Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 237–243

BIOORGANIC & MEDICINAL CHEMISTRY

A New Inhibitor Design Strategy for Carboxypeptidase A as Exemplified by *N*-(2-Chloroethyl)-*N*-methylphenylalanine

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Received 7 June 2000; accepted 25 August 2000

Abstract—*N*-(2-Chloroethyl)-*N*-methylphenylalanine was designed and synthesized in an optically active form as a novel class of mechanism-based inactivator for carboxypeptidase A (CPA). It was anticipated that the chloroethylamino moiety of the CPA bound inhibitor undergoes an intramolecular S_N2 reaction to generate a chemically reactive species (an aziridinium ion) which is expectedly subjected to a nucleophilic attack by the carboxylate of Glu-270, leading to covalent modification of the carboxylate. The irreversible nature of the inhibition of CPA by the inhibitor was supported by the kinetic data: the enzyme lost its enzymic activity in a time-dependent manner in the presence of the inhibitor and the inactivated CPA failed to regain the activity upon dialysis. Interestingly, the (*R*)-isomer that belongs to the D-series was more potent than its enantiomer.

Introduction

The metal assisted substitution reactions that are commonly found in solution chemistry may be of value for designing inhibitors that are effective for metalloproteases.[1–4] Recently, Mobashery and co-workers have successfully exploited the metal (Lewis acid) mediated rate enhancement in the nucleophilic substitution reaction of an unacitvated alkyl halide to design an irreversible inhibitor for carboxypeptidase A (CPA).[5] 2-Haloethylamines readily undergo an intramolecular substitution reaction in an aqueous medium to form aziridinium ions that are known to be relatively stable.[6,7] Such a cyclization reaction may also be facilitated by the coordination of the halide leaving group to a Lewis acid.[8,9] We conceived a novel design strategy for an inactivator of carboxypeptidase A, which takes advantage of the Lewis acid assisted aziridinium formation reaction. This paper describes that *N*-(2-chloroethyl)-*N*-methylphenylalanine is a new class of CPA irreversible inhibitor that illustrates the novel design strategy.

Results and Discussion

CPA is a prototypic zinc-containing exomonopeptidase that cleaves the C-terminal amino acid residue having a hydrophobic side chain.[10,11] Three binding and one catalytic sites have been identified at the active site of CPA.[10,11] The active site zinc ion that is coordinated tightly to the backbone amino acid residues of His-69, Glu-72 and His-196 is essential for the enzymic catalytic hydrolysis reaction of substrate. A molecule of water is bound loosely to the zinc ion as the fourth ligand. The zinc ion activates the water molecule in collaboration with the carboxylate of Glu-270, generating a powerful nucleophilic hydroxyl group that attacks at the carbonyl carbon of the scissile peptide bond. The other important role of the zinc ion is that it stabilizes the tetrahedral transition state that is generated by the hydroxyl attack in the catalytic process. The Arg-145 residue and the S_1' subsite pocket are intimately involved in the binding of substrate to CPA. The guanidinium moiety of Arg-145 forms bifurcated hydrogen bonds with the terminal carboxylate of substrate, and the S_1' subsite pocket accommodates the aromatic ring present in the side chain of the cleavable C-terminal amino acid residue, thus serving as the primary substrate recognition pocket (Fig. 1).

We have previously demonstrated that 2-benzyl-3,4-epoxybutanoic acid (**1**) inactivates CPA very rapidly with extremely high efficiency.[12–14] Analysis of the X-ray crystal structures of the inactivated CPA revealed that, like substrates, the terminal carboxylate in **1** forms hydrogen bonds with the guanidinium moiety of Arg-145 and the hydrophobic phenyl ring anchors in the S_1'

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PII: S0968-0896(00)00239-X

subsite pocket of CPA. The oxirane ring of the CPA bound **1** undergoes a ring cleavage reaction initiated by the nucleophilic attack of the carboxylate of the catalytic residue (Glu-270), leading to covalent modification of the carboxylate with formation of an ester linkage. It occurred to us that the oxirane ring in **1** might be replaced by an aziridinium moiety with retention of the CPA inactivating property. The aziridinium moiety in the potential inhibitor, **2**, is expected to undergo an S_N2 type nucleophilic ring cleavage reaction at the active site of CPA by the attack of the carboxylate of Glu-270 to result in a covalent bond formation, tethering the inhibitor to the Glu-270 carboxylate (Fig. 2). The CPA whose catalytic carboxylate is covalently modified can no longer perform its catalytic function and becomes inactivated.

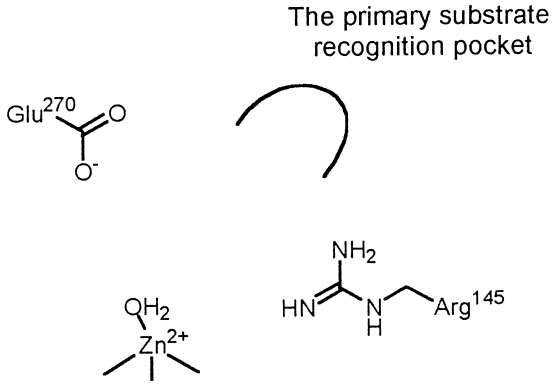


Figure 1. Schematic representation of the active site of CPA.

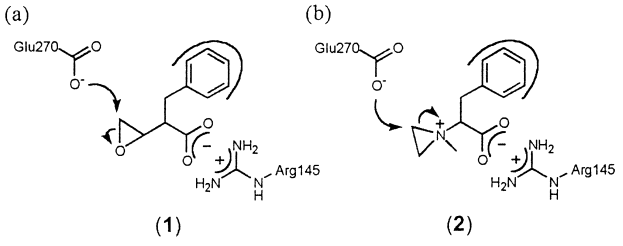
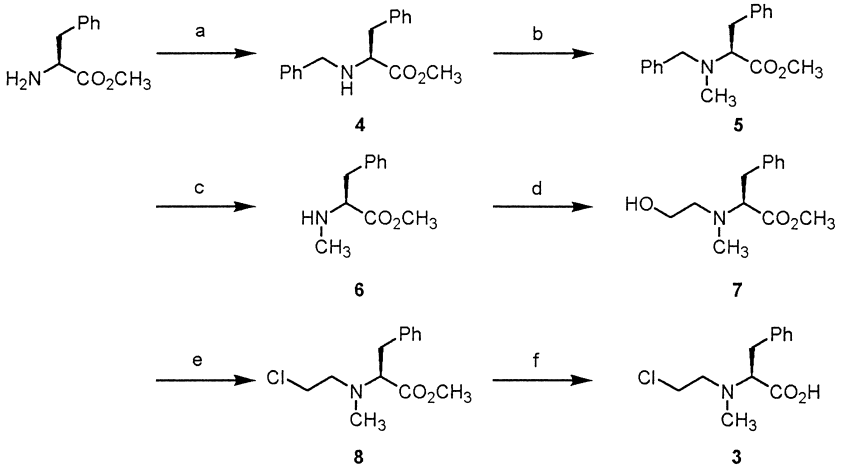


Figure 2. (a) The oxirane ring in the CPA bound 2-benzyl-3,4-epoxybutanoic acid (**1**, BEBA) undergoes a ring cleavage reaction initiated by the nucleophilic attack of the carboxylate of Glu-270, resulting in the covalent modification of the carboxylate. (b) Compound **2** is expected to covalently modify the Glu-270 carboxylate in a similar fashion.

It has been known for a long time that aziridinium ions undergo alkylation reactions with DNA bases.[15–17] Nitrogen mustard anticancer agents such as mechlorethamine (*bis*-dichloroethylmethylamine) cross link DNA via aziridinium intermediates that are generated under physiological conditions. Thus, inhibitor **2** was thought to be generated at the active site of CPA from *N*-(2-chloroethyl)-*N*-methylphenylalanine (**3**). Being a derivative of Phe having the terminal carboxylate and a hydrophobic benzyl group at the position α to the carboxylate, **3** would satisfy the structural requirements for it to bind CPA and forms a Michaelis complex. We envisioned that the chloro group of the CPA bound **3** may rest within the coordination sphere of the active site zinc ion, and its leaving ability in the S_N2 type cyclization reaction would be enhanced, facilitating the formation of the aziridinium ion.[5,8,9] Thus the potential inhibitor (**2**) would be generated at the active site of CPA.

The designed inhibitor, **3**, was prepared in an optically pure form following the route outlined in Scheme 1 starting with (*S*)-phenylalanine methyl ester. Reductive amination of the starting material with benzaldehyde afforded the corresponding *N*-benzyl derivative, (*S*)-**4**, which was allowed to react with iodomethane to give (*S*)-**5**. Hydrogenolysis of (*S*)-**5** in the presence of Pd/C furnished (*S*)-**6**. Treatment of (*S*)-**6** with ethylene oxide in acetonitrile by the method reported by Chini et al. gave (*S*)-**7** in 85% yield.[18] The hydroxyl group in (*S*)-**7** was converted into chloro group by allowing (*S*)-**7** to react with thionyl chloride, giving (*S*)-**8**, which was then treated with concentrated hydrochloric acid to afford the desired compound, (*S*)-**3**, as a hydrochloride salt. (*R*)-**3** was synthesized in a parallel route starting with (*R*)-phenylalanine methyl ester.



Scheme 1. (a) Benzaldehyde, sodium cyanoborohydride (0.7 equiv), Et_3N, MeOH, rt, 2 h, 83%; (b) iodomethane, K_2CO_3, DMF, rt, 7 h, 81%; (c) H_2, Pd/C, MeOH, rt, 5 h, 95%; (d) ethylene oxide, sodium perchlorate, MeCN, 35 °C, 8 h, 85%; (e) $SOCl_2$, $CHCl_3$, rt, 12 h, 95%; (f) concd HCl, reflux, 90%.

Both enantiomeric forms of **3** inhibited CPA in a time-dependent fashion as shown in Figure 3 to suggest that they inhibit the enzyme in an irreversible manner. A protection of the CPA inhibition by **3** was observed when CPA was preincubated with benzylsuccinate, a known active site directed competitive inhibitor of CPA,[19] indicating that the inactivation chemistry takes place at the active site (Fig. 4). The irreversibility of the CPA inhibition by **3** was ascertained by the dialysis experiment: extensive dialysis over 2 days of the inactivated CPA against the kinetic medium failed to regenerate the enzymic activity. It can be concluded from the above kinetic and dialysis studies that the inhibitors bind the enzyme at the active site to form a covalent bond with the catalytic carboxylate of Glu-270 as expected from the design rationale.

The inactivation reaction of CPA by **3** may be represented by Eq. (1), in which E·I represents the Michaelis complex of the enzyme formed with the

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

inhibitor and E-I′ is covalently modified CPA by the inhibitor. In the inactivation assay, if the inhibitor concentration is sufficiently greater than the total concentration of the enzyme, the observed first-order rate constant (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.

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

Eq. (2) may be reduced to Eq. (3) provided the K_I value is much greater than the inhibitor concentration to show that the second-order inhibitory rate constant can be expressed by $k_{obs}/[I]_o$.

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

The reversible binding affinity of **3** to CPA was estimated by the method of Dixon (Fig. 5) and values are listed in Table 1 together with the second-order inhibitory rate constants calculated using Eq. (3).

In contrast to nitrogen mustards, which undergo rapid cyclization in aqueous medium,[20] compound **3** was shown to be fairly stable in the enzyme kinetic medium when examined by NMR spectroscopy. The chemical shift of the methyl group shown at 3.03 ppm remained unchanged for 24 h. The inertness of **3** in the aqueous

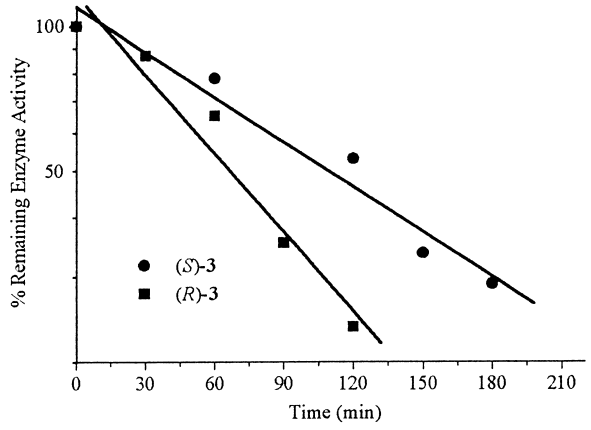


Figure 3. The plot of remaining CPA activity versus time in the presence of (*S*)-**3** or (*R*)-**3** to show that the inhibition occurs in a time-dependent manner.

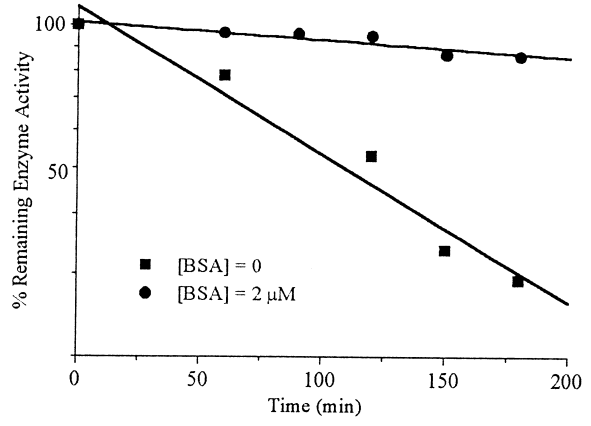


Figure 4. The inhibition of CPA by compound **3** is impeded by 2-benzylsuccinic acid (BSA), an active site directed inhibitor for CPA.

Table 1. Kinetic constants for inhibition of carboxypeptidase A

Inhibitor	K_i (μM)	$k_{obs}/[I]_o$ (M^{-1} s^{-1})
(*S*)-**3**	2.5	0.864
(*R*)-**3**	0.36	1.26
rac-**11**	1.6	–

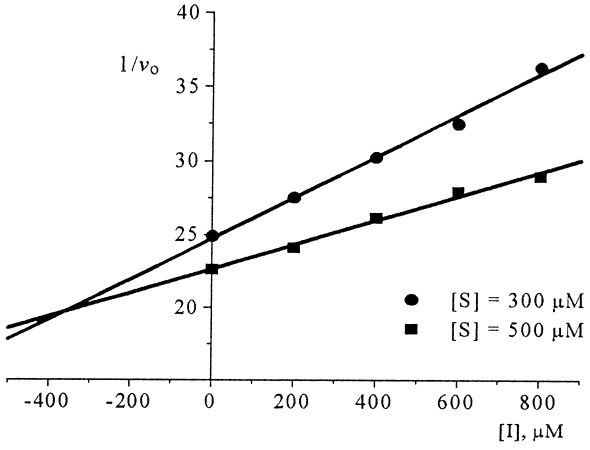


Figure 5. The Dixon plot for the reversible inhibition of CPA by compound **3**.

solution appears to be due to **3** existing mostly in the zwitterionic form with the carboxylate. Nonetheless, a minute amount of the nonzwitterionic form present in equilibrium with the zwitterionic form would bind CPA at the active site and undergoes a covalent bond formation reaction. The alkylation of DNA bases by aziridinium intermediates that are generated in situ from nitrogen mustards is thought to proceed through an S_N1 type reaction.[21] However, it is highly unlikely that the nucleophilic aziridinium ring cleavage at the active site of CPA occurs by the same mode as the alkylaton of DNA bases because the formation of primary carbocation from the aziridinium ion in a nonpolar environment such as at the active site of an enzyme is thought to be a highly energetic process.

It is surprising to note that (*R*)-**3**, which belongs to the D-series, is more effective as a CPA inactiator than its enantiomer, i.e. (*S*)-**3**. This observation may possibly be rationalized as follows: the X-ray crystal structures of native and inhibitor bound CPAs revealed that the active site zinc ion that is liganded by Glu-72, His-69, and His-196 is rested near the core of the enzyme.[22] Inhibitor (*R*)-**3** binds the active site of CPA with its chloroethylamino moiety being projected towards the inside of the enzyme. Thus, the chloro group may possibly be positioned within the coordination sphere of the zinc ion, and as a result the aziridinium ion formation reaction is accelerated. On the other hand, the corresponding chloro group in (*S*)-**3** may not be rested in the region where the chloro group can interact with the active site zinc ion, thus aziridinium ion formation is relatively slow. The overall result is that the inactivation of CPA by (*S*)-**3** is slower compared with that by (*R*)-**3**. The formation of the cyclic aziridinium ion is thought to be the slowest step in the inactivation pathway. Figure 6 depicts schematically the difference in binding mode between (*S*)- and (*R*)-**3** to CPA and the suggested inactivation pathways.

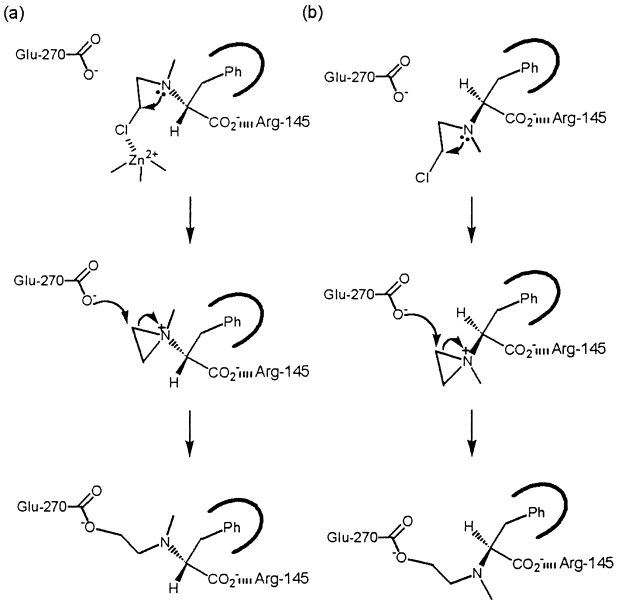
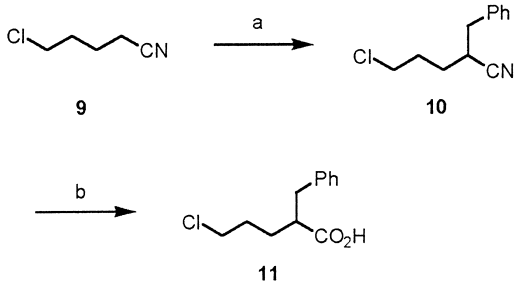


Figure 6. (a) The CPA bound (*R*)-**3** undergoes the aziridinium ion formation readily because the chloro group is coordinated to the active site zinc ion. The aziridinium moiety thus formed reacts with the carboxylate of Glu-270 to result in the covalent modification of the carboxylate. (b) The same inactivation reaction may also occur at the active site of CPA when CPA is treated with (*S*)-**3** but at reduced rate because the aziridinium ion formation is not facilitated by the active site zinc ion.



Scheme 2. (a) LDA (1 equiv), benzyl bromide (1 equiv), THF, 0 °C, 6 h, 54%; (b) concd HCl, reflux, 12 h, 46%.

2-Benzyl-5-chloropentanoic acid (**11**) was prepared in order to verify that **3** is first converted by the enzyme to the chemically reactive aziridinium derivative, **2**, that interacts with a nucleophile present at the active site. That is, **3** is a mechanism-based inactivator of CPA. Compound **11** was synthesized starting with 5-chlorovaleronitrile, which was benzylated in the presence of LDA to give **10**. Treatment of **10** with concentrated hydrochloric acid under reflux conditions afforded **11** as a racemate (Scheme 2). We found that **11** does not function as an irreversible inhibitor for CPA but serves merely as a poor competitive inhibitor for the enzyme with a K_i value of 1.6 mM, which is in accord with the design rationale. Apparently, **11** does not inactivate CPA because it cannot generate the aziridinium ring that is thought to play a critical role in the inactivation of CPA by **3**.

Conclusion

We have designed *N*-(2-chloroethyl)-*N*-methylphenylalanine (**3**) as a novel class of mechanism-based inactivator for CPA. The inhibitor is designed to generate a chemically reactive intermediate **2** at the active site of CPA upon binding to the enzyme. The nucleophilic ring cleavage by the attack of the Glu-270 carboxylate on the aziridinium moiety in the CPA bound **2** would result in tethering of the inhibitor to the catalytic site of the enzyme, which deprives the catalytic activity of the enzyme. Interestingly, (*R*)-**3** that belongs to the D-series is more potent than its enantiomer.

Experimental

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AM 300 (300 MHz) instrument using tetramethylsilane as the internal standard. IR spectra were recorded on a Bruker Equinox 55 FT-IR spectrometer. Mass spectra were obtained with a Micro Mass Platform II 8410E spectrometer. Silica gel 60 (230–400 mesh) was used for

flash chromatography and thin layer chromatography (TLC) was carried out on silica coated glass sheets (Merck silica gel 60 F-254). Elemental analyses were performed at the Center for Biofunctional Molecules, Pohang University of Science and Technology, Pohang, Korea.

(*S*)-*N*-Benzylphenylalanine methyl ester ((*S*)-4). A mixture of (*S*)-phenylalanine methyl ester (10 g, 46.4 mmol), benzaldehyde (5.4 mL, 51.4 mmol), triethylamine (6.5 mL, 46.4 mmol) and sodium cyanoborohydride (2.04 g, 32.5 mmol) in methanol (140 mL) was stirred at room temperature for 2 h. Methanol was removed and the residue was diluted with ethyl acetate, washed with brine (50 mL×3), and dried over anhydrous $MgSO_4$. The solution was concentrated, and the crude product was purified by column chromatography (EA/*n*-Hex = 1/1) to give the product as a colorless oil (10.4 g, 83.2%). $[\alpha]_D = -7.0°$ (*c* 0.6, $CHCl_3$); IR (neat) 1733 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 2.99–3.02 (d, 2H), 3.56–3.60 (t, 1H), 3.68 (s, 3H), 3.74–3.87 (dd, 2H), 7.19–7.39 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 40.2, 52.0, 52.4, 62.5, 127.1, 127.4, 127.9, 128.8, 128.9, 129.4, 137.7, 140.0, 175.4.

(*R*)-*N*-Benzylphenylalanine methyl ester ((*R*)-4). This compound was synthesized from (*R*)-phenylalanine methyl ester in a manner analogous to that used for its enantiomer. $[\alpha]_D = +8.1°$ (*c* 1.0, $CHCl_3$).

(*S*)-*N*-Benzyl-*N*-methylphenylalanine methyl ester ((*S*)-5). A mixture of (*S*)-**4** (8.8 g, 32.7 mmol), potassium carbonate (9.0 g, 65.2 mmol), and methyl iodide (4.06 mL, 65.2 mmol) in dimethylformamide (100 mL) was stirred at room temperature for 7 h. The residue was diluted with ethyl acetate, washed successively with water, 5% sodium thiosulfate (50 mL×3) and brine (50 mL×3), and dried over anhydrous $MgSO_4$. The solution was concentrated, and the crude product was purified by column chromatography (EA/*n*-Hex = 1/4) to give the product as a colorless oil (7.4 g, 80.3%). $[\alpha]_D = -81.6°$ (*c* 0.5, $CHCl_3$); IR (neat) 1732 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 2.31 (s, 3H), 2.98–3.10 (m, 2H), 3.57–3.59 (t, 2H), 3.67 (s, 3H), 3.77–3.82 (d, 1H), 7.15–7.27 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 36.2, 38.3, 51.5, 59.2, 67.7, 126.7, 128.7, 129.1, 129.7, 138.8, 139.6, 172.7.

(*R*)-*N*-Benzyl-*N*-methylphenylalanine methyl ester ((*R*)-5). This compound was synthesized from (*R*)-**4** in a manner analogous to that used for its enantiomer. $[\alpha]_D = +90.8°$ (*c* 0.5, $CHCl_3$).

(*S*)-*N*-Methylphenylalanine methyl ester ((*S*)-6). A mixture of (*S*)-**5** (3.77 g, 13.3 mmol) and 20% acetic acid (3 mL) in methanol (50 mL) was hydrogenated using Pd/C at atmospheric pressure for 5 h. The mixture was filtered, and the filtrate was concentrated and partitioned between ethyl acetate and sodium carbonate solution. Concentration of organic phase gave the product as a pale yellow oil (quantitative) which solidified on standing. Mp 75–76 °C; $[\alpha]_D = +24.9°$ (*c* 0.3, $CHCl_3$); IR (neat) 1744 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 2.35 (s, 3H), 2.93–2.96 (d, 2H), 3.42–3.47 (t, 1H), 3.66 (s, 3H), 7.15–7.31 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 34.8, 39.6, 52.1, 64.7, 127.2, 128.9, 129.5, 137.3, 174.8.

(*R*)-*N*-Methylphenylalanine methyl ester ((*R*)-6). This compound was synthesized from (*R*)-**6** in a manner analogous to that used for its enantiomer. $[\alpha]_D = -25.1°$ (*c* 1.0, $CHCl_3$).

(*S*)-*N*-(2-Hydroxyethyl)-*N*-methylphenylalanine methyl ester ((*S*)-7). A mixture of (*S*)-**6** (2.0 g, 10.3 mmol) and sodium perchlorate (1.27 g, 10.3 mmol) in dried acetonitrile (5 mL) was stirred until a solution was obtained. To the solution was introduced an excess of epoxide within 2 h at room temperature using a gas dispersing tube. The mixture was stirred for 3 h at 35–40 °C, then diluted with water, and extracted with ethyl acetate. The combined extracts were dried over anhydrous $MgSO_4$. Concentration of the organic phase gave the product as a pale yellow oil (2.2 g, 90.0%). $[\alpha]_D = -41.6°$ (*c* 0.5, $CHCl_3$); IR (neat) 1732 cm^{-1}, 3441 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 2.36 (s, 3H), 2.71–2.80 (m, 2H), 2.91–3.13 (m, 2H), 3.43–3.48 (m, 2H), 3.53–3.56 (t, 1H), 3.68 (s, 3H), 7.19–7.33 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 35.9, 37.1, 51.7, 57.0, 58.6, 68.4, 127.0, 128.9, 129.3, 138.7, 172.7.

(*R*)-*N*-(2-Hydroxyethyl)-*N*-methylphenylalanine methyl ester ((*R*)-7). This compound was synthesized from (*R*)-**6** in a manner analogous to that used for its enantiomer. $[\alpha]_D = -40.5°$ (*c* 1.0, $CHCl_3$).

(*S*)-*N*-(2-Chloroethyl)-*N*-methylphenylalanine methyl ester ((*S*)-8). A mixture of (*S*)-**7** (1.36 g, 5.73 mmol) and thionyl chloride (0.84 mL, 11.5 mmol) in chloroform (25 mL) was stirred at room temperature for 12 h. The solution was evaporated and the residue was recrystallized from ether and methanol to give a white powder (1.57 g, 93.5%). Mp 109–110 °C; $[\alpha]_D = -46.9°$ (*c* 0.5, MeOH); IR (neat) 1744 cm^{-1}; ^1H NMR 300 MHz (D_2O) δ 2.92 (s, 3H), 3.15–3.35 (m, 2H), 3.50–3.68 (m, 2H), 3.55 (s, 3H), 3.80–3.83 (m, 2H), 4.40–4.66 (m, 1H), 7.16–7.28 (m, 5H); ^{13}C NMR 300 MHz (D_2O) δ 33.1, 37.8, 39.3, 54.1, 56.4, 68.3, 126.3, 128.4, 129.6, 134.2, 168.7.

(*R*)-*N*-(2-Chloroethyl)-*N*-methylphenylalanine methyl ester ((*R*)-8). This compound was synthesized from (*R*)-**7** in a manner analogous to that used for its enantiomer. $[\alpha]_D = +46.8°$ (*c* 0.4, MeOH).

(*S*)-*N*-(2-Chloroethyl)-*N*-methylphenylalanine ((*S*)-3). A mixture of (*S*)-**8** (0.22 g, 0.75 mmol) and concd HCl (5 mL) was refluxed for 1 h, then evaporated under reduced pressure. The residue was recrystallized from ether and ethanol to give a white powder (quantitative). Mp 142–143 °C; $[\alpha]_D = +23.4°$ (*c* 1.0, MeOH); IR (neat) 1731 cm^{-1}; ^1H NMR 300 MHz (D_2O) δ 2.90 (s, 3H), 3.16–3.25 (m, 2H), 3.47–3.61 (m, 2H), 3.80–3.84 (t, 2H), 4.15 (m, 1H), 7.21–7.29 (m, 5H); ^{13}C NMR 300 MHz (D_2O) δ 33.2, 37.9, 39.1, 56.1, 69.2, 128.2, 129.5, 129.6,

134.8, 170.4. Anal. calcd for $C_{12}H_{17}Cl_2NO_2 \cdot 1/4H_2O$: C, 51.0; H, 6.24; N, 4.96. Found: C, 51.25; H, 6.26; N, 4.94.

(*R*)-*N*-(2-Chloroethyl)-*N*-methylphenylalanine ((*R*)-3). This compound was synthesized from (*R*)-**8** in a manner analogous to that used for its enantiomer. $[\alpha]_D = -21.7°$ (*c* 1.0, MeOH).

2-Benzyl-5-chlorovaleronitrile (10). To an ice chilled solution of diisopropylamine (2.3 mL, 17.8 mmol) in dried THF (100 mL) was added slowly *n*-butyllithium (7.1 mL of 2.5 M solution in *n*-hexane, 17.8 mmol) under nitrogen gas. After the mixture was stirred for 30 min, **9** (2.0 mL, 17.8 mmol) in dried THF (20 mL) was added dropwise over a period of 20 min. The resulting solution was stirred for 40 min, and then benzyl bromide (2.1 mL, 17.8 mmol) was added dropwise. The reaction mixture was stirred for 6 h. The reaction was quenched by the addition of saturated solution of ammonium chloride (50 mL). The organic layer was separated and the aqueous layer was washed with ethyl acetate (3×20 mL). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and evaporated to dryness in vacuo. The crude product was purified by chromatography (EA/*n*-Hex = 1/8) to give the product as an oil (2.0 g, 54%). IR (neat) 2239 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.76 (m, 2H), 1.99 (m, 2H), 2.82 (m, 1H), 2.91 (m, 2H), 3.55 (t, 2H), 7.30 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 29.5, 30.3, 33.7, 38.8, 44.4, 121.7, 127.8, 129.2, 129.4, 137.0.

2-Benzyl-5-chloropentanoic acid (11). A mixture of **10** (1.0 g, 4.8 mmol) and concd HCl (15 mL) was refluxed for 12 h, then evaporated under reduced pressure. The crude product was purified by chromatography (EA/*n*-Hex = 1/2) to give the product as an oil (0.5 g, 46%). IR (neat) 1705 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.73 (m, 2H), 1.81 (m, 2H), 2.72 (m, 1H), 3.00 (dd, 1H), 3.50 (t, 2H), 7.22 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 29.3, 30.6, 38.5, 44.9, 47.1, 127.0, 128.9, 129.3, 139.0, 181.7; HRMS (FAB^+) calcd for ($C_{12}H_{15}O_2Cl$, Na^+): 249.0658; found: 249.0658.

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. Hippuryl-L-phenylalanine (Hipp-L-Phe) purchased from Sigma Chemical Co. was used as substrate for CPA and the decrease in the absorbance at 254 nm was followed at 25 °C. A Perkin–Elmer HP 8453 UV/VIS spectrometer was used in enzyme inhibition studies.

Determination of K_i

Typically, enzyme stock solution was added to various concentrations of inhibitors in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer (1 mL cuvette), and the change in absorbance at 254 nm was measured immediately. Initial velocities were then calculated from the linear initial slopes of the change in absorbance where the amount of substrate consumed was less than 10%. The K_i values were then estimated from the semireciprocal plot of the initial velocity versus the concentration of the inhibitors according to the method of Dixon (Fig. 5).

Determination of k_{obs}

Into a 1 mL cuvette were added 850 μL of buffer solution (0.05 M Tris/0.5 M NaCl, pH 7.5), 100 μL of 5 mM solution of Hipp-L-Phe in the same buffer, and 20 μL of 10 mM stock solution of inactivator in the same buffer. To this cuvette was added 30 μL of 13.7 μM solution of CPA in the Tris buffer, and the change in absorbance at 254 nm was recorded over a time interval of 0–240 min. The values of k_{obs} were calculated from the progress curves using the computer assisted spectrophotometer.

Active site protection test

To demonstrate that the inactivation is active site directed, a competitive experiment with benzylsuccinic acid, a well known competitive CPA inhibitor, was carried out. To a preincubated solution of 2 μM benzylsuccinic acid and 1 μM enzyme in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer for 10 min was added an inactivator and the whole mixture was incubated at room temperature. At some intervals 50 μL samples of the inactivation mixture were removed and added to a 950 μM assay mixture and the activity was monitored at 254 nm. From the data Figure 4 was obtained.

Dialysis

Solutions of 5–20 mM inactivator and 1 μM CPA 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. The enzyme failed to show the proteolytic activity.

Acknowledgement

The authors thank the Korea Science and Engineering Foundation for the generous support of this work.

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