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Optically Active 2-Benzyl-3-methanesulfinylpropanoic Acid: Synthesis and Evaluation as Inhibitors for Carboxypeptidase A

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Abstract—All four possible stereomers of 2-benzyl-3-methanesulfinylpropanoic acid were synthesized and evaluated as inhibitors for carboxypeptidase A to find that the isomer having the (2*S*,4*S*)-configuration is most potent followed by isomers of (2*R*,4*S*)- and (2*S*,4*R*)-configurations. The stereochemical preferences shown by the isomers of the inhibitor in binding to the enzyme suggest that the sulfoxide oxygen in the inhibitor fails to ligate the active site zinc ion but may form a hydrogen bond with the guanidinium moiety of Arg-127 like the carbonyl oxygen of scissile peptide bond of oligopeptide substrate of the enzyme does. It may thus be inferred that a sulfoxide moiety may serve as an isosterer of a carboxamide moiety.

We have been involved in developing design strategies of enzyme inhibitors that are effective against zinc proteases. These proteolytic enzymes are family of enzymes having a catalytically essential zinc ion at the active site and play important roles in the intracellular processing and degradation of proteins.[1] Many of these enzymes contain a conserved consensus sequence, HEXXH that ligates the zinc ion at the enzyme active site. In the endeavor to develop enzyme inhibitor design strategies, we have used carboxypeptidase A (CPA) as a model target enzyme.[2] CPA is a prototypical zinc protease whose active site structure and catalytic mechanism have been well established.[3] The enzyme catalyzes the hydrolysis of the terminal amide bond of peptide substrate and shows specificity for oligopeptide having the C-terminal residue with a hydrophobic side chain, such as Phe and Tyr. The enzyme also hydrolyzes ester bond in a compound, structure of which closely resemble the peptide substrate. At the active site of CPA there is present, in addition to the zinc ion, the S1′ pocket (primary substrate recognition site) which accommodates an aromatic ring such as phenyl and hydroxyphenyl moieties present in Phe and Tyr, respectively. The roles played by the zinc ion are 2-fold: (i) It takes a water molecule as the fourth ligand and activates it by lowering its pK_a, thus it to serve as an effective nucleophile in the scission of peptide bond, and (ii) stabilizes together with the guanidinium moiety of Arg-127 the *gem*-diolate tetrahedral transition state that is generated by the nucleophilic attack of the water molecule on the sessile peptide bond.

Sulfoxide is a polarized molecule having a partial negative charge on the oxygen atom, and thus it is expected that the molecule might be utilized as a zinc coordinating group in the design of CPA inhibitors. In this study, we evaluate 2-benzyl-3-methanesulfinylpropanoic acid (**4**) as a novel CPA inhibitor, in which a sulfoxide moiety is incorporated into a molecular scaffold that can be recognized by CPA and forms a complex. We have synthesized all four possible stereoisomers of **4** and investigated their binding mode to CPA. 2-Benzyl-3-methylthiopropanoic acid (**1**), an intermediate in the synthesis of **4** is also evaluated as a CPA inhibitor to find that it binds the enzyme more strongly than **4**.

Results and Discussion

Scheme 1 outlines the synthesis of CPA inhibitor **4** in an optically active form. 2-Benzyl-3-methylthiopropanoic acid (**1**) prepared by a literature method[4] was converted

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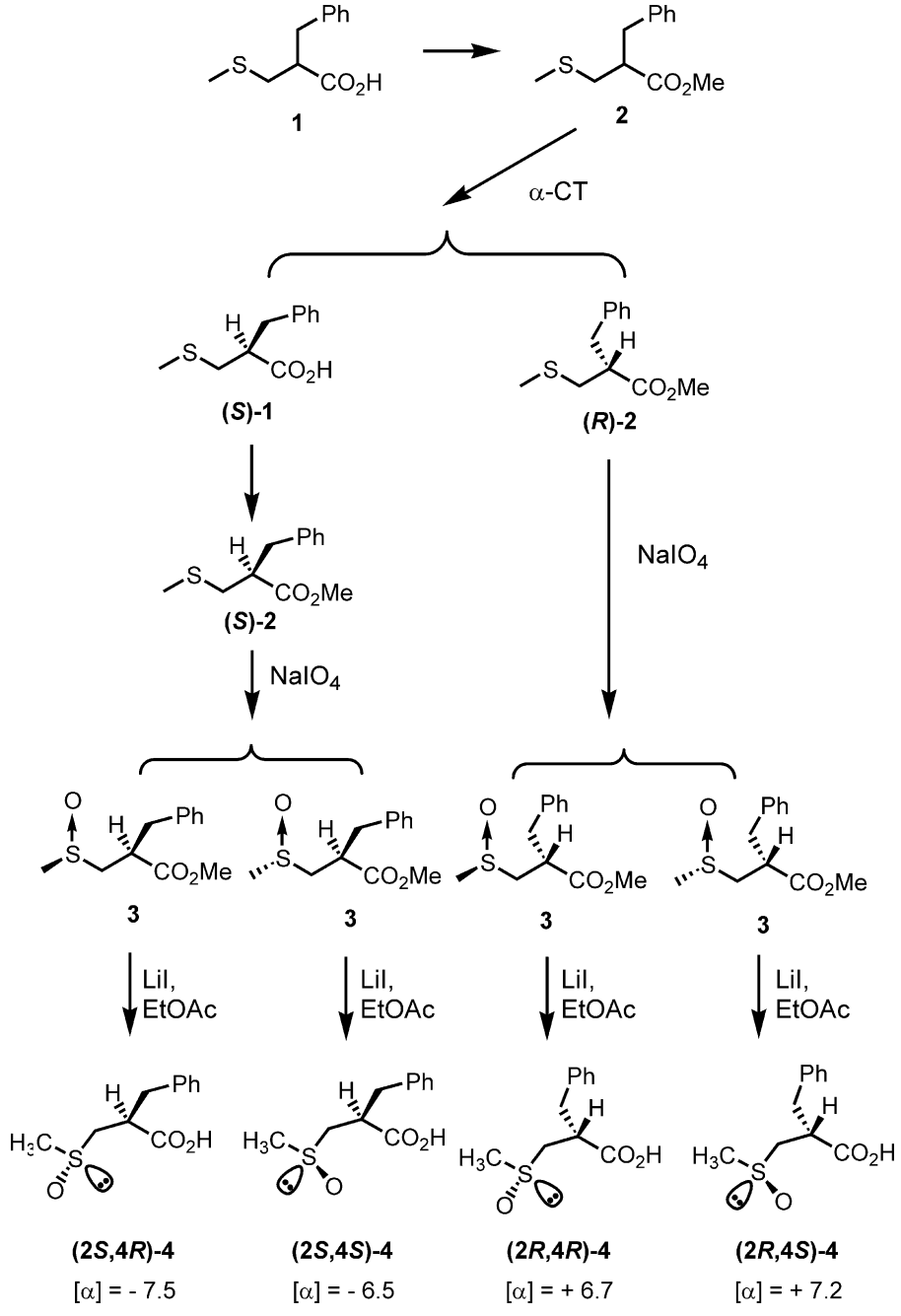
into the corresponding methyl ester (**2**), and the latter was then subjected to enzymatic resolution using α-chymotrypsin, whereby the enantiomer having the (*S*)-configuration was selectively hydrolyzed.[5] The assigned stereochemistry for the acid thus obtained was ascertained by the X-ray crystallographic determination of a final product (**4**) obtained from (*S*)-**1**. The unreacted (*R*)-**2** was treated once more with α-chymotrypsin to improve the optical purity. Since attempts at the oxidation of (*S*)-**1** to obtain directly **4** met with a difficulty in the separation of products, a diastereomeric mixture, (*S*)-**1** was converted into its methyl ester, (*S*)-**2** and the latter was oxidized with sodium periodate to give a mixture of (2*S*,4*R*)- and (2*S*,4*S*)-**3**, which was readily separated on a silica gel column. The hydrolysis of the ester moiety in (2*S*,4*R*)-**3** was effected by following the method of Fisher and Trinkle,[6] which involves treatment of the ester with lithium iodide in refluxing anhydrous ethyl acetate. The method is known to be highly efficacious for ester dealkylation of compounds having an amide carbonyl at the γ-position to the ester moiety. Under the reaction conditions, (2*S*,4*R*)-**3** was converted into (2*S*,4*R*)-**4** without bringing about racemization. Hydrolysis under basic conditions using an aqueous lithium hydroxide solution gave an inseparable mixture, and hydrolysis under acidic conditions (10% HCl) caused partial racemization at the 4-positon to occur.[7] In a similar fashion, remaining three stereoisomers of **4** were synthesized (Scheme 1). The stereochemical assignments for the final products as well as inter-



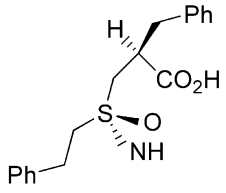
Scheme 1.

mediates in the syntheses are made on the basis of the [α] values and the crystal structure determined for **4** having [α] = −7.5°. The latter compound was shown to have the (2*S*,4*R*)-configuration by the analysis of X-ray diffraction pattern.[8]

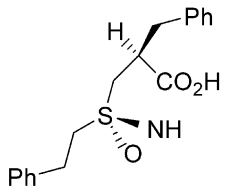
The final compounds as well as an intermediate in the synthesis were assayed for their inhibitory activity against CPA as described in the literature[9] using *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactic acid (Cl-CPL) as the substrate. The K_i values were estimated from the respective Dixon plot and are collected in Table 1. The inhibitory activities exhibited by them are disappointingly weak but nevertheless enough for probing the stereospecificity in binding of these inhibitors to CPA. Inhibitor **4** having the (2*S*,4*S*)-configuration was found to be most potent among the four stereoisomers, but its enantiomer having the (2*R*,4*R*)-configuration failed to show a CPA inhibitory activity up to the concentration of 10 mM. The other two stereoisomers in a mirror image relationship, that is, (2*S*,4*R*)- and (2*R*,4*S*)-**4** bind CPA poorly but interestingly with nearly equal potency. Previously, Mock and coworkers investigated CPA inhibitory activity of **5** having a sulfoximine moiety as the zinc ligating group to reveal that (2*S*,4*R*)-**5** is more potent than (2*S*,4*S*)-**5** by 9-fold.[10] They have attributed the higher potency of (2*S*,4*R*)-**5** over (2*S*,4*S*)-**5** to the ability of its imino group to coordinate the active site zinc ion, suggesting that the S→O group in sulfoximine moiety fails to coordinate the metal ion. This explanation is consistent with the observation that the inhibitory potency of 2-benzyl-3-methanesulfonylpropanoic acid (K_i = 910 μM) is much reduced compared with K_i = 0.26 μM for (2*S*,4*R*)-**5**.[11] Thus, our observation that there operates a stereochemical preference in the CPA inhibition with **4** suggests that although the sulfoxide oxygen fails to interact with the zinc ion, it may form a hydrogen bond with the guanidinium moiety of Arg-127, and the weak binding affinity shown by **4** may be surmised on the ground that a hydrogen bond is much weak compared with the interactions between a metal ion and ligand.[12] Christianson and Lipscomb argued on the basis of X-ray crystallographic analysis of the binding modes of transition state analogue inhibitors of CPA[13] that not the zinc ion but the guanidinium of Arg-127 of CPA interacts, albeit weakly, with the carbonyl oxygen of the scissile peptide bond, and stabilizes the oxyanion of the *gem*-diol tetrahedral transition state generated by the attack of a water molecule on the scissile bond. The arguement has been substantiated by Rutter and coworkers by the site specific mutagenesis study.[14] It may then be arguably noted that the sulfoxide-bearing inhibitors, (2*S*,4*S*)-**4** in particular, is a substrate analogue inhibitor for CPA, binding CPA with its sulfoxide oxygen to form a hydrogen bond with the guanidinium of Arg-127 like peptide substrate does. In this respect, it may be stated that sulfoxide moiety serves as an isosterer of a carboxamide.

Table 1. Inhibition constants (K_i) for CPA

Compd	K_i (mM)
(*RS*)-**1**	0.74
(*R*)-**1**	1.46
(*S*)-**1**	0.1
(2*S*, 4*R*)-**4**	3.86
(2*S*, 4*S*)-**4**	0.56
(2*R*, 4*S*)-**4**	2.79
(2*R*, 4*R*)-**4**	NI



(2*S*,4*R*) -5



(2*S*,4*S*) -5

It is worth noting that **1** shows a significant CPA inhibitory activity (Table 1). In fact, (*S*)-**1**, the stereochemistry of which belongs to the that of the P1′ residue of substrate, i.e., the L-series, was shown to be the most potent CPA inhibitor having the K_i value of 100 μM among the compounds evaluated in the present study. 2-Benzyl-3-mercaptopropanoic acid in which the methylthio moiety in **1** is replaced with a mercapto group is one of the most potent inhibitors of CPA, and it has been suggested that its sulfhydryl group coordinates the active site zinc ion in a deprotonated form.[15] The drastically reduced binding affinity of **1** in comparison with 2-benzyl-3-mercaptopropanoic acid supports the proposition that the thiolate rather than thiol group of mercapto-bearing CPA inhibitors ligates the zinc ion: if the mercapto group ligates the zinc ion with its proton being attached to the sulfur atom, the replacement of the hydrogen with a methyl group would not diminish the binding affinity in such a drastic extent. Replacement of the sulfur atom in **1** with a carbon to obtain 2-benzylpentanoic acid[16] attenuated the inhibitory potency by 3-fold, suggesting that the sulfide moiety is not an effective zinc ligating group.

Conclusion

All four possible stereoisomers of **4** were synthesized and evaluated as competitive inhibitors of CPA to find that (2*S*,4*S*)-**4** binds the enzyme most tightly followed by (2*R*,4*S*)- and (2*S*,4*R*)-**4**. The enantiomer of (2*S*,4*S*)-**4** fails to bind the enzyme. The stereochemical preference shown by these inhibitors suggests that the sulfoxide oxygen of (2*S*,4*S*)-**4** may form a hydrogen bond with the guanidinium of Arg-127 like the carbonyl oxygen of scissile peptide bond of substrate when it forms a Michaelis complex with CPA. It may thus be inferred that a sulfoxide may serve as an isosterer of carboxamide moiety.

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. 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. CPA was purchased from Sigma Chemical Co. (Allan form, twice crystallized from bovine pancrease, aqueous suspension in toluene) and used without further purification. Cl-CPL, the substrate used in the kinetic study, was synthesized as described in the literature.[9] All solutions for kinetic study 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 (ε_{278} = 64,200). The stock assay solutions were filtered (GHP Acrodic syringe filter, pore size 0.2 μm) before use. Perkin-Elmer HP 8453 UV–vis spectrometer was used for UV absorbance measurements.

2-Benzyl-3-methylthiopropanoic acid, methyl ester (2). To a mixture of 2-benzyl-3-methylthiopropanoic acid (2.10 g, 10 mmol) and anhydrous methanol (10 mL) was added thionyl chloride (0.84 mL, 11.5 mmol) and the resulting solution was stirred at room temperature overnight. After removing the solvent, the residue was dissolved in ethyl acetate and the solution was washed with saturated sodium bicarbonate solution, then with brine, dried over $MgSO_4$, and evaporated under reduced pressure to give an oil which was purified by column chromatography (silica gel, EtOAc/*n*-hexane = 1:3) to afford a colorless oil (2.17 g, 97%). IR (film, cm^{-1}) 1736. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30–7.14 (m, 5H), 3.62 (s, 3H), 3.00–2.89 (m, 3H), 2.77 (dd, J = 7.8, 13.0 Hz, 1H). 2.61 (dd, J = 5.2, 13.0 Hz, 1H). 2.07 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.79, 138.93, 129.27, 128.88, 126.95, 52.10, 47.94, 38.18, 36.03, 16.26. HRMS calcd for $C_{12}H_{16}O_2S$; 224.0871, found: 224.0873.

(*S*)-2-Benzyl-3-methylthiopropanoic acid, methyl ester [(*S*)-2]. This was prepared in a similar fashion. (Yield, 95%). $[\alpha]_D^{20} = -4.45$ (*c* 0.23, $CHCl_3$), HRMS calcd for $C_{12}H_{16}O_2S$; 224.0871, found: 224.0871.

(*R*)-2-Benzyl-3-methylthiopropanoic acid, methyl ester [(*R*)-2]. (Yield, 95%). $[\alpha]_D^{20}$ −4.45 (*c* 0.16, $CHCl_3$), HRMS calcd for $C_{12}H_{16}O_2S$; 224.0871, found: 224.0874.

(*S*)-2-Benzyl-3-methylthiopropanoic acid [(*S*)-1]. Racemic **2** (2.68g, 11.96 mmol) was suspended in 0.01 M phosphate buffer (35 mL) to which was added α-chymotrypsin (200 mg) and the resulting mixture was stirred slowly at room temperature. The pH of the mixture was maintained at 7.5 by addition of NaOH solution (0.2 N) using a pH-stat potentiometer. When 12 mL of the alkaline solution was consumed (11 h), the reaction mixture was made acidic with HCl (1.0 N), and saturated with sodium chloride, then extracted with ethyl acetate (25 mL×4). The extract was dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, EtOAc/*n*-hexane = 1:6) to give (*S*)-**1**. Yield, 45%. The spectral data of (*S*)-**1** are identical with those reported in the literature[4] for **1**. Mp 104–105 °C. $[\alpha]_D^{20} = -8.96$ (*c* 0.05, $CHCl_3$). Anal. calcd for $C_{11}H_{14}O_2S$: C, 62.83; H, 6.71. Found: C, 62.66; H, 6.74.

(*R*)-2-Benzyl-3-methylthiopropanoic acid [(*R*)-1]

The crude (*R*)-**2** from the enzymatic resolution of **2** was treated with α-chymotrypsin in 0.01 M phosphate buffer (25 mL) in order to improve the optical purity. When a few mL of NaOH solution (0.2 N) was consumed, the reaction mixture was extracted with ethyl acetate (30 mL×4), and the combined extract was washed with sodium bicarbonate solution (5%), dried over $MgSO_4$, and evaporated under reduced pressure to afford (*R*)-**2** as oil. A portion of the residue (0.8 g) was dissolved in a mixture of dioxane (8 mL) and LiOH solution (1.0 N, 4.0 mL), and stirred at room temperature for 12 h. The solution was then acidified to pH 1 with HCl (1.0 N), and extracted with ether (10 mL×3) and the combined extract was washed with brine, dried over $MgSO_4$, and evaporated in vacuo to give a solid residue which was recrystallized from chloroform and *n*-hexane to give (*R*)-**1**. Yield, 45%. Mp 104–105 °C. $[\alpha]_D^{20} = +8.70$ (*c* 0.05, $CHCl_3$). Anal. calcd for $C_{11}H_{14}O_2S$: C, 62.83; H, 6.71, found: C, 62.71; H, 6.74.

General procedure for oxidation of 2 to obtain 2-benzyl-3-methanesulfinylpropanoic acid, methyl ester (3)

Typically, (*S*)-**2** (1.38 g, 6.15 mmol) was dissolved in a 50% aqueous methanol solution (124 mL), and to the resulting solution was added sodium periodate (1.32 g, 6.15 mmol) under stirring at 0 °C. The reaction mixture was stirred vigorously for 30 min at 0 °C, and most of the organic solvent was removed under reduced pressure at 35–40 °C, then was added to aqueous NaS_2O_3 solution (5%, 40 mL), and extracted with ethyl acetate (30 mL×3). The combined extracts was washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure to give a mixture of diastereomers, which was separated by column chromatography (silica gel, $CHCl_3$/MeOH = 200:3). (2*S*,4*R*)-**3** (560 mg, 38%). Mp 66–68 °C; IR (KBr, cm^{-1}): 1730, 1032. ^{1}H NMR ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33–7.22 (m, 3H), 7.17–7.14 (m, 2H), 3.70 (s, 3H), 3.39–3.33 (m, 1H), 3.12 (dd, J = 6.8, 13.6 Hz, 1H), 3.05–2.92 (m, 2H), 2.70 (dd, J = 2.6, 12.8 Hz, 1H). 2.56 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.13, 137.44, 129.38, 129.12, 127.46, 56.36, 52.55, 41.86, 39.90, 38.85. $[\alpha]_D^{20}$ −8.13 (*c* 0.015, $CHCl_3$). Anal. calcd for $C_{12}H_{16}O_3S$: C, 59.97; H, 6.71, found: C, 59.79; H, 6.74. (2*S*,4*S*)-**3**: (oil) yield, 42%. IR (KBr, cm^{-1}): 1735, 1044. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30–7.25 (m, 3H), 7.23–7.15 (m, 2H), 3.68 (s, 3H), 3.29–3.20 (m, 1H), 3.15–2.96 (m, 3H), 2.75 (dd, J = 7.0, 13.0 Hz, 1H), 2.55 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.01, 137.62, 129.49, 129.07, 127.39, 55.33, 52.58, 41.77,

39.78, 37.71. $[\alpha]_D^{20}$ −7.06 (c, 0.11, $CHCl_3$). HRMS calcd for $C_{12}H_{16}O_3S$: 240.0820. Found: 240.0820. (2*R*,4*S*)-**3**: Mp 66–68 °C. $[\alpha]_D^{20} = +8.39$ (*c* 0.02, $CHCl_3$). Anal. calcd for $C_{12}H_{16}O_3S$: C, 59.97; H, 6.71; found: C, 60.03; H, 6.77. (2*R*,4*R*)-**3**: (oil) yield, 41%. $[\alpha]_D^{20} = +7.34$ (*c* 0.15%, $CHCl_3$). HRMS, calcd for $C_{12}H_{16}O_3S$: 240.0820, found: 240.0820.

General procedure for conversion of 3 into 2-benzyl-3-methanesulfinylpropanoic acid (4)

Typically, lithium iodide (2.77 g, 20.6 mmol) was added to a anhydrous ethyl acetate (75 mL) solution of (2*S*,4*R*)-**3** (920 mg, 3.75 mmol), and the resulting mixture was heated under reflux for 8 h under N_2. After cooling to room temperature, the reaction mixture was treated with water and the water layer was acidified to pH 1.0 with an aqueous citric acid solution (10%), then extracted with ethyl acetate (50 mL×5). The combined extract was washed with saturated $Na_2S_2O_3$ solution (20 mL), dried over $MgSO_4$, and evaporated under reduced pressure to give a pale yellow oil which crystallized on standing to obtain crystalline (2*S*,4*R*)-**4**, (394 mg, 47%). Mp 122–124 °C. IR (KBr, cm^{-1}) 3429, 1718, 1016. ^1H NMR (300 MHz, $CDCl_3$) δ 9.22 (br, 1H), 7.33–7.18 (m, 5H), 3.33–3.29 (m, 1H), 3.20 (dd, *J* = 5.6, 13.8 Hz, 1H), 3.03 (t, *J* = 12.9 Hz, 1H), 2.93 (dd, *J* = 8.3, 13.8 Hz, 1H), 2.74 (dd, *J* = 2.4, 12.9 Hz, 1H), 2.55 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.06, 138.96, 129.90, 129.24, 127.41, 55.90, 42.18, 39.53, 38.41. $[\alpha]_D^{20} = -7.5$ (*c* 0.1%, $CHCl_3$). Anal. calcd for $C_{11}H_{14}O_3S$: C, 58.38; H, 6.24; found: C, 58.20; H, 6.23. (2*S*,4*S*)-**4**: (48%). Mp 109–110 °C. IR (KBr, cm^{-1}) 3440, 2912,1722, 1007. ^1H NMR (300 MHz, $CDCl_3$) δ 10.68 (br, 1H), 7.30–7.19 (m, 5H), 3.29 (dd, *J* = 6.2, 12.6 Hz, 1H), 3.22–3.12 (m, 2H), 3.03–2.99 (m, 1H), 2.74 (dd, *J* = 5.1, 12.6 Hz, 1H), 2.60 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.12, 139.19, 129.84, 129.21, 127.34, 55.83, 42.13, 39.30, 37.56. $[\alpha]_D^{20} = -6.5$ (*c* 0.1%, $CHCl_3$). Anal. calcd for $C_{11}H_{14}O_3S$: C, 58.38; H, 6.24; found: C, 58.30; H, 6.29. (2*R*,4*S*)-**4**: (43%). Mp 122–124 °C. $[\alpha]_D^{20} = +7.2$ (*c* 0.1%, $CHCl_3$). Anal. calcd for $C_{11}H_{14}O_3S$: C, 58.38; H, 6.24. Found: C, 58.23; H, 6.31. (2*R*,4*R*)-4: (41%). Mp 109–110 °C. $[\alpha]_D^{20} = +6.7$ (*c* 0.1%, $CHCl_3$). Anal. calcd for $C_{11}H_{14}O_3S$: C, 58.38; H, 6.24. Found: C, 58.16; H, 6.33.

Determination of K_i value

The enzyme stock solution was added to a solution containing Cl-CPL (final concentrations: 50 and 100 μM) and inhibitor (five different final concentrations in the range of 0.5–2 K_i) in 0.05 M Tris/0.5 M NaCl, pH 7.5 buffer (1-mL cuvette), and the change in absorbance at 320 nm was measured immediately. The final concentration of CPA was 18 nM. Initial velocities were then 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 inhibitor according to the method of Dixon.[17] The correlation coefficients for the Dixon plots were above 0.990.

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