Sulfamide-Based Inhibitors for Carboxypeptidase A. Novel Type Transition State Analogue Inhibitors for Zinc Proteases

Jung Dae Park,[†] Dong H. Kim,[†,*] Seung-Jun Kim,[‡] Joo-Rang Woo,[‡] and Seong Eon Ryu[‡,*]

Center for Integrated Molecular Systems, Division of Molecular and Life Sciences, Pohang University of Science and Technology, San 31 Hyoja-dong, Namku, Pohang 790-784 Korea, and Center for Cellular Switch Protein Structure, Korea Research Institute of Bioscience and Biotechnology, 52 Euh-eun-dong, Yusong-gu, Deajeon 305-806 Korea

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N-Sulfamoylphenylalanine and its derivatives having varied alkyl groups on the terminal amino group were designed rationally as transition state analogue inhibitors for carboxypeptidase A (CPA) and synthesized. In CPA inhibitory assays the parent compound having the (*S*)-configuration, i.e., (*S*)-**1a**, showed potent inhibitory activity with the K_i value of 0.64 μM. Its enantiomer was shown to be much less potent (K_i = 470 μM). Introduction of an alkyl group such as methyl or isopropyl group on the terminal amino group of (*S*)-**1a** lowered the inhibitory potency drastically. Introduction of a methyl group on the internal amino group of (*S*)-**1a** also caused a drastic reduction of the inhibitory activity. The structure of the CPA·(*S*)-**1a** complex determined by single-crystal X-ray diffraction reveals that the sulfamoyl moiety interacts with the zinc ion and functional groups at the active site of CPA, which is reminiscent of the postulated stabilization mode of a tetrahedral transition state in the CPA-catalyzed hydrolysis of a peptide substrate. On the basis of the design rationale and the binding mode of (*S*)-**1a** to CPA shown by X-ray crystallographic analysis, the present inhibitors are inferred to be a novel type of transition state analogue inhibitor for CPA.

Zinc proteases are a family of enzymes having a catalytically essential zinc ion at their active sites. These enzymes play key roles in a wide variety of physiological and pathological processes.[1] Among the enzymes, carboxypeptidase A (CPA) has been most extensively investigated and serves as a leading representative.[2] Furthermore, CPA has been much used as a model target enzyme[3] for developing inhibitor design strategies that can be applied to zinc proteases of pathological importance such as angiotensin converting enzyme[4] and matrix metalloproteases.[5]

CPA cleaves preferentially the peptide bond of the C-terminal amino acid having an aromatic side chain. The X-ray crystal structure of the enzyme has been elucidated to the resolution of 1.54 Å,[6] and its catalytic mechanism has been the subject of numerous investigations.[2] The catalytically essential zinc ion is coordinated to side chain functional groups of His-69, Glu-72, and His-196. A water molecule is loosely bound to the zinc ion as the fourth ligand. The most important residues present at the active site are Glu-270 and Arg-145. The former is intimately involved in the hydrolysis of substrate, and the latter forms hydrogen bonds with the C-terminal carboxylate of substrate. In addition, there is present a hydrophobic pocket, the primary function of which is to accommodate the aromatic ring in the side chain of the P_1' residue of the substrate. It is generally believed that the CPA-catalyzed hydrolysis of peptide substrates is initiated by the attack of the zinc-bound water molecule on the scissile peptide bond to generate a tetrahedral transition state which is known to be stabilized by the zinc ion and the guanidinium moiety of Arg-127.[2,7]

Mechanistic theories for enzymic reactions conspire that chemically stable analogues of a transition state generated along the reaction path of an enzymic reaction would bind tightly to the enzyme, hampering the catalytic activity,[8] and this proposition has been successfully exploited.[9] Thus, for example, in the case of CPA, the substrates in which the scissile peptide bond is replaced with a tetrahedral phosphorus ester or amide moiety were shown to be potent inhibitors for the enzyme. The X-ray crystal structures of the CPA complexes formed with these inhibitors revealed that the phosphorus moieties bear close structural resemblances to the transition state postulated for the enzymic hydrolytic reaction.[10] In addition to the phosphorus ester and amides, sulfoximine,[11] sulfodiimine,[11] and sulfonimidamide[12] moieties have also been incorporated into substrate analogues of CPA to obtain potent inhibitors for CPA. We wish to report herein a novel type of transition state analogue inhibitors for CPA, in which a readily obtainable sulfamide moiety is incorporated into the basic structural frame of CPA substrate.

Results and Discussion

Although sulfamide was first reported in 1892 by Traube who prepared it from sulfuryl chloride and gaseous ammonia,[13] it received only scant attention. Recently, however, the utility of sulfamide as an isostere for a peptide bond has been recognized.[14] Sulfamide is a water soluble and chemically stable compound, and its X-ray crystal structure showed that the compound has the shape of tetrahedron with its sulfur atom occupying the center.[15] The nitrogen atoms in sulfamide are conceivably sufficiently basic to allow metal ion

* To whom correspondence should be addressed (E-mail: dhkim@postech.ac.kr or ryuse@mail.krib.re.kr).
[†] Pohang University of Science and Technology.
[‡] Korea Research Institute of Bioscience and Biotechnology.

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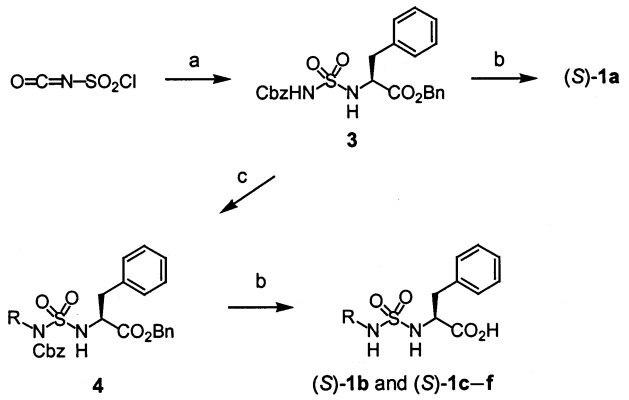
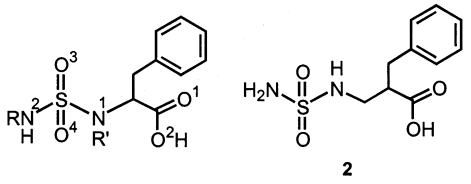
Scheme 1[a]



[a] Reagents, conditions, and yield: (a) (i) BnOH, CH_2Cl_2, 0 °C, 30 min; (ii) phenylalanine benzyl ester, Et_3N, CH_2Cl_2, rt, 1 h, 96%; (b) H_2, Pd–C, MeOH, rt, 1 h, quantitative; (c) ROH (1.1 equiv), PPh_3 (1.5 equiv), DIAD (1.5 equiv), CH_2Cl_2, rt, 1–12 h, 69–93%.

coordination. From the physicochemical properties, molecular geometry, and electronic feature, it was inferred that sulfamide would serve as a viable mimic for the tetrahedral transition state postulated for the proteolysis catalyzed by CPA, and its incorporation into substrate molecule would result in potent inhibitors for the enzyme. Compounds **1a**–**f** have been synthesized and evaluated as inhibitors of CPA. We have also prepared and tested *N*-sulfamoylated derivatives of *β*-phenylalanine, **2**, as inhibitors for CPA.



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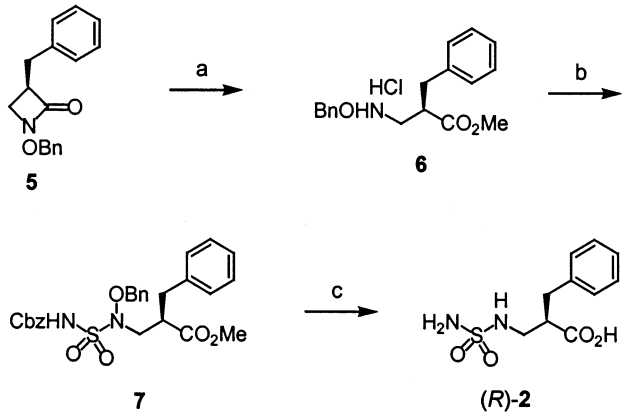
1a: R = H, R′ = H
1b: R = CH_3, R′ = H
1c: R = H, R′ = CH_3
1d: R = $CH(CH_3)_2$, R′ = H
1e: R = CH_2Ph, R′ = H
1f: R = CH_2CH_2Ph, R′ = H

Chemistry. Chlorosulfonyl isocyanate was used for the *N*-sulfamoylation of phenylalanine benzyl ester: Addition of benzyl alcohol to a chlorosulfonyl isocyanate solution in dry methylene chloride generated benzyl *N*-chlorosulfonylcarbamate which was then allowed to react with (*S*)-phenylalanine benzyl ester in the presence of an equivalent amount of triethylamine to obtain **2** in 96% yield. Hydrogenolysis of **2** using 10% Pd–C provided (*S*)-**1a** in quantitative yield (Scheme 1). In a similar fashion, (*R*)-**1a** was prepared. *N*-(*N*-Alkylsulfamoyl)phenylalanines, (*S*)-**1b** and (*S*)-**1d**–**f** were prepared in 69–93% yield by allowing **2** to react with the appropriate alcohol under the Mitsunobu conditions[16] followed by hydrogenolysis. Likewise, (*S*)-**1c** was synthesized using *N*-methylphenylalanine methyl ester[17] in place of phenylalanine benzyl ester in the above reactions. In this case, the Cbz group was removed under the hydrogenolysis conditions and the methyl ester moiety was hydrolyzed with 1 N lithium hydroxide solution. *β*-Phenylalanine required for the synthesis of

Scheme 2[a]



[a] Reagents, conditions, and yield: (a) TMSCl (4 equiv), MeOH, rt, 4 h, 98%; (b) $CbzNH_2SO_2Cl$ prepared from $OCNSO_2Cl$ and BnOH (CH_2Cl_2, 0 °C, 30 min), Et_3N, CH_2Cl_2, rt, 6 h, 88%; (c) (i) H_2, Pd–C, MeOH, rt, 2 h; (ii) 1 N LiOH, MeOH, rt, 2 h, 91%.

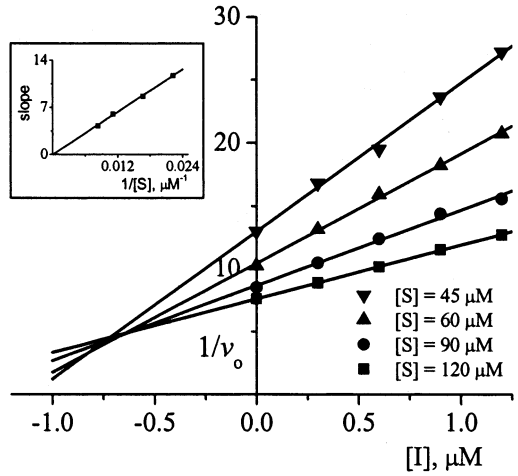


Figure 1. The Dixon plot for the inhibition of CPA-catalyzed hydrolysis of ClCPL by (*S*)-**1a** at pH 7.5. The inset represents the replot of slopes in the Dixon plot against the corresponding 1/[S].

2 was prepared as its methyl ester according to the method reported by Jin and Kim:[18] (3*R*)-*N*-Benzyloxy-3-benzyl-2-azetidine (**5**) that was obtained from 3-phenylpropanoic acid in eight steps was treated with methanol in the presence of TMSCl to give **6**. The latter was then allowed to react with *N*-(benzyloxycarbonyl)aminosulfonyl chloride that was obtained by the reaction of chlorosulfonyl isocyanate with benzyl alcohol to give **7**. The subsequent hydrogenolysis followed by alkaline hydrolysis using lithium hydroxide afforded (*R*)-**2** (Scheme 2). In an analogous fashion (*S*)-**2** was synthesized.

Inhibition of CPA and Structure–Activity Relationships. The compounds thus synthesized were evaluated as inhibitors for CPA using *O*-(*trans*-*p*-chlorocinnamoyl)-L-phenyllactic aicd (ClCPL)[19] as substrate at pH 7.5, and the inhibitory constants (K_i values) were estimated from the respective Dixon plot[20] (Figure 1) and collected in Table 1. As can be seen in the inset of Figure 1, (*S*)-**1a** is a competitive inhibitor for CPA.[21] The unsubstituted *N*-sulfamoylPhe having the (*S*)-configuration, i.e., (*S*)-**1a**, binds CPA most tightly with the K_i value of 0.64 μM. The binding affinity of its enantiomer is reduced dramatically to the K_i value of 470 μM. Since

Table 1. Inhibitory Potencies (K_i values) of *N*-Sulfamoylphenylalanines for CPA Inhibition

compd no.	K_i (μM)[a]
(*S*)-**1a**	0.65 ± 0.05[b]
(*R*)-**1a**	470 ± 110
(*S*)-**1b**	180 ± 50
(*S*)-**1c**	3500 ± 900
(*S*)-**1d**	610 ± 130
(*S*)-**1e**	35 ± 21
(*S*)-**1f**	180 ± 60
(*S*)-**2**	2900 ± 930
(*R*)-**2**	1400 ± 190

[a] K_i values are expressed as mean ± SEM ($n = 3$). [b] K_i value of 1.6 ± 0.3 μM was obtained when Hipp-Phe was used as the substrate.

most of the inhibitory activity of **1** was found to be associated with the enantiomer **1a** that belongs to the L-series, structural modifications for the improvement of the inhibitory activity were performed with *N*-sulfamoylPhe having the (*S*)-configuration. The S_1 site of CPA is known to consist of a cavity that can accommodate a hydrophobic side chain of amino acids such as Phe and Leu.[10c] Accordingly, we have introduced varied alkyl groups on the terminal amino nitrogen of (*S*)-**1**. However, contrary to the expectation, the substitution attenuated the inhibitory activity by 2 orders of magnitude except in the case of the benzyl. The drastic reduction of the binding affinity caused by the alkyl substitution may be due to unfavorable steric interactions of the alkyl group possibly with the zinc ion at the active site. The substitution of the methyl group on the nitrogen with a benzyl group to give **1e**, i.e., an introduction of a benzene ring on the methyl group of **1b**, improved the binding affinity by 5-fold, which may be rationalized on the ground that the benzene ring may undergo favorable interactions with the side chains of the amino acid residues that constitute the S_1 pocket. Introduction of a methyl group at the internal nitrogen to yield (*S*)-**1c** reduced the binding affinity by 3 orders of magnitude. The drastic reduction of the binding affinity shown by (*S*)-**1c** may be explained on the basis that the methyl group would experience a severe steric hindrance possibly by the phenolic hydroxyl of the down positioned Tyr-248 and the carboxylate of Glu-270 upon its binding to the enzyme. Some of β-phenyalanine derivatives are known to be potent inhibitors for CPA.[22] It was thus thought to be of interest to evaluate *N*-sulfamoylated β-phenylalanine (**2**) as a CPA inhibitor. Both enantiomers of **2** were, however, found to be only marginally active as inhibitors for CPA. As with *N*-sulfamoylated β-Phe, (*R*)-**2**, that belongs to the L-series is 2-fold more potent than its enantiomer. The limited study for the SAR tends to suggest that the improvement for the inhibitory activity of the parent compound, (*S*)-**1a**, may only be realized by incorporating substituents that can interact with cavities at the S_1 subsite and beyond.

X-ray Structural Analysis of CPA·(*S*)-1 Complex.[23] Crystal structure of the CPA·(*S*)-**1a** complex was determined at 2.0 Å resolution by using the native CPA structure (pdb code: 5CPA) as the starting model (Table 2). The final model including all residues of CPA and the inhibitor is very well defined with the R_{cryst} and R_{free} values of 17.5% and 20.8%, respectively. Figure 2 depicts the stereoview of the difference electron density in the region of the active site of CPA that is occupied by (*S*)-**1a** and the mode of binding of the inhibitor to CPA. Distances of important interactions between CPA and the inhibitor in the complex are estimated from Figure 2 and listed in Table 3.

Table 2. Crystallographic Data of Inhibitor A

space group	$P2_1$
unit cell	
a, b, c (Å)	51.69, 60.47, 47.26
α, β, γ (°)	90, 82.55, 90
resolution range (Å)	99−2.0
number of unique reflections	19491
overall completeness (%)	99.2
R_{merge} (%)[a]	10.8
number of atoms	2437
protein atoms	165
nonprotein atoms	
R_{cryst} (%)[b]	17.5
R_{free} (%)[c]	20.8
rms deviations	0.005
bonds (Å)	1.2
angles (deg)	23.2
dihedrals (deg)	0.71
impropers (deg)	

[a] $R_{merge} = \Sigma_i|I_i - \langle I\rangle| / \Sigma\langle I\rangle|$, where I is the intensity for the ith measurement of an equivalent reflection with the indices h,k,l. [b] $R_{cryst} = \Sigma||F_o| - |F_c||/\Sigma |F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively. [c] The R_{free} value was calculated from 5% of all data that were not used in the refinement.

The carboxylate of (*S*)-**1a** forms a salt bridge with the guanidinium moiety of Arg-145 with bond distances of 2.93 and 2.73 Å. The amide group of the side chain of Asn-144 forms a hydrogen bond (2.87 Å) to O^2 of the carboxylate. The aromatic side chain of Tyr-248 is found in the "so-called" down position, and its phenolic oxygen is separated from one of the carboxylate oxygen atoms of the inhibitor by 2.60 Å to form a hydrogen bond. This type of hydrogen bonding is commonly observed in X-ray crystal structures of CPA·inhibitor complexes. For example, the phenolic oxygen atom of the down positioned Tyr-248 in the complex of CPA·Gly-L-Tyr, a slowly hydrolyzed substrate of CPA, was shown to be separated by 2.8 Å from one of the terminal carboxylate oxygens of the dipeptide.[24] The benzene ring of (*S*)-**1a** is anchored in the S_1' hydrophobic pocket, the primary substrate recognition site for CPA. One (O^4) of the two oxygen atoms of the sulfamide moiety in (*S*)-**1a** is engaged in a hydrogen bonding with a nitrogen atom of the guanidinium group in Arg-127 with the bond distance of 2.76 Å. The binding mode of sulfamide moiety in (*S*)-**1a** is very reminiscent of those seen in CPA complexes formed with phosphonate-bearing transition state analogues. For example, in the CPA complexes formed with Cbz-Ala-Gly^P(O)Phe, Cbz-Ala-Ala^P-(O)Phe, or Cbz-Phe-Val^P(O)Phe, the guanidinium nitrogen of Arg-127 was shown to interact with one of the phosphinyl oxygens of the phosphonate.[10c] The other oxygen atom in the sulfamide moiety is shown to rest in an open space exposed to bulk water, being separated from the oxygen atoms of Glu-270 carboxylate by 4.13 and 4.28 Å. The Glu-270 carboxylate forms a hydrogen bond with N^1 of the sulfamide: one of the Glu-270 carboxylate oxygens is separated from the N^1 of the sulfamide moiety by 2.87 Å. It is also noteworthy that not an oxygen but the terminal nitrogen atom (N^2) of sulfamide coordinates to the zinc ion at the active site of CPA with the bond distance of 1.92 Å. The binding mode of (*S*)-**1a** to CPA is schematically depicted in

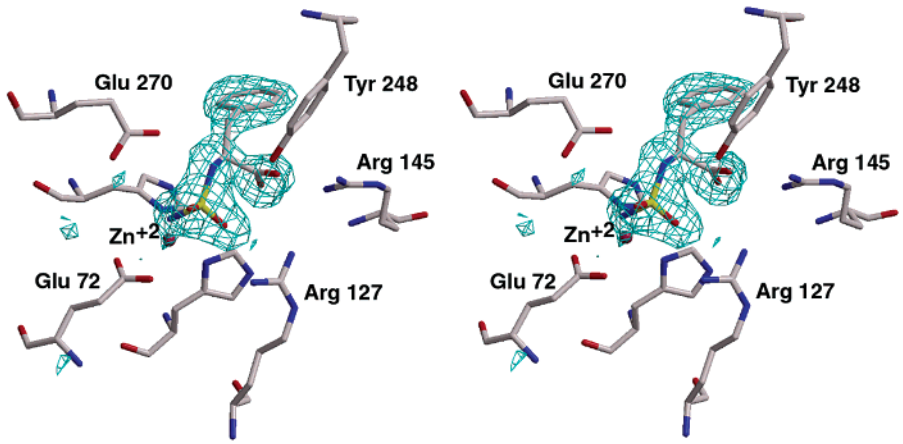


Figure 2. Difference electron density map for CPA·(*S*)-**1a** complex generated with Fourier coefficient $|F_o| - |F_c|$ phases calculated from the final model omitting the bound inhibitor.

Table 3. Selected CPA·(*S*)-**1a** Interactions

atom in (*S*)-**1a**	enzyme residue	separation (Å)
O^1	Arg-145 guanidinium N^1	2.73
O^2	Arg-145 guanidinium N^2	2.73
O^1	Asn-144 side chain amide N	2.87
O^2	Try-248 phenolic O	2.60
O^2	Arg-127 guanidinium N^1	3.54
O^3	Glu-270 carboxylate O^1	4.13
O^3	Glu-270 carboxylate O^2	4.28
O^4	Arg-127 guanidinium N^1	3.22
O^4	Arg-127 guanidinium N^2	2.76
O^4	Zn^{2+}	3.19
N^1	Glu-270 carboxylate O^1	2.87
N^1	Try-248 phenolic O	3.16
N^2	Zn^{2+}	1.92

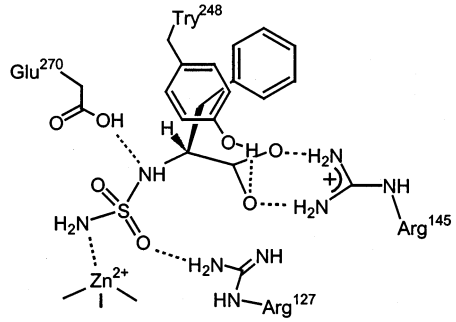
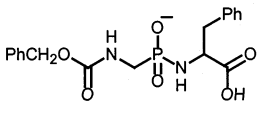


Figure 3. Schematic representation of the binding mode of (*S*)-**1a** to CPA.

Figure 3. The binding mode of (*S*)-**1a** to CPA is significantly different from those of phosphorus transition state analogue inhibitors: In the binding of phosphoramidate transition state analogue such as **8**[10a] the two oxygen atoms of its tetrahedral phosphinyl group coordinate to the zinc ion and one of these oxygens interacts with Arg-127. The phosphonate transition state analogue inhibitor such as **9**[10b] binds CPA with the both oxygen atoms of its phosphinyl group coordinating to the zinc ion. In this case, one of the phosphinyl oxygens is also engaged in hydrogen bonding with the carboxylate of Glu-270.



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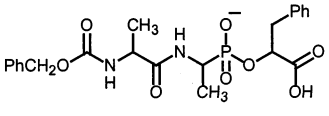

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Table 4. Bond Angles and Bond Lengths between Atoms in the Sulfamide Moiety of (*S*)-**1**

bond angle (deg)		bond length (Å)	
N^1-S-O^3	109.9	N^1-S	1.75
N^1-S-O^3	107.5	N^2-S	1.70
N^2-S-O^3	102.3	O^3-S	1.43
N^2-S-O^4	97.5	O^4-S	1.43
N^1-S-N^2	117.7		
O^3-S-O^4	122.1		

The sulfamoyl moiety in (*S*)-**1a** bears an ill-shaped tetrahedral configuration. Bond distances and angles in the moiety are collected in Table 4. On the basis of the structural feature and the binding mode of the sulfamoyl moiety in (*S*)-**1a** to CPA, it may be concluded that (*S*)-**1a** is a transition state analogue inhibitor for CPA as it has been so designed. The observed stereochemical preference in the binding of **1a** to CPA, i.e., the (*S*)-isomer of **1a** that belongs to the L-series binds CPA more tightly than the (*R*)-diastereoisomer by 2 orders of magnitude is in agreement with the stipulation that transition state analogue inhibitors exhibit the same stereochemical preference as that of substrate in inhibiting the target enzyme.[25] The inhibitor-bound CPA shows no significant changes in its conformation from that of the native enzyme except that the rotation along the Cα−Cβ bond of Tyr-248 to cause its aromatic ring to move down from the surface of the enzyme molecule toward the opening of the S_1' pocket and the movement of Arg-127 side chain toward the sulfamide moiety to form hydrogen bonds with one of sulfamide oxygens of the inhibitor.

Conclusion

Transition state analogue inhibitors constitute an important class of inhibitors whose binding affinities usually surpass those of the other classes of reversible inhibitors.[26] This study demonstrated that the sulfamide moiety would serve as a viable transition state mimic when incorporated judiciously into substrate molecules to afford potent inhibitors for zinc proteases. The prototypical sulfamide-bearing transition state analogue inhibitor, (*S*)-**1a** is 3-fold more potent in the inhibition of CPA than the corresponding phosphonamidate type transition state analogue inhibitor,[9b] suggesting that the sulfamide moiety may possibly better mimic the tetrahedral transition state postulated for the proteolytic

reaction path than the phosphonamidate. Sulfamide bearing inhibitors are easily prepared and stable under physiological conditions, which are important requirements with respect to developing enzyme inhibitors of therapeutic uses. Investigation for the application of the present design protocol to pathologically important zinc proteases with the aim of obtaining therapeutically useful agents is in progress.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AM 300 (300 MHz) instrument using tetramethylsilane as the internal standard. IR spectra were recorded on a Bruker Equinox 55 FT-IR spectrometer. Silica gel 60 (230−400 mesh) was used for flash chromatography and thin-layer chromatography (TLC) was carried out on silica coated glass sheets (Merck silica gel 60 F-254). Mass spectra were obtained at Korea Basic Science Institute, Daejeon, Korea. Elemental analyses were performed at Center for Biofunctional Molecules, Pohang University of Science and Technology, Pohang, Korea and results were within ± 0.4% of the theoretical values.

All solutions for kinetic study were prepared using doubly distilled and deionized water. Stock assay solutions were filtered (GHP Acrodic syringe filter, pore size 0.2 μm) before use. Carboxypeptidase A was purchased from Sigma Chemical Co. (Allan form, twice crystallized from bovine pancreas, 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. *O*-(*trans*-*p*-Chlorocinnamoyl)-L-phenyllactic acid (ClCPL) was synthesized by the literature method[19] and hippuryl L-phenylalanine (Hipp-L-Phe) was purchased from Sigma Chemical Co. An HP 8453 UV/VIS spectrometer was used in enzyme inhibition kinetic studies.

(*S*)-*N*-(*N*-(Benzyloxycarbonyl)sulfamoyl)phenylalanine Benzyl Ester ((*S*)-3). Benzyl alcohol (2.4 mL, 23 mmol) was added slowly at 0 °C to the stirring chlorosulfonyl isocyanate (2 mL, 23 mmol) solution in anhydrous dichloromethane (20 mL), and the stirring was continued for 30 min at 0 °C. Triethylamine (10 mL) in anhydrous dichloromethane was added to the solution. The resulting mixture was then added dropwise to an ice-chilled suspension of (*S*)-phenylalanine benzyl ester *p*-toluenesulfonate (10.3 g, 23 mmol) and triethylamine (6 mL) in anhydrous dichloromethane (50 mL). The solution thus obtained was stirred for 2 h and evaporated under reduced pressure. The residue was dissoloved in ethyl acetate (50 mL), washed successively with 1 N HCl solution (50 mL × 3) and brine (50 mL × 3), and dried over anhydrous $MgSO_4$. The ethyl acetate solution was concentrated in vacuo to afford a white solid, which was recrystallized from dichloromethane to give (*S*)-**3** as a white crystal (10.7 g, 96%): mp 109−110 °C; $[\alpha]^{25}_D$ +5.2° (*c* 1.06, $CHCl_3$); IR (neat) 1360, 1741 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.07 (d, 2H), 4.55 (q, 1H), 5.03 (s, 2H), 5.06 (d, 2H), 5.29 (s, 1H), 5.77 (d, 1H), 7.02−7.63 (m, 15H); ^{13}C NMR (D_2O, 300 MHz) δ 39.4, 58.1, 68.1, 68.9, 127.7, 128.8, 129.1, 129.9, 135.0, 135.1, 151.3, 171.0. Anal. ($C_{24}H_{24}N_2O_6S$) C, H, N.

(*R*)-*N*-(*N*-(Benzyloxycarbonyl)sulfamoyl)phenylalanine Benzyl Ester ((*R*)-3). Compound (*R*)-**3** was prepared from (*R*)-phenylalanine benzyl ester *p*-toluenesulfonate in a manner analogous to that used for the preparation of (*S*)-**3**. $[\alpha]^{25}_D$ −5.8° (*c* 1.06, $CHCl_3$). Mp and spectral data are identical with those of (*S*)-**3**. Anal. ($C_{24}H_{24}N_2O_6S$) C, H, N.

General Procedure for Alkylation of (*S*)-*N*-(*N*-(Benzyloxycarbonyl)sulfamoyl)phenylalanine Benzyl Ester To Prepare (*S*)-4b and (*S*)-4d−4f. To an ice-chilled solution of triphenylphosphine (1.5 equiv) in anhydrous dichloromethane (20 mL) was added slowly diisopropyl azodicarboxylate (1.5 equiv). To the resulting solution was added the mixture of (*S*)-**3** and the appropriate alcohol (1.1 equiv) in anhydrous dichloromethane (10 mL). The solution was stirred for 1 h at room temperature and concentrated in vacuo. The crude product was purified by column chromatography (EtOAc/*n*-hexane = 1/8) and recrystallized from ethyl acetate and *n*-hexane to afford a white crystalline solid.

(*S*)-*N*-(*N*-(Benzyloxycarbonyl)-*N*-methylsulfamoyl)-phenylalanine Benzyl Ester ((*S*)-4b). Compound (*S*)-**4b** was prepared from (*S*)-**3** and methanol. Yield, 69%; $[\alpha]^{20}_D$ +18.3° (*c* 0.65, $CHCl_3$); IR (neat) 1379, 1738 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.05 (d, 2H), 3.13 (s, 3H), 4.40 (m, 1H), 5.02 (m, 2H), 5.14 (m, 2H), 5.78 (d, 1H), 7.02−7.38 (m, 15H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 34.8, 39.5, 58.2, 68.0, 69.4, 127.8, 128.7, 129.1, 129.8, 135.0, 135.2, 170.8. HRMS (FAB+) $(M + H)^+$: calcd for $C_{25}H_{26}N_2O_6S$, 483.1590; found 483.1590.

(*S*)-*N*-(*N*-(Benzyloxycarbonyl)-*N*-isopropylsulfamoyl)-phenylalanine Benzyl Ester ((*S*)-4d). Compound (*S*)-**4d** was prepared from (*S*)-**3** and isopropyl alcohol. Yield, 80%; mp 94−95 °C; $[\alpha]^{20}_D$ +20.7° (*c* 1.03, $CHCl_3$); IR (neat) 1366, 1740 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.31 (t, 6H), 3.03−3.06 (m, 2H), 4.03−4.35 (m, 1H), 4.52−4.57 (m, 1H), 5.02 (s, 2H), 5.13 (q, 2H), 5.87 (d, 1H), 7.00−7.36 (m, 15H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 21.4, 21.5, 39.7, 52.7, 58.0, 68.0, 69.3, 127.7, 128.7, 129.0, 129.1, 129.2, 129.8, 135.0, 135.1, 135.2, 153.2, 170.7. Anal. ($C_{27}H_{30}N_2O_6S$) C, H, N.

(*S*)-*N*-(*N*-Benzyl-*N*-(benzyloxycarbonyl)sulfamoyl)-phenylalanine Benzyl Ester ((*S*)-4e). Compound (*S*)-**4e** was prepared from (*S*)-**3** and benzyl alcohol. Yield, 93%; mp 82−83 °C; $[\alpha]^{20}_D$ −4.7° (*c* 0.98, $CHCl_3$); IR (neat) 1368, 1735 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.93 (m, 2H), 4.09 (m, 1H), 4.64−4.85 (m, 2H), 4.99 (s, 2H), 5.07−5.19 (m, 2H), 5.76 (d, 2H), 6.93−7.32 (m, 20H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 39.4, 46.1, 51.3, 57.7, 67.9, 69.6, 127.7, 128.3, 128.8, 129.0, 129.8, 135.1, 137.3, 153.2, 170.6. Anal. ($C_{31}H_{30}N_2O_6S$) C, H, N.

(*S*)-*N*-(*N*-(Benzyloxycarbonyl)-*N*-phenethylsulfamoyl)-phenylalanine Benzyl Ester ((*S*)-4f). Compound (*S*)-**4f** was prepared from (*S*)-**3** and phenethyl alcohol. Yield, 90%; mp 82.5−83 °C; $[\alpha]^{20}_D$ +13.9° (*c* 1.03, $CHCl_3$); IR (neat) 1368, 1729 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.84 (t, 2H), 3.03 (d, 2H), 3.75 (t, 2H), 3.36 (q, 1H), 5.00 (m, 2H), 5.09 (q, 2H), 5.77 (d, 1H), 7.00−7.35 (m, 20H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 36.5, 39.5, 49.9, 58.2, 68.0, 69.4, 121.4, 126.3, 127.0, 127.7, 128.2, 128.7, 129.1, 129.4, 129.8, 135.1, 135.2, 138.2, 153.0, 170.7. Anal. ($C_{32}H_{32}N_2O_6S$) C, H, N.

General Procedure for the Synthesis of *N*-(*N*-Alkyl)-sulfamoylphenylalanines, ((*S*)-1a, (*R*)-1a, (*S*)-1b, and (*S*)-1d−f). A solution of (*S*)-**3**, (*R*)-**3**, (*S*)-**4b**, or (*S*)-**4d**−**f** in MeOH (20 mL) was stirred for 1 h under hydrogen atmosphere in the presence of 10% Pd−C. The resulting mixture was filtered and the filtrate was evaporated under reduced pressure to give (*S*)-**1a**, (*R*)-**1a**, (*S*)-**1b**, and (*S*)-**1d**−**f**, respectively.

(*S*)-*N*-Sulfamoylphenylalanine Benzyl Ester ((*S*)-1a). Compound (*S*)-**1a** was prepared from (*S*)-**3** (quantitative). mp 113.5−114 °C; $[\alpha]^{20}_D$ +16.4° (*c* 0.22, MeOH); IR (neat) 1358, 1634 cm^{-1}; ^{1}H NMR (D_2O, 300 MHz) δ 2.85−3.07 (m, 2H), 4.13 (q, 1H), 7.18−7.28 (m, 5H); ^{13}C NMR (D_2O, 300 MHz) δ 38.2, 57.8, 127.6, 129.0, 129.8, 136.6, 175.8. Anal. ($C_9H_{12}N_2O_4S$) C, H, N.

(*R*)-*N*-Sulfamoylphenylalanine ((*R*)-1a). Compound (*R*)-**1a** was prepared from (*R*)-**3** (quantitative). $[\alpha]^{20}_D$ −16.0° (*c* 0.53, MeOH). Mp and spectral data are identical with those of (*S*)-**1a**. Anal. ($C_9H_{12}N_2O_4S$) C, H, N.

(*S*)-*N*-(*N*-Methylsulfamoyl)phenylalanine ((*S*)-1b). Compound (*S*)-**1b** was prepared from (*S*)-**4b**. mp 93−94 °C; $[\alpha]^{20}_D$ −22.7° (*c* 0.3, $CHCl_3$); IR (neat) 1331, 1746 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.35 (s, 3H), 2.97 (m, 1H), 3.21 (dd, 1H), 4.26 (q, 1H), 5.08 (d, 1H), 7.24−7.35 (m, 5H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 29.0, 38.9, 57.7, 127.6, 129.1, 130.0, 136.4, 176.6. Anal. ($C_{10}H_{14}N_2O_4S\cdot{}^1/_4H_2O$) C, H, N.

(*S*)-*N*-(*N*-Isopropylsulfamoyl)phenylalanine ((*S*)-1d). Compound (*S*)-**1d** was prepared from (*S*)-**4d** (quantitative) and isolated as a dicyclohexylamine salt. mp 198−200 °C; $[\alpha]^{20}_D$ +1.3° (*c* 0.54, $CHCl_3$); IR (neat) 1371, 1730 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.95−1.09 (dd, 6H), 2.95−3.20 (m, 2H), 3.25 (m, 1H), 4.22 (q, 1H), 5.13 (d, 1H), 5.29 (d, 1H), 7.22−

7.34 (m, 5H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 23.8, 23.9, 39.1, 46.7, 57.5, 127.7, 129.1, 130.0, 136.0, 176.6. Anal. ($C_{24}H_{41}N_3O_4S$) C, H, N.

(*S*)-*N*-(*N*-Benzylsulfamoyl)phenylalanine ((*S*)-1e). Compound (*S*)-**1e** was prepared from (*S*)-**4e** (quantitative) and isolated as a dicyclohexylamine salt. mp 201−202 °C; $[\alpha]^{20}_D$ −5.2° (*c* 0.52, $CHCl_3$); IR (neat) 1324, 1729 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.88−3.15 (m, 2H), 3.70−3.96 (dd, 2H), 4.25 (q, 1H), 5.13 (d, 1H), 5.29 (d, H), 7.13−7.32 (m, 10H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 38.9, 47.2, 57.4, 127.8, 128.3, 129.1, 129.2, 130.0, 135.8, 136.8, 176.5. Anal. ($C_{28}H_{41}N_3O_4S$) C, H, N.

(*S*)-*N*-(*N*-Phenethylsulfamoyl)phenylalanine ((*S*)-1f). Compound (*S*)-**1f** was prepared from (*S*)-**4f**. mp 116.5−117.5 °C; $[\alpha]^{20}_D$ −3.4° (*c* 1.04, $CHCl_3$); IR (neat) 1317, 1731 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.64 (m, 2H), 2.89−3.17 (m, 4H), 4.19 (m, 1H), 5.00 (d, 1H), 5.29 (d, 1H), 7.08−7.32 (m, 10H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 35.9, 39.0, 44.3, 57.3, 127.2, 127.8, 129.1, 129.9, 135.7, 138.2, 176.6. Anal. ($C_{17}H_{20}N_2O_4S$) C, H, N.

(*S*)-*N*-Methyl-*N*-sulfamoylphenylalanine ((*S*)-1c). Benzyl alcohol (0.4 mL, 3.9 mmol) was added slowly to an ice-chilled chlorosulfonyl isocyanate (0.36 mL, 3.9 mmol) solution in anhydrous dichloromethane (10 mL). After being stirred for 30 min, the solution and triethylamine (1.8 mL) in anhydrous dichloromethane were concurrently added dropwise to an ice-chilled solution of *N*-methylphenylalanine benzyl ester (0.75 g, 3.9 mmol) in dichloromethane (10 mL). The resulting solution was stirred for 2 h and then evaporated under reduced pressure. The residue was dissoloved in ethyl acetate (30 mL), washed successively with 1 N HCl solution (50 mL × 3) and brine (50 mL × 3), and dried over anhydrous $MgSO_4$. The solution was concentrated in vacuo to give (*S*)-*N*-(*N*-(benzyloxycarbonyl)sulfamoyl)-*N*-methylphenylalanine methyl ester as an oil (1.3 g, 82%): $[\alpha]^{25}_D$ −18.5° (*c* 0.41, $CHCl_3$); IR (neat) 1359, 1746 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.93 (q, 1H), 3.00 (s, 3H), 3.28 (q, 1H), 3.63 (s, 3H), 4.88 (q, 1H), 5.09 (s, 2H), 7.19−7.38 (m, 10H); ^{13}C NMR (D_2O, 300 MHz) δ 32.2, 36.0, 52.9, 61.8, 68.7, 127.6, 128.8, 129.1, 129.4, 136.3, 151.2, 171.1.

A solution of (*S*)-*N*-(*N*-(benzyloxycarbonyl)sulfamoyl)-*N*-methylphenylalanine methyl ester (1.3 g, 3.2 mmol) in anhydrous MeOH (20 mL) was stirred under hydrogen atmosphere in the presence of 10% Pd−C (100 mg) for 2 h. The resulting mixture was filtered, and the filtrate was evaporated under reduced pressure to give *N*-methyl-*N*-sulfamoylphenylalanine methyl ester as an oil. The ester was dissolved in a mixture of anhydrous MeOH (20 mL) and 1 N lithium hydroxide solution (3.2 mL), and the resulting mixture was stirred for 1 h, then acidified with 1 N HCl solution and extracted with ethyl acetate. The organic layer was evaporated under reduced pressure to afford a white solid, which was recrystallized from ethyl acetate to give a white crystal (0.82 g, quantitative). mp 152−153 °C; $[\alpha]^{20}_D$ −108.8° (*c* 1, $CHCl_3$); IR (neat) 1310, 1731 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.61 (s, 3H), 3.08 (m, 1H), 3.2 (m, 1H), 4.06 (q, 1H), 7.22−7.35 (m, 5H); ^{13}C NMR (D_2O, 300 MHz) δ 33.2, 37.1, 69.2, 127.9, 129.2, 130.0, 135.4, 168.2. Anal. ($C_{10}H_{14}N_2O_4S\cdot 1/3H_2O$) C, H, N.

(*R*)-2-Benzyl-3-(*N*-benzyloxy)aminopropanoic Acid Methyl Ester((*R*)-6). To the solution of (*R*)-**5**[17] (2.2 g, 8.2 mmol) in anhydrous MeOH (20 mL) was added slowly TMSCl (4.18 mL, 32.8 mmol) under nitrogen atmosphere. The resulting solution was stirred for 2 h and evaporated under reduced pressure to afford a white solid which was recrystallized from methanol and ether to give a white crystal (2.7 g, 98%). mp 152.5−153 °C; $[\alpha]^{20}_D$ +25.4° (*c* 1.1, EtOH); IR (KBr) 1726 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.87 (m, 2H), 3.16 (m, 1H), 3.23 (m, 1H), 3.38 (s, 3H), 3.42 (m, 1H), 5.01 (s, 2H), 7.12−7.37 (m, 10H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ 36.6, 43.7, 50.4, 52.5, 75.6, 127.5, 129.3, 129.4, 129.7, 129.9, 138.5, 173.8. Anal. ($C_{18}H_{22}ClNO_3$) C, H, N.

(*S*)-2-Benzyl-3-(*N*-benzyloxy)aminopropanoic Acid Methyl Ester((*S*)-6) Compound (*S*)-**6** was prepared from (*S*)-**5**[17] in a manner analogous to that used for the preparation of (*R*)-**6**. $[\alpha]^{20}_D$ −25.7° (*c* 0.8, EtOH). Mp and spectral data are identical with those of (*R*)-**6**. Anal. ($C_{18}H_{22}ClNO_3$) C, H, N.

(*R*)-2-Benzyl-3-(*N*-benzyloxy-*N*-(*N*-benzyloxycarbonyl)sulfamoyl)aminopropanoic Acid Methyl Ester((*R*)-7). Benzyl alcohol (0.13 mL, 1.2 mmol) was added slowly to an ice-chilled chlorosulfonyl isocyanate (0.11 mL, 1.2 mmol) solution in anhydrous dichloromethane (5 mL). After being stirred for 30 min, the solution and triethylamine (0.5 mL) in anhydrous dichloromethane were concurrently added dropwise to an ice-chilled solution of (*R*)-**6** (0.4 g, 1.2 mmol) in dichloromethane (10 mL). The resulting solution was stirred for 6 h and then evaporated under reduced pressure. The residue was dissoloved in ethyl acetate (20 mL), washed successively with 1 N HCl solution (30 mL × 3) and brine (30 mL × 3), and dried over anhydrous $MgSO_4$. The solution was concentrated in vacuo to give the product as an oil (0.53 g, 88%): $[\alpha]^{25}_D$ −18.9° (*c* 1.1, $CHCl_3$); IR (neat) 1386, 1738 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.80 (m, 1H), 2.91 (m, 1H), 3.04 (m, 1H), 3.44 (d, 1H), 3.81 (q, 1H), 5.00 (d, 2H), 5.15 (s, 2H), 7.10−7.39 (m, 15H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 36.5, 46.3, 52.3, 55.5, 69.4, 80.0, 127.2, 129.0, 129.1, 129.2, 129.4, 129.5, 130.0, 134.8, 134.9, 136.8, 139.4, 153.2, 174.5.

(*S*)-2-Benzyl-3-(*N*-benzyloxy-*N*-(*N*-benzyloxycarbonyl)sulfamoyl)aminopropanoic Acid Methyl Ester ((*S*)-7). Compound (*S*)-**7** was prepared from (*S*)-**6** in a manner analogous to that used for the preparation of (*R*)-**7**. $[\alpha]^{20}_D$ −19.6° (*c* 0.94, $CHCl_3$). Mp and spectral data are identical with those of (*R*)-**7**.

(*R*)-2-Benzyl-3-(*N*-sulfamoyl)aminopropanoic Acid ((*R*)-2). A solution of (*R*)-**7** (0.53 g, 1.03 mmol) in anhydrous MeOH (10 mL) was stirred under hydrogen atmosphere in the presence of 10% Pd−C (30 mg) for 2 h. The resulting mixture was filtered, and the filtrate was evaporated under reduced pressure to give (*R*)-2-benzyl-3-(*N*-sulfamoyl)aminopropanoic acid methyl ester as an oil. The ester was dissolved in a mixture of anhydrous MeOH (5 mL) and 1 N lithium hydroxide solution (1.1 mL), and the resulting mixture was stirred for 2 h, then acidified with 1 N HCl solution and extracted with ethyl acetate. The organic layer was evaporated under reduced pressure to afford a white solid, which was recrystallized from ethyl acetate to give a white solid (0.24 g, 91%). mp 111−113 °C; $[\alpha]^{20}_D$ −60.4° (*c* 0.65, $CHCl_3$); IR (neat) 1341, 1693 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 2.70 (m, 2H), 2.88 (m, 1H), 3.08 (m, 2H), 6.56 (s, 2H), 7.14−7.29 (m, 5H), 7.88 (t, 1H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ 35.0, 42.5, 42.6, 127.6, 129.3, 129.5, 137.4, 173.2. Anal. ($C_{10}H_{14}N_2O_4S$) C, H, N.

(*S*)-2-Benzyl-3-(*N*-sulfamoyl)aminopropanoic Acid ((*S*)-2). Compound (*S*)-**2** was prepared from (*S*)-**7** in a manner analogous to that used for the preparation of (*R*)-**2**. $[\alpha]^{20}_D$ −59.1° (*c* 1.1, $CHCl_3$). Mp and spectral data are identical with those of (*R*)-**2**. Anal. ($C_{10}H_{14}N_2O_4S$) C, H, N.

Determination of K_i Value. Typically, the enzyme stock solution was added to a solution containing ClCPL (final concentrations: 50 μM and 100 μM) and inhibitor (five different final concentrations in the range of 0.5K_i to 2K_i μM) 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 12 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.[20] The correlation coefficients for the Dixon plots were above 0.990.

X-ray Crystallography. The complex of CPA and (*S*)-**1a** was prepared by incubating the protein and the inhibitor in a 1:10 molar ratio overnight at 4 °C. Crystals were grown by the microdialysis method by equilibrating the protein−inhibitor complex in a solution of 1.2 M LiCl and 20 mM Tris-HCl buffer (pH 7.5) against a reservoir containing 0.2 M LiCl and 20 mM Tris-HCl buffer (pH 7.5) as described previously.[6] Single crystals grew in the $P2_1$ space group with one molecule in the asymmetric unit. Crystallographic data were collected

using a Rigaku RU300 rotating-anode X-ray generator operating at 40 kV × 100 mA and a R-axis IIc imaging plate detector system. Diffraction images were processed with the program MOSFLM[27] and the CCP4 program suite.[28]

Model refinement was carried out with the programs O[29] and CNS.[30] The structure of native CPA (pdb code: 5CPA) was used as the starting model for refinement. A cycle of rigid-body refinement was followed by cycles of simulated annealing and individual B factor refinement with model rebuilding. The extra density accounting for the inhibitor was apparent from the initial stage of the refinement. During the refinement, the randomly selected 5% of data were set aside for the R_{free} calculation. Water molecules were gradually added to the model with the WATERPICK routine in the program CNS.[30] The inhibitor model was included in the last stage of the refinement. The final model includes all residues of CPA (residues 1−307, 2437 atoms), 16 inhibitor atoms, a zinc atom, and 148 water molecules.

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