Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 4685–4691

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Evaluation of α,α-Disubstituted-3-mercaptopropanoic Acids as Inhibitors for Carboxypeptidase A and Implications with Respect to Enzyme Inhibitor Design

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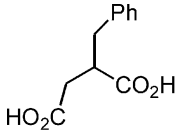
Received 2 May 2003; accepted 15 August 2003

Abstract—2-Ethyl-2-methyl-3-mercaptopropanoic acid (**6**) and 2-benzyl-2-methyl-3-mercaptopropanoic acid (**7**) were synthesized and evaluated as inhibitors for carboxypeptidase A (CPA), a prototypical zinc protease with the expectation that the binding affinities of these inhibitors would be augmented over those of 2-ethyl-3-methylsuccinic acid (**2**) and 2-benzyl-3-methylsuccinic acid (**3**), respectively, in light of the fact that the sulfhydryl group is a better zinc coordinating moiety than the carboxylate group. Contrary to the expectation, however, the inhibitory potency of **6** was not improved and that of **7** was rather attenuated by the replacement. A probable explanation for the unexpected results is offered.

Introduction

Zinc proteases that are characterized by having a catalytically essential zinc ion at their active site constitute a major family of proteolytic enzymes.[1] Physiologically and etiologically important enzymes such as angiotensin converting enzyme[2] and matrix metalloproteases[3] belong to this family, and thus zinc proteases have received much attention as target enzymes for drug development. Captopril[4] and enalapril[5] that are widely prescribed for the treatment of hypertension, congestive heart failure and myocardial infarction are inhibitors of angiotensin converting enzyme.[2] Of numerous zinc proteases, carboxypeptidase A (CPA)[6] is most extensively studied and has served as a model target enzyme for the development of inhibitor design strategies[7] that can be applied to zinc proteases of medicinal interest. 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 such as Phe.[6]

It has been known that 2-benzylsuccinic acid (**1**) and closely related compounds are effective inhibitors for CPA.[8] The X-ray crystal structure of the CPA complex formed with L-2-benzylsuccinic acid revealed that the inhibitor binds CPA with its carboxylate at the β-position coordinating the zinc ion at the enzyme active site in an asymmetric bidentate fashion.[9] The other carboxylate forms a salt link with the guanidinium moiety of Arg-145 and also engages in hydrogen bonding with the phenolic hydroxyl of Tyr-248 and the amide group of Asn-144. The side chain aromatic ring of **1** is accommodated in the S1′ pocket of CPA. Recently, Asante-Appiah et al.[10] reported that 2-ethyl-2-methylsuccinic acid (**2**) is a highly potent inhibitor for CPA. The X-ray



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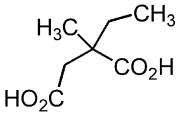

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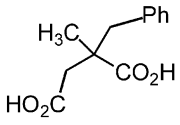

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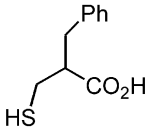

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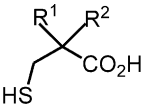
doi:10.1016/j.bmc.2003.08.017

crystal structure of the complex of CPA formed with (*R*)-**2** showed that the methyl group of the inhibitor rests in a small hydrophobic cavity present next to the S1′ pocket. They have proposed to call the cavity a 'methyl hole' and attributed the unexpectedly high inhibitory potency of **2** to the additional binding interactions resulted from its 2-methyl group occupying the cavity.[10] We have evaluated 2-benzyl-2-methylsuccinic acid (**3**) as a CPA inhibitor, expecting it to be more potent than **2**, but found that the compound is slightly less potent than **2**.[11]

Sulfhydryl group has been extensively utilized as a zinc ligating functionality in designing inhibitors for zinc proteases including CPA. 2-Benzyl-3-mercaptopropanoic acid (**4**) is a CPA inhibitor whose inhibitory potency exceeds that of **1** by at least 50-fold.[12] The enhanced CPA inhibitory activity of **4** compared with **1** is attributable to the thiol group that forms a stronger coordinative bond to the active site zinc ion than carboxylate does.[13] It was, therefore, thought to be of interest to evaluate 2-ethyl-2-methyl-3-mercaptopropanoic acid (**6**) and 2-benzyl-2-methyl-3-mercaptopropanoic acid (**7**) as inhibitors for CPA. It was expected that the replacement of the carboxylate at the 3-positon of **2** and **3** with a thiol group to give **6** and **7**, respectively, would improve the inhibitory potency due to the increased ligating affinity of the sulfhydryl to the active site zinc ion. This paper reports syntheses of 2-ethyl-3-mercaptopropanoic acid (**5**) and **6** as well as racemic and optically active **7**, and their evaluation as inhibitors for CPA.



5: $R^1 = H$, $R^2 = CH_2CH_3$
6: $R^1 = CH_3$, $R^2 = CH_2CH_3$
7: $R^1 = CH_3$, $R^2 = CH_2Ph$

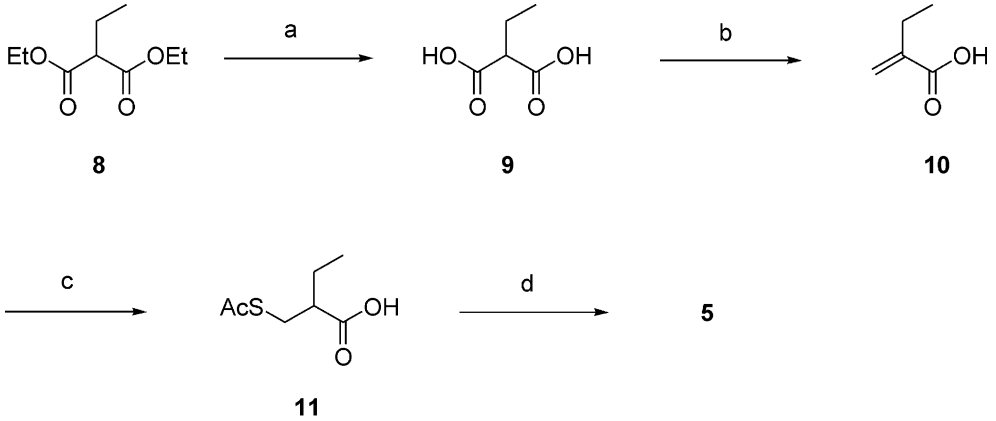
Results and Discussion

Compound **5** was prepared according to the synthetic route described in the literature[12] starting with diethylmalonate as outlined in Scheme 1.

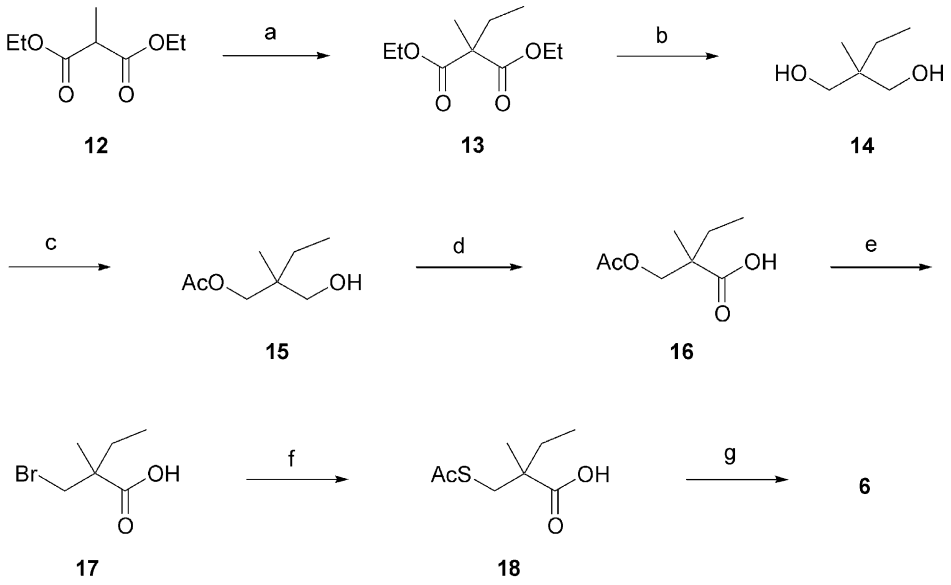
Scheme 2 represents the synthetic route for the preparation of **6**. Treatment of **12** with sodium hydride followed by addition of ethyl iodide afforded **13** in an excellent yield. The latter was then reduced with lithium aluminumhydride to obtain **14**. When **14** was allowed to react with trimethyl orthoacetate in the presence of *p*-toluenesulfonic acid yielded **15** which was oxidized with the Jones reagent to give **16**. The latter was converted into **17** by treatment with hydrobromic acid under reflux. The treatment of **17** with potassium thioacetate afforded **18**, and subsequent treatment of **18** with 6 N hydrochloric acid yielded **6**. By an analogous route, compound **7** was synthesized.

Synthesis of optically active **7** is illustrated in Scheme 3. Compound **19**, an intermediate in the synthesis of **7** was treated with 2 N sodium hydroxide solution in methanol to give **20** which was then allowed to react with (*S*)-phenylethylamine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI) to obtain **21** and its diastereoisomer as an 1:1 diastereoisomeric mixture. The mixture was readily separated by flash column chromatography to give optically pure **21**, the absolute stereochemistry of which was established by the X-ray crystallographic method.[14] The diastereoisomer **21** thus separated was treated with concentrated hydrobromic acid under reflux to give (*S*)-**22**, which was then converted into (*S*)-**7** by the same route as that was used for the conversion of **17** into **7**. (*R*)-**7** was similarly prepared from the diastereoisomer of **21**.

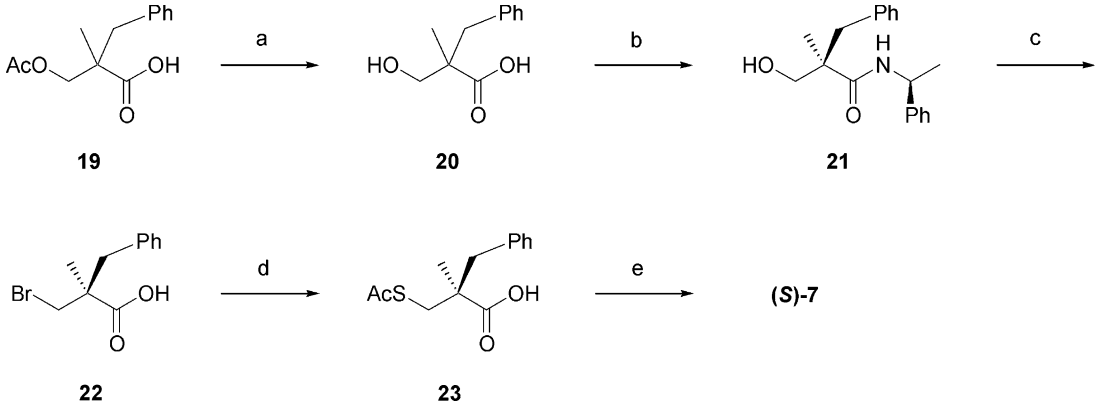
The synthesized compounds were assayed as competitive inhibitors for CPA by a standard method using hippuryl-L-phenylalanine (Hipp-L-Phe) as substrate at pH 7.5. Inhibitory constants (K_i values) were estimated from the respective Dixon plot[11,15] and are collected in Table 1. It can be seen from Table 1 that the substitution of 3-carboxylate in **2** with a sulfhydryl group failed



Scheme 1. (a) 4 N KOH, reflux, 4 h, 94%; (b) HCHO, Et_2NH HCl, H_2O, reflux, 4 h, 64%; (c) CH_3COSH, benzene, reflux, 12 h, 82%; (d) HCl, reflux, 4 h, 97%.



Scheme 2. (a) NaH, EtI, DMF, reflux, 2 h, 98%; (b) LAH, diethyl ether, 4 h, 96%; (c) $CH_3C(OCH_3)_3$, *p*-TsOH, CH_2Cl_2, 1 h, 91%; (d) Jones reagent, acetone, 2 h, 71%; (e) HBr, reflux, 1 day, 85%; (f) KSAc, Et_3N, THF, 3 h, 90%; (g) 6 N HCl, reflux, 4 h, 95%.



Scheme 3. (a) 2 N NaOH, MeOH, reflux, 4 h, 97%; (b) (*S*)-phenylethylamine, EDCI, HOBT, Et_3N, CH_2Cl_2, 1 h, 97%, then chromatographic separation; (c) concd HBr, reflux, 1 day, 78%; (d) KSAc, Et_3N, THF, 2 h, 90%; (e) 6 N HCl, 4 h, 95%.

to improve the binding affinity. The replacement of 3-carboxylate in **3** with a sulfhydryl group to obtain **7** did not enhance the binding affinity but rather attenuated the affinity slightly. However, when there is present no methyl group at the 2-positon, surprisingly the replacement of the zinc liganting carboxylate with a sulfhydryl moiety improved the binding affinity drastically: the binding affinity of **5** corresponds to 250-fold that of 2-ethylsuccinic acid.[11] These observations indicate that the methyl group in **6** and **7** effects adversely in binding of the inhibitors to CPA, which may be reconciled with the assumption that due to the tight binding of the sulfhydryl group to the active site zinc ion these inhibitor molecules reside closer towards the zinc ion than molecules of **2** and **3** in forming complexes with CPA, and as a consequence the 2-methyl group in these inhibitors cannot fit in the methyl hole, bringing about lowering of the binding affinity of these inhibitors. In this conjunction, the lack of stereochemical preference shown by the enantiomeric pair of **7** in inhibiting CPA is noteworthy and may be accounted for by invoking a proposition that the methyl group in both the enantiomers find a room for its resting by pointing towards bulk water. On the other hand, in cases of **2** and **3**, due to the relatively weak ligating propensity of the carboxylate to the zinc ion, these molecules may have considerable freedom of movement in forming complexes with the enzyme, and as a result, their methyl group would fit in the small methyl hole, reinforcing the binding interactions of the inhibitors to CPA. Especially, in the binding of **2** to CPA, since the S1′ pocket is occupied by a methyl group of the 2-ethyl chain,[10] there remains in the pocket a sufficient room for the inhibitor molecule to maneuver to form an enzy-

Table 1. K_i values for CPA inhibition

Inhibitor	K_i (μM)
(*RS*)-**1**	0.55[11] (0.2)[24]
(*RS*)-**2**	0.11[10]
(*RS*)-**3**	0.28[10]
(*RS*)-**4**	0.011[12]
(*RS*)-**5**	0.10±0.019
(*RS*)-**6**	0.11±0.017
(*RS*)-**7**	0.38±0.030
(*R*)-**7**	0.43±0.073
(*R*)-**7**	0.37±0.059

mei[currency]inhibitor complex with its 2-methyl group being accommodated in the methyl hole.

We have previously reported that an introduction of a methyl group at the 2-position of 2-benzylsuccinic acid improves the binding affinity by 2-fold.[11] In this study, we have observed that such an introduction of a methyl group into **4** to obtain **7** deminished the binding affinity by 35-fold. This attenuation of the binding affinity by the methyl introduction into **4** may as well be accounted for by the proposition discussed above: in the case of **7** that bears a methyl group at the 2-position, the 2-methyl group deters the binding of the molecule to the active site as discussed above, but in the binding of **4**, since there is no methyl group at the 2-position, no such adverse effect is in operation, and **4** can form a complex with CPA much more tightly than **7**.

Conclusion

The replacement of the zinc coordinating carboxylate in CPA inhibitors such as **2** and **3** with a sulfhydryl group, a stronger zinc ligating moiety than carboxylate, failed to improve the binding affinity. The present investigation together with our previous studies[16,17] tends to suggest that the expectation that one may design CPA inhibitors of improved potency by taking advantage of the methyl hole at the active site is hardly achievable especially when the zinc ligating moiety has a high binding affinity towards the active site zinc ion.

Experimental

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and were uncorrected. IR spectra were recorded on a Bruker Equinox 55 FT-IR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were obtained with a Bruker AM 300 (300 MHz) NMR spectrometer using tetramethylsilane as the internal standard. Mass spectra were obtained with a Micro Mass Platform II 8410E spectrometer and high resolution mass spectra were obtained at Korea Basic Science Institute, Daejeon, Korea. 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 Pohang University of Science and Technology, Pohang, Korea.

2-Ethylmalonic acid (9). A mixture of diethyl ethylmalonate (10 mL, 53.2 mmol) and 4 N KOH solution (50 mL) was refluxed for 2 h. The solution was diluted with water, washed with ether, acidified with 6 N HCl, and extracted with ether. The organic phase was dried over $MgSO_4$, and concentrated under reduced pressure to give **9**[18] as a white solid (6.61 g, 94%). Mp 108–110 °C; ^{1}H NMR (MeOH-d_4, 300 MHz) δ 0.95 (t, 3H), 1.85 (m, 2H), 3.20 (t, 1H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 11.20, 22.42, 53.57, 172.33.

2-Ethylacrylic acid (10). To a mixture of **9** (1.00 g, 7.6 mmol) and diethylamine hydrochloride (1.24 g, 11.4 mmol) in water was added 37% aqueous formaldehyde solution (1.2 mL, 15.2 mmol) and the resulting mixture was refluxed for 12 h. The solution was extracted with ether, and the organic phase was dried over $MgSO_4$ and concentrated under reduced pressure to give **10**[18] as an oil (0.48 g, 64%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.11 (t, 3H), 2.34 (q, 2H), 5.66 (d, 1H), 6.29 (d, 1H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 13.03, 24.83, 126.34, 141.95, 172.85.

2-Acetylsufanylmethylbutyric acid (11). A mixture of **10** (0.54 g, 4.5 mmol) and thioacetic acid (1.2 mL, 17.0 mmol) in benzene was refluxed for 12 h. The solution was concentrated under reduced pressure, diluted with ethyl acetate, and washed with 1 N HCl. The organic phase was dried over $MgSO_4$, and concentrated under reduced pressure to give **11**[18] as a pale yellow oil (0.65 g, 82%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.00 (t, 3H), 1.71 (m, 2H), 2.35 (s, 3H), 2.58 (m, 1H), 3.03 (dd, 1H), 3.14 (dd, 1H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 11.72, 25.36, 29.98, 31.00, 47.36, 180.87, 195.89.

2-Mercaptomethylbutyric acid (5). Compound **11** (400 mg, 2.27 mmol) was suspended in 6 N HCl and refluxed for 4 h. The reaction mixture was cooled to room temperature and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give a crude product which was purified by column chromatography (hexane/EtOAc = 3/1) to yield **5** as a colorless oil (295 mg, 97%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.98 (t, 3H), 1.55 (t, 1H), 1.72 (m, 2H), 2.55 (m, 1H), 2.63–2.83 (m, 2H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 11.79, 24.69, 25.58, 51.00, 181.30. FAB HRMS calcd for $C_5H_{11}O_2S$ (MH^+) 135.0480, found 135.0479.

Diethyl 2-ethyl-2-methylmalonate (13). Diethyl methylmalonate (10.0 mL, 58 mmol) was added to the suspension of NaH (60% dispersion in mineral oil, 2.55 g, 64 mmol) in DMF at 0 °C. When hydrogen gas evolution was ceased, iodoethane (5.62 mL, 70 mmol) was added to the solution and the reaction mixture was stirred for 2 h at 100 °C. The mixture was cooled to room temperature and diluted with ethyl acetate. The solution was washed with 5% aqueous $Na_2S_2O_3$ solution to remove DMF. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash column chromatography (hexane/EtOAc = 10/1) to give **13**[19] (11.4 g, 97%) as a colorless oil. ^{1}H NMR 300 MHz ($CDCl_3$) δ 0.87 (t, 3H), 1.25 (t, 6H), 1.39 (s, 3H), 1.91 (q, 2H), 4.18 (q, 4H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 9.30, 14.69, 19.97, 29.22, 54.78, 61.65, 127.27, 173.06.

Diethyl 2-benzyl-2-methylmalonate.[20] This was similarly prepared from diethyl methylmalonate and benzyl bromide in 98% yield as a colorless oil. ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.25 (t, 6H), 1.34 (s, 3H), 3.23 (s, 2H), 4.19 (q, 4H), 7.10–7.26 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 14.42, 20.12, 41.50, 55.22, 61.70, 127.27, 128.56, 130.60, 136.64, 172.35.

2-Ethyl-2-methyl-1,3-propanediol (14). An ice-chilled solution of $LiAlH_4$ (6.07 g, 160 mmol) in diethyl ether was treated dropwise with a solution of **13** (8.08 g, 40 mmol) in diethyl ether. After stirring for 4 h at room temperature, the reaction mixture was treated successively by the careful dropwise addition of 5.8 mL of H_2O, 11.7 mL of 15% aqueous NaOH solution, and 17.5 mL of H_2O. The mixture was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 3/1) to give **14** (4.44 g, 94%) as a colorless oil. ^{1}H NMR 300 MHz ($CDCl_3$) δ 0.81 (s, 3H), 0.87 (t, 3H), 1.37 (q, 2H), 2.72 (br, 2H), 3.53 (dd, 4H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 7.97, 18.24, 26.60, 39.23, 70.59.

2-Benzyl-2-methyl-1,3-propanediol.[21] This was similarly prepared in 96% yield as a white solid. Mp 53–55 °C; ^{1}H NMR 300 MHz ($CDCl_3$) δ 0.76 (s, 3H), 2.58 (br, 2H), 2.70 (s, 2H), 3.55 (s, 4H), 7.20–7.29 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 18.97, 40.23, 40.51, 70.39, 126.55, 128.41, 130.98, 138.20.

2-Acetoxymethyl-2-methylbutanol (15). A mixture of **14** (3.00 g, 25.4 mmol), trimethylorthoacetate (3.56 mL, 27.9 mmol), and a catalytic amount of *p*-toluenesulfonic acid monohydrate (0.48 g, 2.5 mmol) in CH_2Cl_2 was stirred for 1 h at room temperature. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (EtOAc/hexane = 1/5) to give **15** (3.70 g, 91%) as a colorless oil. IR (neat) 3450, 2967, 1740 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 0.85 (m, 6H), 1.31 (m, 2H), 2.09 (s, 3H), 3.33 (dd, 2H), 3.96 (dd, 2H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 7.79, 18.32, 21.16, 26.80, 39.20, 44.30, 66.96, 68.40, 172.02; MS (EI) *m*/*z* 161 (M^+). HRMS (FAB+) $(M+H)^+$: calcd for $C_8H_{16}O_3$, 161.1178; found 161.1174.

2-Acetoxymethyl-2-methyl-3-phenylbutanol. This was similarly prepared in 90% yield as a colorless oil. IR (neat) 3470, 2927, 1737 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 0.83 (s, 3H), 2.13 (s, 3H), 2.34 (br, 1H), 2.60 (dd, 2H), 3.32 (dd, 2H), 3.95 (dd, 2H), 7.16–7.32 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 18.93, 21.35, 40.33, 40.55, 66.57, 68.08, 126.73, 128.48, 130.99, 137.40, 172.32; MS (EI) *m*/*z* 223 (M^+). HRMS (FAB+) $(M+H)^+$: calcd for $C_{13}H_{19}O_3$, 223.1334; found 223.1333.

2-Acetoxymethyl-2-methylbutyric acid (16). To an ice-cooled solution of **15** (3.20 g, 20.0 mmol) in acetone was added slowly the Jones reagent until brownish color of the solution remains over 20 min, then 2-propanol was added until the solution became clear. The precipitate was filtered and the filtrate was concentrated under reduced pressure. The residue was diluted with ethyl acetate and extracted with saturated aqueous $NaHCO_3$ solution. The aqueous layer was acidified with 6 N HCl and extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give **16** (2.47 g, 71%) as a colorless oil. IR (neat) 2975, 1746, 1705 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 0.92 (t, 3H), 1.22 (s, 3H), 1.55–1.76 (m, 2H), 2.07 (s, 3H), 4.09–4.23 (dd, 2H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 8.87, 19.29, 21.20, 28.94, 46.71, 68.83, 171.35, 181.93; MS (EI) *m*/*z* 175 (M^+). HRMS (FAB+) $(M+H)^+$: calcd for $C_8H_{14}O_4$, 175.0970; found 175.0966.

2-Acetoxymethyl-2-methyl-3-phenylpropanoic acid. This was similarly prepared in 71% yield as a white. Mp 98–100 °C (solid diethyl ether and hexane); IR (KBr) 2978, 1747, 1699 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.22 (s, 3H), 2.10 (s, 3H), 2.97 (dd, 2H), 4.13 (dd, 2H), 7.13-7.29 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 19.93, 21.12, 41.60, 47.44, 68.22, 127.34, 128.69, 130.55, 136.36, 170.93, 180.03. Anal. calcd for $C_{13}H_{16}O_4$: C, 66.09; H, 6.83. Found: C, 66.18; H, 6.82.

2-Bromomethyl-2-methylbutyric acid (17). Compound **16** (1.70 g, 9.8 mmol) was suspended in concentrated hydrobromic acid and refluxed for 24 h. The reaction mixture was cooled to room temperature, diluted with water, and extracted with EtOAc. The organic layer was dried over $MgSO_4$, and concentrated under reduced pressure to give a crude oil which was purified by column chromatography (hexane/EtOAc = 5/1) to yield **17**[22] (1.58 g, 83%) as a white solid which was recrystallized from hexane. Mp 48–50 °C; ^{1}H NMR 300 MHz ($CDCl_3$) δ 0.93 (t, 3H), 1.31 (s, 3H), 1.66–1.83 (m, 2H), 3.44–3.64 (dd, 2H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 9.21, 14.55, 21.14, 30.71, 39.45, 48.11, 65.25, 181.47.

2-Bromomethyl-2-methyl-3-phenylpropanoic acid. This was similarly prepared in 85% yield as a white solid which was recrytallized from hexane. Mp 115–116 °C; IR (KBr) 2939, 1696 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.33 (s, 3H), 3.04 (s, 2H), 3.43 (d, 1H), 3.57 (d, 1H), 7.21–7.32 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 21.94, 39.07, 42.53, 48.98, 127.55, 128.82, 130.53, 136.30, 181.08. Anal. calcd for $C_{19}H_{21}NO$: C, 51.38; H, 5.10. Found: C, 51.28; H, 5.01.

2-Acetylsulfanylmethyl-2-methylbutanoic acid (18). Potassium thioacetate (322 mg, 2.82 mmol) was added to a stirred solution of **17** (500 mg, 2.56 mmol) and triethylamine (393 μL, 2.82 mmol) in THF, and the reaction mixture was stirred for 3 h at room temperature. The mixture was diluted with EtOAc and washed with 1 N HCl. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give a crude oil which was purified by column chromatography (hexane/EtOAc = 4/1) to yield **18**[23] (439 mg, 90%) as a colorless oil. ^{1}H NMR 300 MHz ($CDCl_3$) δ 0.91 (t, 3H), 1.18 (s, 3H), 1.61–1.77 (m, 2H), 2.35 (s, 3H), 3.19 (dd, 2H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 9.31, 20.89, 30.95, 31.81, 36.03, 47.29, 182.51, 195.63.

2-Acetylsulfanylmethyl-2-methyl-3-phenylpropanoic acid. This was similarly prepared in 90% yield as a colorless oil. IR (neat) 3029, 1699 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.21 (s, 3H), 2.36 (s, 3H), 2.97 (dd, 2H), 3.19 (dd, 2H), 7.13–7.30 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 20.37, 30.08, 35.32, 44.14, 47.52, 126.55, 127.86, 129.61, 135.72, 181.02, 194.48; MS (EI) *m*/*z* 253 (M^+). HRMS (EI): calcd for $C_{13}H_{17}O_3S$, 252.0820; found 252.0819.

2-Mercaptomethyl-2-methylbutanoic acid (6). Compound **18** (250 mg, 1.31 mmol) was suspended in 6 N HCl and refluxed for 4 h. The reaction mixture was cooled to room temperature and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give a crude oil which was purified by column chromatography (hexane/EtOAc = 3/1) to yield **6** (185 mg, 95%) as a colorless oil. ^{1}H NMR 300 MHz ($CDCl_3$) δ 0.90 (t, 3H), 1.24, (s, 3H), 1.43 (t, 1H), 1.60–1.79 (m, 2H), 2.58 (dd, 1H), 2.86 (dd, 1H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 9.28, 20.46, 31.19, 32.58, 48.65, 182.85. FAB HRSM calcd for $C_6H_{13}O_2S$ (MH^+) 149.0636, found 149.0643.

2-Mercaptomethyl-2-methyl-3-phenylpropanoic acid (7). This was similarly prepared in 95% yield as a crystalline solid. Mp 62–64 °C; IR (KBr) 3244, 1731, 1696 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.25 (s, 3H), 1.50 (t, 1H), 2.58 (dd, 1H), 2.84 (dd, 1H), 2.99 (dd, 2H), 7.15–7.31 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 21.11, 32.40, 43.71, 49.67, 127.32, 128.69, 130.58, 136.82, 181.77. Anal. calcd for $C_{19}H_{21}NO$: C, 62.83; H, 6.71. Found: C, 63.20; H, 6.65.

2-Hydroxymethyl-2-methyl-3-phenylpropanoic acid (20). Compound **19** (4.00 g, 16.9 mmol) was dissolved in MeOH containing 2 N NaOH (17 mL) and the solution was refluxed for 4 h. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residue was acidified with 6 N HCl and extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give **20** (3.19 g, 97%) as a white solid which was recrystallized from the mixed solvent of diethyl ether and hexane. Mp 103–105 °C; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.14 (s, 3H), 2.95 (dd, 2H), 3.60 (dd, 2H), 7.18–7.30 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 19.60, 40.99, 48.97, 67.03, 127.21, 128.64, 130.81, 136.60, 182.75. Anal. calcd for $C_{11}H_{14}O_3$: C, 68.02; H, 7.27. Found: C, 68.10; H, 7.30.

(2*R*,1′*S*)- and (2*S*,1′*S*)-2-hydroxymethyl-2-methyl-3-phenyl-*N*-(1′-phenylethyl)-propionamide [(2*R*,1′*S*)- and (2*S*,1′*S*)-21]. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI, 2.87 g, 14.9 mmol), 1-hydroxybenzotriazole hydrate (2.01 g, 14.9 mmol), and triethylamine (2.29 mL, 16.4 mmol) were added to the stirred solution of **20** (2.90 g, 14.9 mmol) in CH_2Cl_2 at 0 °C, and the solution was stirred for 10 min. (*S*)-Phenylethylamine (2.11 mL, 16.4 mmol) was added to the reaction mixture at 0 °C, and the mixture was stirred for 1 h at room temperature. The solution was washed with 10% aqueous solution of citric acid, saturated aqueous $NaHCO_3$ solution, and brine, and the organic layer was dried over $MgSO_4$. The dried solution was concentrated under reduced pressure to give the product (4.31 g, 97%) as a diastereomeric mixture which was separated by flash column chromatography (hexane/EtOAc = 4/1 to 2/1) to give the optically pure product, (2*R*,1′*S*)-**21** (1.95 g) and (2*S*,1′*S*)-**21** (2.08 g). The analytical sample was prepared by recrystallization from the mixed solvent of diethyl ether and hexane.

(2*R*,1′*S*)-21. Mp 129–131 °C; $[\alpha]_D$ −50.8° (*c* 1.89, MeOH); IR (KBr) 3250, 2915, 1635, 1545 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.05 (s, 3H), 1.34 (d, 3H), 2.80 (d, 1H), 3.03 (d, 1H), 3.53–3.61 (m, 3H), 5.06 (m, 1H), 6.20 (br, 1H), 7.14–7.31 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 19.72, 22.13, 42.26, 47.70, 49.03, 68.94, 126.35, 127.03, 127.68, 128.56, 129.08, 130.80, 137.44, 143.66, 176.40. Anal. calcd for $C_{19}H_{23}NO_2$: C, 76.73; H, 7.80; N, 4.71. Found: C, 76.82; H, 7.88; N, 4.73.

(2*S*,1′*S*)-21. Mp 95–97 °C; $[\alpha]_D$ −81.5° (*c* 1.34, MeOH); IR (KBr) 3292, 2929, 1631, 1545 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$) δ 1.04 (s, 3H), 1.45 (d, 3H), 2.80 (d, 1H), 3.03 (d, 1H), 3.30 (dd, 1H), 3.59 (m, 2H), 5.10 (m, 1H), 6.09 (br, 1H), 7.07–7.32 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 19.66, 22.06, 42.20, 47.71, 49.07, 69.08, 126.57, 126.89, 127.69, 128.52, 129.01, 130.71, 137.29, 143.29, 176.27. Anal. calcd for $C_{19}H_{23}NO_2$: C, 76.73; H, 7.80; N, 4.71. Found: C, 76.73; H, 7.82; N, 4.73.

(*R*)-2-Bromomethyl-2-methyl-3-phenyl-propanoic acid [(*R*)-22]. Compound (2*R*,1′*S*)-**21** (800 mg, 2.7 mmol) was suspended in concentrated hydrobromic acid and refluxed for 24 h. The reaction mixture was cooled to room temperature, diluted with water, and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give a colorless oil which was purified by column chromatography (hexane/EtOAc = 5/1) to yield (*R*)-**22** (630 mg, 78%) as a white solid which was recrystallized from hexane. Mp 115–116 °C; $[\alpha]_D$ +3.37° (*c* 1.33, MeOH). Anal. calcd for $C_{11}H_{13}BrO_2$: C, 51.38; H, 5.10. Found: C, 51.29; H, 4.75.

(*S*)-2-Bromomethyl-2-methyl-3-phenyl-propanoic acid [(*S*)-22]. This was similarly prepared from (2*S*,1′*S*)-**21** in 77% yield as a crystalline solid. Mp 115-116 °C; $[\alpha]_D$ −3.32° (*c* 1.17, MeOH). Anal. calcd for $C_{11}H_{13}BrO_2$: C, 51.38; H, 5.10. Found: C, 51.59; H, 5.21.

(*R*)-2-Acetylsulfanylmethyl-2-methyl-3-phenylpropanoic acid [(*R*)-23]. Potassium thioacetate (151 mg, 1.32 mmol) was added to a stirred solution of (*R*)-**22** (225 mg, 0.88 mmol) and triethylamine (0.134 mL, 0.96mmol) in THF and stirred for 3 h at room temperature. The reaction mixture was diluted with EtOAc and washed with 1 N HCl. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give an yellowish oil which was purified by column chromatography (hexane/EtOAc = 4/1) to yield (*R*)-**23** (199 mg, 90%) as an oil. $[\alpha]_D$ +9.7° (*c* 1.00, $CHCl_3$). HRMS (EI): calcd for $C_{13}H_{16}O_3S$, 252.0820; found 252.0823.

(*S*)-2-Acetylsulfanylmethyl-2-methyl-3-phenylpropanoic acid [(*S*)-23]. This was similarly prepared from (*S*)-**22** in 90% yield as an oil. $[\alpha]_D$ −10.3° (*c* 1.43, $CHCl_3$). HRMS (EI): calcd for $C_{13}H_{16}O_3S$, 252.0820; found 252.0824.

(*R*)- and (*S*)-2-Mercaptomethyl-2-methyl-3-phenylpropanoic acid [(*R*)- and (*S*)-7]. These were prepared from (*R*)- and (*S*)-**23**, respectively, in 95% yield in an analogous

fashion to that used for preparation of (*R*,*S*)-**7** from (*R*,*S*)-**23**.

(*R*)-7. Mp 62–64 °C; $[\alpha]_D$ −5.5° (*c* 0.87, $CHCl_3$). Anal. calcd for $C_{19}H_{21}NO$: C, 62.83; H, 6.71. Found: C, 63.05; H, 6.62.

(*S*)-7. Mp 62–64 °C; $[\alpha]_D$ +5.5° (*c* 0.70, $CHCl_3$). Anal. calcd for $C_{19}H_{21}NO$: C, 62.83; H, 6.71. Found: C, 63.15; H, 6.72.

General remarks for enzyme kinetic experiments

CPA was purchased from Sigma Chemical Co. (Allan form, twice crystallized from bovine pancrease, aqueous suspension in toluene) and used without further purification. Hippuryl-L-phenylalanine (Hipp-L-Phe) purchased from Sigma Chemical Co. was used as substrate. All solutions were prepared by dissolving in doubly distilled and deionized water. CPA stock solutions were prepared by dissolving CPA in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer solution and their concentrations were estimated from the absorbance at 278 nm ($\varepsilon_{278} = 64{,}200$). The stock assay solutions were filtered (GHP Acrodic syringe filter, pore size 0.2 μm) before use. A Perkin-Elmer HP 8453 UV–vis spectrometer was used for UV absorbance measurements.

Determination of K_i values

Typically, an aliquot of the enzyme stock solution was added to the solution of the inhibitor and the substrate in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer solution containing 0.1 mM glutathione (1 mL cuvette), and the absorption increase at 254 nm was recorded immediately. The initial velocities were obtained from the linear plot of the substrate hydrolysis monitored by the increase of absorbance at 254 nm. The K_i values were then estimated from the semi-reciprocal plot of the initial velocity versus the concentrations of the inhibitors according to the method of Dixon. Two concentrations of substrate of Hipp-L-Phe were used.

Acknowledgements

The authors express their thanks to the Korean Science and Engineering Foundation for the financial support of this work and the Ministry of Education and Human resources for the BK21 fellowship to HSL.

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