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Hippuryl-α-methylphenylalanine and Hippuryl-α-methylphenyllactic Acid as Substrates for Carboxypeptidase A. Syntheses, Kinetic Evaluation and Mechanistic Implication

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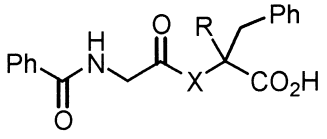
Abstract—(*R*)- and (*S*)-Hippuryl-α-methylphenylalanine [(*R*)- and (*S*)-Hipp-α-MePhe] and (*S*)-hippuryl-α-methylphenyllactic acid [(*S*)-Hipp-α-MeOPhe] were synthesized and evaluated as substrates for carboxypeptidase A (CPA) in an effort to shed further light on the catalytic mechanism of the enzyme. The rate of CPA-catalyzed hydrolysis of (*S*)-Hipp-α-MePhe was reduced by 105-fold compared with that of (*S*)-Hipp-Phe, but the hydrolysis rate of (*S*)-Hipp-OPhe was lowered by only 6.8-fold by the introduction of a methyl group at the α-position. (*R*)-Hipp-α-MePhe failed to be hydrolyzed initially, then started to undergo hydrolysis in about 2 h at a much reduced rate. The results of present study may be envisioned on the basis of the proposition that while peptide substrate is hydrolyzed via a tetrahedral transition state formed by the attack of the zinc-bound water molecule at the peptide carbonyl carbon, ester hydrolysis takes the path that involves an anhydride intermediate generated by the attack of the carboxylate of Glu-270 at the ester carbonyl carbon. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Carboxypeptidase A (CPA) is a prototypic zinc-containing proteolytic enzyme, representing a large family of physiologically important zinc proteases such as angiotensin converting enzyme and matrix metalloproteases.[1] The enzyme catalyzes the hydrolysis of the C-terminal amide bond of peptide substrate and shows specificity for oligopeptide having the C-terminal residue with a hydrophobic side chain.[1] CPA also catalyzes the hydrolysis of esters having a structural feature similar to the peptide substrate.[1] Thus, the enzyme cleaves esters of α-hydroxy acid such as β-phenyllactic acid. As a representative zinc protease, the enzyme has received much attention as a model enzyme for devising design protocols of inhibitors that are effective against zinc proteases of medicinal interest. Recently, Asante-Appiah et al. reported that at the active site of CPA there exists a small hydrophobic cavity that can accommodate a methyl group present at the α-position of substrate-like inhibitors of the enzyme.[2] Accordingly, it was thought to be of interest to know the effect that might be brought about by the introduction of a methyl group at the α-position of a substrate such as hippuryl-phenylalanine,[3] a substrate used widely for inhibition kinetics. In this respect, hippuryl-α-methylphenyllactic acid was thought to be of interest to study as well. This paper describes syntheses and kinetic evaluations of these compounds as substrates for CPA and their implications to the catalytic mechanism of the enzyme.



	X	R
1	NH	H
2	NH	CH_3
3	O	H
4	O	CH_3

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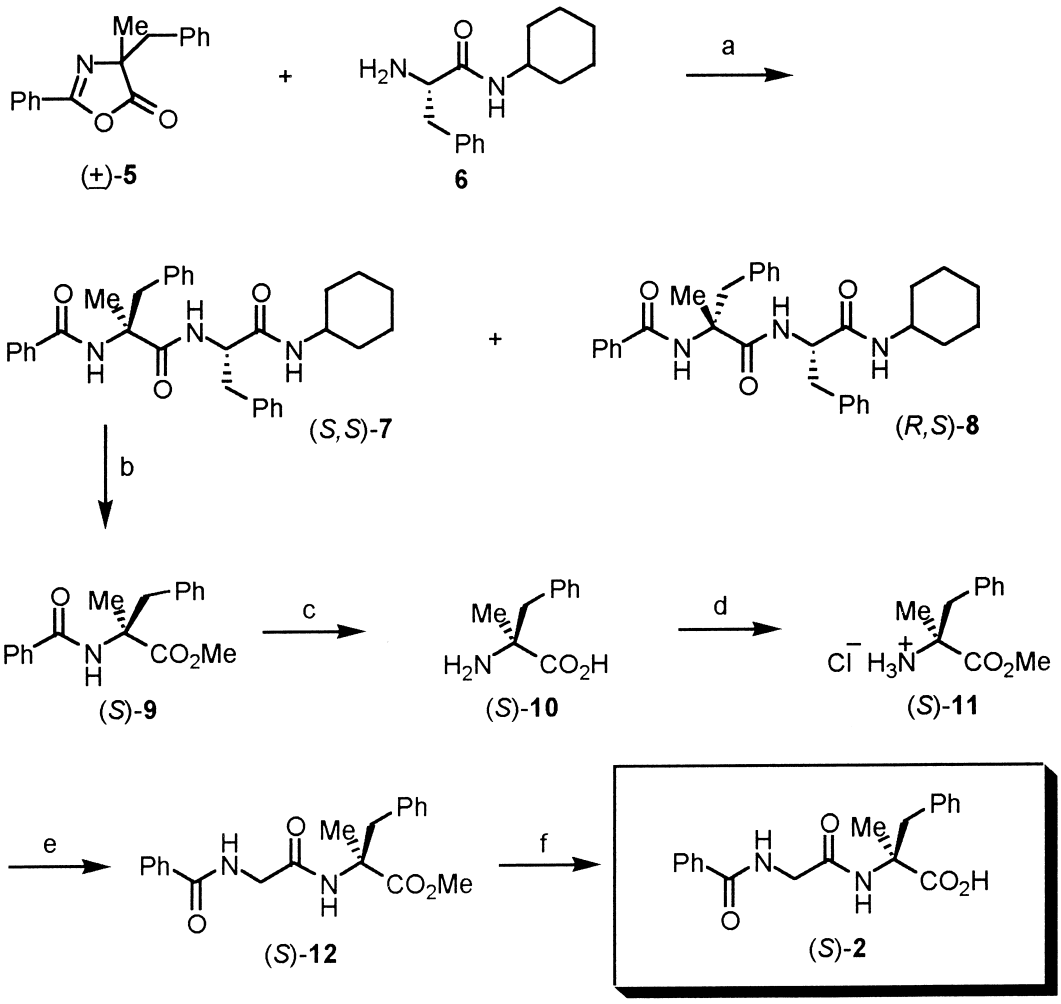


Scheme 1. (a) NMP, 70 °C, 24 h, (*S,S*)-**7**, 39%; (*R,S*)-**8**, 38%; (b) CF_3SO_3H, MeOH, 80 °C, 20 h, 70%; (c) HCl/dioxane, 100 °C, 20 h; propylene oxide, EtOH, reflux, 0.5 h, 90%; (d) H^+, MeOH, reflux, 12 h, 90%; (e) hippuric acid, NMM, isobutyl chloroformate, THF, −10 °C to rt, 12 h, 52%; (f) LiOH·H_2O (3 equiv), MeOH/H_2O/THF, rt, 24 h, 90%.

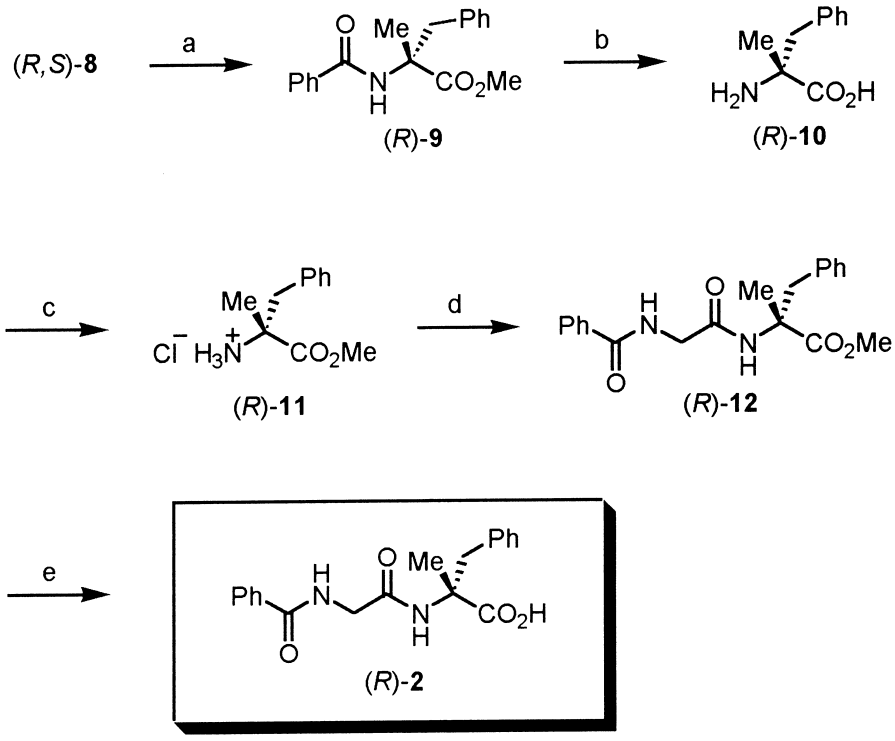
Results and Discussion

Synthesis of (*S*)-Hipp-α-MePhe (**2**) is outlined in Scheme 1. The key intermediate, (*S*)-α-MePhe (**10**) was prepared following the route described by Obrecht et al.[4] Compound **6** was obtained by benzylation of 4-methyl-2-phenyl-1,3-oxazol-5(4*H*)-one according to the literature method.[5] Treatment of racemic **5** with (*S*)-phenylalanine cyclohexylamide in *N*-methylpyrolidin-2-one gave a mixture of diastereomers in the ratio of 1:1, which was readily separated by column chromatography. Diastereomer, (*S,S*)-**7** having $[\alpha]_D = +1.8°$ (EtOH) was treated with trifluoromethanesulfonic acid in methanol to yield (*S*)-**9**. The stereochemistry of the diastereomer has been previously established by X-ray crystallography to be the (*S,S*)-configuration.[4] Acidic hydrolysis of (*S*)-**9** and subsequent esterification gave (*S*)-α-MePhe methyl ester (**11**) which was coupled with hippuric acid by the mixed anhydride method to give (*S*)-**12**. The desired compound was obtained by alkaline hydrolysis of (*S*)-**12** with lithium hydroxide in an overall yield of 10% from **5**.

Compound (*R*)-**2** was prepared from (*R,S*)-**8** with $[\alpha]_D = +58.0°$ (EtOH) following the same sequences of reactions as used for the preparation of (*S*)-**2** (Scheme 2).

Scheme 3 depicts the synthetic route for the preparation of **5**. (*S*)-α-MeOPhe (**17**) was prepared by the general route described by Seebach et al.[6] with a minor modification. Thus, the conversion of **15** into **16** was carried out under the conditions of Greiner and Ortholand.[7] The coupling of **18** with *N*-*t*-Boc-glycine to give **19** was achieved in a 62% yield by the method of Zhao et al.,[8] which is known to be highly effective for esterification of sterically hindered *tert*-alcohols. Removal of the protection group in **19** followed by the *N*-benzoylation and then hydrogenolysis produced **5** in an overall yield of 12% from **13**.

The unnatural substrates thus synthesized were assayed as substrates of CPA. The rate of the hydrolysis of (*S*)-**2** was found to be lower considerably compared with that of **1**. The kinetic parameters for the enzymic hydrolysis reaction were obtained from the Lineweaver–Burk plot[9]



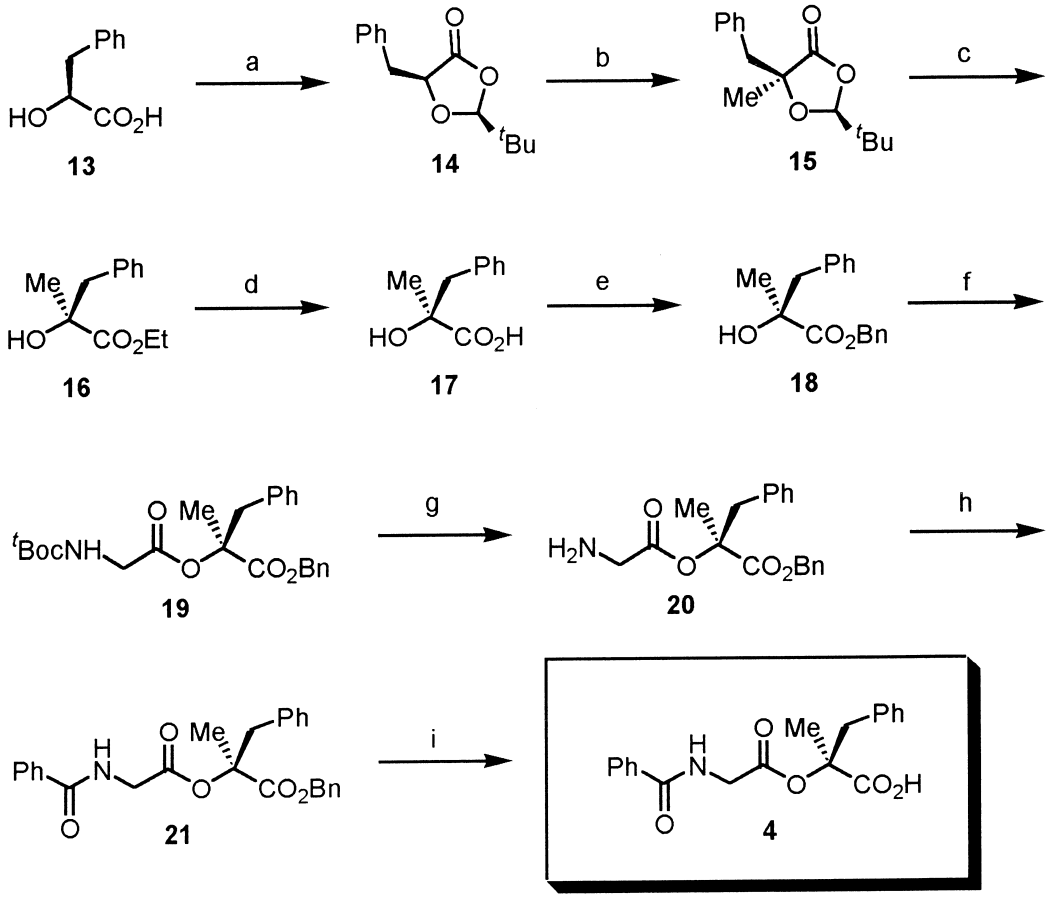
Scheme 2. (a) CF_3SO_3H, MeOH, 80 °C, 20 h, 70%; (b) HCl/dioxane, 100 °C, 20 h; propylene oxide, EtOH, reflux, 0.5 h, 90%; (c) H^+, MeOH, reflux, 12 h, 90%; (d) hippuric acid, NMM, isobutyl chloroformate, THF, −10 °C to rt, 12 h, 52%; (e) $LiOH \cdot H_2O$ (3 equiv), $MeOH/H_2O/THF$, rt, 24 h, 90%.

(Fig. 1) and are collected in Table 1. The k_{cat} value was reduced by 245-fold by the replacement of the hydrogen at the α-position in **1** with a methyl group, although the binding affinity is improved. The apparent second-order rate constant (k_{cat}/K_m) was reduced by 105-fold. On the other hand, the enantiomer of (*S*)-**2** failed to be hydrolyzed by the enzyme initially, then started to undergo hydrolysis in about 2 h at a much reduced rate (Fig. 2). As expected, (*R*)-**2** inhibited the enzymic hydrolysis of (*S*)-Hipp-Phe with the K_i value of 405 μM when CPA was added to the incubation mixture of the substrate and (*R*)-**2** (Fig. 3).

The hydrolysis rate of **4** that carries a methyl group at the α-position of the ester (**3**) was also reduced compared with that for **3** but much less profoundly: it was reduced by 6.8-fold. The kinetic parameters were obtained from the Lineweaver–Burk plot[9] (Fig. 4) and are listed in Table 2.

Asante-Appiah et al. reported that 2-ethyl-2-methylsuccinic acid is a potent competitive inhibitor for CPA.[2] The X-ray crystal structure of the complex of CPA formed with the *R*-form of the inhibitor revealed that the α-methyl group occupies a small hydrophobic cavity. The small cavity (methyl hole) located next to the primary recognition pocket of the enzyme is, however, not filled when substrates bind the enzyme, and thus it was proposed that its function is to provide a space needed for the protonated carboxylate of Glu-270 to transfer a proton to the amino group of the tetrahedral transition state in the catalytic process (Fig. 5(a)).[2,12] The decrease in the enzymic hydrolysis rate observed with (*S*)-**2** may be envisioned on the ground of the hypothesis proposed by Asante-Appiah et al. Thus, in the case of (*S*)-**2**, the space required for the proton transfer is occupied by the α-methyl group upon forming the complex and as a result the proton delivery to the departing amino group becomes prohibitive, causing impedence of the catalytic activity (Fig. 5(b)) as manifested by the lowering of the k_{cat}/K_m value by 105-fold. Details of the CPA catalytic mechanism will be discussed below. The observation that (*R*)-**2** commences to undergo CPA-catalyzed hydrolysis in about 2 h may be a reflection of the inherent propensity of an enzyme to exert its catalytic action on the bound ligand. The CPA molecule may possibly undergo an induced-fit conformational change upon forming a complex with (*R*)-**2**, and begins to catalyze the hydrolysis reaction.

The catalytic mechanism of CPA remains to be the subject of much debate in spite of intensive research over the last several decades. The promoted-water pathway has been proposed by Christianson and Lipscomb mostly based on the X-ray structural studies of CPA complexes formed with slowly hydrolyzing substrates and transition state analogue inhibitors.[13,14] According to them, the carboxylate of Glu-270 functions as a base, abstracting a proton from the zinc-bound water molecule to generate a nucleophilic hydroxide ion, which, in turn, attacks at the carbonyl carbon of the scissile peptide bond, generating a tetrahedral transition state (Fig. 5(a)). The scissile peptide bond of the enzyme bound substrate is thought to be activated for the nucleophilic attack by forming its carbonyl oxygen a bifurcated hydrogen bond with the guanidinium of



Scheme 3. (a) Pivalaldehyde (1.1 equiv), $BF_3{\cdot}Et_2O$ (3 equiv), ether, 0 °C, 4 h, 94%; (b) LDA (1.05 equiv), MeI (1.5 equiv), THF:hexane (9:1), −78 °C to −20 °C, 3 h, 47%; (c) NaOEt (cat.), EtOH, reflux, 10 min, 66%; (d) $LiOH{\cdot}H_2O$ (3 equiv), THF:MeOH:H_2O (3:1:1), rt, 12 h, 96%; (e) Cs_2CO_3 (1 equiv), MeOH, rt, 30 min; BnBr (1 equiv), DMF, rt, 6 h, 86%; (f) *t*Boc-Gly-OH (3 equiv), $Sc(OTf)_3$ (0.6 equiv), DMAP (3 equiv), rt, −10 °C, 30 min; EDCI (3 equiv), CH_2Cl_2, −10 to 40 °C, 48 h, 62%; (g) CF_3CO_2H, rt, 1 h, 90%; (h) BzCl (1 equiv), Et_3N (1 equiv), CH_2Cl_2, 0 °C to rt, 1 h, 91%; (i) Pd/C, H_2, MeOH, rt, 1 h, 95%.

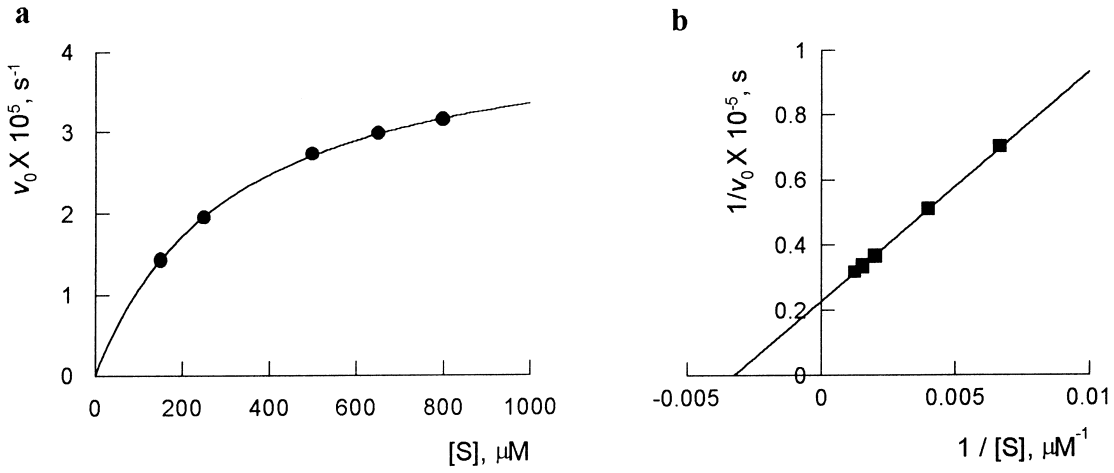


Figure 1. The progress curve (a) and the Lineweaver–Burk plot (b) for CPA catalyzed hydrolysis of (*S*)-Hipp-α-MePhe (Tris buffer of pH 7.5 at 25 °C).

Arg-127.[15] The abstracted proton is then delivered to the amino nitrogen of the leaving amino acid residue at the transition state, making the collapse of the transition state to products facile (Fig. 5(a)). On the other hand, Makinen and others were able to detect spectroscopically anhydride-type acyl-enzyme intermediates in the CPA-catalyzed hydrolysis of ester type substrates under low temperature conditions.[16–21] On the basis of these observations Makinen proposed that the catalytic mechanism of CPA is initiated by the nucleophilic attack of the carboxylate of Glu-270 on the carbonyl carbon of the bound substrate to form an anhydride

Table 1. Kinetic parameters for carboxypeptidase A catalyzed hydrolysis reactions

	(*S*)-Hipp-Phe (**1**)	(*S*)-Hipp-α-MePhe ((*S*)-**2**)	(*R*)-Hipp-α-MePhe ((*R*)-**2**)
Initial velocity[a]	1.39×10^{-3}	1.95×10^{-5}	9.23×10^{-7}
k_{cat} (s^{-1})	81 (67)[b]	0.33	ND[c]
K_m (μM)	700 (690)[b]	313	ND
k_{cat}/K_m (M^{-1} s^{-1})	11.6×10^4 (9.7×10^4)[b]	0.11×10^4	ND
K_i (μM)			405[d]

[a]Initial velocity is defined as the increase of UV absorbance at 254 nm per second at the initial stage of the enzymic reaction ([E] = 600 nM, [S] = 250 μM).
[b]ref 11.
[c]ND denotes that the kinetic parameter was not determined.
[d]The K_i value was obtained for the inhibition of the hydrolysis of (*S*)-Hipp-Phe by CPA.

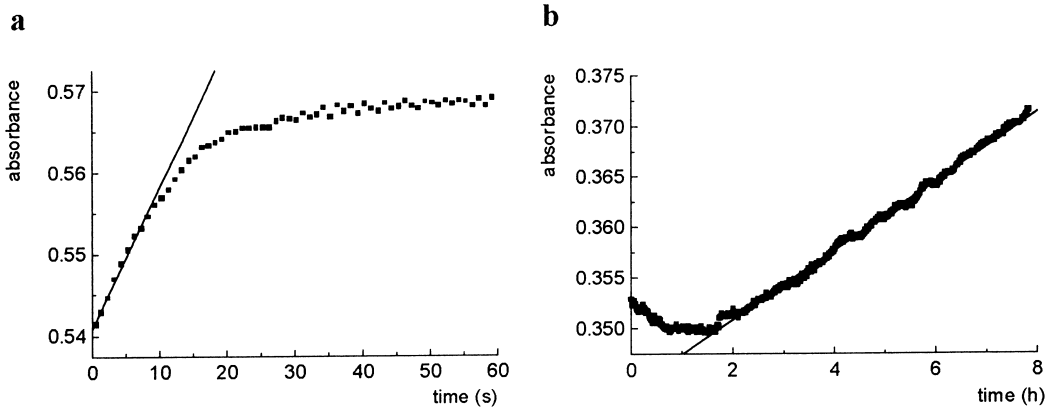


Figure 2. Graphs showing the product formation against time for CPA catalyzed hydrolysis of (*S*)-Hipp-Phe (a) and (*R*)-Hipp-α-MePhe (b) at [S] = 250 μM and [CPA] = 600 nM (Tris buffer of pH 7.5 at 25 °C).

intermediate (Fig. 6).[16,17,20] The anhydride intermediate is then hydrolyzed by the active site zinc bound water molecule to yield products with regeneration of CPA. This reaction path is generally referred to as the anhydride mechanism. The accumulated experimental data[13–21] pertaining to the enzymic mechanism of the CPA catalyzed hydrolysis reactions may be reconciled by proposing that there may operate two different catalytic reaction paths depending on the nature of substrate: natural substrates of peptide are hydrolyzed by the promoted-water pathway, but in the hydrolysis of esters the anhydride intermediates are involved.

It is now well established that the carbonyl oxygen of peptide substrate forms a hydrogen bond with the guanidinium moiety of Arg-127 as demonstrated by the X-ray crystal structure study[15] and subsequently confirmed by the site specific mutagenesis experiment.[22] However, no X-ray structural date that can establish the binding mode for ester substrates is available. It is not unreasonable to expect that esters may bind differently from peptides in the complex formation in light of their molecular conformational differences. While a peptide bears a planar conformation, ester moiety is known to have a bent molecular shape[23] with the dihedral angle of 54.5°. Thus, in forming the Michaelis complex with CPA, the carbonyl oxygen of ester substrates may not hydrogen bond to guanidinium of Arg-127, but instead ligates to the zinc ion. The finding of Auld and Holmquist that binding of esters requires the presence of the zinc ion, whereas binding of a peptide does not, supports the proposition that the ester coordinates to the active site zinc ion in binding to CPA.[24] The altered binding mode is thought to be responsible for the esters taking the anhydride pathway. As an ester, **4** would also bind CPA with its carbonyl oxygen being coordinated to the zinc ion, and is hydrolyzed by the anhydride pathway that does not require the proton transfer process

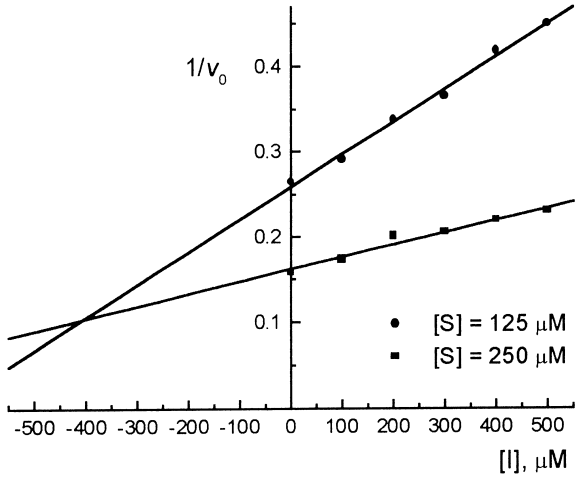


Figure 3. The Dixon plot[10] for the inhibition of the CPA catalyzed hydrolysis of (*S*)-Hipp-Phe by (*R*)-Hipp-α-MePhe (Tris buffer of pH 7.5 at 25 °C).

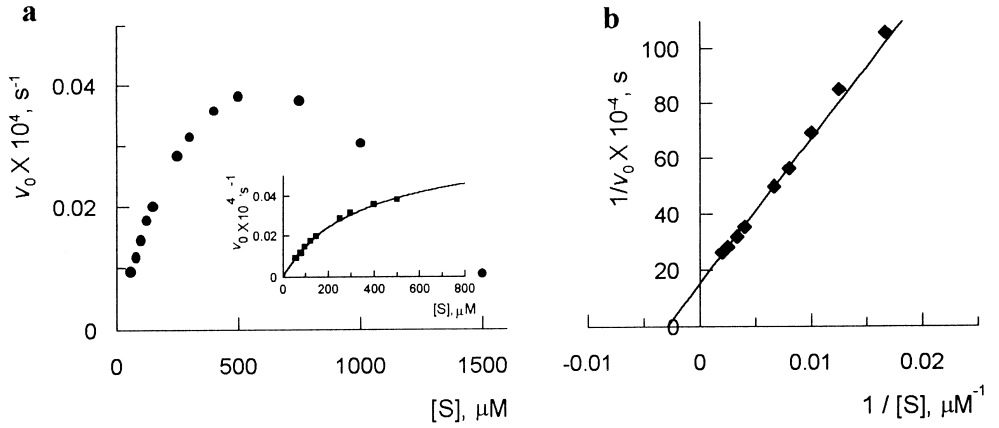


Figure 4. The progress curve (a) and the Lineweaver–Burk plot (b) for CPA catalyzed hydrolysis of (*S*)-Hipp-α-MeOPhe (Tris buffer of pH 7.5 at 25 °C).

Table 2. Kinetic parameters for carboxypeptidase A catalyzed hydrolysis reactions

	(*S*)-Hipp-OPhe (**3**)	(*S*)-Hipp-α-MeOPhe (**4**)
k_{cat} (s^{-1})	682 (424)[a]	477
K_m (μM)	72 (53)[a]	344
k_{cat}/K_m (M^{-1} s^{-1})	9.5×10^6 (8.0×10^6)[a]	1.4×10^6

[a]ref 3.

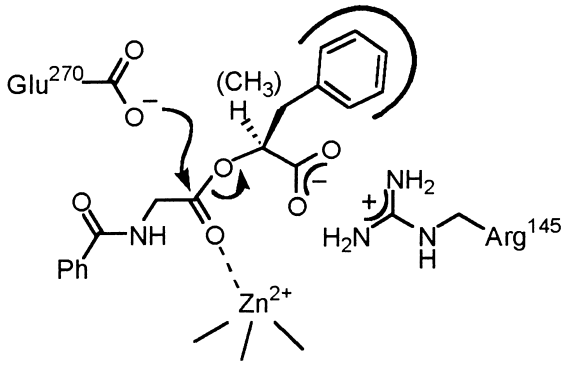


Figure 6. Schematic representation showing a proposed reaction path for the CPA-catalyzed hydrolysis of ester-type substrates, for example, **3** and **4**.

which is critical for the peptide substrate. Therefore, the reaction path of the CPA-catalyzed hydrolysis of **4** is not perturbed by the α-methyl group, and the hydrolysis proceeds rapidly (Table 2) albeit not at the optimal rate as observed with **3** (Fig. 6).

The K_m value obtained for **4** is augmented compared with that for **3** (Table 2), indicating that the α-methyl group tends to deter the binding of **4** to the enzyme. This observation suggests that the minor cavity next to the primary substrate recognition pocket of CPA is not optimally situated for accommodating the methyl group at the α-position of the substrate as expected from the proposition that its primary function is to provide a space for the proton transfer in the enzymic hydrolysis of peptide substrates. The surprising finding of Asante-Appiah et al.,[2] that 2-ethyl-2-methylsuccinic acid binds CPA with extremely high affinity may be explained. The ethyl group that is considerably smaller than the aromatic side chain present in the P_1' residue of normal substrates can fit freely in the primary recognition pocket. The inserted ethyl group may have sufficient room for maneuvering inside the pocket and enable the 2-methyl moiety to fit fully in the small cavity resulting in the strengthening of the binding of 2-ethyl-2-methylsuccinic acid to CPA.

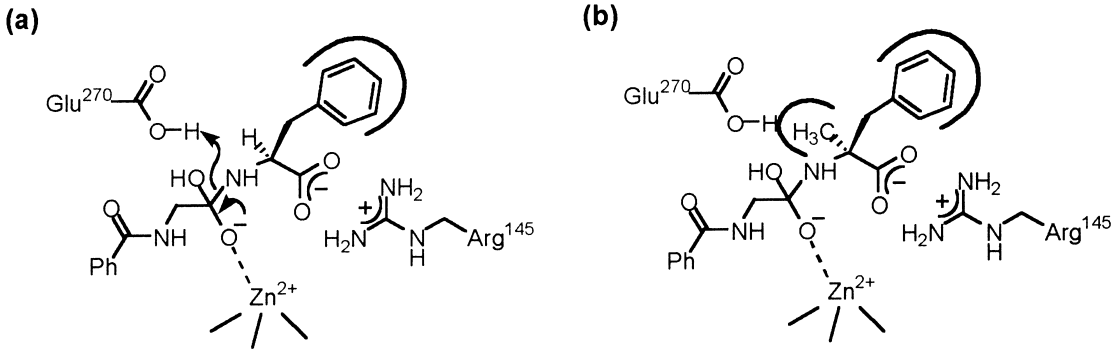


Figure 5. Schematic representations showing the tetrahedral transition state proposed for the CPA-catalyzed hydrolysis of normal peptide substrate (**1**) (a) and unnatural substrate (**2**) whose P_1' residue carries a methyl group at the α-position.

Conclusion

The substitution of the proton at the α-position of (*S*)-Hipp-Phe with a methyl group causes the substrate to be resistant to the enzymic hydrolysis, resulting in the decrease of the second-order rate constant by 105-fold. This is probably due to blocking of the approach of the protonated carboxylate of Glu-270 to the departing amino group in the transition state of the bond cleavage process by the methyl group. In the case of the ester substrate, the hydrolysis rate was lowered by only 6.8-fold by the introduction of a methyl at the α-position. The different effects exhibited by the introduction of an α-methyl group in the two different classes of substrates on the enzymic reaction may be explained on the basis that they do not bind in the same mode at the enzyme active site, and as a result take a different reaction path. The peptide bond is cleaved via a tetrahedral transition state generated by the attack of the zinc-bound activated water molecule on the scissile amide bond, but ester hydrolysis takes the path involving an anhydride intermediate formed by the attack of the carboxylate of the catalytic Glu-270 residue at the ester carbonyl carbon. The result of the present study is in accord with the suggestion made by Breslow that there might exist two different pathways in the CPA catalyzed reaction.[25]

Experimental

Melting points (mp) were taken on a Thomas–Hoover capillary mp apparatus and were uncorrected. ^{1}H 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. 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 Basic Science Center, Kyungbook National University, Taegu, Korea. A Perkin–Elmer HP 8453 UV–vis spectrometer was used in enzyme inhibition studies.

(*S*)-Methyl hippuryl-α-methylphenylalaninate (12). To a mixture of hippuric acid (192 mg, 1.1 mmol) and *N*-methylmorpholine (NMM, 120 μL, 1.1 mmol) in dry THF (5 mL) was added isobutyl chloroformate (140 μL, 1.1 mmol) at −15 °C. (*S*)-α-Methylphenylalanine methyl ester hydrochloride ((*S*)-**11**, 250 mg, 1.1 mmol) was neutralized with NMM (120 μL, 1.1 mmol) in DMF (1 mL), and added slowly to the reaction mixture. The resulting mixture was allowed to warm up to room temperature and stirred for 12 h. The hydrochloride of NMM was removed by filtration and washed with THF. The combined filtrate and washings were concentrated under reduced pressure and diluted with ethyl acetate. The solution was washed with 0.5 N $NaHCO_3$, 0.5 N HCl, water, and brine, then dried ($MgSO_4$), and evaporated under reduced pressure. The residue was purified by column chromatography to give (*S*)-**12** as an oil (150 mg, 52%). $[\alpha]_D$ −72.1° (*c* 0.5, MeOH); IR (neat) 1723, 1652, 1647 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.60 (s, 3H), 1.96 (br. s, 1H), 3.19–3.40 (dd, 2H), 3.72 (s, 3H), 4.06–4.07 (d, 2H), 6.89–7.80 (m, 11H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 23.48, 41.99, 44.26, 53.13, 61.39, 127.59, 128.76, 128.98, 130.26, 132.24, 133.93, 136.21, 137.59, 168.07, 168.80, 174.35.

(*S*)-Hippuryl-α-methylphenylalanine ((*S*)-2). Lithium hydroxide (46 mg, 1.1 mmol) solution in water (500 μL) was added to a solution of **12** (130 mg, 0.37 mmol) dissolved in THF:MeOH (3:1, 1.5 mL). The reaction mixture was stirred at room temperature for 24 h. After evaporation of the solvent under reduced pressure, the residue was acidified with 2 N HCl (2 mL) and extracted with ethyl acetate (3×5 mL). The combined extracts were washed with brine and dried over $MgSO_4$. Evaporation of the solvent gave **2** (110 mg, 90%) which was recrystallized from ethyl acetate, diethyl ether and hexane: mp = 164.5–166 °C; $[\alpha]_D$ −49.0° (*c* 0.95, MeOH); IR (KBr) 3412, 1715, 1677 cm^{-1}; ^{1}H NMR 300 MHz (D_2O) δ 1.60 (s, 3H), 3.26–3.37 (dd, 2H), 3.99–4.18 (m, 2H), 6.96–7.82 (m, 12H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 18.39, 41.57, 44.17, 61.14, 127.43, 127.77, 128.71, 130.46, 132.45, 133.47, 136.14, 168.77, 169.97, 176.71. Anal. calcd for $C_{19}H_{20}N_2O_4$: C, 67.05; H, 5.92; N, 8.23. Found: C, 66.82; H, 5.89; N, 7.95.

(*R*)-Hippuryl-α-methylphenylalanine ((*R*)-2). The synthesis was carried out as described for (*S*)-hippuryl-α-methylphenylalanine starting from (*R,S*)-**8**: mp = 165–166 °C; $[\alpha]_D$ +48.5° (*c* 1.0, MeOH).

(*S*)-Ethyl α-methylphenyllactate (16). Ethanol solution of **15** (3.3 g, 13.3 mmol) and a catalytic amount of sodium ethoxide was refluxed for 10 min, then evaporated under reduced pressure to afford 1.8 g (66%) of **16** as an oil. $[\alpha]_D$ −20.9° (*c* 2.1, $CHCl_3$); IR (neat) 3300, 1762 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.23–1.27 (t, 3H), 1.49 (s, 3H), 2.90–3.10 (dd, 2H), 3.23 (br. s, 3.23), 4.11–4.19 (q, 2H), 7.19–7.30 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 14.59, 26.27, 46.79, 62.18, 75.43, 127.30, 128.55, 130.55, 136.47, 176.53.

(*S*)-α-Methylphenyllactic acid (17). Lithium hydroxide solution obtained by dissolving lithium hydroxide (0.725, 17.3 mmol) in water (3 mL) was added to a solution of **16** (1.8 g, 8.6 mmol) in THF:MeOH (3:1, 12 mL). The reaction mixture was stirred at room temperature for 12 h. After evaporation of the solvent under reduced pressure, the residue was acidified with 2 N HCl (10 mL) and extracted with ethyl acetate (3×5 mL). The combined extracts were washed with brine and dried over $MgSO_4$. Evaporation of the solvent gave **17** (1.5 g, 96%) which was recrystallized from diethyl ether and hexane. Mp = 114–116 °C (lit.[26] 117.5–119 °C); $[\alpha]_D$ −32.1° (*c* 1.0, $CHCl_3$) (lit.[26] −16.4° (*c* 5.7, dioxane); IR (KBr) 3446, 1652 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.47 (s, 3H), 2.89–3.16 (dd, 2H), 7.25–7.28 (m, 6H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 26.28, 46.38, 75.00, 127.04, 128.35, 130.72, 136.90, 178.64.

(*S*)-Benzyl α-methylphenyllactate (18). To a solution of **17** (0.62 g, 3.4 mmol) dissolved in 10 mL of MeOH was added Cs_2CO_3 (1.12 g, 3.4 mmol) and stirred for 30 min.

After the evaporation of the solvent under the reduced pressure, cesium salt of lactate was suspended in 10 mL DMF. Benzyl bromide (430 μL, 3.6 mmol) was added dropwise to the reaction mixture and stirred for 6 h at room temperature. The reaction mixture was poured onto ice (20 g) and extracted with ethyl acetate (3×20 mL). The combined extracts were washed with water (30 mL), 0.5 N HCl (30 mL), and brine (30 mL), dried over $MgSO_4$, and evaporated under the reduced pressure to give a white solid. The crude product was recrystallized from petroleum ether to give 0.80 g (86%) of **18** (white crystal). Mp = 59.5–61.0 °C; $[\alpha]_D$ −70.8° (*c* 0.54, $CHCl_3$); IR (KBr) 3446, 1733 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.55 (s, 3H), 2.92–3.13 (dd, 2H), 5.16 (s, 2H), 7.10–7.42 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 26.27, 46.83, 67.94, 75.67, 126.29, 127.33, 128.62, 128.95, 129.08, 130.48, 135.51, 136.19, 176.36.

(*S*)-Benzyl *t*-Boc-glycyl-α-methylphenyllactate (19). A stirring mixture of **18** (0.90 g, 3.3 mmol), scandium triflate (0.98 g, 2.0 mmol), *t*-Boc-Gly-OH (1.75 g, 10.0 mmol), and DMAP (1.22 g, 10.0 mmol) in anhydrous CH_2Cl_2 (7 mL) was cooled to −10 °C in an ice-salt bath for 30 min. EDCI (1.98 g, 10.0 mmol) was added and the stirring was continued for 30 min. The resulting mixture was allowed to warm up to room temperature over the period of 2 h and stirred at 40 °C for 48 h. The reaction mixture was diluted with 20 mL of CH_2Cl_2 and washed with 2×20 mL of 0.1 N HCl, 2×20 mL of 0.1 N $NaHCO_3$, 20 mL of brine, then 20 mL of water. The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The product was purified by chromatography on a silica gel column to give **19** (1.00 g, 62%) as a colorless oil. $[\alpha]_D$ −20.8° (*c* 0.80, $CHCl_3$); IR (neat) 1739, 1717, 1652 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.45 (s, 9H), 1.56 (s, 3H), 3.03–3.30 (dd, 2H), 3.89–3.92 (m, 2H), 4.93 (s, 1H), 5.11 (s, 2H), 7.10–7.36 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 21.63, 28.73, 42.98, 44.18, 67.67, 80.40, 82.30, 126.30, 127.61, 128.67, 128.72, 128.95, 130.91, 134.88, 135.66, 155.943, 169.76, 171.65.

(*S*)-Benzyl glycyl-α-methylphenyllactate (20). To a solution of **19** (900 mg, 0.75 mmol) in CH_2Cl_2 (5 mL) was added CF_3CO_2H (3 mL) and stirred at room temperature for 1 h. Evaporation of the excess trifluoroacetic acid and solvent yielded **20** (680 mg 90%) as a yellow oil. $[\alpha]_D$ −14.7° (*c* 0.80, $CHCl_3$); IR (neat) 1750, 1684 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.59 (s. 3H), 3.07–3.22 (dd, 2H), 3.84–3.87 (m, 2H), 5.01–5.11 (m, 2H), 7.00–7.41 (m, 10H), 7.82–7.93 (br. s, 2H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 21.32, 41.05, 44.40, 68.28, 84.66, 127.93, 128.79, 128.82, 129.04, 129.35, 130.66, 133.95, 135.04, 166.65, 171.32.

(*S*)-Benzyl hippuryl-α-methylphenyllactate (21). To a solution of **20** (640 mg, 1.95 mmol) dissolved in 5 mL CH_2Cl_2 was added triethylamine (300 μL, 2.15 mmol) and benzoyl chloride (200 μL, 2.15 mmol) alternatingly at 0 °C. The reaction mixture was stirred for 1 h at room temperature and then washed with water, 0.1 N HCl, 0.1 N $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The product was purified by chromatography on a silica gel column to give **21** (770 mg 91%) as a colorless oil. $[\alpha]_D$ −30.0° (*c* 0.40, $CHCl_3$); IR (neat) 1739, 1652, 1539 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.64 (s, 3H), 3.10–3.36 (dd, 2H), 4.13–4.32 (m, 2H), 5.16 (s, 2H), 7.09–7.86 (m, 15H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 21.68, 42.38, 44.20, 67.71, 82.67, 127.55, 127.66, 128.72, 128.81, 128.97, 129.01, 130.49, 130.92, 132.24, 134.03, 134.79, 135.62, 167.83, 169.59, 171.61.

(*S*)-Hippuryl-α-methylphenyllactic acid (4). A methanol (3 mL) solution containing **21** (700 mg, 1.6 mmol) and a catalytic amount of Pd/C was stirred for 1 h at room temperature. The catalyst was removed by filtration, and the solvent was evaporated under the reduced pressure to give a resin (526 mg, 95%). $[\alpha]_D$ −39.5° (*c* 1.3, $CHCl_3$); IR (KBr) 1733, 1652, 1538 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.54 (s, 3H), 3.01–3.35 (dd, 2H), 4.21–4.23 (m, 2H), 7.14–7.38 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 18.24, 21.56, 42.46, 43.63, 82.19, 127.60, 127.74, 128.69, 129.01, 131.32, 132.39, 133.60, 134.93, 168.86, 169.86, 175.07. Anal. calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.56; H, 5.93; N, 3.85.

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 Tirs/0.5 M NaCl, pH 7.5 buffer solution. Enzyme concentrations were estimated from the absorbance at 278 nm (ϵ_{278} = 64,200). (*S*)-Hippuryl-phenylalanine ((*S*)-Hipp-Phe) was purchased from Sigma Chemical Co. (S)-Hippuryl-phenyllactic acid ((*S*)-Hipp-OPhe) was prepared according to the literature method.[27]

Determination of k_{cat} and K_m

Data obtained by repeated measurements of the change in absorbance upon complete hydrolysis of several known concentrations of each substrate was converted to initial velocities by using observed Δε ((*S*)-Hipp-OPhe) = 332 M^{-1} cm^{-1}, Δε ((*S*)-Hipp-α-MeOPhe) = 318 M^{-1} cm^{-1}, Δε ((*S*)-Hipp-α-MePhe) = 210 M^{-1} cm^{-1} at 254 nm. The k_{cat} and K_m values were then estimated from the double reciprocal plot of the initial velocity versus the concentration of the substrate.[9] Typically, enzyme stock solution was added to various concentrations of substrates in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer (1 mL cuvette), and the initial rates were measured immediately.

Determination of K_i

The K_i values were estimated from the semireciprocal plot of the initial velocity versus the concentration of the inhibitors according to the method of Dixon.[10] Two

concentrations of the substrate were used. Typically, enzyme stock solution was added to various concentrations of inhibitors in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer (1 mL cuvette), and the initial rates were measured immediately using a microcomputer-interfaced UV spectrometer.

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