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Syntheses and Kinetic Evaluation of Racemic and Optically Active 2-Benzyl-2-methyl-3,4-epoxybutanoic Acids as Irreversible Inactivators for Carboxypeptidase A

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Abstract—Racemic and optically active 2-benzyl-2-methyl-3,4-epoxybutanoic acids were synthesized and evaluated as inactivators for carboxypeptidase A, a representative zinc-containing proteolytic enzyme. Only the *threo*-form of the inactivator is effective and its potency in terms of k_{inact}/K_I value is lower by 42-fold compared with 2-benzyl-3,4-epoxybutanoic acid, indicating that the α-methyl group affects adversely in the inactivation contrary to the expectation that it would enhance the inactivation activity of the inhibitor through additional interactions of the methyl group with a small cavity (α-methyl hole) present next to the S_1' hydrophobic pocket. Of the enantiomeric pair, the inactivator having the (2*S*,3*R*)-configuration is more potent than its enantiomer by 44-fold. The observed kinetic results may be rationalized on the basis that the methyl group in the inactivator having the (2*R*,3*S*)-configuration experiences the van der Waals repulsive interactions with the bottom of the active site crevice in binding to CPA, casting a doubt on the presence of the so-called α-methyl hole at the active site of carboxypeptidase A.

Introduction

Zinc metalloproteases constitute an important class of proteolytic enzymes and have received much attention.[1] These proteases play vital roles in numerous biological processes.[1] Of these enzymes, carboxypeptidase A (CPA) is the most extensively studied and well characterized protease which cleaves the C-terminal amino acid residue having a hydrophobic side chain from protein substrate.[2] The zinc ion that is essential for the catalytic activity is coordinated to His-69 His-196 and Glu-72 and a molecule of water that attacks the scissile peptide bond to generate a tetrahedral transition state. The carboxylate of Glu-270 is known to function as a general base that activates the zinc bound water molecule by abstracting a proton to form a hydroxide ion that attacks the scissile peptide bond.[2] However, in the case of ester hydrolysis, the carboxylate was shown to attack directly the carbonyl carbon of the substrate, which is activated by the zinc ion.[3,4] At the active site of CPA, there are present three binding sites, that is Arg-145, a large hydrophobic pocket at the S_1' subsite, and Arg-127. The Arg-145 forms bifurcated hydrogen bonds with the terminal carboxylate of substrates, and the S_1' subsite pocket accommodates the side chain of the C-terminal amino acid residue thus to serve as the primary substrate recognition pocket.[5] The guanidinium moiety of Arg-127 forms a hydrogen bond to the carbonyl oxygen of the scissile peptide bond.[6]

Recently, Asante-Appiah et al. reported that 2-ethyl-2-methylsuccinic acid (**1**) is a potent inhibitor for CPA.[7] The X-ray crystal structure of CPA·**1** complex led them to propose that the high inhibitory potency of **1** is due to additional interactions that are in effect between the methyl group of **1** and a small hole next to the hydrophobic pocket at the active site of CPA. These authors suggested that improvement of potency of a CPA inhibitor may be brought about by the incorporation of a methyl group at its α-position. 2-Benzyl-3,4-epoxybutanoic acid (**2**) is one of the most potent irreversible inhibitor reported for CPA.[8] The epoxide ring in the enzyme-bound **2** is believed to be activated by the zinc ion at the active site and undergoes a S_N2 type ring cleavage by the attack of the carboxylate of Gly-270 to result in covalent modification of the carboxylate.[9,10]

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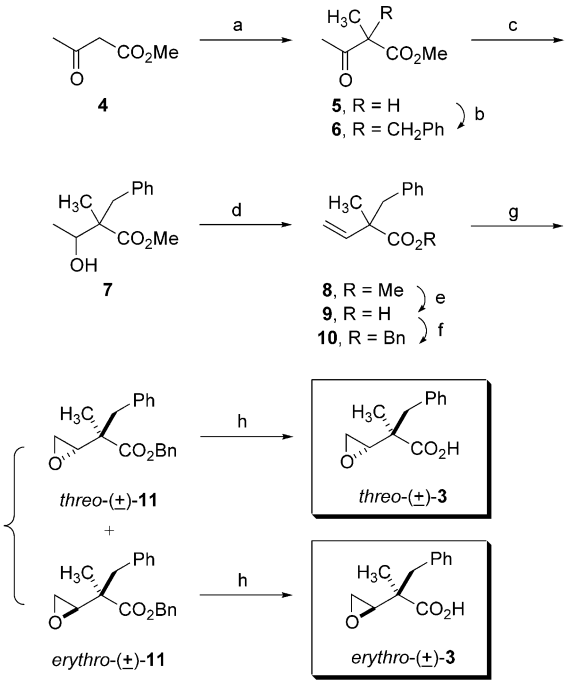
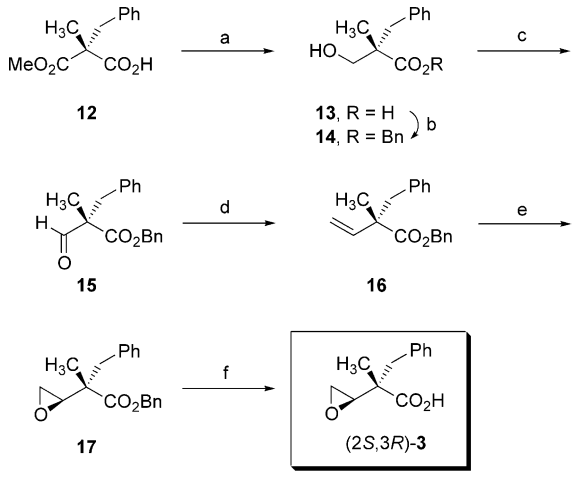


Scheme 1. (a) MeI, K_2CO_3, MeOH, reflux, 87.5%; (b) BnBr, K_2CO_3, MeOH, reflux, 88.5%; (c) $NaBH_4$, MeOH/H_2O, 0 °C to rt, 85.5%; (d) $SOCl_2$, pyridine, 0–50 °C, 68.5%; (e) LiOH·H_2O, MeOH/H_2O/THF, rt, 85.9%; (f) (i) Cs_2CO_3, MeOH, rt; (ii) BnBr, DMF, rt, 88.2%; (g) *m*CPBA, K_2HPO_4, CH_2Cl_2, 0 °C to rt, *threo*, 55%; *erythro*, 30%; (h) H_2, Pd/C, MeOH, rt, 95%.

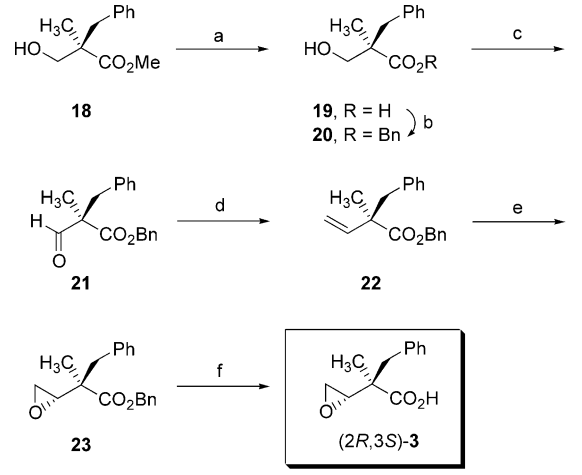
It was thought therefore that introduction of a methyl group at the α-position of **2** would enhance the binding affinity for CPA leading to improvement of the inactivation potency. This paper describes syntheses of 2-benzyl-2-methyl-3,4-epoxybutanoic acid (**3**) as racemic and optically active forms and their kinetic evaluation as irreversible inhibitors for CPA.

Chemistry

The synthesis of (±)-**3** was started with methyl acetoacetate (**4**) which was subjected to methylation followed by benzylation[11] to afford methyl 2-benzyl-2-methyl-3-oxobutanoate (**6**). The latter was then reduced with sodium borohydride in MeOH to give **7**. The dehydration of **7** was carried out in the presence of thionyl chloride and pyridine to yield a 1:1 mixture of **8** and methyl 2-benzyl-2-methyl-3-cholorobutanoate.[12] Alkaline hydrolysis of this mixture with lithium hydroxide gave 2-benzyl-2-methylbut-3-enoic acid (**9**). Methyl 2-benzyl-2-methyl-3-cholorobutanoate that resisted to be hydrolyzed could be separated from the product. 2-Benzyl-2- methylbut-3-enoic acid (**9**) was protected as a benzyl ester to give **10**, which was then subjected to expoxidation using *m*-chloroperbenzoic acid (*m*CPBA) under the condition reported by Albeck and Persky.[13] The diastereoisomeric mixture thus obtained was separated by column chromatography on silica gel to afford *threo*-(±)- and *erythro*-(±)-**11**, respectively in the ratio of 2:1. Catalytic hydrogenolysis of *threo*-(±)- and *erythro*-(±)-**11** gave the target compounds, *threo*-(±)- and *erythro*-(±)-**3**, respectively (Scheme 1).



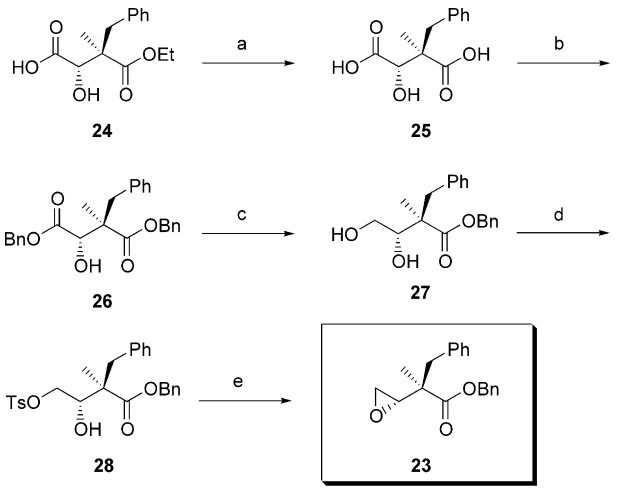
Scheme 2. (a) BH_3·SMe_2, DMF, 0 °C, 97%; (b) Cs_2CO_3, MeOH; BnBr, DMF, rt, 60%; (c) DMSO, $(COCl)_2$; Et_3N, −78 °C to rt, 89%; (d) Ph_3PMeI, KHMDS, THF, −78 °C–40 °C, 45%; (e) *m*CPBA, K_2HPO_4, CH_2Cl_2, 0 °C to rt, 55%; (f) H_2, Pd/C, MeOH, rt, 95%.



Scheme 3. (a) LiOH·H_2O, MeOH/THF/H_2O, rt, 95%; (b) (i) Cs_2CO_3, MeOH; ii) BnBr, DMF, rt, 60%; (c) DMSO, $(COCl)_2$; Et_3N, −78 °C to rt, 85%; (d) Ph_3PMeI, KHMDS, THF, −78–40 °C, 45%; (e) *m*CPBA, K_2HPO_4, CH_2Cl_2, 0 °C to rt, 50%; (f) H_2, Pd/C, MeOH, rt, 95%.

The synthetic route for the preparation of (2*S*,3*R*)-**3** is depicted in Scheme 2. Enzymatic hydrolysis of racemic benzylmethylmalonic dimethylester using α-chymotrypsin according to the literature method[14] afforded the corresponding monoester, **12** having the (*R*)-configuration. The methyl ester moiety in **12** was selectively reduced with BH_3·SMe_2 to give hydroxymethyl acid, **13** of which the carboxylate was converted into benzyl ester to yield **14**.[15] Oxidation of the primary alcohol moiety in **15** to the corresponding aldehyde (**15**) was accomplished by the method of Swern[16] and the subsequent reaction with methyltriphenylphosphine bromide under the Wittig conditions gave **16**.[17] The conversion of **16** to (2*S*,3*R*)-**3** was accomplished by the same sequence of reactions as those used for the preparation of (±)-**3.**

The synthesis of (2*R*,3*S*)-**3** was commenced with the alkaline hydrolysis of **18** that was prepared by the method of Lee et al.[18] to give hydroxymethyl ester (**19**).



Scheme 4. (a) HBr (aq), n-Bu_4NBr (cat), reflux, 74%; (b) (i) *n*-Bu_4NOH, MeOH; (ii) BnBr, DMF/acetonitrile, rt, 79%; (c) BH_3·SMe_2, $NaBH_4$ (cat), 0 °C to rt, 67%; (d) TsCl, pyridine, CH_2Cl_2, rt, 68%; (e) K_2CO_3, MeOH, rt, 60%.

The conversion of **19** to (2*R*,3*S*)-**3** was accomplished following the same sequences of reactions as those used for the preparation of (2*S*,3*R*)-**3**, as illustrated in Scheme 3.

The stereochemistry of the epoxidation product (**23**) was confirmed by an alternative synthesis that starts with **24**[19] as depicted in Scheme 4.

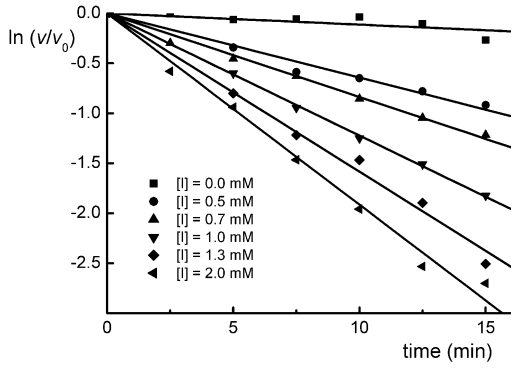


Figure 1. Time dependent loss of CPA activity by *threo*-(±)-**3** (substrate, Hip-L-Phe, [S] = 250 μM; [E] = 1.3 μM; Tris buffer of pH 7.5; temperature, 25 °C).

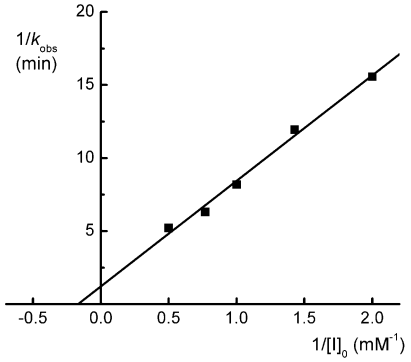


Figure 2. The double reciprocal plot of k_{obs} versus $[I]_0$ for the inactivation of CPA by *threo*-(±)-**3**.

(2*R*,3*S*)-**24** was prepared by the method reported by Renaud et al.[19] The hydrolysis of **24** in aqueous HBr gave hydroxysuccinic acid (**25**) which was converted to benzyl ester in the presence of *n*-Bu_4NOH and BnBr.[20] The subsequent four functional group interconversions to obtain epoxybutanoate (**23**) were accomplished according to the method described by Lee et al.[21] The conversion of **26** into **27** was effected in moderate yield by the regioselective reduction by means of borane–dimethyl sulfide complex in the presence of a catalytic amount of sodium borohydride in tetrahydrofuran.[22] When **27** was treated with *p*-toluenesulfonyl chloride in the presence of pyridine, the primary hydroxyl group was selectively tosylated. The subsequent treatment of the tosylate (**29**) thus obtained with potassium carbonate in methanol afforded (2*R*,3*S*)-**23** whose spectral data were identical with those of **23** that was obtained from the epoxidation of **22**, establishing the stereochemistry of **23** and its hydrogenolysis product to be (2*R*,3*S*) configuration.

Biological Evaluation

The compounds thus synthesized were assayed for CPA inhibitory activity at 25 °C in Tris buffer (0.05 M) of pH 7.5 using hippuryl-L-phenylalanine (Hip-L-Phe) as the substrate. *Threo*-(±)-**3**, (2*S*,3*R*)-**3**, and (2*R*,3*S*)-**3** inhibited CPA in a time-dependent fashion as shown in Figure 1 to suggest that they inhibit the enzyme in an irreversible manner. The inactivated CPA by *threo*-**3** failed to gain its enzymic activity upon dialysis for 2 days, supporting the irreversible nature of the inhibitions. Kinetic parameters for the irreversible inhibitions,

Table 1. Kinetic parameters for the inactivation of carboxypeptidase A

Compds	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I ($M^{-1}min^{-1}$)	K_i (mM)
(2*S*,3*R*)-**2**[a]	1.6	0.19	8400	
(2*R*,3*S*)-**2** [a]	1.1	0.34	3300	
Threo-(±)-**3**	0.85±0.3	5.9±2	140±7	
Erythro-(±)-**3**			NI[b]	
(2*S*,3*R*)-**3**	0.71±0.2	2.2±0.6	320±28	1.1±0.07
(2*R*,3*S*)-**3**	0.03±0.003	5.4±0.5	7.2±0.3	5.7±0.4

[a]Ref. 21.
[b]NI means no irreversible inhibition.

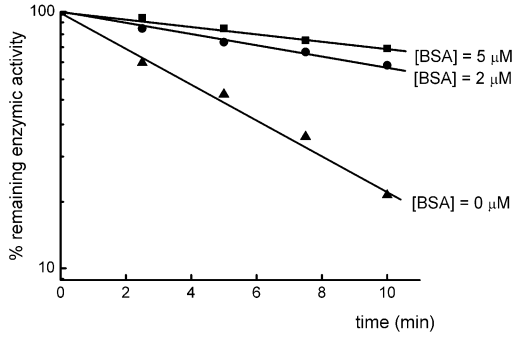


Figure 3. Time dependent loss of CPA activity by *threo*-(±)-**3** in the absence and presence of (±)-2-benzylsuccinic acid (substrate, Hip-L-Phe, [S] = 250 μM; [E] = 1.3 μM; Tris buffer of pH 7.5; temperature, 25 °C).

K_I and k_{inact} were estimated from the respective double reciprocal plot according to the method of Kitz and Wilson[23] as exemplified by Figure 2, and are listed in Table 1. A protection of the CPA inhibition by *threo*-**3** was observed when CPA was preincubated with (±)-2-benzylsuccinic acid, a known active site directed competitive inhibitor of CPA,[24] indicating that the inactivation by *threo*-**3** takes place at the active site (Fig. 3). (2*S*,3*R*)-**3** and (2*R*,3*S*)-**3** were assayed as competitive inhibitors for CPA as well and the inhibition constant K_i values were estimated from the Dixon plot[25] (Fig. 4). *Erythro*-(±)-**3** failed to show the time-dependent loss of the enzymic activity.

Results and Discussion

The *threo* form of **2** are rationally designed fast acting and highly potent pseudomechanism-based inactivators of CPA,[8] which inhibits the enzyme irreversibly by covalent modification of the carboxylate of Glu-270.[9,10] In the present study, we have evaluated an analogue of **2** in which the α-H is replaced with a methyl group, expecting that the new inhibitor would have improved inactivating property for CPA. This expectation was based on the proposition set forth by Asante-Appiah et al.[7] that there is present a small cavity at the active site of CPA. The cavity referred by them to as the α-methyl hole is situated next to the substrate recognition pocket at the S_1 subsite of CPA and thus can accommodate a methyl group at the α-position of C-terminal residue of substrate and inhibitor. The unexpectedly high CPA inhibitory potency ($K_i = 0.11$ μM) shown by **1** has been attributed to the additional interactions of the methyl group with the cavity.[7]

Like inactivator **2**, only the *threo* form of **3** showed CPA inactivating activity. As can be seen from Table 1, *threo*-(±)-**3** is less active than the parent inhibitor, that is *threo*-(±)-**2** by 42-fold[26] in terms of the second order inactivation rate constant (k_{inact}/K_I), which reflects effectiveness of an enzyme inctivator. *Erythro*-(±)-**3** did not exhibit the CPA inactivation activity, which is in line with the observation made with inhibitor **2**. It was thought to be of interest to examine inhibitory potency of each enantiomer of *threo*-**3** and the effect of the α-methyl group on the inactivation of CPA. We have found that (2*S*,3*R*)-**3** is more potent than its enantiomer by 44-fold, indicating that the effect of the α-methyl group on the CPA inactivation is stereospecific, that is, the methyl group in (2*R*,3*S*)-**3** brings about much more profound effect than that in (2*S*,3*R*)-**3**. In the case of **2**, the stereoisomer having the (2*S*,3*R*)-configuration is more active than its enantiomer by 2.6-fold.[21] The (2*S*)-configuration in these inhibitors corresponds to the D-series. The replacement of the α-H in (2*S*,3*R*)-**2** with a methyl group renders increase of the K_I value by 12-fold and decrease of the k_{inact} by 2.3-fold with resultant decrease of the k_{inact}/K_I value by 26-fold. In comparison, the methyl substitution at the α-position of (2*R*,3*S*)-**2** caused increase of the K_I value by 16-fold but there was a profound 28-fold decrease of the k_{inact} value, indicating that the covalent modification reaction is retarded markedly by the substitution.

Covalent modification reaction leading to inactivation of an enzyme takes place in the complex that is formed by binding the inhibitor to the enzyme at the active site. In the inactivation of CPA by (2*R*,3*S*)- and (2*S*,3*R*)-**2** the carboxylate of Glu-270 is known to attack at the 3-position of the oxirane heterocycle of the enzyme-bound inhibitor.[9,10] In this S_N2 type ring cleavage of the oxirane ring, the carboxylate is known to attack the electrophilic center from the direction of 180° to the cleavable C–O bond of the ring. Therefore, in order for the covalent bond formation reaction to take place at the active site of CPA, the carboxylate nucleophile should be disposed spatially on the same line of the cleavable C_3–O bond of the oxirane ring in the enzyme-bound inhibitor. Apparently, in the inactivation of CPA by (2*R*,3*S*)- and (2*S*,3*R*)-**2**, the requisite spatial arrangement for the nucleophilic cleavage of the oxirane ring is well satisfied. The kinetic results obtained with (2*R*,3*S*)- and (2*S*,3*R*)-**3** may be rationalized by the proposition that the methyl group of (2*R*,3*S*)-**3** may not fit in the so-called α-methyl hole when the inhibitor binds CPA and as a result the half of the molecule including the oxirane ring would be pushed outward compared with that of the enzyme-bound (2*R*,3*S*)-**2.** As a consequence, the oxirane ring in (2*R*,3*S*)-**3** and the carboxylate of Glu-270 are no longer optimally aligned for the S_N2 type ring cleavage reaction. Accordingly, the rate of the covalent modification reaction of the catalytic carboxylate, that is, the k_{inact}, value would be diminished. Figure 5A depicts schematically the binding mode of (2*R*,3*S*)-**3** to CPA and the ensuing covalent modification reaction of the carboxylate. On the other hand, in binding of (2*S*,3*R*)-**3** to CPA the α-methyl group would

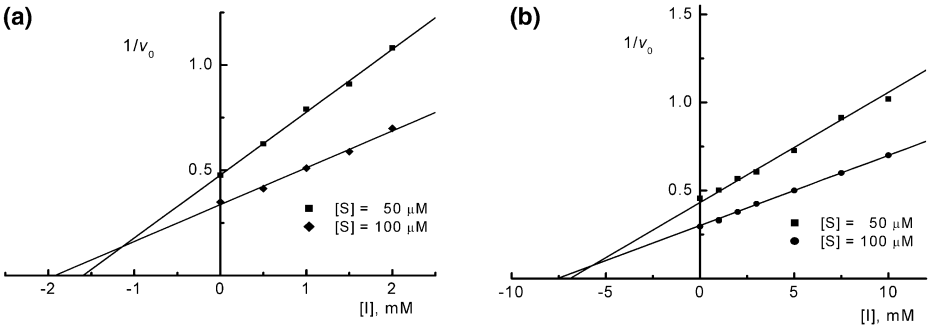
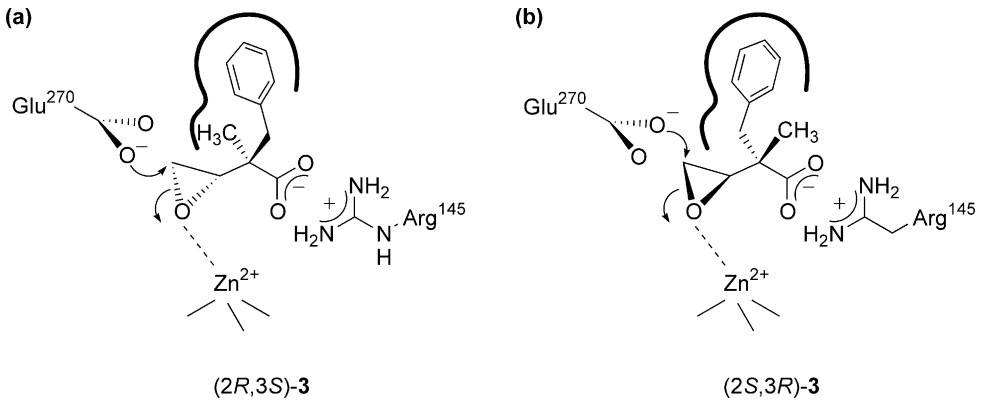


Figure 4. The Dixon plot for the inhibition of CPA with (2*S*,3*S*)-**3** (a) and (2*R*,3*S*)-**3** (b). (substrate, Cl-CPL; Tris buffer of pH 7.5; temperature, 25 °C).



(2*R*,3*S*)-**3** (2*S*,3*R*)-**3**

Figure 5. Proposed binding modes of (2*R*,3*S*)- and (2*S*,3*R*)-**3** to CPA and ensuing nucleophilic attack of the Glu-270 carboxylate on the oxirane of the enzyme-bound inhibitors, leading to covalent modification of the enzyme.

not experience the van der Waals repulsive interactions since the methyl group is expected to rest in the open space filled with water. Thus, the spatial alignment required for the nucleophilic ring cleavage reaction is maintained in this CPA· (2*S*,3*R*)-**3** complex. The ring opening reaction for the covalent modification of CPA would proceed at the rate comparable to that for the inactivation of CPA by (2*S*,3*R*)-**2** (Fig. 5B). As a consequence, (2*S*,3*R*)-**3** is 44-fold more potent than (2*R*,3*S*)-**3** in terms of the k_{inact}/K_I value. The lower K_i value of 1.1 mM obtained with (2*S*,3*R*)-**3** compared with that (5.7 mM) for (2*R*,3*S*)-**3** tends to support the proposition that the reduced binding affinity of (2*R*,3*S*)-**3** is due to the methyl group projected toward the bottom of the active site cleft preventing the inhibitor from binding to the enzyme active site. The K_i value represents reversible inhibitory constant that reflects the affinity of an inhibitor for enzyme. Recently, we have reported about the effect of the α-methyl group on the CPA-catalyzed hydrolysis of ester substrate: Hippuryl-L-phenyllactic acid and hippuryl-L-α-methylphenyllactic acid were found to hydrolyze essentially at the same rate, suggesting that the attack of the carboxylate of Glu-270 of CPA is not deterred by the α-methyl group.[27] This observation suggests that the diminution of the CPA inactivation rate caused by the introduction of a methyl group at the α-position of (2*R*,3*S*)-**2** may not be due to the methyl group blocking the carboxylate to attack the electrophilic center of the oxirane ring in the inhibitor but likely due to disarranging the spatial alignment of the two reaction centers for the covalent modification of the Glu-270 carboxylate.

Conclusion

Contrary to the expectation that the substitution of the α-H of **2**, a pseudomechanism-based inactivator for CPA, with a methyl group would augment the inhibitory potency through the additional interactions of the methyl group with the so-called α-methyl hole at the active site of CPA, the inactivation potency of the α-methylated inhibitor was lowered. For example, the α-methylation of (2*R*,3*S*)-**2** results in the decrease of the second order rate constant by 453-fold. This diminution of the second order inactivation rate constant may be rationalized by the proposition that the two reaction centers for the covalent bond formation reaction, that is, the carboxylate of Glu-270 and the oxirane of the inhibitor are disarranged from the optimal alignment for the nucleophilic ring cleavage reaction due to the van der Waals repulsive interactions between the methyl group in the inhibitor and the bottom of the active site crevice of CPA. The present study highlights that the proper alignment of the two reaction centers is crucial in order for a chemical reaction to take place at the active site of an enzyme. Furthermore, this study casts a doubt on the validity for the presence of the so-called α-methyl hole at the active site of CPA and efforts to improve the potency of existing inhibitors for CPA by introduction of a small alkyl group at the α-position to its carboxylate may not be fruitful.

Experimental

All chemicals were of reagent grade obtained from Aldrich Chemical Co. and solvents were purified before use. Flash chromatography was performed on silica gel 60 (230–400 mesh) and thin layer chromatography (TLC) was carried out on silica coated glass sheets (Merck silica gel 60 F-254). All melting points were determined on a Thomas-Hoover capillary melting point apparatus and were not corrected. ^{1}H NMR and ^{13}C NMR spectra were measured 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. Optical rotations were measured on a Rudolp Research Autopol III digital polarimeter. Mass spectra and elemental analyses were performed at Central Machine and Facilities Shop, Pohang University of Science and Technology, Pohang, Korea. High resolution mass spectra were performed by Mass Spectrometry Analysis Team, Korea Basic Science Institute, Taegu, Korea.

Methyl 2-methyl-3-oxobutanoate (5). A mixture of methyl iodide (9.4 mL, 0.15 mol), methyl acetoacetate (16.2 mL, 0.15 mol) and anhydrous powdered K_2CO_3 (20.7 g, 0.15 mol) in MeOH (100 mL) was refluxed. As the refluxing started, the evolution of carbon dioxide was observed. When the gas evolution ceased (~3 h), the reaction mixture was poured on to cold water (30

mL) and the resulting mixture was concentrated to about 50 mL under reduced pressure. The residue was extracted with ethyl acetate (3×50 mL). The combined extracts were washed with 0.5 N HCl (50 mL), dried over $MgSO_4$, and evaporated. The residue thus obtained was purified by column chromatography to give a colorless oil (14.6 g, 87.5%). IR (neat) 1738, 1720 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.24 (d, $J=1.61$ Hz, 3H), 2.12 (s, 3H), 3.42 (q, $J=1.61$ Hz, 1H), 3.62 (s, 3H).

Methyl 2-benzyl-2-methyl-3-oxobutanoate (6). The benzylation was carried out in the same manner as that used for the synthesis of **5** using benzyl bromide instead of iodomethane. The crude product was recrystallized from hexane to give colorless crystal in 88.5% yield. Mp = 58–59 °C; IR (KBr) 1743, 1715 cm^{-1}; FAB MS *m/z* 221 (MH^+); FAB HRMS calcd for $C_{13}H_{17}O_3$ (MH^+) 221.1178, found 221.1171; ^1H NMR 300 MHz ($CDCl_3$) δ 1.26 (s, 3H), 2.12 (s, 3H), 3.01, 3.25 (2d, $J=13.7$ Hz, 2H), 3.67 (s, 3H), 7.03–7.25 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 19.3, 26.8, 40.9, 52.7, 61.2, 127.2, 128.6, 130.5, 136.8, 173.2, 205.5. Anal. calcd for $C_{13}H_{16}O_3$: C, 70.89; H, 7.32. Found: C, 71.17; H, 7.11.

Methyl 2-benzyl-2-methyl-3-hydroxybutanoate (7). To a solution of **6** (2.2 g, 10 mmol) in MeOH (10 mL) was added $NaBH_4$ (380 mg, 10 mmol) in water (0.5 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h and quenched with 2 N HCl (10 mL) followed by extraction with ethyl acetate (3×20 mL). The combined extracts were washed with brine (30 mL), dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by column chromatography to give a colorless oil (1.9 g, 85.5%). IR (neat) 3418, 1725 cm^{-1}; FAB MS *m/z* 239 (MH^+), 221 (MH^+-H_2O); ^1H NMR 300 MHz ($CDCl_3$) δ 1.06, 1.08 (2s, 3H), 1.19 (d, $J=6.3$ Hz, 3H), 2.76, 2.83 (2d, AB, $J=13.2$ Hz, 2H), 3.06, 3.19 (2d, AB, $J=13.2$ Hz, 1H), 3.60, 3.66 (2s, 3H), 3.75–3.81, 3.98–4.02 (m, 1H), 7.07–7.27 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 16.2, 17.6, 41.8, 42.9, 52.0, 52.9, 72.0, 126.9, 128.5, 130.4, 137.4, 138.8, 176.9, 177.4.

Methyl 2-benzyl-2-methylbut-3-enoate (8). To an ice-chilled solution obtained by dissolving **7** (1.8 g, 8.1 mmol) in pyridine (2 mL) was added $SOCl_2$ (3.5 mL, 24.3 mmol) and then the reaction mixture was stirred at 50 °C for 24 h. The reaction was quenched by pouring into 30 mL of ice water and extracted with ethyl acetate (3×10 mL). The organic layer was dried over $MgSO_4$ and evaporated to afford yellowish oil. The crude product was purified by column chromatography to give 1:1 mixture of **8** and methyl 2-benzyl-2-methyl-3-chlorobutanoate (1.2 g, 68.5%). **8**: IR (neat) 1722 cm^{-1}; FAB HRMS calcd for $C_{13}H_{17}O_2$ (MH^+) 205.1229, found 205.1219; ^1H NMR 300 MHz ($CDCl_3$) δ 1.24, 1.25 (2s, 3H), 1.28 (s, 3H), 1.49, 1.58 (2d, AB, $J=6.6$, 6.8 Hz, 3H), 2.68–3.20 (m, 2H), 2.89, 3.12 (2d, AB, $J=13.2$ Hz, 2H), 3.62, 3.64 (2s, 3H), 3.68 (s, 3H), 4.46, 4.55 (2q, $J=6.8$, 6.6 Hz, 1H), 5.11 (d, $J=17.6$ Hz, 1H), 5.16 (d, $J=10.8$, 1H), 6.13 (dd, $J=17.6$, 10.8 Hz, 1H), 7.11–7.31 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 15.3, 19.0, 20.5, 21.7, 43.5, 45.1, 45.9, 50.4, 52.2, 54.0, 54.4, 63.3, 63.4, 114.4, 127.0, 127.4, 128.4, 128.7, 130.4, 130.7, 137.0, 137.2, 137.6, 141.8, 174.6, 176.0.

2-Benzyl-2-methylbut-3-enoic acid (9). Lithium hydroxide (960 mg, 33 mmol) solution in water (10 mL) was added to a solution of 1:1 mixture of **8** and methyl 2-benzyl-2-methyl-3-chlorobutanoate (2.4 g, 11 mmol) dissolved in THF/MeOH (3:1, 30 mL). The reaction mixture was stirred at room temperature for 24 h. After evaporation of the solvent under reduced pressure, the residue was extracted with ethyl acetate (3×30 mL). The combined extracts were washed with brine, dried over $MgSO_4$, and evaporated to afford methyl 2-benzyl-2-methyl-3-chlorobutanoate (1.1 g, 80%). The basic aqueous solution was then acidified with 2 N HCl (40 mL) and extracted with ethyl acetate (3×30 mL). The combined extracts were washed with brine and dried over $MgSO_4$. Evaporation of the solvent gave the desired product (0.9 g, 85.9%). **9**: IR (neat) 1703 cm^{-1}; FAB HRMS calcd for $C_{12}H_{15}O_2$ (MH^+) 191.1072, found 191.1069; ^1H NMR 300 MHz ($CDCl_3$) δ 1.28 (s, 3H), 2.89, 3.12 (2d, AB, $J=13.2$ Hz, 2H), 5.10 (d, $J=17.5$ Hz, 1H), 5.16 (d, $J=10.8$ Hz, 1H), 6.13 (dd, $J=10.8$, 17.8 Hz, 1H), 7.11–7.15 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 20.2, 45.7, 50.3, 115.2, 127.2, 128.5, 130.9, 137.3, 141.2, 182.8. methyl 2-benzyl-2-methyl-3-chlorobutanoate: ^1H NMR 300 MHz ($CDCl_3$) δ 1.24, 1.25 (2s, 3H), 1.49, 1.58 (2d, AB, $J=6.6$, 6.8 Hz, 3H), 2.68–3.20 (m, 2H), 3.62, 3.64 (2s, 3H), 4.46, 4.55 (2q, $J=6.8$, 6.6 Hz, 1H), 7.11–7.31 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 15.3, 19.0, 21.7, 43.5, 45.1, 52.2, 54.0, 54.4, 63.3, 63.4, 127.4, 128.7, 130.4, 137.0, 137.2, 174.6.

Benzyl 2-benzyl-2-methylbut-3-enoate (10). To a stirred solution obtained by dissolving **9** (0.50 g, 2.65 mmol) in 10 mL of MeOH was added Cs_2CO_3 (0.86 g, 2.65 mmol) and the stirring continued for 30 min. The mixture was concentrated in vacuo and the resulting white solid was suspended in 10 mL of DMF. To this solution, benzyl bromide (330 μL, 2.78 mmol) was added and the resulting mixture was stirred overnight at room temperature. The reaction mixture was diluted with water (20 mL) and extracted with ethyl acetate (3×10 mL). The combined extracts were washed with water, 0.5 N HCl, and brine, dried over $MgSO_4$, and evaporated under reduced pressure to give a crude product which was purified by column chromatography to afford **10** (0.65 g, 88.2%). IR (neat) 1722 cm^{-1}; FAB HRMS calcd for $C_{18}H_{21}O_3$ (MH^+) 285.1491, found 285.1485; ^1H NMR 300 MHz ($CDCl_3$) δ 1.29 (s, 3H), 2.89, 3.12 (2d, AB, $J=13.2$ Hz, 2H), 5.10 (d, $J=17.4$ Hz, 1H), 5.14 (s, 2H), 5.15 (d, $J=10.7$ Hz, 1H), 6.14 (dd, $J=10.7$, 17.4 Hz, 1H), 7.07–7.38 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 17.2, 45.9, 50.4, 66.9, 114.6, 127.0, 128.4, 128.5, 128.9, 130.7, 136.4, 137.5, 141.7, 175.4.

Benzyl 2-benzyl-2-methyl-3,4-epoxybutanoate (*threo*-(±)-11) and *erythro*-(±)-11). To a mixture of **10** (560 mg, 2.0 mmol) in CH_2Cl_2 (40 mL) and K_2HPO_4 (480 mg, 12.0 mmol) in water (0.5 mL) was added *m*CPBA (3.76 g, 12.0 mmol, 50–60%) at 0 °C. The solution was stirred vigorously at rt for 48 h. The white precipitate was

filtered off and washed with CH_2Cl_2. The filtrate was washed with 5% aqueous $NaHCO_3$ solution, water and brine, then dried over $MgSO_4$ and evaporated under reduced pressure. The residue (a mixture of four diastereomers) was separated into *threo* and *erythro* isomers by column chromatography on silica gel to give 180 mg (30%) of *erythro*-(±)-**11**, and 330 mg (55%) of *threo*-(±)-**11**. *threo*-(±)-**11**: IR (neat) 3028, 1722 cm^{-1}; FAB MS *m*/*z* 297 (MH^+); FAB HRMS calcd for $C_{19}H_{21}O_3$ (MH^+) 297.1491, found 297.1491; ^1H NMR 300 MHz ($CDCl_3$) δ 1.02 (s, 3H), 2.56 (m, 1H), 2.72 (t, $J=4.2$ Hz, 1H), 2.96, 3.04 (2d, AB, $J=13.4$ Hz, 2H), 3.34 (m, 1H), 7.16–7.31 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 15.3, 42.4, 43.9, 47.7, 55.9, 65.9, 126.9, 128.3, 128.7, 130.3, 136.0, 136.5, 174.5. *erythro*-(±)-**11**: IR (neat) 3028, 1722 cm^{-1}; FAB MS *m*/*z* 297 (MH^+); FAB HRMS calcd for $C_{19}H_{21}O_3$ (MH^+) 297.1491, found 297.1489; ^1H NMR 300 MHz ($CDCl_3$) δ 1.04 (s, 3H), 2.71–2.76 (m, 2H), 2.97, 3.08 (2d, AB, $J=13.3$ Hz, 2H), 3.19 (t, $J=3.4$ Hz, 1H), 5.11 (s, 2H), 7.09–7.35 (m, 10 H), 9.90 (br s, 1H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 17.1, 44.0, 45.0, 48.0, 55.2, 67.0, 127.1, 128.6, 128.7, 129.0, 130.5, 136.0, 137.0, 174.5.

***threo*-(±)-2-Benzyl-2-methyl-3,4-epoxybutanoic acid (*threo*-(±)-3).** A methanol (3 mL) solution containing *threo*-(±)-**11** (300 mg, 1.0 mmol) and a catalytic amount of Pd/C was stirred under an atmosphere of H_2 gas at rt. After 2 h, the reaction mixture was filtered through Celite to remove the catalyst and concentrated in vacuo to give *threo*-(±)-**3** (200 mg, 95%) as an colorless oil. IR (neat) 3028, 1706 cm^{-1}; FAB HRMS calcd for $C_{12}H_{15}O_3$ (MH^+) 207.1021, found 207.1011; ^1H NMR 300 MHz ($CDCl_3$) δ 1.02 (s, 3H), 2.55–2.57 (m, 1H), 2.72 (t, $J=4.2$ Hz, 1H), 2.96, 3.04 (2d, AB, $J=13.4$ Hz, 2H), 3.33–3.36 (m, 1H), 7.16–7.31 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 15.9, 42.4, 43.9, 47.7, 55.9, 66.8, 127.4, 128.7, 130.7, 136.4, 181.0.

***erythro*-(±)-2-Benzyl-2-methyl-3,4-epoxybutanoic acid (*erythro*-(±)-3).** The hydrogenolysis was carried out as described for the preparation of *threo*-(±)-**3**. IR (neat) 3028, 1706 cm^{-1}; FAB HRMS calcd for $C_{12}H_{15}O_3$ (MH^+) 207.1021, found 207.1010; ^1H NMR 300 MHz ($CDCl_3$) δ 1.11 (s, 3H), 2.80–2.85 (m, 2H), 3.05, 3.14 (2d, AB, $J=13.3$ Hz, 2H), 3.24 (t, $J=3.2$ Hz, 1H), 7.22–7.36 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 15.9, 42.5, 44.5, 47.7, 56.2, 127.4, 128.7, 130.7, 136.4, 181.0.

(*S*)-2-Benzyl-2-methyl-3-hydroxypropananoic acid (13). To a solution of **12**[14] (4.0 g, 18.0 mmol) in dry THF (45 mL) was added dropwise borane dimethylsulfide (12.6 mL of 2 M solution in THF, 21.6 mmol) at 20 °C. The mixture was stirred for 6 h, then chilled at 0 °C, quenched with water (30 mL), and extracted with ethyl acetate (3×10 mL). The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated. The resulting product was purified by column chromatography on silica gel followed by recrystallization from ether and hexane (3.4 g, 97%). mp = 73.5–74.5 °C; °C$[\alpha]_D^{25}$ −12.6°(*c* 0.54, MeOH); IR (KBr) 3440, 3030, 1735 cm^{-1}; FAB MS *m*/*z* 195 (MH^+), 177 (MH^+-H_2O); FAB HRMS calcd for $C_{11}H_{15}O_3$ (MH^+) 195.1021, found 195.1019; ^1H NMR 300 MHz ($CDCl_3$) δ 1.16 (s, 3H), 2.89–3.01 (dd, 2H), 3.58–3.59 (d, 2H), 7.18–7.32 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 19.5, 41.1, 49.0, 67.3, 127.1, 128.6, 130.8, 136.7, 181.8. Anal. calcd for $C_{11}H_{14}O_3$: C, 68.02; H, 7.27. Found: C, 68.28; H, 7.11.

Benzyl (*S*)-2-benzyl-2-methyl-3-hydroxypropanate (14). To a MeOH (30 mL) solution of **13** (1.7 g, 8.75 mmol) was added Cs_2CO_3 (2.85 g, 8.75 mmol) and the stirring was continued for 30 min. The mixture was concentrated in vacuo and the resulting white solid was suspended in 30 mL of DMF. To this solution, benzyl bromide (1.0 mL, 8.75 mmol) was added and the resulting mixture was stirred overnight at rt. The reaction mixture was diluted with water (50 mL) and extracted with ethyl acetate (3×30 mL). The combined extracts were washed with water, 0.5 N HCl, and brine, then dried over $MgSO_4$, and evaporated under reduced pressure to give a crude product which was purified by column chromatography to afford **14** (1.50 g, 60%) as an oil. $[\alpha]_D^{25}$ −3.0°(*c* 1.0, $CHCl_3$); IR (neat) 3450, 1735 cm^{-1}; FAB MS *m*/*z* 285 (MH^+), 267 (MH^+-H_2O); ^1H NMR 300 MHz ($CDCl_3$) δ 1.24 (s, 3H), 3.01 (s, 2H), 3.63, 3.72 (2d, AB, $J=11.13$ Hz, 2H), 5.20 (s, 2H), 7.17–7.44 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 19.8, 41.5, 49.5, 67.0, 127.1, 128.6, 129.1, 130.8, 136.2, 176.9.

Benzyl (*S*)-2-benzyl-2-methyl-2-formylacetate (15). To a solution of oxalyl chloride (360 μL, 4.2 mmol) in anhydrous CH_2Cl_2 (3 mL) was added dropwise a solution of DMSO (390 μL, 5.6 mmol) in CH_2Cl_2 (2 mL) at −63 °C ($CHCl_3$/dry ice) over a period of 15 min and the mixture was further stirred for 10 min at −63 °C. To this solution, the solution of **14** (0.79 g, 2.8 mmol) in CH_2Cl_2 (20 mL) was then added dropwise over 10 min at −63 °C. After stirring the resulting slightly cloudy solution for 10 min at −63 °C, Et_3N (1.5 mL, 11.2 mmol) in CH_2Cl_2 (3 mL) was added dropwise over 15 min. The reaction mixture was stirred overnight at rt and the reaction was quenched by adding water (4.2 mL) to the rapidly stirred reaction solution at −63 °C. The resulting slurry was immediately poured into hexane (30 mL) and washed with 100 mL of 20% saturated aqueous $KHSO_4$. The organic layers was collected and the aqueous layer was back extracted with ether (2×30 mL). The combined organic layers were washed with saturated aqueous $NaHCO_3$, water, and brine, then dried over $MgSO_4$, and concentrated in vacuo to provide the crude product which was purified by column chromatography to give **15** (0.70 g, 89%) as an oil. $[\alpha]_D^{25}$ −18.9°(*c* 1.0, $CHCl_3$); IR (neat) 1740, 1735 cm^{-1}; FAB MS *m*/*z* 283 (MH^+), 254 (MH^+-H_2O); FAB HRMS calcd for $C_{18}H_{19}O_3$ (MH^+) 283.1334, found 283.1358; ^1H NMR 300 MHz ($CDCl_3$) δ 1.28 (s, 3H), 3.07, 3.24 (2d, AB, $J=13.6$ Hz, 2H), 5.20 (s, 2H), 7.09–7.30 (m, 5H), 9.76 (s, 1H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 17.3, 40.7, 52.9, 59.3, 49.5, 69.6, 127.4, 128.7, 130.4, 135.8, 172.5, 199.7.

Benzyl (*S*)-2-benzyl-2-methylbut-3-enoate (16). KHMDS (11.8 mL of 0.5 M solution in toluene, 5.22 mmol) was added using a cannula to an ice-cooled solution of methyltriphenylphosphonium bromide (1.86 g, 5.25

mmol) in THF (10 mL). The mixture was stirred vigorously for 1 h, at which time it was cooled to −78 °C. Compound, **15** (0.70 g, 2.5 mmol) dissolved in THF (5 mL) was then added to the mixture using a cannula. After being stirred at −78 °C for 15 min, the mixture was allowed to slowly warm to rt and then heated at 40 °C for 12 h. The mixture was then cooled to rt and quenched with methanol (1.0 mL) followed by addition of aqueous solution of 20% Rochelle salts (5 mL). The mixture was extracted with ethyl acetate (3×20 mL). The combined extracts were washed with water, brine, and dried over $MgSO_4$, and concentrated. The resulting crude alkene was purified by chromatography on silica gel to give **16** (310 mg, 45%) as a colorless oil. $[\alpha]_D^{25}$ −4.0°(*c* 1.0, $CHCl_3$); IR (neat) 3027, 1722 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.28 (s, 3H), 3.07, 3.24 (2d, AB, *J* = 13.6 Hz, 2H), 5.20 (s, 2H), 7.09–7.30 (m, 5H), 9.76 (s, 1H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 17.2, 45.9, 50.3, 66.9, 114.6, 127.0, 128.4, 128.5, 128.9, 130.7, 136.4, 137.5, 141.7, 175.4.

Benzyl (2*S*,3*R*)-2-benzyl-2-methyl-3,4-epoxybutanoate (17). The synthesis was carried out as described for the preparation of *threo*-(±)-**11**. $[\alpha]_D^{25}$ −20.3°(*c* 1.2, $CHCl_3$); IR (neat) 3028, 1722 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.02 (s, 3H), 2.55–3.57 (m, 1H), 2.72 (t, *J* = 4.2 Hz, 1H), 2.96, 3.04 (2d, AB, *J* = 13.4 Hz, 2H), 3.33–3.36 (m, 1H), 5.18 (s, 2H), 7.16–7.31 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 16.2, 42.7, 44.3, 47.9, 56.2, 67.1, 126.9, 128.3, 128.7, 130.3, 136.0, 136.5, 174.9.

(2*S*,3*R*)-2-Benzyl-2-methyl-3,4-epoxybutanoic acid ((2*S*,3*R*)-3). The synthesis was carried out as described for the preparation of *threo*-(±)-**3** and obtained as an oil. $[\alpha]_D^{25}$ −19.2° (*c* 0.5, $CHCl_3$); IR (neat) 3028, 1706 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.02 (s, 3H), 2.55–2.57 (m, 1H), 2.72 (t, *J* = 4.2 Hz, 1H), 2.96, 3.04 (2d, AB, *J* = 13.4 Hz, 2H), 3.33–3.36 (m, 1H), 7.16–7.31 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 15.9, 42.4, 43.9, 47.7, 55.9, 127.4, 128.7, 130.7, 136.4, 181.0.

(*R*)-2-Benzyl-2-methyl-3-hydroxypropananoic acid (19). An aqueous lithium hydroxide solution (0.324 g of LiOH in 3 mL of water, 7.7 mmol) was added to a solution of **18**[18] (1.33 g, 6.4 mmol) dissolved in THF/MeOH (3:1, 12 mL). The reaction mixture was stirred at rt 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 **19** (1.18 g, 95%) which was recrystalized from diethyl ether and hexane. Mp = 76–77 °C; $[\alpha]_D^{25}$ +12.5°(*c* 0.5, MeOH).

Benzyl (*R*)-2-benzyl-2-methyl-3-hydroxypropanate (20). Compound **20** was obtained from **19** following the procedure used for the preparation of **14** in 60% yield. $[\alpha]_D^{25}$ +2.95°(*c* 1.0, $CHCl_3$).

Benzyl (*R*)-2-benzyl-2-methyl-3-formylacetate (21). Compound **21** was obtained from **20** following the procedure used for the preparation of **15** in 85% yield. $[\alpha]_D^{25}$ +19.0°(*c* 1.0, $CHCl_3$).

Benzyl (*R*)-2-benzyl-2-methylbut-3-enoate (22). Compound **22** was obtained from **21** following the procedure used for the preparation of **16** in 45% yield. $[\alpha]_D^{25}$ +4.1° (*c* 1.0, $CHCl_3$).

Benzyl (2*R*,3*S*)-2-benzyl-2-methyl-3,4-epoxybutanoate (23). Compound **23** was obtained from **22** following the procedure used for the preparation of **17** in 50% yield. $[\alpha]_D^{25}$ +20.4° (*c* 1.1, $CHCl_3$).

(2*R*,3*S*)-2-Benzyl-2-methyl-3,4-epoxybutanoic acid ((2*R*,3*S*)-3). Compound (2*R*,3*S*)-**3** was obtained from **19** following the procedure used for the preparation of (2*S*,3*R*)-**3** in 95% yield. $[\alpha]_D^{25}$ +18.8° (*c* 0.1, MeOH).

(2*R*,3*S*)-2-Benzyl-2-methyl-3-hydroxysuccinic acid (25). Compound (2*R*,3*S*)-2-benzyl-2-methyl-3-hydroxysuccinic acid 1-ethyl ester (**24**,[19] 2.50 g, 8.5 mmol) was dissolved in 48% aqueous solution of HBr (10 mL) in the presence of *n*-Bu_4NBr (500 mg, 1.5 mmol), and the resulting mixture was refluxed for 4 h, then extracted with ethyl acetate (3×10 mL). The combined organic layers were washed with water and brine, dried over $MgSO_4$, and evaporated under reduced pressure to give the crude product which was recrystallized from methanol and ethyl acetate (1.5 g, 74%). Mp = 203–205 °C; $[\alpha]_D^{25}$ −34.6° (*c* 0.95, MeOH); IR (KBr) 3028, 1706 cm^{-1}[21]; FAB HRMS calcd for $C_{12}H_{15}O_5$ (MH^+) 239.0920, found 239.0925; ^1H NMR 300 MHz ($CDCl_3$) δ 1.12 (s, 3H), 3.03, 3.17 (2d, AB, *J* = 13.3 Hz, 2H), 4.32 (s, 1H), 4.61 (br s, 2H), 7.21–7.27 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 16.8, 41.5, 51.5, 75.3, 126.9, 128.3, 130.7, 137.1, 174.8, 177.3. Anal. calcd for $C_{12}H_{14}O_5$: C, 60.50; H, 5.92. Found: C, 60.68; H, 5.71.

Dibenzyl (2*R*,3*S*)-2-benzyl-2-methyl-3-hydroxysuccinate (26). A solution of *n*-Bu_4NOH (11.0 mL of 1.0 M solution in MeOH, 11.0 mmol) was added to a solution of **25** (1.3 g, 5.5 mmol) in methanol (20 mL). The mixture was concentrated in vacuo and the white residue was suspended in 30 mL of dry DMF/acetonitrile (3:1). Benzyl bromide (1.3 mL, 11.0 mmol) was added and the mixture was stirred for 21 h at rt. The reaction mixture was diluted with ether (100 mL), washed with 5% $Na_2S_2O_3$ solution, 10% citric acid, 5% $NaHCO_3$, and brine. The organic solution was dried over $MgSO_4$, and evaporated under reduced pressure to give a crude product which was purified by column chromatography followed by recrystallization from petroleum ether and hexane to afford **26** (1.8 g, 79% yield). Mp = 57–58 °C; $[\alpha]_D^{25}$ +11.4° (*c* 0.95, $CHCl_3$); IR (KBr) 1725, 3450 cm^{-1}; FAB HRMS calcd for $C_{26}H_{27}O_5$ (MH^+) 419.1859, found 419.1847; ^1H NMR 300 MHz ($CDCl_3$) δ 1.10 (s, 3H), 2.97, 3.12 (2d, AB, *J* = 13.3 Hz, 2H), 4.24–4.29 (m, 1H), 4.98–5.20 (m, 4H), 7.09–7.34 (m, 5H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 17.9, 41.6, 51.7, 67.3, 68.0, 75.8, 127.2, 128.6, 129.1, 129.2, 130.9, 135.3, 135.9, 136.6, 173.0, 174.7. Anal. calcd for $C_{26}H_{26}O_5$: C, 74.62; H, 6.26. Found: C, 74.55; H, 6.05.

Benzyl (2*R*,3*S*)-2-benzyl-2-methyl-3,4-dihydroxybutanoate (27). Borane–dimethyl sulfide complex (1.6 mL of 2.0 M solution in THF, 3.2 mmol) was added dropwise

to a solution of **26** (1.4 g, 3.3 mmol) in dried THF (15 mL) at 0 °C under nitrogen atmosphere, and the mixture was stirred for 1 h. Sodium borohydride (0.05 g) was slowly added to the mixture and stirring was continued for 30 min at rt and for 1 h at 30 °C. The reaction was quenched by slow addition of methanol (1.5 mL). The mixture was stirred for a further 30 min and then evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel and recrystallized from petroleum ether and diethyl ether to give **27** as a white solid (0.7 g, 67% yield). Mp = 55.5–56.5 °C; $[\alpha]_D^{25}$ +28.3° (*c* 1.0, $CHCl_3$); IR (KBr) 3350, 1720 cm^{-1}; EI MS *m*/*z* 314 (M^+); FAB HRMS calcd for $C_{19}H_{22}O_4$ (MH^+) 315.1596, found 315.1586; ^1H NMR 300 MHz ($CDCl_3$) δ 1.11 (s, 3H), 2.93, 3.12 (2d, AB, *J* = 13.2 Hz, 2H), 3.49–3.76 (m, 3H), 5.09 (s, 2H), 7.11–7.35 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ?18.1, 43.0, 50.0, 63.6, 67.3, 76.5, 126.3, 127.2, 128.6, 128.78, 129.0, 130.7, 135.7, 136.6, 176.9. Anal. calcd for $C_{19}H_{22}O_4$: C, 72.59; H, 7.05. Found: C, 72.79; H, 6.90.

Benzyl (2*R*,3*S*)-2-benzyl-2-methyl-3,4-epoxybutanoate (23). To a solution of **27** (0.6 g, 1.9 mmol) and freshly distilled pyridine (290 μL, 3.8 mmol) in CH_2Cl_2 (5 mL) was added *p*-toluene sulfonylchloride (362 mg, 1.9 mmol) at 0 C under nitrogen atmosphere and the mixture was stirred for 48 h at rt. Ethyl acetate (10 mL) was added to the reaction mixture, and the organic phase was washed with 3 N aqueous hydrochloric acid, water, and brine. The organic phase was then dried over $MgSO_4$ and evaporated under reduced pressure. The residue (**28**) was used directly in the next reaction. Powdered potassium carbonate (190 mg, 1.4 mmol) was added to a solution of **28** (0.7 g, 1.5 mmol) in methanol (10 mL) while being chilled in an ice bath, and the resulting mixture was stirred for 10 min at rt. The reaction was quenched with water and extracted with ethyl acetate (3×10 mL). The combined extracts were washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure and the residue was purified by flash column chromatography on silica gel to give **23** as a colorless oil (340 mg, 60%). $[\alpha]_D^{25}$ +19.9° (*c* 0.75, $CHCl_3$); IR (neat) 1725, 1270 cm^{-1}; ^1H NMR 300 MHz ($CDCl_3$) δ 1.02 (s, 3H), 2.53–2.56 (m, 1H), 2.69 (t, *J* = 4.2 Hz, 1H), 2.91, 3.02 (2d, AB, *J* = 13.3 Hz, 2H), 3.31–3.34 (m, 1H), 5.16 (s, 2H), 7.05–7.34 (m, 10H); ^{13}C NMR 300 MHz ($CDCl_3$) δ 16.2, 42.7, 44.3, 47.9, 56.3, 67.2, 127.2, 128.5, 128.6, 130.0, 136.1, 136.6, 174.9.

General remarks for kinetic experiments

All solutions were prepared by dissolving in double distilled and deionized water. Stock assay solutions were filtered before use. Carboxypeptidase A was purchased from Sigma Chemical Co. (Allan form, twice crystallized from bovine pancrease, aqueous suspension in toluene) and used without further purification. CPA stock solutions were prepared by dissolving the enzyme in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer solution. Enzyme concentrations were estimated from the absorbance at 278 nm (ε_{278} = 64,200). Hippuryl-L-phenylalanine (Hip-L-Phe) was purchased from Sigma Chemical Co. *O*-(*trans*-*p*-chlorocinnamyl)-L-β-phenyllactate (Cl-CPL) was synthesized according to the method reported by Suh et al.[28] A Perkin-Elmer HP 8453 UV–vis spectrometer was used for UV absorbance measurement in kinetic experiments.

Determination of k_{inact} and K_I

A series of inactivation experiments were carried out with inactivation concentrations within the range of 0.5 to 2.0 mM for (±)-*threo*-**3**, 0.5–1.4 mM for (2*S*,3*R*)-**3**, and 2.0–5.0 mM for (2*R*,3*S*)-**3**. A solution of the inactivator in 1:1 solution of DMSO and 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer was added to an enzyme solution in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer to afford a final concentration of 1.3 μM enzyme in 15% DMSO for any given inactivator concentration. The solutions were incubated at 25 °C. At 2.5 min intervals for (±)-*threo*-**3** and (2*S*,3*R*)-**3**, and at 30 min intervals for (2*R*,3*S*)-**3**, 50 μL aliquots of the inactivation mixture were taken and added to a 950 μL assay mixture containing 50 μL of 5 mM solution of Hip-L-Phe. The increase in the absorbance at 254 nm was monitored for the first 40 s of the reaction immediately. A plot of the natural log of the residual enzymic activity versus inactivation time gave a straight line with a slope of $-k_{obs}$ (Fig. 1). The values of K_I and k_{inact} were calculated from the double reciprocal plot of the k_{obs} versus concentration of inhibitors according to the method of Kitz–Wilson (Fig. 2).[23]

Active site protection test[24]

CPA (1.3 μM) was incubated with (±)-2-benzylsuccinic acid (2 and 5 μM) for 10 min at 25 C. Subsequently, (2*S*,3*R*)-**3** (or (2*R*,3*S*)-**3**) was added to the mixture to give a final inactivator concentration of 500 μM. At 2.5-min intervals, 50 μL aliquots of the incubation mixture were removed and added to a 950 μL assay mixture containing 250 μM of Hip-L-Phe and the remaining enzymic activity was monitored at 254 nm at 25 °C (Fig. 3).

Determination of K_i

Two different concentrations of Cl-CPL (50 and 100 μM) were used. A series of assay mixtures containing both the substrate (Cl-CPL) and various concentrations of the inactivator [in the range 0.5–2.0 mM for (2*S*,3*R*)-**3** and 1.0–10.0 mM for (2*R*,3*S*)-**3**] were prepared in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer. Enzyme stock solution was added to assay mixture to afford a final enzyme concentration of 20 nM in a total volume of 1000 μL. The initial rates of enzymic reaction were measured immediately using a microcomputer-interfaced UV spectrometer. The K_i values were then estimated from the semireciprocal plot of the initial velocity versus the concentration of the inhibitors according to the method of Dixon (Fig. 4).[25]

Dialysis

Solutions of the inactivators (1–10 mM) and CPA (1 μM) in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer were incubated at 4 °C for 48 h. The mixture was then dialyzed for 24 h at rt against 0.05 M Tris/0.5 M NaCl, pH

7.5 buffer. The buffer was changed every 6 h. After dialysis, 50 μL aliquots of inactivation mixture were removed and added to 950 μL of assay mixture. The remaining enzymic activity was monitored at 254 nm. The enzyme failed to show the proteolytic activity.

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