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2-Benzyl-2-methylsuccinic Acid as Inhibitor for Carboxypeptidase A. Synthesis and Evaluation

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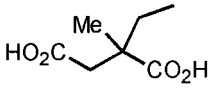
Abstract—Recently, Asante-Appiah et al. (Asante-Appiah, E.; Seetharaman, J.; Sicheri, F.; Yang, D. S.-C.; Chan, W. W.-C. *Biochemistry* **1997**, *36*, 8710–8715) reported that 2-ethyl-2-methylsuccinic acid is a highly potent inhibitor for carboxypeptidase A (CPA), a prototypic zinc protease. The X-ray crystal structure of the complex of the enzyme formed with 2-ethyl-2-methylsuccinic acid revealed that at the active site of CPA there is present a small cavity which accommodates the methyl group of the inhibitor. These investigators postulated that incorporation of a methyl group at the α-position to the carboxylate of existing inhibitors of CPA would improve the inhibitory potency. We have synthesized racemic and optically active 2-benzyl-2-methylsuccinic acids and evaluated their inhibitory activities for CPA to find the K_i values to be 0.28, 0.15, and 17 μM for racemic form, (*R*)-, and (*S*)-enantiomer, respectively. Contrary to the expectation, the effect on the binding affinity by the incorporation of the methyl group is minimal. The validity of the proposition that the small cavity may be utilized for the improvement of the inhibitory potency appears questionable.

Introduction

Carboxypeptidase A (CPA), a well studied zinc protease, selectively cleaves off the C-terminal amino acid residue having a hydrophobic side chain from protein substrate.[1] The enzyme has been served as a model in devising design strategies for inhibitors that are effective for zinc containing proteases (for example, refs 2–5). Recently, Asante-Appiah et al. reported that 2-ethyl-2-methylsuccinic acid (**1**) is a potent inhibitor for carboxypeptidase A (CPA).[6] The X-ray crystal structure of the complex of CPA formed with (*R*)-**1** revealed that the methyl group at the 2-position of the inhibitor occupies a small cavity of hydrophobic nature. They proposed to call the cavity a 'methyl hole' and attributed the high inhibitory potency of **1** to the additional interactions that result from the α-methyl group in **1** occupying the cavity.[6] These investigators invited evaluation of 2-benzyl-2-methylsuccinic acid (**2**) that resembles more closely optimal substrates by having a benzyl side chain at the α-position, expecting it would be an inhibitor of considerably improved potency over **1**. This paper reports syntheses of racemic and optically active **2** as well as 2-ethylsuccinic acid, and their evaluation as inhibitors for CPA in an effort to probe the proposition that the methyl hole may be utilized to obtain inhibitors of enhanced potency in designing inhibitors for CPA.



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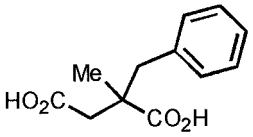

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Results and Discussion

Racemic **2** was prepared in three steps starting with commercially available 2-benzylpropanoic acid. The latter was converted into the corresponding *tert*-butyl ester. The enolate that was generated by the treatment of the *tert*-butyl ester with LDA was allowed to react with *tert*-butyl bromoacetate to afford 2-benzyl-2-methylsuccinic acid di-*tert*-butyl ester which upon treatment with trifluoroacetic acid gave **2** in an overall yield of 47% (Scheme 1).

(*R*)-Benzylmethylpropanedioic acid monomethyl ester (**6**) that was obtained by enzymatic hydrolysis of dimethyl benzylmethylmalonate using α-chymotrypsin

Key words: Zinc proteases; carboxypeptidase A; enzyme inhibitors; inhibitor design.

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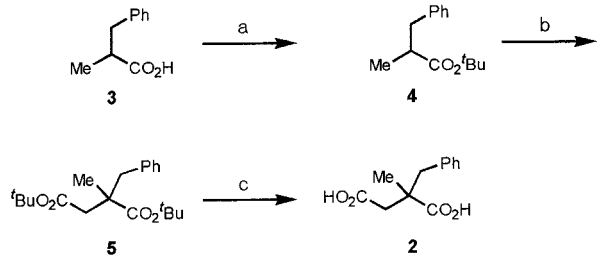


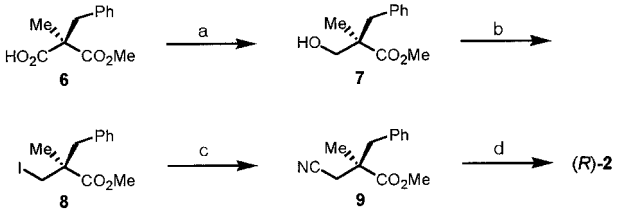
Scheme 1. (a) Isobutylene, H_2SO_4, CH_2Cl_2, rt, 3 d, 90%; (b) LDA (1.2 equiv), THF, 0°C; butyl bromoactate (2.0 equiv), 0°C to rt, 4 h, 65%; (c) CF_3CO_2H, rt, 1 h, 80%.

according to the literature method[7] was regioselectively reduced to give **7** following the method of Martinez et al.[8] The hydroxyl group in **7** was converted into iodo by the method of Garegg and Samuelsson[9] with a minor modification to give **8** which was then transformed to **9** by treatment with KCN. Hydrolysis of **9** under alkaline conditions afforded (*R*)-**2** in an overall yield of 47% (Scheme 2).

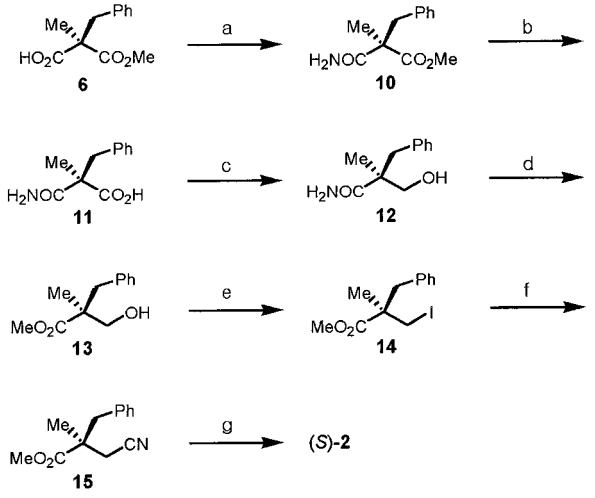
The synthesis of (*S*)-**2** is outlined in Scheme 3. The carboxylate of **6** was converted into carboxamide to give **10** which upon treatment with lithium hydroxide in a solution of THF–MeOH–water afforded **11**. The carboxylate in **11** was reduced to hydroxymethyl and the carboxamide was converted into methoxycarbonyl following the method reported in the literature[10] to give **13**. The hydroxyl in **13** was converted into carboxylate to give (*S*)-**2** in three steps in an analogous manner that was used for the synthesis of (*R*)-**2**. The overall yield for this synthesis amounted to 25%.

2-Ethylsuccinic acid (**18**) was synthesized as outlined in Scheme 4, starting with 2-(triphenylphosphoranylidene)succinic acid 1-methyl ester (**16**) which was prepared as described in the literature.[11] Treatment of **16** with acetaldehyde followed by alkaline hydrolysis afforded **17** which upon hydrogenation in the presence of palladium-charcoal gave the target compound (**18**).

The synthesized compounds were assayed for their inhibitory activities for CPA. As expected, they are found to be competitive inhibitors for CPA as shown by the Dixon plot (Fig. 1).[12] The inhibitory constants of these compounds were estimated from the respective Dixon plot[12] and are listed in Table 1. The (*R*)-form of **2**, which corresponds to the stereochemistry of the L-series of α-amino acids is bound 113-fold more tightly



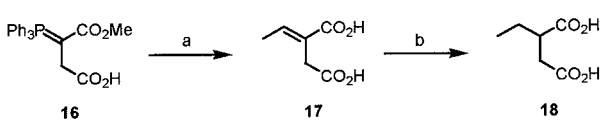
Scheme 2. (a) Isobutyl chloroformate (1 equiv), NMM (1 equiv), DME, −15°C, 15 min; $NaBH_4$ (3 equiv), −15°C, 10 min, 65%; (b) PPh_3 (1.2 equiv), I_2 (1.2 equiv), imidazole (1.2 equiv), toluene, 80°C, 18 h, 90%; (c) KCN, 18-crown-6, DMSO, 80°C, 10 h, 90%; (d) 20% NaOH, 90°C, 24 h, 90%.



Scheme 3. (a) Isobutyl chloroformate (1 equiv), NMM (1 equiv), DME, −15°C, 15 min; NH_4OH (5 equiv), −15°C to rt, 2 h, 64%; (b) LiOH, THF/MeOH/H_2O, rt, 12 h, 95%; (c) isobutyl chloroformate (1 equiv), NMM (1 equiv), DME, −15°C; $NaBH_4$ (3 equiv), −15°C to rt, 1 h, 70%; (d) MeOH, HCl, reflux, 24 h, 80%; (e) PPh_3 (1.2 equiv), I_2 (1.2 equiv), imidazole (1.2 equiv), toluene, 80°C, 18 h, 89%; (f) KCN, 18-crown-6, DMSO, 80°C, 10 h, 90%; (g) 20% NaOH, 90°C, 24 h, 90%.

than its enantiomer. This stereochemistry which is consistent with the stereospecificity of the enzyme shown for peptide substrates is in agreement with the observation of Asante-Appiah et al. who found that the stereochemistry of the CPA-bound **1** bears the *R*-configuration.[6] It was expected that **2** which resembles more closely the substrate than **1** of Asante-Appiah et al. by having a benzyl group instead of the ethyl at the 2-position would be much more potent inhibitor for CPA than **1**.[6] Contrary to the expectation, however, **2** exhibited the K_i value of 0.28 μM,[13] which corresponds to the potency improvement of 2.0-fold.[14] This may reflect that the binding force involved in the interaction of the aromatic ring of **2** with S_1' pocket (primary substrate recognition pocket) is minimal, if at all. The not so significant binding interaction between the aromatic ring and the pocket is inconsistent with the view that the primary role of S_1' pocket is simply to provide an anchoring space for the aromatic side chain of P_1' residue of substrate, and the anchored ring is held by the side chain of Tyr-248.[1,15] It has been well established that Tyr-248 moves downward to the mouth of the primary substrate recognition pocket upon substrates and inhibitors bind the enzyme, and holds physically the ligands in place at the active site.[1,14]

2-Benzylsuccinic acid (BSA) is a prototypic CPA inhibitor designed by Byers and Wolfenden.[16] The X-ray crystal structure of the CPA complex formed with BSA has been reported.[17] It is instructive to compare the K_i



Scheme 4. (a) PPh_3, acetone, rt, 83%; (b) MeOH, rt, 24 h, 75%; (c) (i) CH_3CHO, benzene, rt, 24 h; (ii) 1 N NaOH, rt, 24 h, 80%; (d) Pd/C, MeOH, rt, 3 h, 95%.

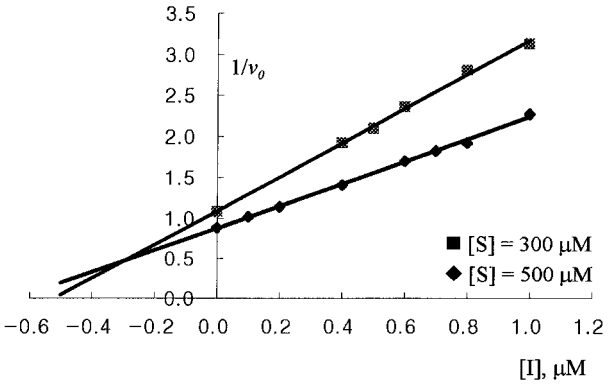


Figure 1. The Dixon plot for the inhibition of CPA with racemic 2-benzyl-2-methylsuccinic acid (substrate, Hipp-L-Phe; Tris buffer of pH 7.5; temperature, 25°C).

Table 1. Inhibitory constants determined for CPA

Inhibitor	K_i (μM)
(*R,S*)-2-Ethylsuccinic acid (**18**)	25
(*R,S*)-2-Ethyl-2-methylsuccinic acid (**1**)	0.11[a]
(*R,S*)-2-Benzylsuccinic acid	0.55[b] (0.2[c], 1.1[d])
(*R*)-2-Benzylsuccinic acid	0.27[b] (0.45[d])
(*S*)-2-Benzylsuccinin acid	3[d]
(*R,S*)-2-Benzyl-2-methylsuccinic acid (**2**)	0.28
(*R*)-2-Benzyl-2-methylsuccinic acid	0.15
(*S*)-2-Benzyl-2-methylsuccinic acid	17

[a] ref 6.
[b] These are values obtained under the same kinetic conditions as those used for compound **2** and ethylsuccinic acid (**18**).
[c] ref 22.
[d] ref 14.

value of **2** for the CPA inhibition with that of 0.55 μM obtained for BSA because the difference in the inhibitory constants reflects the effect that brings about on the binding affinity as a result of replacing the proton at the 2-position of BSA with a methyl group. The introduction of a methyl group, however, brought an improvement of the binding affinity only by 2.0-fold. In the case of (*R*)-2-benzylsuccinic acid the improvement by the introduction of a methyl group is 1.8-fold. On the basis of the binding modes of BSA and **1** to CPA established by the X-ray crystal structure analyses,[6,17] it is expected that the methyl group of **2** would be positioned at the locus where the α-hydrogen of BSA lies, namely the methyl hole, although it may not fit in perfectly.

In order to examine the effect of the methyl group in **1** on the binding affinity, we have synthesized 2-ethylsuccinic acid (**18**) and evaluated its inhibitory activity for CPA. The K_i value of 25 μM which we obtained with **18** reflects that the introduction of a methyl group at the 2-position of the ethylsuccinic acid improves the binding affinity by 227-fold.

Considering the minor increase of the binding affinity resulted by the introduction of a methyl group on the 2-benzylsuccinic acid as described above, the 227-fold improvement is remarkable. This dramatic increase in the binding affinity may, however, be explained on the basis of the distinctive binding mode of **1** to CPA as revealed by the X-ray structural analysis.[6] The X-ray crystal structure of the enzyme complex formed with inhibitor **1** is noticeably different from the crystal structure of complex formed with BSA: in the case of CPA-**1** complex the aromatic side chain of Tyr-248 is appeared as if it is pushed outward by the ethyl group. Furthermore, it is noteworthy that whereas the carboxylate at the 1-position of BSA forms a hydrogen bond with the phenolic hydrogen of Tyr-248, the carboxylate of **1** hydrogen bonds to Arg-127 instead, in addition to the hydrogen bondings that both carboxylates form with Arg-145 and Asn-144. This difference in the binding mode between the two molecules, i.e. **1** is being pushed outward compared with BSA in the complex formation appears to be arisen as a result of the less than satisfactory fitting of the methyl group in the small cavity. That is the methyl hole is most probably not sufficiently large or deep enough to accommodate a methyl group snuggly. It is expected that the binding of **2** which carries a bulkier benzyl group than the ethyl in **1** would push the Tyr-248 side chain further outward. This would result in diminished binding interactions, and as a consequence no significant improvement is effected in the binding of **2** to CPA compared with that for binding of **1**. An alternative interpretation may be offered:[8] The methyl hole is sufficiently large so that the methyl group may be readily accommodated, but by doing so the ring would not be fitted in snugly in the S_1' pocket. As a result, the optimum contribution of the phenyl ring to the overall binding affinity in the formation of the enzyme complex would not be achieved.

The role of the small cavity in the enzymic machinery is not apparent presently, but it is highly unlikely that the small cavity is designed to accommodate any specific group in binding of substrate. However, it is conceivable, as Asante-Appiah et al. postulated,[6] that the cavity serves as a space for the protonated carboxylate of Glu-270 to traverse to the leaving amino group of the P_1' residue transferring its acidic proton to the amino group of the departing P_1' residue in the catalytic process. In a proposed reaction path of the enzymic catalytic reaction of CPA,[1] it has been postulated that a transient intermediate is generated by the nucleophilic attack of an activated water molecule on the carbonyl carbon of the scissile peptide bond of substrates. The subsequent collapse of the transition state to the products is thought to be facilitated by the protonation of the amino group by the protonated carboxylate of Glu-270. The proton delivery from the carboxylate to the departing amino group of the tetrahedral transition state in the catalytic process may be achieved in the small cavity.

Conclusion

Asante-Appiah et al. invited people to investigate zinc protease inhibitors which bear a *gem*-dialkyl structure, postulating that an introduction of a small alkyl group preferably of a methyl onto known inhibitors at an appropriate position (the α-position next to the terminal

carboxylate in the case of inhibitors for CPA) would result in an improvement of the potency.[6] The postulation of Asante-Appiah et al. was made on the basis of their unexpected observation that **1** shows high binding affinity for CPA and the X-ray crystal structure of CPA-**1** complex which reveals the presence of a small cavity that accommodates the methyl group at the active site of CPA. However, the present study suggests that the high binding affinity exhibited by **1** is an exceptional occurrence unique to the compound rather than a general phenomenon. Accordingly, the validity of the proposition of Asante-Appiah et al. that the small cavity at the active site of CPA may be utilized for designing CPA inhibitors of improved potency appears questionable.

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) 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 of Biofunctional Molecules, Pohang University of Science and Technology, Pohang, Korea.

***tert*-Butyl (±)-α-methylhydrocinnamate (4).** To a solution of (±)-α-methylhydrocinnamate (**3**) (1.64 g, 10 mmol) in dichloromethane (20 mL), concentrated sulfuric acid (0.5 mL) was added and the mixture was saturated with isobutylene at −78°C. This resulted in an increase of the volume to about 30 mL. The flask was stoppered and kept at room temperature for three days. After the addition of a solution of sodium carbonate (1 g) in water (15 mL), the two layers were separated and the organic phase was washed with water (3×10 mL), brine and dried under anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure yielded the desired product (**4**) as an oil (1.98 g, 90%). IR (neat) 1720 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.10–1.12 (d, 3H), 1.36 (s, 9H), 2.59–2.66 (m, 2H), 2.92–3.01 (m, 1H), 7.16–7.30 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 17.38, 28.39, 40.27, 42.69, 80.43, 126.30, 126.56, 128.62, 129.46, 175.89.

Di-*tert*-butyl 2-benzyl-2-methylsuccinate (5). Compound **4** (1.50 g, 6.8 mmol) dissolved in THF (10 mL) was added dropwise to a solution of lithium diisopropylamide (8.2 mmol) at 0°C. The mixture was stirred for 2 h, then warmed slowly to room temperature. *tert*-Butyl bromoacetate (2.0 mL, 13.7 mmol) was added dropwise to the reaction mixture at 0°C and the resultant mixture was stirred at room temperature for 4 h. The reaction was quenched by the addition of saturated solution of ammonium chloride (25 mL). The organic layer was separated and the aqueous layer was washed with ethyl acetate (3×10 mL). The combined organic layers were washed with brine, dried under anhydrous $MgSO_4$ and evaporated in vacuo to dryness. The crude product thus obtained was purified by chromatography (silica gel) to give the title compound (**5**) as an oil (1.48 g, 65%). IR (neat) 1725 cm^{-1}; EI-MS *m*/*z* 334; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.18 (s, 3H), 1.41–1.45 (m, 18H), 2.26–2.56 (dd, 2H), 2.87–2.97 (m, 2H), 7.12–7.2 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 22.69, 28.33, 28.42, 43.02, 44.42, 45.59, 126.91, 128.36, 131.01, 137.64, 171.12, 175.54.

2-Benzyl-2-methylsuccinic acid (2). Compound **5** (1.40 g, 4.2 mmol) dissolved in trifluoroacetic acid (3.2 mL, 42 mmol) was stirred at room temperature for 1 h. Evaporation of the excess trifluoroacetic acid yielded **2** (0.74 g, 80%) which was recrystallized from diethyl ether and hexane. Mp = 144–146°C (lit.[19] 117–119°C); IR (neat) 3300, 1652 cm^{-1}; EI-MS *m*/*z* 222; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.24 (s. 3H), 2.37–2.69 (dd, 2H), 2.98 (s, 2H), 7.16–7.28 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 27.09, 46.64, 49.12, 49.76, 131.35, 132.80, 135.32, 142.26, 178.60, 183.48; Anal. calcd for $C_{12}H_{14}O_4$: C, 64.85; H, 6.35. Found: C, 64.47; H, 6.47.

(*R*)-Methyl 2-benzyl-2-methyl-3-hydroxypropanate (7). To a cold (−15°C) solution of (*R*)-2-benzyl-2-methylmalonic acid monomethyl ester (**6**)[7] (2.22 g, 10 mmol) dissolved in 1,2-dimethoxyethane (DME, 10 mL), were added successively *N*-methyl morpholine (1.1 mL, 10 mmol) and isobutyl chloroformate (1.3 mL, 10 mmol). The resulting mixture was stirred in an ice-salt bath for 15 min. To the chilled stirring mixture was added at once a solution of sodium borohydride (1140 mg, 30 mmol) dissolved in water (10 mL), whereby a strong evolution of gas was observed. The mixture was stirred for 1 h. The reaction was quenched with 0.5 N HCl (100 mL), then was extracted with diethyl ether (3×30 mL). The combined extracts were washed with brine and dried under anhydrous $MgSO_4$. After evaporation of the solvent, the residue was purified by silica gel column chromatography to give a colorless oil (1.67 g, 86%). $[\alpha]_D = +16.3°$ (*c* 2.1, MeOH); IR (neat) 3418, 1725 cm^{-1}; EI-MS *m*/*z* 190 (M–H_2O); ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.15 (s, 3H), 2.89–3.01 (dd, 2H), 3.58–3.59 (d, 2H), 3.73 (s, 3H), 7.15–7.33 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 19.66, 41.41, 49.31, 52.26, 67.48, 127.01, 128.52, 130.64, 137.08, 177.53.

(*S*)-Methyl 2-benzyl-2-methyl-3-iodopropanate (8). Triphenylphosphine (2.27 g, 8.6 mmol) was added to iodine (2.19 g, 8.6 mmol) solution dissolved in toluene (1.50 mL) and the mixture was heated at 60°C for 0.5 h. To the resulting solution was added **7** (1.50 g, 7.2 mmol) dissolved in toluene (20 mL) followed by the addition of imidazole (0.59 g, 8.6 mmol). The mixture was then stirred at 60°C for 18 h. After completion of the reaction, the mixture was diluted with ethyl acetate, washed with water, sodium thiosulfate (10%), and brine, and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was purified by silica gel column chromatography to give a colorless oil (2.06 g, 90%).

$[\alpha]_D = -0.76°$ (*c* 0.8, MeOH); IR (neat) 1734 cm^{-1}; EI-MS *m/z* 318; ^1H NMR 300 MHz ($CDCl_3$) δ 1.29 (s, 3H), 2.92–3.06 (dd, 2H), 3.22–3.46 (dd, 2H), 3.71 (s, 3H), 7.13–7.30 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 15.43, 23.52, 44.03, 48.51, 52.58, 127.46, 128.69, 130.34, 136.86, 174.35.

(*R*)-Methyl 2-benzyl-2-methyl-3-cyanopropanate (9). The mixture of **8** (2.00 g, 6.3 mmol), potassium cyanide (0.49 g, 7.5 mmol), and catalytic amount of 18-crown-6 in DMSO (5 mL) was stirred at 80°C for 10 h. After completion of the reaction, the mixture was diluted with ethyl acetate (50 mL), washed with water (5×10 mL) and brine and dried over anhydrous $MgSO_4$. Evaporation of the solvent yielded **9** as an oil (1.30 g, 95%). $[\alpha]_D = +3.30°$ (*c* 0.5, MeOH); IR (neat) 2247, 1734 cm^{-1}; EI-MS *m/z* 217; ^1H NMR 300 MHz ($CDCl_3$) δ 1.45 (s, 3H), 2.46–2.62 (dd, 2H), 2.91–3.17 (dd, 2H), 3.75 (s, 3H), 7.12–7.34 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 23.75, 25.56, 44.13, 46.37, 52.85, 118.19, 127.78, 129.02, 130.10, 136.03, 174.78.

(*R*)-2-Benzyl-2-methylsuccinic acid ((*R*)-2). Compound **9** (1.00 g, 4.6 mmol) dissolved in NaOH solution (20%, 5 mL) was stirred at 90°C for 24 h. After acidifing with 2 N HCl, the mixture was extracted with ethyl acetate (3×10 mL). The combined extracts were washed with brine and dried over anhydrous $MgSO_4$. Evaporation of the solvent yielded (*R*)-**2** (0.86 g, 95%), which was recrystallized from diethyl ether and hexane. Mp = 159–160°C; $[\alpha]_D = +0.7°$ (*c* 5.0, dioxane); IR (neat) 3300, 1652 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.22 (s. 3H), 2.34–2.66 (dd, 2H), 2.96 (s, 2H), 7.15–7.28 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 27.15, 46.73, 49.17, 49.75, 131.65, 133.11, 135.59, 142.39, 178.28, 183.05.

(*R*)-Methyl 2-benzyl-2-methylmalonamate (10). To a cold (−15°C) solution of **6**[6] (2.22 g, 10 mmol) dissolved in 1,2-dimethoxyethane (DME, 10 mL), were added successively *N*-methyl morpholine (1.1 mL, 10 mmol) and isobutyl chloroformate (1.3 mL, 10 mmol). The resulting mixture was stirred in an ice-salt bath for 15 min. To the chilled stirring mixture was added at once NH_4OH solution (30%, 3.5 mL, 30 mmol). The mixture was stirred for 2 h. The reaction mixture was diluted with diethyl ether (30 mL), the organic layer was collected and was washed with brine and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was recrystallized from diethyl ether to a give white solid product (1.50 g, 64%). Mp = 104–106°C; $[\alpha]_D = +9.8°$ (*c* 0.5, $CHCl_3$); IR (neat) 3358, 1732, 1673 cm^{-1}; EI-MS *m/z* 221; ^1H NMR 300 MHz ($CDCl_3$) δ 1.43 (s, 3H), 3.08–3.37 (dd, 2H), 3.73 (s, 3H), 5.59 (br s, 1H). 6.87 (br s, 1H), 7.11–7.29 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 21.19, 44.02, 53.00, 55.48, 127.43, 128.67, 130.27, 136.78, 173.89, 175.05.

(*R*)-2-Benzyl-2-methylmalonic acid monoamide (11). An aqueous lithium hydroxide solution (0.324 g of $LiOH{\cdot}H_2O$ in 3 mL of water, 7.7 mmol) was added to a solution of **10** (1.50 g, 6.4 mmol) dissolved 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 anhydrous $MgSO_4$. Evaporation of the solvent gave **11** (1.23 g, 95%) which was recrystallized from diethyl ether and hexane. Mp = 120–121°C; $[\alpha]_D = -4.4°$ (*c* 0.5, MeOH); IR (KBr) 3412, 1715, 1677 cm^{-1}; EI-MS *m/z* 207; ^1H NMR 300 MHz ($CDCl_3$) δ 1.36 (s, 3H), 3.06–3.27 (dd, 2H), 6.22 (br s, 1H), 7.14–7.50 (m, 6H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 21.70, 43.98, 54.85, 127.20, 128.49, 130.34, 137.10, 175.49, 176.46.

(*S*)-2-Benzyl-2-methyl-3-hydroxypropamide (12). The synthesis was carried out as described for **7**. Compound **12** (white solid, 0.70 g) was obtained in 70% yield from **11** (1.00 g, 4.9 mmol). Mp = 88–89°C; $[\alpha]_D = -21.0°$ (*c* 0.5, MeOH); IR (KBr) 3358, 1655 cm^{-1}; EI-MS *m/z* 193; ^1H NMR 300 MHz ($CDCl_3$) δ 1.08 (s, 3H), 2.86–3.01 (dd, 2H), 3.07 (br s, 1H), 3.54–3.63 (dd, 2H), 5.46 (br s, 1H), 5.93 (r s, 1H), 7.19–7.31 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 19.80, 42.03, 48.20, 68.44, 126.30, 127.10, 128.60, 130.78, 137.12.

Methyl (*S*)-2-benzyl-2-methyl 3-hydroxypropanate (13). To dry methanol (10 mL) saturated with gaseous HCl, **12** (0.70 g, 3.4 mmol) was added and refluxed for 24 h. The solution is poured into ice water (20 mL). A saturated aqueous solution of sodium bicarbonate is added until the mixture is faintly alkaline. The mixture was extracted with diethyl ether (3×20 mL), washed with brine and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was purified by silica gel column chromatography to give an oily product (0.56 g, 80%). $[\alpha]_D = -16.3°$ (*c* 0.5, MeOH); IR (neat) 3418, 1725 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.15 (s, 3H), 2.89–3.01 (dd, 2H), 3.58–3.59 (d, 2H), 3.73 (s, 3H), 7.15–7.33 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 19.66, 41.41, 49.31, 52.26, 67.48, 127.01, 128.52, 130.64, 137.08, 177.53.

Methyl (*R*)-2-benzyl-2-methyl-3-iodopropanate (14). The synthesis was carried out as described for **8**, using **13** (0.50 g, 2.4 mmol) to give **14** (oil, 0.68 g, 89%). $[\alpha]_D = +0.76°$ (*c* 0.5, MeOH); IR (neat) 1734 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.29 (s, 3H), 2.92–3.06 (dd, 2H), 3.22–3.46 (dd, 2H), 3.71 (s, 3H), 7.13–7.30 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 15.43, 23.52, 44.03, 48.51, 52.58, 127.46, 128.69, 130.34, 136.86, 174.35.

Methyl (*S*)-2-benzyl-2-methyl-3-cyanopropanate (15). The synthesis was carried out as described for **9**, using **14** (0.60 g, 1.9 mmol) to give **15** (oil, 0.39 g, 95%). $[\alpha]_D = -3.30°$ (*c* 0.5, MeOH); IR (neat) 2247, 1734 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.41 (s, 3H), 2.42–2.59 (dd, 2H), 2.87–3.12 (dd, 2H), 3.71 (s, 3H), 7.09–7.31 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 23.69, 25.57, 44.15, 46.35, 52.82, 118.18, 127.76, 129.00, 130.10, 136.04, 174.74.

(*S*)-2-Benzyl-2-methylsuccinic acid ((*S*)-2). The synthesis was carried out as described for (*R*)-**2** from **15** (0.30 g, 1.4 mmol) in 90% yield (white solid, 0.28 g).

Mp = 152–154°C; $[\alpha]_D = -0.7°$ (*c* 5.0, dioxane); IR (KBr) cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.27 (s, 3H), 2.39–2.71 (dd. 2H), 3.02 (s, 2H), 7.20–7.32 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 27.19, 46.79, 49.21, 49.77, 131.65, 133.37, 135.60, 142.41, 180.90, 184.86.

2-Ethylidene succinic acid (17). Acetaldehyde (7.0 mL, 125 mmol) was added to a benzene solution (10 mL) containing 2-(triphenylphosphoranylidene)succinic acid 1-methyl ester (**16**)[11] (4.92 g, 12.5 mmol). After stirring for 24 h, the mixture was washed with water, and the ethylidene compound was extracted with a 1 N NaOH solution (30 mL). The alkaline solution was stirred at room temperature for 24 h, washed with diethyl ether, acidified with 4 N HCl solution, and extracted with ethyl acetate (3×30 mL). The collected organic layers were washed with water, brine and dried over anhydrous $MgSO_4$. The solvent was concentrated in vacuo. The residue was recrystallized from ethyl acetate and hexane to give a white powder (1.44 g, 80%). Mp = 166–168°C (lit.[20] mp 165–167°C); ^1H NMR (300 MHz) δ 1.82–1.84 (d. 3H), 3.31 (s, 2H), 7.03–7.06 (q, 1H); ^{13}C NMR (300 MHz) δ 19.72, 36.97, 132,42, 145.16, 145.61, 174.18, 178.10.

2-Ethylsuccinic acid (18). Compound **17** (1.44 g, 10 mmol) was dissolved in 95% methanol (50 mL) and hydrogenated in the presence of a Pd/C (10%) catalyst. When no more starting material could be detected by TLC, the catalyst was removed by filtration, and the solvent was concentrated in vacuo. The residue was recrystallized from diethyl ether and hexane to give a white powder (1.39 g, 95%). Mp = 97–98.5°C (lit.[21] mp 97°C); ^1H NMR (300 MHz) δ 0.93–0.98 (t, 3H), 1.57–1.71 (m, 2H), 2.35–2.44 (m, 1H), 2.63–2.78 (m, 2H); ^{13}C NMR (300 MHz) δ 16.44, 29.91, 40.56, 47.53, 179.51, 182.33.

General remarks for kinetic experiments

All solutions were prepared by dissolving in doubly distilled and deionized water. Stock assay solutions were filtered before use. Carboxypeptidse 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

Initial velocities were 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.[12] Two concentrations of the substrate of Hipp-L-Phe 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.

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