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N-(Hydroxyaminocarbonyl)phenylalanine: A Novel Class of Inhibitor for Carboxypeptidase A

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Abstract—*N*-(Hydroxyaminocarbonyl)phenylalanine (**1**) was designed rationally as a new type of inhibitor for carboxypeptidase A (CPA). The designed inhibitor was readily prepared from phenylalnine benzyl ester in two steps and evaluated to find that *rac*-**1** inhibits CPA in a competitive fashion with the K_i value of 2.09 μM. Surprisingly, inhibitor **1** having the D-configuration is more potent ($K_i = 1.54$ μM) than its antipode by about 3-fold. A possible explanation for the stereochemistry observed in the inhibition of CPA with **1** is presented.

Introduction

The zinc peptidases constitute an important class of proteolytic enzymes from both the mechanistic and biological standpoint. Among these enzymes, carboxypeptidase A (CPA) that cleaves the C-terminal amino acid residue having a hydrophobic side chain has been most intensively studied and serves as a prototypic zinc peptidase.[1] CPA has also been used as a model target enzyme for the development of inhibitor design strategies that can be useful for designing inhibitors of other zinc proteases of medicinal interest such as angiotensin converting enzyme and matrix metalloproteases.[2–5] In principle, inhibitors of zinc proteases are obtained by incorporating a zinc ligating functionality into the structural frame of a substrate-like molecule that can be recognized and accommodated by the target enzyme. Hydroxamate moiety is one of the representative zinc ligating motifs and has been extensively utilized for the design of inhibitors for numerous zinc metalloproteases since Nishino and Powers[6] have reported its use as a zinc ligating functionality in designing thermolysin inhibitors.[7–9] *N*-Hydroxyurea can be considered as hydroxamate of carbamic acid and thus is expected to chelate metal ions like hydroxamate does. Hydroxyurea and 1-ethyl-3-hydroxyurea were reported to form complexes with Fe(III) and Cu(II) ions.[10] Some of hydroxyurea derivatives are potent 5-lipoxygenase inhibitors and under clinical evaluation as anti-asthma agents.[11] These hydroxyurea derivatives have been speculated to interact with the heme iron that plays an important role in the catalytic pathway of 5-lipoxygenase.[12,13] We wish to describe in this paper design and synthesis of a new type of potent CPA inhibitors by exploiting the metal chelating property of hydroxyurea.

Results and Discussion

CPA is a much studied and well characterized zinc containing metalloprotease which catalyzes the cleavage of the C-terminal amino acid residue having an aromatic side chain from peptide substrate.[1] Three binding and one catalytic sites have been identified at the active site of CPA. The presence of a zinc ion that is coordinated tightly to the backbone amino acid residues of His-69, Glu-72 and His-196 is essential for the catalytic hydrolysis reaction. 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 hydroxyl group that attacks at the carbonyl carbon of the scissile peptide bond. The other important role that the zinc ion plays in the catalytic process is to stabilize the tetrahedral transition state that is generated by the hydroxyl attack on the peptide carbonyl in the reaction process. The other principal binding sites are Arg-145 and a substrate recognition pocket at the S_1' subsite (S_1' pocket). The latter that is known as the primary substrate recognition pocket is shaped complementary to an aromatic ring present in the side chain of amino acids such as Phe, and

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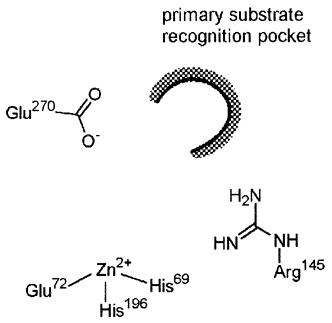
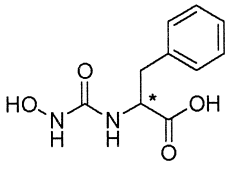


Figure 1. Schematic representation of the active site of CPA.

the former is involved in forming bifurcated hydrogen bonds with the terminal carboxylate of substrate (Fig. 1).[1]

The X-ray crystal structure of CPA complex formed with Gly-L-Tyr, a slowly hydrolyzing substrate for CPA, revealed that the terminal amino group and the carboxamide carbonyl group of the CPA bound Gly-L-Tyr form a bidentate coordinative bond to the active site zinc ion.[14] The distance between one of carboxylate oxygens of Glu-270 and α-amino nitrogen of the bound ligand is found to be 2.99 Å, suggesting that there might be formed a hydrogen bond between them.

We envisioned that the replacement of Gly in Gly-L-Tyr with hydroxyaminocarbonyl to give a *N*-substituted hydroxyurea would convert the substrate into inhibitor of CPA. Since hydroxyurea has been known to form chelation complexes with metal ions such as Fe(III) and Cu(II), compound **1** is thought to bind CPA with its hydroxyaminocarbonyl moiety forming a bidentate coordinative bond to the active site zinc ion.[10] The carboxylate and phenyl ring in **1** would interact with the guanidinium moiety of Arg-145 and the S_1' pocket of CPA, respectively. Hydrogen bonding between the Glu-270 carboxylate and the NH of the hydroxyurea moiety was anticipated on the basis of the X-ray crystal structure of CPA·Gly-L-Tyr complex[14] and such an interaction was deemed to strengthen the binding of **1** to CPA. Figure 2 depicts schematically the possible binding mode of **1** to CPA, leading to inhibition of the enzymic activity.



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Inhibitor **1** was readily prepared in racemic and optically active forms by allowing phenylalanine benzyl ester to react with *O*-benzylhydroxylamine in the presence of carbonyldiimidazole (CDI) followed by removal of the benzyl groups by hydrogenolysis in the presence of Pd-C catalyst (Scheme 1).

The prepared compounds were evaluated as CPA inhibitors by a standard procedure to find that they inhibit the enzyme in a competitive reversible fashion as can be seen from Figure 3.

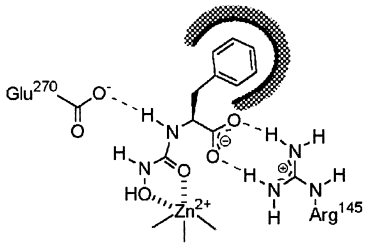
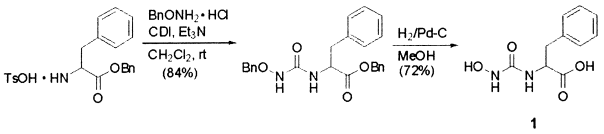


Figure 2. Schematic representation showing the design rationale for **1** as an inhibitor of CPA.



Scheme 1.

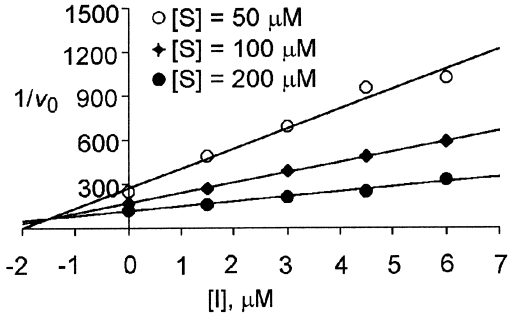


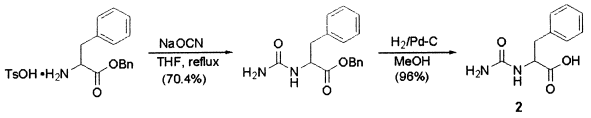
Figure 3. The Dixon plot.

Their inhibitory constants (K_is) obtained from Dixon's plots (Fig. 3)[15] are listed in Table 1. The CPA inhibitory potency of **1** is comparable to that of 2-benzylsuccinic acid, a well known CPA inhibitor but less than that of 2-benzyl-3-mercaptopropanoic acid by about two order of magnitude (Table 1). Inhibitor **1** binds CPA with higher binding affinity than 3-hydroxyaminocarbonyl-2-benzylpropanoic acid ($K_i = 24.2\,\mu M$)[16] that bears a strong zinc chelating functionality of hydroxamate by about 12-fold. This observation supports the design

Table 1. Inhibitory constants for CPA inhibition

Inhibitor	K_i (μM)[a]
rac-**1**	2.09±0.16
L-**1**	4.56±0.38
D-**1**	1.54±0.10
rac-**2**	58.50±2.94
L-**2**	18.98±1.1
D-**2**	—[b]
HBPA[c]	24.2[d]
BSA[e]	1.1±0.3[f]
BMPA[g]	0.011[h]

[a] K_i values were calculated using GraFit®.
[b] No inhibition was observed up to 3.6 mM of D-**2**.
[c] 3-Hydroxyaminocarbonyl-2-benzylpropanoic acid.
[d] ref 16.
[e] *rac*-2-Benzylsuccinic acid.
[f] ref 22.
[g] *rac*-2-Benzyl-3-mercaptopropanoic acid.
[h] ref 23.



Scheme 2.

rationale that the α-NH in **1** would enhance the binding affinity through hydrogen bonding with the carboxylate of Glu-270.

In order to ascertain the conjectural role of the *N*-hydroxyaminocarbonyl moiety in **1** to form a chelate coordinative bond to the zinc ion in binding of the inhibitor to CPA, we have decided to evaluate the urea derivative of Phe, i.e., **2**. Compound **2** was prepared simply by refluxing the mixture of phenylalanine benzyl ester and sodium cyanate in THF followed by hydrogenolysis to remove the benzyl group (Scheme 2).[17]

The K_i value of *rac*-**2** is increased by 28-fold compared with that of *rac*-**1** to be 58.5 μM, which suggests strongly that the hydroxy group in the hydroxyurea moiety plays a critical role in binding of **1** to CPA. Although the literature[10] reports that hydroxyurea forms chelation complexes with metal ions such as Fe(III) and Cu(II), it was thought to be of interest to probe whether the hydroxyurea moiety in **1** also coordinates to the active site zinc ion in a bidentate fashion when the inhibitor binds CPA. There are two acidic groups in **1** and we found two pKa values of 2.22 and 6.16 that may be attributed to the protonated carboxylate and the ureahydroxy groups, respectively. The pK_a value of 6.16 for the hydroxyurea suggests that under the assay conditions the hydroxyurea moiety ligates the active site zinc ion in a monodentate fashion rather than chelating the metal ion as originally thought.

As can be seen from Table 1, D-**1** is more potent than L-**1** by 3-fold. The reversal of stereochemistry in the inhibition in comparison with stereochemistry of substrate is of interest, although some precedences of such reversal have been reported. Holmquist and Vallee reported that *N*-mercaptoacetylphenylalanine obtained from D-Phe is more potent than its L-enantiomer by about 23-fold.[18] More recently, Jin and Kim[19] reported that *N*-formyl-*N*-hydroxy α-amino acid methyl esters derived from D-Leu is about 70-fold more potent than that prepared from L-Leu in inhibition of thermolysin,[20] a zinc containing protease whose active site structure and catalytic mechanism resemble those of CPA. However, in the case of compound **2** where the *N*-hydroxy is lacking, the L-isomer was found to be much potent than its enantiomer (Table 1), which suggests that the binding of **2** is resulted primarily from the interactions of the carboxylate and the phenyl ring with the guanidinium moiety of Arg-145 and the substrate recognition pocket, respectively, as does substrate.

The observed reversal of stereochemistry in the inhibition of CPA by the hydroxyurea inhibitors may be envisioned on the basis of the unique position that the zinc ion takes in the active site. The zinc ion that is coordinated

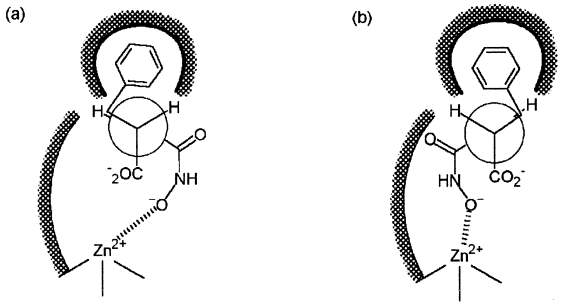


Figure 4. Schematic representation of binding modes of (a) L-**1** and (b) D-**1** to the active site of CPA. In both cases, the hydroxyl group in the form of anion forms a salt bridge with the active site zinc ion. In the case of D-**1**, however, the hydroxyurea moiety experiences steric interactions with the wall of the catalytic hole and as a result the hydroxyl is pushed towards the zinc ion to have a stronger interaction with the zinc ion than the corresponding interaction in the CPA·L-**1** complex.

to the imidazoles of His-69 and His-196, the carboxylate of Glu-72, and a molecule of water is rested near the core of CPA.[21] It appears that the hydroxyurea moiety of D-**1** would project towards the core of the enzyme when it binds CPA, but may hit the wall of the catalytic hole. As a result, the moiety may be pushed towards the zinc ion at the catalytic site and rest closer to the zinc ion (Fig. 4b). The electrostatic interactions between the deprotonated ureahydroxy and the zinc ion become fortified. On the other hand, the hydroxyurea moiety of the CPA bound L-**1** orients near the entrance of the catalytic hole again its hydroxyl being positioned towards the zinc ion, but in this case it is not being pushed towards the zinc ion because the moiety may be placed in open space (Figure 4a). Accordingly, the two interacting species are more distanced compared with that in the CPA complex formed with D-**1** and the binding force is reduced correspondingly. Figure 4 illustrates schematically the observed inhibitory stereochemistry. The rear atom in the Newman projection in Figure 4 represents the α-amino nitrogen.

In summary, racemic and optically active *N*-hydroxyurea derivatives of phenylalanine, **1** were designed and synthesized as inhibitors of CPA. The inhibitory kinetics showed that they are indeed potent competitive inhibitors for CPA. Surprisingly, D-**1** is more potent than L-**1**. Hydorxyurea derivatives of an amino acid or peptide that can be readily prepared may thus be potentially useful in the design of inhibitors that are effective for zinc proteases of medicinal interest.

Experimental

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

***N*-(Hydroxyaminocarbonyl)phenylalanine (1).** A solution of carbonyldiimidazole (810 mg, 5 mmol), *O*-benzylhydroxylamine hydrochloride (800 mg, 5 mmol), and triethylamine (1.4 mL, 10 mmol) in dichloromethane (40 mL) was stirred at room temperature for 1 h, and then phenylalanine benzyl ester *p*-toluenesulfonate (2.15 g, 5 mmole) was added. After stirring for additional 4 h, the reaction mixture was washed with 1 N HCl, saturated aqueous solution of sodium bicarbonate, brine, and dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue was chromatographed on a silica gel column (30% ethyl acetate in hexane) to afford **1** (1.7 g, 84%) as benzyl ester. A solution of the benzyl ester (1.5 g, 3.7 mmole) in methanol was stirred under hydrogen atmosphere in the presence of 10% Pd-C for 2 h at room temperature and filtered. The filtrate was concentrated under the reduced pressure and ether was added slowly to afford a crystalline product, which was recrystallized from methanol-ether to give analytically pure **1** (700 mg, 84%). Mp 132–134 °C; IR (KBr) 3290, 1654, 1635, 1541 cm^{-1}; ^1H NMR (300 MHz) δ 8.46 (s, 1H), 7.27–7.16 (m, 5H), 6.52 (d, $J = 8.26$ Hz, 1H), 4.37 (ddd, $J = 13.27, 7.85, 5.53$ Hz, 1H), 3.08–2.94 (m, 2H); ^{13}C NMR 174.28, 161.61, 138.46, 130.03, 129.06, 127.27, 54.08, 37.70. Anal. Calcd. for $C_{10}H_{12}N_2O_4$: C, 53.57; H, 5.39; N, 12.49. Found: C, 53.93; H, 5.52; N, 12.40.

Compound **1** was prepared in the same fashion as the above, starting with optically active phenylalanine benzyl ester *p*-toluenesulfonate. (*S*)-**1**: mp 136–138 °C; $[\alpha]_D^{20}$ +42.17° (*c* 0.54, EtOH). (*R*)-**1**: mp 136–138 °C; $[\alpha]_D^{20}$ −42.78° (*c* 1.2, EtOH);

***N*-(Aminocarbonyl)phenylalanine (2).** A mixture of phenylalanine benzyl ester *p*-toluenesulfonate (4.27 g, 10 mmole) and sodium cyanate (2.6 g, 40 mmole) in THF was refluxed for 4 h, and the solvent was evaporated under reduced pressure. The residue was dissolved in hot ethyl acetate and the insoluble material was removed by filtration. The filtrate was chilled in an ice–water bath to afford a crystalline solid, which was recrystallized from ethyl acetate to give **2** as its benzyl ester (2.1 g, 70.4%). A solution of the benzyl ester (1.1 g, 3.69 mmole) in MeOH was stirred under hydrogen atmosphere in the presence of 10% Pd-C (200 mg) for 2 h and filtered. The filtrate was concentrated to give a solid residue, which was recrystallized from water to give an analytically pure **2** (740 mg, 96%). Mp 182–183 °C; IR (KBr) 3455, 3313, 1697, 1636, 1558 cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.76 (s, 1H), 7.41–7.27 (m, 5H), 6.29 (d, $J = 8.22$ Hz, 1H), 5.75 (s, 2H), 4.44 (ddd, $J = 13.14, 7.88, 5.34$ Hz, 1H), 3.12 (dd, $J = 13.73, 5.12$ Hz, 1H), 2.96 (dd, $J = 13.70, 7.88$ Hz, 1H); ^{13}C NMR 174.02, 158.21, 137.56, 129.28, 128.22, 126.44, 53.75, 37.63. Anal. calcd for $C_{10}H_{12}N_2O_3$: C, 57.68; H, 5.81; N, 13.45. Found: C, 57.32; H, 6.16; N, 13.46.

Optically active **2** was prepared in the same fashion as the above, starting with optically active phenylalanine benzyl ester *p*-toluenesulfonate. (*S*)-**2**: mp 191–192 °C; $[\alpha]_D^{20}$ + 16.99° (*c* 0.36, DMF). (*R*)-**2**: mp 191–192 °C; $[\alpha]_D^{20}$ −16.95° (*c* 0.59, DMF).

Determination of pK_a

The pK_a value of **1** was determined using a AT-400 Potentiometric Titrator (Kyoto Electronics Manufacturing Co.). A solution of **1** (22.4 mg, 0.1 mmole) in water (60 mL) was adjusted to pH 1.85 with 1 N HCl, and titrated with 0.05 N aqueous NaOH solution. The pKa values were obtained directly from the titrator.

General remarks for kinetic experiments

All solutions were prepared by dissolving in 0.5 M NaCl, pH 7.5, 0.05 M Tris buffer solution. Stock assay solutions were filtered before use. Carboxypeptidase A was purchased from Sigma Chemical Co. (Allan form, twice recrystallized from bovine pancreas, aqueous suspension in toluene) and used without further purification. CPA stock solutions were prepared by dissolving the enzyme in the buffer. Hippuryl-L-phenylalnine (Hipp-L-Phe) purchased from Sigma Chemical Co. was used as substrate for CPA. The change in the absorbance at 254 nM that is due to the generation of hippuric acid form the substrate was followed at 25 °C on a Perkin–Elmer HP 8453 UV–vis spectrometer.

Determination of K_i

The initial velocities were obtained from the linear plot of the substrate hydrolysis monitored by following the increase of absorbance at 254 nM. 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. Two or three concentrations of the substrate were used. Typically, the enzyme stock solution was added to the solution of the inhibitor and the substrate in the buffer (1 mL cuvette), and the rate of absorption increase at 254 nM was recorded immediately.

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