hold the promise of a viable approach for designing selective inactivators of metalloproteases.

Experimental Section

General Methods. Melting points were measured on a capillary melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a 300 MHz NMR

(41) Wright, S. W.; Hageman, D. L.; Wright, A, S.; Mcclure, L. D. *Tetrahedron Lett.* **1997**, *38*, 7345−7348.

(42) Compound **14a** has been reported previously but the assigned stereochemistry was not established (Maruoka, O.; Tanabe, G.; Sano, K.; Minematsu, T.; Momese, T. *J. Chem. Soc., Perkin Trans 1* 1994, 1883−1845.).

spectrometer using tetramethylsilane as an internal standard. ESI mass spectra were recorded on CPA (native and inactivated by (αS,4*R*)-**1**)) that was dissolved in formic acid and then diluted with acetonitrile. Flash chromatography was performed on silica gel 60 (230−400 mesh). Elemental analyses were performed at the Center for Biofunctional Molecules, Pohang University of Science and Technology, Pohang, Korea, and the Korea Basic Science Center, Taegu, Korea. All chemicals were of reagent grade and obtained from Aldrich Chemical Co. The solvents were purified before use.

(2*R*,3*S*)-*N*-*tert*-Butyloxycarbonyl-3-benzylaspartic Acid Dibenzyl Ester (3a). To a solution of hexamethyldisilazane (58.6 mL, 50 g, 278 mmol) in THF (400 mL) was added *n*-butyllithium in hexane (10 M, 24.5 mL, 245 mmol) at 0 °C under nitrogen atmosphere and the mixture was stirred for 10 min, then cooled to −78 °C. A solution of *N*-*tert*-butyloxycarbonyl-L-aspartic acid dibenzyl ester (**2a**) (46 g, 111.2 mmol) in THF (200 mL) was added slowly to the mixture and the reaction mixture was allowed to stir for 3 h at the same temperature. Benzyl bromide (26.4 mL, 38 g, 222 mmol) was added and the reaction mixture was stirred for an additional 4 h, and quenched by addition of 3 N hydrochloric acid to pH ∼3. The aqueous layer was saturated with sodium chloride, and then extracted with ethyl acetate (200 mL × 2). The combined organic layer was dried over anhydrous magnesium sulfate and evaporated under reduced pressure to give an oily residue which crystallized on standing. The product was recrystallized from methanol to afford white crystalline **3a** (26 g, 46%): mp 82.5−84 °C; $[\alpha]_D$ −0.9° (*c* 1.0, $CHCl_3$); IR (KBr) 3396 (NH), 1733 (C═O), 1717 (C═O), 1503 (C═O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9H), 2.84 (dd, 1H), 3.08 (dd, 1H), 3.42 (sextet, 1H), 4.50 (d, 1H), 4.92−5.10 (m, 4H), 5.56 (d, 1H) and 7.10−7.32 (m, 15H); MS−FAB *m*/*z* 504 (MH^+) and 526 ($M + Na^+$). Anal. Calcd for $C_{30}H_{33}NO_6$: C, 71.55; H, 6.60; N, 2.78. Found: C, 71.44; H, 6.72; N, 2.83.

(2*S*,3*R*)-*N*-*tert*-Butyloxycarbonyl-3-benzylaspartic Acid Dibenzyl Ester (3b) was obtained in 53% yield from *N*-*tert*-butyloxycarbonyl-D-aspartic acid dibenzyl ester (**2b**) following the procedure used for the preparation of **3a**: mp 82.5−84 °C; $[\alpha]_D$ +1.0° (*c* 1.0, $CHCl_3$).

(2*R*,3*S*)-*N*-*tert*-Butyloxycarbonyl-3-benzylaspartic Acid *β*-Benzyl Ester (4a). To an ice-cooled solution of **3a** (21.36 g, 42.4 mmol) in 80% aqueous dioxane (530 mL) was added an aqueous solution of 1 N sodium hydroxide (42 mL, 42 mmol) and 80% aqueous dioxane (540 mL). The resultant solution was stirred at room temperature for 24 h, acidified with 3 N hydrochloric acid at 0 °C to pH ∼3, and extracted with ether (200 mL × 3). The combined extract was dried over anhydrous magnesium sulfate, and filtered. Dicyclohexylamine (44 mmol) was added to the filtrate, and chilled in a refrigerator for 24 h to afford a fine crystalline solid (20.1 g, 80%). The solid was collected and treated with cold 1 N hydrochloric acid (60 mL) and extracted with ether (100 mL × 3). The combined extract was dried over anhydrous magnesium sulfate. Evaporation of the organic solvent afforded an oily residue which crystallized on standing. Recrystallization from benzene-petroleum ether gave white crystalline **4a** (12 g, 68.5%): mp 108−110 °C; $[\alpha]_D$ +24° (*c* 1, $CHCl_3$); IR (KBr) 3263 (NH), 1740 (C═O), 1706 (C═O), 1647 (C═O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9H), 2.88 (dd, 1H), 3.13 (dd, 1H), 3.42 (sextet, 1H), 4.44 (dd, 1H), 5.08 (dd, 2H), 5.70 (d, 1H) and 7.20−7.41 (m, 10 H); MS-FAB *m*/*z* 414 (MH^+). Dicyclohexylamine salt: mp 178−180 °C; IR (KBr) 3422 (NH), 1734 (C═O), 1712 (C═O) and 1635 (C═O) cm^{-1}; ^1H NMR (300 MHz; DMSO-d_6) δ 1−1.5 (m, 19H), 1.57 (d, 2H), 1.70 (d, 4H), 1.95 (d, 4H), 2.69 (dd, 1H), 2.73−3.0 (m, 3H), 3.20 (m, 1H), 4.08 (d, 1H), 4.99 (dd, 2H), 5.95 (d, 1H) and 7.00−7.40 (10H, m); MS−FAB *m*/*z* 595 (MH^+). Anal. Calcd for $C_{23}H_{27}NO_6$: C, 66.81; H, 6.58; N, 3.39. Found: C, 67.12; H, 6.55; N, 3.39.

(2*S*,3*R*)-*N*-*tert*-Butyloxycarbonyl-3-benzylaspartic acid *β*-benzyl ester (4b) was prepared from **3b** in 75% yield following the procedure used for the preparation of **4a**: mp 108−110 °C; $[\alpha]_D$ −26° (*c* 1, $CHCl_3$).

(α*R*,4*S*)-α-Benzyl-2-oxo-1,3-oxazolidine-4-acetic Acid Benzyl Ester (6a). To a cooled solution of **4a** (4.7 g, 11.38 mmol) in 1,2-dimethoxyethane (30 mL) were successively added 1-ethylmorpholine (1.6 mL, 1.44 g, 12.5 mmol) and isobutyl chloroformate (1.61 mL, 1.7 g, 12.5 mmol) at −15 °C. After the mixture was stirred for 3 min, a solution of sodium borohydride (0.82 g, 34.16 mmol) in water (9 mL) was added in one portion. After a few minutes, the reaction mixture was quenched by the addition of water (500 mL). The resulting solution was extracted with ethyl acetate (200 mL × 3), and the combined organic layer was dried over anhydrous magnesium sulfate. Evaporation of the organic solvent under reduced pressure afforded a reduction product **5a**, which was added in a dried pyridine solution (20 mL) containing an excess amount of *p*-toluenesulfonyl chloride (7 g). The reaction mixture was stirred at room temperature for 7 days and diluted with ether (200 mL). The ether solution was washed with 10% sulfuric acid (50 mL × 2), water, and brine and dried over anhydrous magnesium sulfate. The oily residue thus obtained from evaporation of the solvent was purified via flash chromatography (10% methanol in chloroform). The product which was slowly crystallized on evaporation of the solvent was recrystallized from ethanol to afford white crystalline **6a** (1.2 g, 33%): mp 105−106 °C; $[\alpha]_D$ +19.2° (*c* 0.5, $CHCl_3$); IR (KBr) 3296 (NH), 1749 (C═O), 1722 (C═O) cm^{-1}; ^1H NMR (300 MHz; $CDCl_3$) δ 2.88 (m, 3H), 4.01 (m, 2H), 5.04 (s, 2H), 5.72 (s, 1H), 6.90−7.30 (m, 10H); EIMS *m*/*z* 325 (M^+). Anal. Calcd for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.88; N, 4.30. Found: C, 70.05; H, 5.81; N, 4.54.

(α*R*,4*S*)-α-Benzyl-2-oxo-1,3-oxazolidine-4-acetic acid benzyl ester (6b) was prepared from **4b** in 30% yield following the procedure used for the preparation of **6a**: mp 105−106 °C; $[\alpha]_D$ −19.2° (*c* 0.5, $CHCl_3$).

(α*R*,4*S*)-α-Benzyl-2-oxo-1,3-oxazolidine-4-acetic Acid ((α*R*,4*S*)-1). A solution of **6a** (1 g, 3 mmol) and 10% palladium on charcoal (0.2 g) in methanol (10 mL) was stirred under hydrogen atmosphere at room temperature for 2 h and filtered. Concentration of the filtrate under reduced pressure gave the solid residue which was recrystallized from water to afford white crystalline product (560 mg, 80%): mp 178−179 °C; $[\alpha]_D$ −2.4° (*c* 0.5, EtOH); IR (KBr) 3277 (NH), 3500−2500 (acid OH) 1716 (C═O) and 1700 (C═O) cm^{-1}; ^1H NMR (300 MHz; DMSO-d_6) δ 2.75 (m, 2H), 2.85 (dd, 1H), 3.97 (quintet, 1H), 4.24 (dd, 1H), 4.37 (t, 1H), 7.16−7.30 (m, 5H) 7.93 (s, 1H) and 12.45 (s, 1H); EIMS *m*/*z* 191 ($M^+ - CO_2$), 235 (M^+). Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.29; H, 5.53; N, 5.96. Found: C, 61.05; H, 5.54; N, 5.91.

(α*S*,4*R*)-α-Benzyl-2-oxo-1,3-oxazolidine-4-acetic acid ((α*S*,4*R*)-**1**) was prepared from **6b** in 85% yield following the procedure used for the preparation of (α*R*,4*S*)-**1**: mp 178−179 °C; $[\alpha]_D$ +2.4° (*c* 0.5, EtOH). Anal. Calcd for $C_{12}H_{13}NO_4 \cdot {}^1/_5H_2O$: C, 60.37; H, 5.66; N, 5.87. Found: C, 60.66; H, 5.50; N, 5.50.

(3*S*,4*S*)-3-Benzyl-4-(*tert*-butyloxycarbonylamino)-*γ*-butyrolactone (8a). To a stirred solution of lithium diisopropylamide (50 mmol) in THF (40 mL)-HMPA (40 mL) at −78 °C was added dropwise a solution of compound (*S*)-4-(*tert*-butyloxycarbonylamino)-*γ*-butyrolactone (**7a**) (4.1 g, 20.4 mmol) in THF (50 mL). After 2 h benzyl bromide (5.1 g, 30 mol) was added slowly at the same temperature and the resulting mixture was stirred for 3 h, acidified with 10% aqueous citric acid to pH 3 and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (100 mL × 3). The combined organic layer was washed with 10% aqueous citric acid (100 mL × 3), saturated aqueous $NaHCO_3$ (100 mL × 2), brine (100 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure. Purification via flash chromatography (10∼25% ethyl acetate in hexane) gave **8a** (4.33 g, 73%) as white crystals: mp 89−90 °C (recrystallized from ether−hexane); $[\alpha]_D$ −20.3° (*c* 0.62, $CHCl_3$); IR ($CHCl_3$) 3300 (NH), 1776 (C═O), 1712 (C═O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.32−7.19 (m, 5H), 4.55, (br s, 1H), 4.29−4.15 (m, 2H), 3.88 (dd, J = 8.72, 6.41 Hz, 1H), 3.19 (dd, J = 14.04, 4.23 Hz, 1H), 2.95 (dd, J = 13.40, 7.09 Hz, 1H), 2.78 (dd, J = 7.38,

5.14 Hz, 1H), 1.37 (s, 9H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 176.64, 155.29, 137.38, 129.55, 127.50, 80.75, 71.36, 52.41, 47.25, 34.9, 28.64; EIMS *m/z* 291 (M^+), 235 ($M^+ - C_4H_8$). Anal. Calcd for $C_{16}H_{21}NO_4$: C, 65.96; H, 7.27; 4.81. Found: C, 65.90; H, 7.39; 4.57.

(3*R*,4*R*)-3-Benzyl-4-(*tert*-butyloxycarbonylamino)-γ-butyrolactone (8b) was prepared from (*R*)-4-(*tert*-butyloxycarbonylamino)-γ-butyrolactone (**7b**) by the same method as the preparation of **8a**: mp 91−92 °C; $[\alpha]_D$ +18.4° (*c* 0.63, $CHCl_3$). Anal. Calcd for $C_{16}H_{21}NO_4$: C, 65.96; H, 7.27; 4.81. Found: C, 66.18; H, 7.35; 4.68.

(2*S*,3*S*)-2-Benzyl-3-(*tert*-butyloxycarbonylamino)-4-(methanesulfonyloxy)butanoic Acid *tert*-Butyl Thioester (9a). *tert*-Butylthiol (1.16 mL, 10.3 mmol) was added to a solution of trimethylaluminum (10.3 mmol) in dichloromethane (8.15 mL) at 0 °C, and the resultant mixture was allowed to warm to room temperature over 20 min with stirring. A solution of compound **8a** (1 g, 3.43 mmol) in dichloromethane (8 mL) was added and the mixture was stirred overnight. The mixture was cooled to −78 °C and quenched with ether (20 mL) followed by careful addition of 1 N hydrochloric acid (30 mL). The aqueous layer was extracted with ether (30 mL × 2). The combined organic layer was washed with 1 N cold hydrochloric acid (30 mL), saturated aqueous sodium bicarbonate (50 mL), and brine (50 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure to give an oily product (1.37 g). To a solution of the oil (1.37 g, 3.4 mmol) in dichloromethane (60 mL) at 0 °C were successively added triethylamine (0.98 mL, 7 mmol) and methanesulfonyl chloride (0.48 mL, 7 mmol). The mixture was stirred for 20 min at room temperature, and then washed with water (20 mL), 0.5 N cold hydrochloric acid (20 mL), 5% aqueous sodium bicarbonate (20 mL), brine (20 mL), dried over anhydrous sodium sulfate and concentrated. Purification of the residue via flash chromatography (20% ethyl acetate in hexane) gave **9a** (1.4 g, two step yield: 88%) as white crystals: mp 129.5−130.5 °C (recrystallized from ether−hexane); $[\alpha]_D$ − 9.6° (*c* 0.57, $CHCl_3$); IR ($CHCl_3$) 3370 (NH), 1720−1660 (C═O) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28−7.11 (m, 5H), 4.89 (d, 8.1 Hz, 1H), 4.30−4.25 (m, 3H), 3.02 (s, 3H), 3.02−2.91 (m, 3H), 1.45 (s, 9H), 1.32 (s, 9H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 201.2, 155.6, 138.1, 129.6, 128.8, 127.0, 80.7, 70.0, 57.5, 51.9, 49.2, 37.8, 36.5, 29.8, 28.7; MS−FAB *m/z* 460 (MH^+), 404 ($MH^+ - C_4H_8$). Anal. Calcd for $C_{21}H_{33}NO_6S_2$: C, 54.88; H, 7.24; N, 3.05. Found: C, 54.88; H, 7.51; N, 3.42.

(2*S*,3*S*)-2-Benzyl-3-(*tert*-butyloxycarbonylamino)-4-(methanesulfonyloxy)butanoic acid *tert*-butyl thioester (9b) was prepared from **8b** by the same method as that used for the preparation of **9a**: mp 125−126 °C; $[\alpha]_D$ +47.1° (*c* 0.50, $CHCl_3$). Anal. Calcd for $C_{21}H_{33}NO_6S_2$: C, 54.88; H, 7.24; N, 3.05. Found: C, 54.54; H, 7.23; N, 2.95.

(α*S*,4*S*)-α-Benzyl-2-oxo-1,3-oxazolidine-4-acetic Acid *tert*-Butyl Thioester (10a). A solution of **9a** (1 g, 2.18 mmol) in pyridine (50 mL) was stirred for 3 h at 90 °C, cooled to room temperature, and concentrated under reduced pressure. The resulting oily residue was dissolved in ethyl acetate (200 mL), and the mixture was washed with 0.5 N cold hydrochloric acid (50 mL), brine (50 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure. Purification of the residue via flash chromatography (30% ethyl acetate in hexane) gave **10a** (430 mg, 64%) as white crystals: mp 144−145 °C (recrystallized from ethyl acetate−hexane); $[\alpha]_D$ −88.6° (*c* 0.54, $CHCl_3$); IR ($CHCl_3$) 3300 (NH), 1752 (C═O) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.25−7.06 (m, 5H), 6.21 (s, 1H), 4.39 (t, *J* = 8.84 Hz, 1H), 4.20 (dd, *J* = 8.99, 5.67 Hz, 1H), 4.03 (m, 1H), 2.94 (dd, *J* = 14.49, 3.97 Hz, 1H), 2.87−2.81 (m, 2H), 1.30 (s, 9H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 200.8, 159.6, 137.2, 129.3, 128.8, 127.1, 68.2, 60.1, 54.0, 49.3, 35.7, 29.7; EIMS *m/z* 307 (M^+). Anal. Calcd for $C_{16}H_{21}NO_3S$: C, 62.51; H, 6.89; N, 4.56. Found: C, 62.89; H, 7.17; N, 4.69.

(α*R*,4*R*)-α-Benzyl-2-oxo-1,3-oxazolidine-4-acetic acid *tert*-butyl thioester (10b) was prepared from **9b** by the same method as that used for the preparation of **10a**: mp 147−148 °C; $[\alpha]_D$ +84.2° (*c* 0.50, $CHCl_3$). Anal. Calcd for $C_{16}H_{21}NO_3S$: C, 62.51; H, 6.89; N, 4.56. Found: C, 62.41; H, 6.85; N, 4.57.

(α*S*,4*S*)-α-Benzyl-2-oxo-1,3-oxazolidine-4-acetic Acid ((α*S*,4*S*)-1). A solution of **10a** (492 mg, 1.6 mmol) and mercury (II) trifluoroacetate (1.36 g, 3.2 mmol) in 80% aqueous dioxane (40 mL) was stirred for 3 h at 50 °C and then cooled to 0 °C. Hydrogen sulfide freshly prepared from Na_2S and sulfuric acid was introduced into the reaction mixture for 15 min to give a black precipitation of mercury sulfide which was filtered off through Celite pad. The filtrate was concentrated and purified by flesh column chromatography (silica gel, 50−100% ethyl acetate in hexane) followed by recrystallization from ethyl acetate−hexane to give analytically pure (α*S*,4*S*)-**1** (240 mg, 64%): mp 152.5−153.5 °C; $[\alpha]_D$ −19.6° (*c* 0.52, EtOH); IR (KBr) 3292 (NH), 1734, 1690 (C═O) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 7.44 (s, 1H), 7.36−7.18 (m, 5H), 4.47 (t, *J* = 8.82 Hz, 1H), 4.24 (dd, *J* = 8.85, 5.70, 1H, 4.03 (m, 1H), 3.00 (m, 2H), 2.83 (t, *J* = 7.35 Hz, 1H); ^{13}C NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ 174.0, 159.3, 137.9, 129.0, 128.5, 126.7, 68.2, 53.1, 51.8, 34.5; MS-FAB *m/z* 236 (MH^+). Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.95. Found: C, 61.31; H, 5.60; N, 5.88.

(4*R*,α*R*)-α-Benzyl-2-oxo-1,3-oxazolidine-4-acetic acid ((4*R*,α*R*)-**1**) was prepared from **10b** by the same method as that used for the preparation of **1c**: mp 149−150 °C; $[\alpha]_D$ +20.0° (*c* 0.63, EtOH). Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.95. Found: C, 61.37; H, 5.56; N, 5.93.

(±)-2-Benzyl-3-butenoic Acid *tert*-Butyl Ester (12). Concentrated sulfuric acid (1.9 mL, 34 mmol) was added to a vigorously stirred suspension of anhydrous magnesium sulfate (16.4 g, 136 mmol) in dichloromethane (70 mL). After the mixture was stirred for 15 min 2-benzyl-3-butenoic acid (**11**) (6.0 g, 34 mmol) and 2-methyl-2-propanol (16 mL, 167 mmol) were added successively. The reaction flask was sealed tightly and stirred for 18 h at 25 °C. The reaction mixture was then quenched with saturated aqueous sodium bicarbonate (50 mL) and stirred until all magnesium sulfate had dissolved. The organic phase was separated, washed with brine, dried over magnesium sulfate, and evaporated under reduced pressure to give the crude *tert*-butyl ester which was purified by column chromatography (10% ethyl acetate in *n*-hexane) to give **12** as a colorless oil (5.5 g, 70%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35 (s, 9H), 2.87−2.85 (dd, 1H), 3.01−3.08 (dd, 1H), 3.20−3.25 (q, 1H), 5.06−5.13 (m, 2H), 5.80−5.92 (m, 1H), 7.17−7.30 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 28.3, 38.9, 53.2, 81.0, 117.4, 126.7, 128.6, 129.5, 136.5, 139.3, 173.0.

(±)-2-Benzyl-3,4-epoxybutanoic Acid *tert*-Butyl Ester (13a and 13b). A solution of **12** (3.0 g, 13 mmol), *m*-chloroperbenzoic acid (7.4 g, 26 mmol), and dibasic potassium phosphate (4.5 g, 26 mmol) in dichloromethane was stirred at room temperature for 4 days. The resulting mixture was quenched with 5% aqueous sodium bisulfite, and partitioned between dichloromethane and water. The combined organic extracts were washed with 5% aqueous sodium bicarbonate, brine, and then dried over magnesium sulfate. After evaporation of the solvent the resulting diastereomeric mixture was separated by column chromatography (5% ethyl acetate in *n*-hexane) to give **13a** (1.7 g, 53%) and **13b** (0.9 g, 28%) as colorless oil. **13a**: IR ($CHCl_3$) 2979, 1725, 1455 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.42 (s, 9H), 2.31−2.42 (m, 2H), 2.69−2.72 (t, 1H), 2.85−2.92 (dd, 1H), 3.00−3.07 (dd, 1H), 3.16−3.20 (m, 1H), 7.19−7.32 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 28.4, 35.7, 46.7, 51.8, 52.9, 81.7, 127.0, 128.8, 129.4, 138.6, 172.4. **13b**: IR ($CHCl_3$) 2979, 2931, 1725, 1497 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35 (s, 9H), 2.38−2.45 (q, 1H), 2.70−2.72 (dd, 1H), 2.82−2.85 (dd, 1H), 3.07−3.13 (m, 3H), 7.20−7.32 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 28.3, 36.4, 47.0, 51.8, 53.0, 81.7, 126.9, 128.7, 128.8, 129.5, 138.7, 172.4.

(±)-*cis*-3-Benzyl-4-hydroxytetrahydrofuran-2-one (14a). TFA (1 mL) was added to stirred solution of **13a** (80 mg, 0.32 mmol) dissolved in methylene chloride (5 mL) at 0 °C. After the reaction mixture was being stirred for 90 min at 0 °C, it was evaporated under reduced pressure and the residue was purified by column chromatography (hexane/ethyl acetate = 3:1) to give **14a** (10 mg, 16%). IR ($CHCl_3$) 3448, 1761 cm^{-1}; ^{1}H

NMR (CDCl3) δ 1.99 (br s, 1H), 2.84 (m, 1H), 2.99 (dd, 1H), 3.25 (dd,1H), 4.29 (d, 2H), 4.41 (m, 1H), 7.23−7.37 (m, 5H); ^{13}C NMR δ 29.8, 47.7, 69.2, 74.8, 127.0, 129.0, 129.3, 139.0, 180.0.

(±)-4-Azido-2-benzyl-3-hydroxybutanoic Acid *tert*-Butyl Ester (15a). To a solution of **13a** (0.50 g, 2 mmol) in 60% ethanol were added sodium azide (0.65 g, 10 mmol) and ammonium chloride (0.22 g, 4 mmol). The mixture was heated at 60 °C for 2 h, and the solvent was removed under reduced pressure. The residue was suspended in ether and washed with water. The organic layer was dried over magnesium sulfate and concentrated to dryness under reduced pressure. The resulting crude product was recrystallized from ether−hexane to give **14a** (0.56 g, 96%) as white crystals: mp 80−81 °C; IR ($CHCl_3$) 3445, 2103, 1715, 1645 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.37 (s, 9H), 2.71−2.84 (m, 1H), 2.93−3.05 (m, 2H), 3.32−3.43 (m, 2H), 3.65 (br s, 1H), 3.79−3.82 (q, 1H), 7.21−7.34 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 28.3, 36.1, 50.4, 55.8, 71.5, 82.5, 127.1, 128.9, 129.6, 138.4, 174.3.

(±)-4-Azido-2-benzyl-3-hydroxybutanoic acid *tert*-butyl ester (15b) was prepared in 97% yield from **13b** (0.80 g, 3.2 mmol) following the same procedure as that described for **15a**: colorless oil; IR ($CHCl_3$) 3449, 2103, 1719 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.30 (s, 9H), 2.77−2.84 (m, 1H), 2.89−2.97 (dd, 1H), 3.04−3.10 (m, 2H), 3.36−3.46 (m, 1H), 3.97−4.03 (q, 1H), 7.20−7.32 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 28.2, 34.5, 51.9, 55.0, 71.6, 82.1, 126.9, 128.8, 129.5, 139.0, 173.4.

(±)-4-Amino-2-benzyl-3-hydroxybutanoic Acid *tert*-Butyl Ester, Acetic Acid Salt (16a). To a solution of **15a** (0.52 g, 1.8 mmol) in 50% aqueous methanol was added a catalytic amount of 10% Pd/C and acetic acid (0.16 mL). The resulting mixture was stirred under hydrogen atmosphere for 5 h. The catalyst was removed by filtration and the filtrate was evaporated under reduced pressure. The crude product was recrystallized from methanol−ether to give **16a** (0.35 g, 60%) as white crystals: mp 149−150 °C; IR ($CHCl_3$) 3418, 1714, 1644 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.30 (s, 9H), 1.93 (s, 3H), 2.71−2.78 (m, 1H), 2.88−2.96 (m, 3H), 3.04−3.09 (dd, 1H), 3.95−4.01 (m, 1H), 7.17−7.29 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 24.1, 28.3, 35.2, 44.3, 52.5, 70.1, 81.8, 126.8, 128.7, 129.5, 138.9, 173.2, 178.9.

(±)-4-Amino-2-benzyl-3-hydroxybutanoic acid *tert*-butyl ester, acetic acid salt (16b) was prepared in 60% yield from **15b** (0.82 g, 2.8 mmol) following the same procedure as that used for **16a** (0.55 g,): mp 89−90 °C; IR ($CHCl_3$) 3418, 1722, 1631 1520 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.27 (s, 9H), 1.99 (s, 3H), 2.68−2.76 (m, 1H), 2.83−3.03 (m, 3H), 3.16−3.21 (dd, 1H), 3.85−4.03 (m, 1H), 7.17−7.28 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 24.4, 28.2, 35.6, 43.9, 53.7, 69.7, 81.8, 126.7, 128.6, 129.5, 139.1, 173.1, 180.5

(±)-α-Benzyl-2-oxo-1,3-oxazolidine-5-acetic Acid *tert*-Butyl Ester (17a). Carbonyldiimidazole (0.10 g, 0.6 mmol) was added to a suspension of **16a** (0.20 g, 0.6 mmol) in dichloromethane. After being stirred at room temperature for 5 h, the reaction mixture was washed with 5% sodium bicarbonate and brine and dried over magnesium sulfate. The residue resulted from evaporation of the solvent was recrystallized from dichloromethane−hexane to give **17a** (0.13 g, 74%) as white crystals: mp 91−92 °C; IR ($CHCl_3$) 2978, 1752, 1644, 1493 cm^{-1}; ^{1}H NMR (30 MHz, $CDCl_3$) δ 1.34 (s, 9H), 2.82−2.99 (m, 3H), 3.45−3.51 (t, 1H), 3.59−3.65 (t, 1H), 4.75−4.82 (m, 1H), 5.80 (br, 1H), 7.18−7.31 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 28.2, 34.0, 44.0, 52.6, 76.6, 82.3, 127.2, 129.0, 129.5, 137.9, 159.9, 170.8.

(±)-α-Benzyl-2-oxo-1,3-oxazolidine-5-acetate *tert*-butyl ester (17b) was prepared in 73% yield from **16b** (0.50 g, 1.5 mmol) following the same procedure as that used for **17a**: mp 155−156 °C; IR ($CHCl_3$) 3271, 2981, 1755, 1720 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.33 (s, 9H), 2.94−3.05 (m, 2H), 3.14−3.22 (m, 1H), 3.41−3.46 (t, 1H), 3.69−3.75 (t, 1H), 4.67−4.75 (m, 1H), 6.10 (br s, 1H), 7.20−7.33 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 28.3, 35.2, 44.9, 52.9, 76.3, 82.4, 127.1, 128.8, 129.7, 137.7, 159.8, 170.0.

(±)-α-Benzyl-2-oxo-1,3-oxazolidine-5-acetic Acid (*threo*-18). A solution of **17a** (0.10 g, 0.3 mmol) in trifluoroacetic acid (0.3 mL, 3 mmol) and dichloromethane was stirred at room temperature for 5 h. The volatile materials were evaporated under reduced pressure and the remaining residue was recrystallized from methanol-ether to give *threo*-**18** (60 mg, 73%) as white crystals: mp 197−199 °C; ^{1}H NMR (30 MHz, MeOH-d_4) δ 2.84−2,97 (m, 3H), 3.44−3.50 (dd, 1H), 3.64−3.70 (t, 1H), 4.77−4.85 (m, 1H), 7.17−7.30 (m, 5H); ^{13}C NMR (MeOH-d_4) δ 33.8, 44.0, 52.2, 77.1, 126.7, 128.5, 129.0, 138.4, 160.7, 174.0. Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.95. Found: C, 61.18; H, 5.47; N, 5.68.

(±)-α-Benzyl-2-oxo-1,3-oxazolidine-5-acetic acid (*erythro*-18) was prepared in 74% yield from **17b** (0.25 g, 0.9 mmol) following the same procedure as that used for the preparation of *threo*-**18**: mp 90−91 °C; ^{1}H NMR (30 MHz, MeOH-d_4) δ 2.93−3.12 (m, 3H), 3.42−3.50 (dd, 1H), 3.58−3.71 (t, 1H), 4.68−4.75 (m, 1H), 7.21−7.31 (m, 5H); ^{13}C NMR (MeOH-d_4) δ 34.0, 44.4, 51.5, 76.1, 126.8, 128.5, 129.0, 138.1, 160.7, 173.7. Anal. Calcd for $C_{12}H_{13}NO_4$: C, 61.27; H, 5.57; N, 5.95. Found: C, 61.22; H, 5.68; N, 5.97.

Enzyme Assays. Carboxypeptidase A (Sigma Chemical Co. Type II, twice recrystallized) showed one band when analyzed by 15% SDS polyacrylamide gel electrophoresis.[43] Hippuryl-L-phenylalanine and Tris acid/base were purchased from the Sigma Chemical Co. Tris buffer of pH 7.5 (0.05 M Tris) containing 0.5 M NaCl was used. The commercial CPA (50 μM) was suspended in the buffer (950 μL) and its concentration was determined by optical density measurements at 278 nm (ϵ_{278} = 64 200 M^{-1} cm^{-1}). The enzyme stock solution (1.6 μM) was prepared by dilution of the enzyme solution in the buffer. The inhibitor stock solutions (40 mM) were prepared by dissolution of each inhibitor (9.4 mg, 40 μmol) in DMSO (260 μL) followed by addition of the buffer (740 μL). Enzyme activity was measured by monitoring hydrolysis of hippuryl-L-phenylalanine (substrate) at 254 nm using a computer-assisted UV spectrophotometer (Hewlett-Packard 8453 diode array spectrophotometer).

Measurements of the Time-Dependent Loss of the Enzymic Activity. A portion of the inhibitor stock solution was added to the same volume of the enzyme stock solution in an ice−water bath to give an incubation mixture of the enzyme (1.6 μM). While the mixture was incubated at room-temperature aliquots (20 μL) of the mixture were taken, at 5 min intervals, into the buffer solution (980 μL) of the substrate to give 300 μM and 32 nM of final concentrations of the substrate and the enzyme, respectively. The remaining enzyme activity was measured by monitoring the hydrolysis of the substrate at 254 nm for 60 s. Values of k_{obs} (the apparent inactivation rate constant) were calculated from semilogarithm plots of residual enzymic activity vs time.

Determination of Partition Ratios. A series of solutions (100 μL each) containing CPA (250 μM) and various molar equivalent amount of the inhibitor were prepared to give $[I]_0/[E]_0$ ratios of 0 to 200 in Tris buffer containing DMSO (13%) and were incubated at 4 °C for 24 h. The incubated mixtures were dialyzed in the same buffer at 4 °C for 48 h during which the buffer solution was changed five times. A 30 μL of each dialyzed mixture was added to 970 μL of assay mixture (final $[S]_0$ = 500 μM) and the activity was measured immediately. A solution of no inhibitor was used as the reference setting 100%. A plot of the percent enzyme activity remaining vs the ratio of the molar equivalents of the inhibitors for CPA was constructed, in which the extrapolation to the ratio gives turnover number (The plots not shown). The turnover number minus 1 gives partition ratio.

Irreversibility of the Inhibition. Incubation mixtures of CPA and the inhibitors were prepared as described above. A reference mixture was prepared analogously by mixing the CPA and the buffer containing DMSO (26%). When the mixtures were incubated for 3 days at 4 °C the mixtures

(43) A comparative activity assay of the commercial enzyme with homogeneous CPA obtained by overexpressing bovine CPA gene in *E. coli* (Cho, J. H.; Kim, D.-H.; Lee, K. J.; Kim, D. H.; Choi, K. Y. *Biochemistry*, in press) showed that both enzymes are essentially equipotent.

containing inhibitors showed less than 5% of remaining activity against the reference. The mixtures were transferred into dialysis bags and dialyzed in the buffer for 3 days. The enzyme activity after the dialysis was measured.

Active Site Protection by (*R*)-2-Benzylsuccinic Acid. A mixture of the inhibitor (40 mM) and (*R*)-2-benzylsuccinic acid (40 μM) in the buffer containing DMSO (26%) was added to the same volume of the enzyme stock solution. The mixture was incubated at room temperature, and the remaining activity at certain time intervals was measured as described above.

Acknowledgment. We express our sincere thanks to the Korea Science and Engineering Foundation for the financial support of this work. S.C. is a recipient of BK21 stipend.

JO010421E